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Detection and Isolation of *Rickettsia tillamookensis* (Rickettsiales: Rickettsiaceae) From *Ixodes pacificus* (Acari: Ixodidae) From Multiple Regions of California

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Abstract

The western black-legged tick (*Ixodes pacificus*) is the most frequently identified human-biting tick species in the western United States and the principal vector of at least three recognized bacterial pathogens of humans. A potentially pathogenic *Rickettsia* species, first described in 1978 and recently characterized as a novel transitional group agent designated as *Rickettsia tillamookensis*, also exists among populations of *I. pacificus*, although the distribution and frequency of this agent are poorly known. We evaluated DNA extracts from 348 host-seeking *I. pacificus* nymphs collected from 9 locations in five California counties, and from 916 *I. pacificus* adults collected from 24 locations in 13 counties, by using a real-time PCR designed specifically to detect DNA of *R. tillamookensis*. DNA of *R. tillamookensis* was detected in 10 (2.9%) nymphs (95% CI: 1.6–5.2%) and 17 (1.9%) adults (95% CI: 1.2–3.0%) from 11 counties of northern California. Although site-specific infection rates varied greatly, frequencies of infection remained consistently low when aggregated by stage, sex, habitat type, or geographical region. Four novel isolates of *R. tillamookensis* were cultivated in Vero E6 cells from individual adult ticks collected from Alameda, Nevada, and Yolo counties. Four historical isolates, serotyped previously as 'Tillamook-like' strains over 40 yr ago, were revived from long-term storage in liquid nitrogen and confirmed subsequently by molecular methods as isolates of *R. tillamookensis*. The potential public health impact of *R. tillamookensis* requires further investigation.

Key words: *Ixodes pacificus*, western black-legged tick, *Rickettsia tillamookensis*, transitional group *Rickettsia*

The western black-legged tick (*Ixodes pacificus* Cooley and Kohls, 1943) is the most frequently identified human-biting tick species in California, Oregon, and Washington, as well as in British Columbia, Canada (Lane 1990, Clover and Lane 1995, Xu et al. 2019, Salkeld et al. 2019, Morshed et al. 2021). *Ixodes pacificus* is also the principal vector in the western United States of several pathogens of humans, including *Borrelia burgdorferi* (Burgdorfer et al. 1985, Lane et al. 1999), *Anaplasma phagocytophilum* (Richter et al. 1996), and *Borrelia miyamotoi* (Padgett et al. 2014). Western black-legged ticks can be infected simultaneously with several of these agents (Lane et al. 2004, Eschoo et al. 2015, Dykstra et al. 2020, Lehane et al. 2021), or with other bacterial and protozoan

species of recognized or suspected pathogenicity to humans or animals, including *Spiroplasma ixodetis*, *Ehrlichia chaffeensis Borrelia bissettiae*, and *Babesia odocoilei* (Tully et al. 1995, Kramer et al. 1999, Girard et al. 2011, Eschoo et al. 2015).

In 1967, a field survey of western black-legged ticks in western Oregon allowed the eventual isolation in embryonated chicken eggs of a *Rickettsia* species from triturated pools of adult, host-seeking *I. pacificus* ticks collected in Tillamook and Josephine counties (Hughes et al. 1976). Additional isolates, referred to as 'Tillamook-like' strains, were established subsequently from host-seeking *I. pacificus* ticks collected from Oregon during 1975–1976 (Philip et al. 1978a), and from Sonoma and Monterey counties in California during 1979–1980 (Lane et al. 1981, Philip et al. 1981). Although Tillamook-like strains reacted with a fluorescein-conjugated antibody to spotted fever group *Rickettsia* species (Philip et al. 1978a), these isolates were distinct from classical spotted fever and typhus group *Rickettsia* species by mouse serotyping (Philip et al. 1978a, b; Philip 1981). Despite preliminary evidence of pathogenicity by the original Tillamook strain to mice and guinea pigs (Hughes et al. 1976), this *Rickettsia* species remained largely ignored for the subsequent 40 yr, and no confirmed cases of infections of humans with this agent have been identified.

In 2021, whole genome sequencing of the original type strain (Tillamook 23^T) revealed a unique transitional group *Rickettsia* species, designated *Rickettsia tillamookensis* (Gautier et al. 2021). Herein, we document the distribution and occurrence of *R. tillamookensis* among specimens of nymphal and adult *I. pacificus* ticks collected from multiple locations in California, validate the identities of historical Tillamook-like strains, and describe the cultivation of contemporary isolates of this recently characterized and potentially pathogenic tick-borne bacterium in the western United States.

