VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 11, Number 7, 2011

DOI: 10.1089/vbz.2010.0077

Molecular Detection and Identification of *Rickettsia* Species in *Ixodes pacificus* in California

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Abstract

We amplified *16S* rRNA, *gltA*, and *ompA* genes from *Ixodes pacificus* by polymerase chain reaction. Sequencing, BLAST analysis, and phylogenetic constructions indicated that two *Rickettsia* phylotypes are present in *I. pacificus*. While phylotype G021 has high homology to *Ixodes scapularis* endosymbiotic *Rickettsia*, phylotype G022 is a deeply branched novel spotted fever group *Rickettsia*.

Key Words: *Ixodes pacificus* ticks—*Rickettsia*—Vector-borne.

Introduction

The western black-legged tick, *Ixodes pacificus*, is the most widely distributed tick in the Pacific West Coast region of the United States. It is a primary vector of human diseases, including Lyme borreliosis and anaplasmosis (Burgdorfer et al. 1985). Although *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever, has never been detected in *I. pacificus*, Hughes et al. (1976) identified Tillamook and Grants Pass strains, the spotted fever group agent of rickettsiae from *I. pacificus*, in western Oregon. Later, Philip et al. (1981) reported that *I. pacificus* in western California possesses a *Rickettsia* species that is similar to the Tillamook stain. Alhough several lines of evidence have been collected that support the presence of the *Rickettsia* species in *I. pacificus* using serological tests (Hughes et al. 1976, Philip et al. 1981), investigators have not yet used DNA analysis to determine the presence of rickettsiae in the tick. In the present study, we report detection of rickettsial DNA in *I. pacificus* ticks that were collected in the Napa Valley of California.

Materials and Methods

Tick collection and identification

To detect the presence of rickettsiae in questing *I. pacificus*, 96 adult *I. pacificus* Cooley and Kohls ticks were collected in 2003 in the Napa Valley by dragging a white cloth flag along trails with grass vegetation. Genus and species of *I. pacificus* ticks were confirmed by standard taxonomic characteristics.

DNA extraction and polymerase chain reaction amplification

To isolate DNA, individual ticks were frozen in liquid nitrogen and pulverized using a fitted pestle. Total genomic DNA was extracted from individual ticks using DNeasy Tissue Kit (Qiagen, Valencia, CA), as previously described by Jasinskas et al. (2007). Mock extractions were carried out as a negative control. To detect if rickettsiae were present in the total tick DNA extracts of *I. pacificus*, aliquots of 96 DNA extracts were mixed; the pool of the total genomic DNA was used as a template for detecting the presence of rickettsiae by polymerase chain reaction (PCR) using 16S rRNA, *gltA* (encoding citrate synthase protein), and *ompA* (encoding outer membrane protein A) primer sets. PCR amplification was performed using 50 ng of total genomic DNA of *I. pacificus* in a 50 μL reaction, containing PCR Master Mix (Promega Corporation, Madison, WI) and 0.1 mM of forward and reverse primers. The primer sequences of the 16S rRNA, *gltA*, and *ompA* are 5′-TAAGGAGGTAATCCAGCC-3′ and 5′-CCTG GCTCAGAACGAA-3′, 5′-GGCTAATGAAGCGGTAATAA ATATGCTT-3′ and 5′-TTTGCGACGGTATACCCATAGC-3′, and 5′-CACYACCTCAACCGCAGC-3′ and 5′-AAAGTTA TATTTCCTAAACCYGTATAAKTATCRGC-3′, respectively. The primers were designed as a result of multiple sequence alignments of each gene of rickettsiae. The PCRs were performed in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) with the following conditions: denaturation at 95°C for 5 min, and then 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 1 min, and elongation at 72°C for 2 min for 16S rRNA or 30 s for *gltA* and *ompA* genes, respectively.

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Cloning and sequencing

The PCR products of 16S rRNA, *gltA*, and *ompA* genes of rickettsiae were cloned into StrataClone™ PCR cloning vector pSC-A (Agilent Technologies, La Jolla, CA) and sequenced at the MicroChemical Core Facility of San Diego State University using M13 reverse primer (5′-GGAAACAGCTATGACCA TG-3′) and custom oligonucleotide primers derived from the 16S rRNA gene (Integrated Device Technology, Coralville, IA). The sequences are listed in GenBank under the accession numbers EU072493 and EU072494 for the 16S gene, GQ375159 and GQ375160 for the *gltA* gene, GQ375161 and GQ375162 for the *ompA* gene of the two *Rickettsia* phylotypes, and GU556973 for the actin gene sequence of *I. pacificus*.

Phylogenetic analysis

To study evolutionary relatedness of identified rickettsiae, phylogenetic trees of 16S rRNA, *gltA*, and *ompA* genes were constructed as described by Jasinskas et al. (2007). Briefly, multisequence alignments were performed using ClustalX version 1.83.1 (University College Dublin); phylogeny of each gene was constructed by PHYLO_WIN (version 2). Galtier and Gouy's method was used to determine evolutionary distance values; the values were then used to construct phylograms by a neighbor-joining method.

Transmission electron microscopy

To observe bacteria in *I. pacificus* ticks, midgut from an adult male *I. pacificus* was imaged using a transmission electron microscope. The dissected tick midgut was fixed in 3% glutaraldehyde in 50 mM cacodylate buffer (pH 7.0). Samples were chilled to 10°C, and microwaved for 40 s at a power setting that produced a temperature increase of 10°C–15°C using a PELCO™ Microwave System (Ted Pella, Redding, CA). Specimens were again chilled, and microwaved for 40 s. Samples were washed 3× with 50 mM cacodylate buffer (pH 7.0), and then postfixed with 1.5% aqueous osmium tetroxide in the microwave (40 s, 2×). After osmication, tissues were rinsed three times with distilled water, and then dehydrated in a graded ethanol series, 40 s of microwave energy per grade, and temperature restriction of 37°C. Next, samples were infiltrated with a 1:1 mixture of ethanol ERL4206 (ERL)-Quetol (3,4-epoxy cyclohexyl methyl 3,4-epoxy cyclohexyl carboxylale) resin (Ted Pella) for 15 min using the microwave set at a temperature restriction of 45°C. Infiltration was completed by replacing the 1:1 mixture of ethanol and resin with 100% ERL-Quetol for 15 min in the microwave with the same temperature restriction, and then repeating this step a second time. Samples were embedded in 100% ERL-Quetol and polymerized overnight at 60°C in a vacuum oven. Specimens were sectioned on a Reichert ultramicrotome using a Diatome diamond knife, stained with aqueous uranyl acetate followed by Reynold's lead citrate. The sections were examined using a transmission electron microscope (Phillips EM 208S; FEI, Hillsboro, OR).

Results

16S rRNA, *gltA*, and *ompA* genes were detected by PCR in pooled *I. pacificus* tick DNA (Fig. 1). "Classifier" analysis at the Ribosomal Database Project II Web site (http://rdp.cme.msu.edu/index.jsp) placed the amplified 16S rRNA sequences in this study in the genus *Rickettsia*. Sequencing of 10 clones for each gene determined that the size of the *gltA* clone is 341 base pairs (bp). However, the sizes of 16S rRNA and *ompA* clones are 1482 and 1483 bp, and 444 and 438 bp, respectively. The sequences of the two 16S rRNA amplicons shared 98% of their nucleotide-sequence identity. Similar nucleotide sequence identity (97%) was observed in the *gltA* sequences. When the *ompA* nucleotide sequences were compared with each other, a significantly low nucleotide identity of 81% was observed. Comparison at the amino acid level revealed 72% identity between the two translated *ompA* nucleotide sequences. BLAST analysis of the sequences of the 16S rRNA, *gltA*, and *ompA* genes against the NCBI DNA database showed nucleotide sequence similarities with spotted fever group *Rickettsia.* The closest matches of the 16S rRNA nucleotide sequences exhibited 98% and 99% nucleotide sequence identities to 16S rRNA of *Rickettsia massiliae* MTU5 (accession number CP000683.1). One of the *gltA* sequences exhibited 100% nucleotide sequence identities with the *gltA* gene of *Rickettsia raoultii* strain Khabarovsk (accession number DQ365804.1), whereas the other *gltA* sequence was most identical (99% nucleotide sequence identities) to the *gltA* gene sequence of *Rickettsia monacensis* strain CN45Kr (accession number FJ009429.1). The *ompA* sequences were also compared with sequences of the *ompA* gene of *Rickettsia* species in GenBank. One of the *ompA* sequences had 99% nucleotide sequence identity to the *ompA* gene of *Rickettsia* sp. CAMWD07-2 from *I. pacificus* (accession number EU544298.1) and 98% nucleotide sequence identity to the *gltA* gene of *Ixodes scapularis* endosymbiont TX125 (accession number