Materials and Methods

Tick Collection and Site Characteristics

During 2016–2021, host-seeking nymphs and adults of *I. pacificus* were collected from vegetation using flannel cloth flags and drags from sites in northern, central, and southern California (Table 1). Nymphal specimens were collected during previous investigations (Lawrence et al. 2018, Sambado et al. 2020). Most specimens were collected along hiking trails in city, state, or regional parks and in open space preserves. Habitat sites were categorized as: evergreen woodlands, represented predominantly by coast live oak (*Quercus agrifolia*) and California laurel (*Umbellularia californica*); deciduous woodlands, comprising mixtures of valley oak (*Quercus lobata*), California black oak (*Quercus kelloggii*), blue oak (*Quercus douglasii*), California buckeye (*Aesculus californica*), California sycamore (*Platanus racemosa*), or bigleaf maple (*Acer macrophyllum*); mixed woodlands, including one or more *Quercus* spp., *U. californica*, *A. californica*, coast redwood (*Sequoia sempervirens*) or madrone (*Arbutus menziesii*); coastal sage scrub, predominated by California sagebrush (*Artemisia californica*), coyote bush (*Baccharis pilularis)*, and poison oak (*Toxicodendron diversilobum*), or; savannah, comprising predominantly native grassland with occasional *Quercus, Umbellularia*, or *Pinus* species.

Ticks were stored in 70 or 95% ethanol at room temperature or maintained live for subsequent attempts to isolate *Rickettsia* species in cell culture. Specimens were identified using morphological characteristics (Furman and Loomis 1984, Keirans and Clifford 1978). Live specimens were washed sequentially for 5 min each in 5% Micro-Chem Plus (National Chemical Laboratories, Philadelphia, PA), 1.7% sodium hypochlorite, and 3% hydrogen peroxide, with a final rinse in sterile, cell-culture-grade water (Paddock et al. 2018). Washed specimens were bisected longitudinally using sterile scalpel blades. One half was placed in 200 µl of sucrose phosphate glutamate (SPG) buffer and frozen at −80°C and the other half was processed for DNA extraction. DNA was extracted from individual whole nymphal or adult specimens that were preserved in ethanol, and from halved and frozen adult specimens processed for isolation. Nymphs were crushed using sterile pestles and adults were minced using sterile scalpel blades. Samples were processed using a DNAeasy Blood and Tissue kit (QIAGEN, Valencia, CA) and eluted into final volumes of 100 µl each.

Development of a Real-Time PCR Assay for *R. tillamookensis*

Because conventional *omp*A assays do not detect *R. tillamookensis* (Gautier et al. 2021), a unique molecular target, comprising a 157-bp segment of the aspartate tRNA ligase gene (*asp*S), was selected from the annotated genome of *R. tillamookensis* strain Tillamook 23^T (GenBank accession number CP060138). The component concentrations of each reaction included 2 µl of DNA template, 0.1 µM each of primer Till4F (5'-GCTTGCTGATTTAAAGGAAATGCA-3') and Till4R (5'-AGGAA GTTGATAAGAGATTTGGGG-3'), 0.25 µM of probe Till_P (5'-FAM-CCATGCGGCGGTGCTCCAAACT-BHQ1-3'), 10 µl of QuantiTect Probe PCR Master Mix (Qiagen, Valencia, CA), and sterile, PCR-grade water to adjust the final reaction volume to 20 µl. Reactions were performed on a CFX96 Thermal Cycler (Bio-Rad, Hercules, CA) with 15 min at 95°C followed in turn by 40 cycles of 30 s at 95°C and then 60 s at 60°C. To assess the sensitivity of the assay, a control plasmid containing a segment of the *asp*S of *R. tillamookensis* strain Tillamook 23^T was constructed using an Invitrogen TOPO TA Cloning kit. The sequence of the insertion site was confirmed using primers T7 Promoter and M13 Reverse. A 10-fold serial dilution series of the plasmid, from 100,000 copies to 1 copy per 2 µl sample, was created to determine the limit of detection of the assay. All samples were tested in triplicate. The specificity of the assay was evaluated using a panel of DNA extracts representing *Rickettsia* species cultivated in Vero E6 cells (*Rickettsia africae*, *Rickettsia akari*, *Rickettsia australis*, *Rickettsia amblyommatis*, *Rickettsia bellii*, *Rickettsia canadensis*, *Rickettsia conorii*, *Rickettsia heilongjiangensis*, *Rickettsia helvetica*, *Rickettsia honei*, *Rickettsia massiliae*, *Rickettsia montanensis*, *Rickettsia monteiroi*, *Rickettsia parkeri*, *Rickettsia peacockii*, *Rickettsia prowazekii*, *Rickettsia raoultii*, *Rickettsia rhipicephali*, *Rickettsia rickettsii*, *Rickettsia* 364D, *Rickettsia slovaca*, *Rickettsia sibirica*, *Rickettsia tamurae*, and *Rickettsia typhi*), or ISE6 cells (*Rickettsia* endosymbiont of *I. pacificus* strain Humboldt (G021), *Rickettsia felis*, and *Rickettsia asembonensis*). Tick extracts that tested positive for *R. tillamookensis* were additionally evaluated by a conventional PCR assay for *omp*A (Table 2) to detect *Rickettsia* spp. G021 or G022 (Cheng et al. 2013a, b).

Isolation of *R. tillamookensis* in Cell Culture

Remaining halves of selected ticks with a corresponding *R. tillamookensis asp*S-positive extract were thawed and minced individually in 200 µl of SPG using sterile scalpel blades. Each inoculum was added to a semi-confluent monolayer of Vero E6 cells in a vented T25 tissue culture flask (Corning, Corning, NY) containing 5 ml of cell culture medium (Minimum Essential Medium with 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum) and 10 units/ml penicillin, 10 µg/ml streptomycin sulfate with 0.25 µg/ml amphotericin B (Gibco, Grand Island, NY). Cultures were incubated at 32 °C in a 5% CO_2-in-air atmosphere. The cell culture medium

Table 1. Distribution and frequency of *Rickettsia tillamookensis* among host seeking, adult and nymphal *Ixodes pacificus* ticks collected from California, 2016–2021

County	Location	Coordinates	Elevation (m)	Month/year of collection	No. of ticks evaluated	No. (%, 95% CI) with *R. tillamookensis*
Alameda	Del Valle Regional Park	37.59449, –121.70894	271	11/2016	40	0
	Garin Regional Park	37.62095, –122.02090	102	12/2016	41	0
	Pleasanton Ridge Regional Park	37.61541, –121.88237	83	12/2016	40	2 (5, 1.4–16.5)
	Anthony Chabot Regional Park	37.75192, –122.11145	279	11/2016	19	3 (15.8, 5.5–37.6)
				01/2017	20	0
Contra Costa	Tilden Regional Park	37.90389, –122.25778	182	05/2019	11[a]	1 (9.1, 1.6–37.7)
	Lafayette	37.88394, –122.13472	180	04/2018	7[a]	0
El Dorado	Gold Bug Park	38.74266, –120.79954	638	02/2021	6	0
Humboldt	Green Diamond Property	40.86361, –123.97028	48	05/2020	60	0
		40.85917, –123.84389	917	05/2020	20	0
Marin	Tiburon Upland	37.88931, –122.45141	110	06/2016	22[a]	0
				05/2018	20[a]	0
	China Camp State Park	38.00131, –122.48889	29	05/2016	29[a]	0
				04/2018	12[a]	0
				04/2019	16[a]	0
				02/2021	61	1 (1.6, 0.3–8.7)
	Olompali State Historic Park	38.15301, –122.57495	96	03/2017	81	1 (1.2, 0.2–6.7)
			29	05/2016	29[a]	0
Monterey	Fort Ord Natural Reserve	36.68553, –121.77503	33	01/2021	9	0
	Big Creek Natural Reserve	36.07016, –121.59829	46	01/2021	10	0
				03/2021	10	0
Napa	Bothe-Napa Valley State Park	38.54083, –122.53778	304	01/2020	75	2 (2.7, 0.7–9.2)
Nevada	South Yuba River State Park	39.29157, –121.09764	374	12/2020	32	1 (3.1, 0.5–15.7)
		39.29377, –121.19414	215	02/2021	25	0
	Round Mountain	39.30569, –121.02678	788	02/2021	31	2 (6.5, 1.8–20.7)
	Scotts Flat Reservoir	39.28207, –120.92553	936	02//2021	10	0
Placer	Auburn State Recreation Area	38.96683, –120.95176	488	02/2021	49	0
	Stevens Trail	39.10584, –120.94713	727	02/2021	26	0
San Luis Obispo	Rancho Marino Reserve	35.53624, –121.08302	40	04/2021	10	0
				05/2021	10	0
Santa Barbara	Santa Cruz Island Reserve	34.00138, –119.71451	70	04/2021	20	0
	Sedgwick Reserve	34.71622, –120.05077	454	03/2021	20	0
Santa Clara	Heintz Open Space Preserve	37.22730, –121.92351	207	05/2016	5[a]	1 (20, 3.6–62.4)
				04/2018	15[a]	0
				04/2019	8[a]	0
San Mateo	Water Dog Lake Park	37.50311, –122.29899	106	05/2019	20[1]	1 (5, 0.9–23.6)
	Windy Hill Open Space Preserve	37.36475, –122.22176	217	05/2018	15[a]	1 (6.7, 1.2–30)
				04/2019	27[a]	0
	Filoli	37.46946, –122.31639	130	05/2018	15[a]	0
				05/2019	20[a]	2 (10, 2.7–30.1)
Solano	Rockville Hills Regional Park	38.25045, –122.14269	130	02/2021	93	2 (2.1, 0.6–7.5)
Sonoma	Spring Lake Regional Park	38.45225, –122.64833	146	04/2018	19[a]	2 (10.5, 2.9–31.4)
				05/2019	80[a]	2 (2.5, 0.7–8.7)