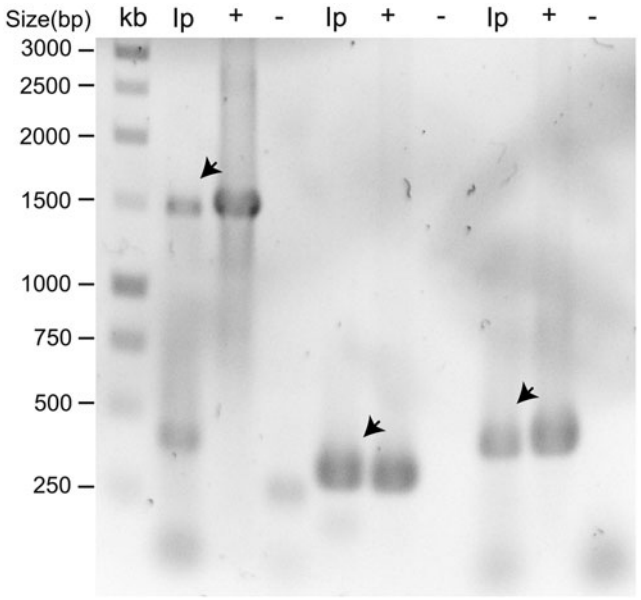

FIG. 1. Detection of 16S rRNA, *gltA*, and *ompA* genes by PCR amplification of *Ixodes pacificus* tick extracts. Ninety-six ticks were collected in the Napa Valley, California. Tick DNA was extracted, pooled, and used as template for PCR amplification. The PCR products were electrophoresed and observed by staining with ethidium bromide. kb, 1 kb DNA ladder (Promega). Ip, *I. pacificus* tick extract; (+), *Rickettsia conorii* DNA positive control; (−), no template negative control; arrow, PCR amplicons. PCR, polymerase chain reaction.

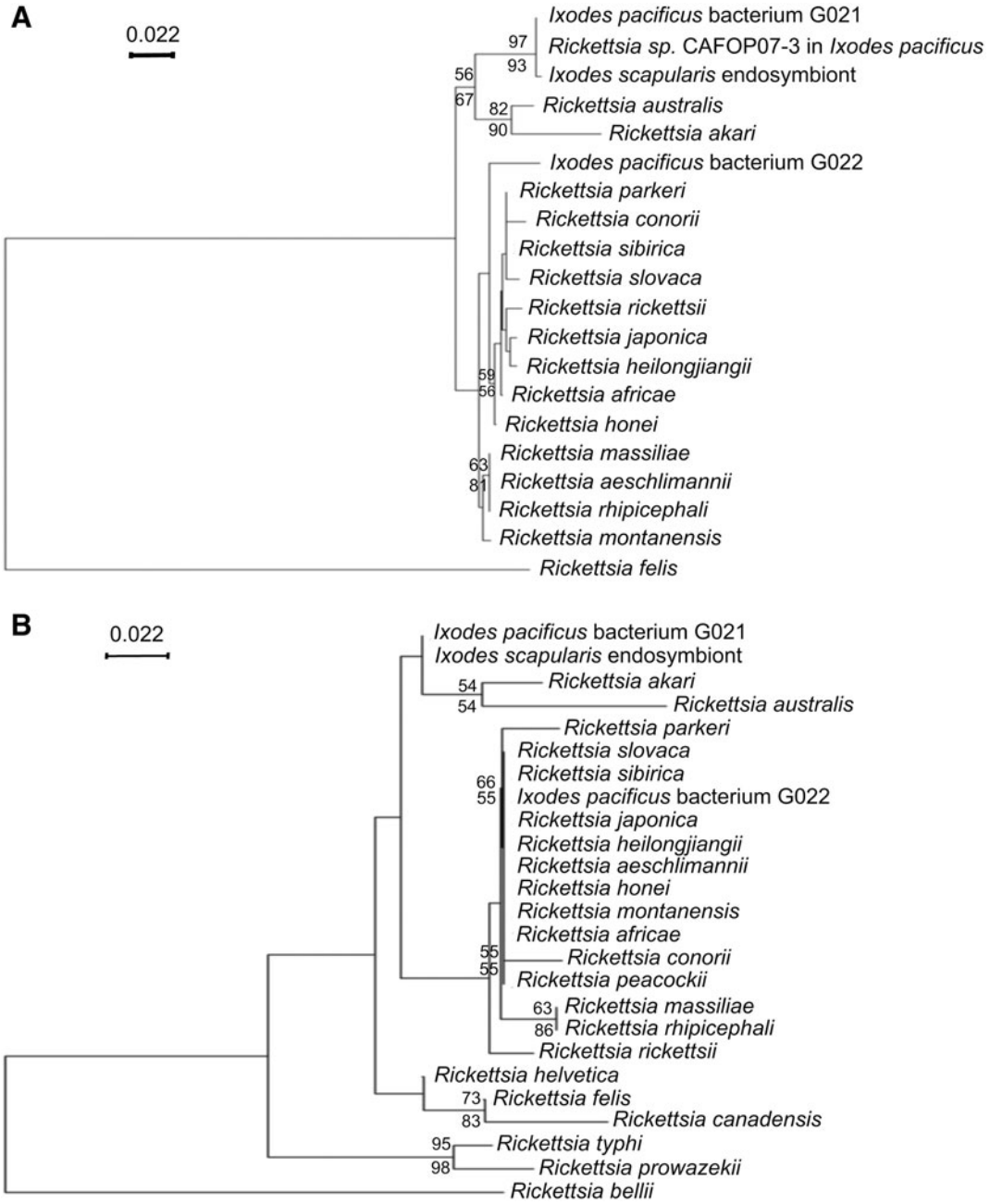

FIG. 2. Phylogenetic trees of *ompA* **(A)** and *gltA* **(B)** genes of the two *Rickettsia* phylotypes in *I. pacificus*. Other selected sequences on the phylogenetic tree of the *ompA* gene are *Rickettsia* sp. CAFOP07-3 (EU544297.1), *Ixodes scapularis* endosymbiont (AB002268.1), *Rickettsia aeschlimannii* (DQ379981.1), *Rickettsia massiliae* (DQ212707.1), *Rickettsia rhipicephali* (EU109177.1), *Rickettsia australis* (AF149108.1), *Rickettsia montanensis* (AF045223.1), *Rickettsia sibirica* (AABW01000001.1), *Rickettsia rickettsii* (AY319293.1), *Rickettsia slovaca* (EU622810.1), *Rickettsia honei* (AF018075.1), *Rickettsia africae* (EU622980.1), *Rickettsia parkeri* (EU715288.1), *Rickettsia heilongjiangii* (AF179362.2), *Rickettsia conorii* (U43794.1), *Rickettsia felis* (AY727036.1), *Rickettsia peacockii* (AY319292.1), *Rickettsia japonica* (U83442.1), and *Rickettsia akari* (L01461.1). Other selected sequences on the phylogenetic tree of the *gltA* gene are *I. scapularis* endosymbiont (EF662058.1), *Rickettsia felis* (CP000053.1), *Rickettsia helvetica* (DQ821857.1), *Rickettsia akari* (U59717.1), *Rickettsia canadensis* (BAF79997.1), *Rickettsia australis* (U59718.1), *Rickettsia africae* (CP001612.1), *Rickettsia japonica* (AY743327.1), *Rickettsia parkeri* (EF102236.1), *Rickettsia slovaca* (AY129301.1), *Rickettsia montanensis* (U74756.1), *Rickettsia honei* (U59726.1), *Rickettsia sibirica* (DQ124930.1), *Rickettsia typhi* (U59714.1), *Rickettsia heilongjiangensis* (AY285776.1), *Rickettsia bellii* (NC_007940.1), *Rickettsia prowazekii* (NC_000963.1), *Rickettsia rickettsii* (DQ150689.1), *Rickettsia conorii* (NC_003103.1), *Rickettsia aeschlimannii* (DQ235776.1), *Rickettsia massiliae* (U59719.1), *Rickettsia rhipicephali* (U59721.1), and *Rickettsia peacockii* (DQ100162.1). The tree is inferred from the comparison of either *ompA* or *gltA* sequences by the neighbor-joining method using PHYLO_WIN. The numbers at nodes are the bootstrap values obtained from 1000 replicates. Bootstrap values >50 are shown at the nodes. Bootstrap values determined by the neighbor-joining method and maximum-parsimony are shown above the line and below the line, respectively. Bar, nucleotide distance.

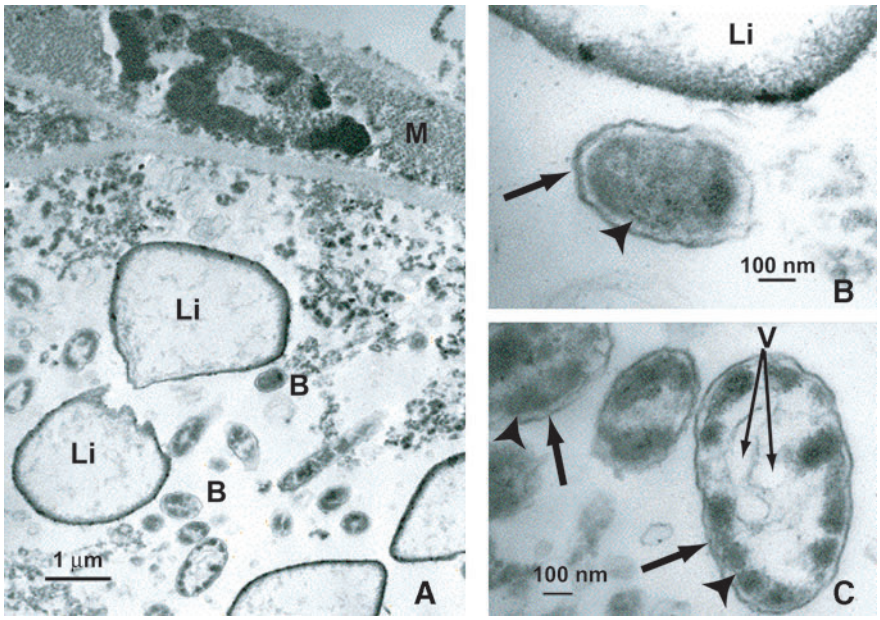

FIG. 3. Transmission electron micrographs of the midgut tissue from an *Ixodes pacificus* tick. **(A)** Longitudinal view of the midgut tissue: B, bacteria; Li, lipid inclusion. **(B, C)** Higher magnification of bacteria: thick arrows point to the outer membrane; arrowheads point to the inner membrane. Notice the electron-lucent vacuole-like structures (V) within the bacteria.

EF689735.1), whereas the other *ompA* sequence had 88% nucleotide sequence identity to the *gltA* gene of *Rickettsia amblyommii* species TNLBL06-3 (accession number EU544295.1).

The neighbor-joining tree shown in Figure 2 was based on the results of a distance matrix analysis of all available *gltA* and *ompA* sequences of rickettsiae in GenBank. The phylogenetic trees revealed that two PCR sequences of each gene, which were amplified from *I. pacificus* in this study, clustered with species of the spotted fever group of the genus *Rickettsia*. Since the two PCR sequences of each gene belong to different lineages, as shown in Figure 2, we have named the rickettsiae in *I. pacificus* as G021 and G022 phylotypes. The neighbor-joining tree indicated that the *ompA* gene sequence of the phylotype G021 was related to known *Rickettsia australis* and *Rickettsia akari*. However, the sequence was more closely related to the *ompA* gene sequence of *Rickettsia* sp. CAFOP07-3 in *I. pacificus*, and *I. scapularis Rickettsia* endosymbiont. All of the above *Rickettsia* species, including the phylotype G021, formed a monophyletic group that is distinct from the rest of spotted fever group rickettsiae. On the other hand, the *ompA* gene of the phylotype G022 constituted a distinct and deeply branched phylogenetic lineage within the spotted fever group of *Rickettsia*. The phylogeny placed the phylotype G022 as a sister lineage to the clade of members of rickettsiae in the spotted fever group, including *Rickettsia slovaca*, *Rickettsia heilongjiangii*, *Rickettsia japonica*, *Rickettsia rickettsii*, *Rickettsia parkeri*, *Rickettsia sibirica*, *Rickettsia africae*, *Rickettsia honei*, and *Rickettsia conorii* (Fig. 2A). Although the branching order of the two *Rickettsia* phylotypes failed to be resolved with other *Rickettsia* species based on the 16S rRNA sequences (data not shown), results of the phylogeny of the *gltA* gene sequences supported the phylogeny of the *ompA* gene sequences; the phylotype G021 formed a monophyletic group with *R. australis*, *R. akari*, and the endosymbiotic *Rickettsia* species in *I. scapularis*, whereas the phylotype G022 had close relatives with members of the spotted fever group of rickettsiae (Fig. 2B). Similar tree topologies were obtained when either maximum-likelihood or maximum-parsimony was used for tree constructions.

Transmission electron microscope observations of the midgut of *I. pacificus* revealed intracellular microorganisms in the cytoplasm of the cells in the midgut. The microorganisms appeared to be rod-shaped bacteria, with distinct gram-negative cell walls that included an outer membrane and a cytoplasmic membrane. The bacteria varied in shape from straight to curved and the size ranged from 0.3 to 0.6 μm wide by 0.5 to 0.9 μm long. The bacterial cells also possessed intracellular inclusions, and some of them appeared to be electron dense, whereas other intracellular inclusions were electron transparent. The number of the rod-shaped bacteria varied among cells, ranging from none to hundreds per cell (Fig. 3). No ultrastructural evidence of the presence of more than one type of bacteria was found in the midgut of *I. pacificus*.

Discussion

In this study, we detected and identified two *Rickettsia* phylotypes in *I. pacificus* ticks from Napa Valley, California. The two phylotypes belong to different lineages: while the phylotype G021 is a close relative of a *Rickettsia* symbiont in *I. scapularis*, the phylotype G022 is a deeply branched novel spotted fever group *Rickettsia*. Although there were a couple of studies identifying *Rickettsia* species in *I. pacificus* using less-sensitive serological tests (Hughes et al., 1976, Philip et al. 1981), our investigation documents the presence of *Rickettsia* species using molecular biology tools.

Rickettsia species have been repeatedly isolated from *Ixodes* ticks in the United States; more than 20 novel species of spotted fever group rickettsiae have been reported in *Ixodes* tick species other than *I. pacificus* (Fournier et al. 2003). Using a hemolymph test or direct immunofluorescence, spotted fever group rickettsiae have been detected in *I. scapularis* (Mag-

narelli et al. 1979) and *Ixodes cookei* nymphs (Magnarelli et al. 1985). Later, PCR detected that a rickettsial endosymbiont is present in *I. scapularis* (Moreno et al. 2006) and that *R. rickettsii* is present in *Ixodes texanus* (Anderson et al. 1986). Recently, a bacterial symbiont of the genus *Rickettsia* was identified in *I. scapularis* in the tick genome project (Nene 2009). All of the previous descriptions of spotted fever group rickettsiae in association with *Ixodes* ticks represent the great genetic diversity within the spotted fever group rickettsiae.

The pathogenicity of *Rickettsia* phylotypes G021 and G022 remains to be determined. The discovery of these phylotypes also prompts future research on their transmission and prevalence in tick populations.

Acknowledgments

We thank Dr. Alan G. Barbour at the University of California, Irvine for providing *I. pacificus* tick samples. We thank Dr. David H. Walker at the University of Texas Medical Branch for providing genomic DNA of *R. conorii.* We also thank Dr. Patricia L. Siering for providing comments on the manuscript. This work was supported by Dr. Jianmin Zhong's NIH R15 grant 1 R15 AI 82515-01 and Humboldt State University New Faculty Seed Grant and by Dr. Jacob P. Varkey's Howard Hughes Medical Institute grant 52005127.

Disclosure Statement

No competing financial interests exist

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