Table 1. Continued

County	Location	Coordinates	Elevation (m)	Month/year of collection	No. of ticks evaluated	No. (%, 95% CI) with *R. tillamookensis*
Yolo	Cache Creek Canyon	38.913001, –122.27217	218	02/2021	28	2 (7.1, 2.0–22.6)
Yuba	Bullards Bar Reservoir	39.42897, –121.108561	591	02/2021	50	1 (2.0, 0.4–10.5)
Total	Adults				916	17 (1.9, 1.2–3.0)
	Nymphs				348	10 (2.9, 1.6–5.2)

[a]Nymphs; all other specimens were adults.

Table 2. Genetic loci, primer designations and sequences, annealing temperatures, and product sizes of amplicons associated with conventional PCR assays used to evaluate DNA extracted from *Ixodes pacificus* ticks and isolates of *Rickettsia tillamookenesis*

Locus	Primer designation	Primer sequence	Annealing temperature (°C)	Product size (bp)	Reference
*omp*A	*Rr*190.70p	ATGGCGAATATTTCTCCAAAA	57	629	Regnery et al. 1991
	*Rr*190.701	GTTCCGTTAATGGCAGCATCT			Roux et al. 1996
*glt*A	*Rp*CS.877p	GGGGGCCTGCTCACGGCGG	54	382	Regnery et al. 1991
	*Rp*CS.1258n	ATTGCAAAAAGTACAGTGAACA			
17 kDa antigen	R17-122	CAGAGTGCTATGAACAAACAAGG	55	403	Massung et al. 2001
	R17-500	CTTGCCATTGCCCATCAGGTTG			
Rtill_00381	Rtill_00381F.112	GCAGGTCTAGCGGCTAGT	65	498	This study
	Rtill_00381R.610	CGGGCTCTGCTTCTATCTTCT			
Rtill_01168	Rtill_01168F.2193	CGCATTTCATAAAATCACGCCT	63	502	This study
	Rtill_01168R.2695	GATCCGCTAGGTGTTGCAC			

was replaced the following day with 5 ml of medium containing the same antibiotics, and again one day later with antibiotic-free cell culture medium. Cultures were monitored weekly for evidence of infection by examining cytospin preparations fixed in absolute methanol and stained with a 0.1 g/liter solution of acridine orange (Becton, Dickinson and Company, Sparks, MD). Cultures without discernible intracellular bacteria were monitored for a minimum of four weeks before discarding. Isolates with intracellular bacteria confirmed as a *Rickettsia* sp. were maintained continuously for a minimum of three passages in culture before considered established. The identity of each isolate was confirmed by conventional PCR amplification and sequencing of segments of the 17 kDa antigen and *glt*A genes, as well as Rtill_00381 and Rtill_01168, two unique hypothetical protein genes identified from the genome of *R. tillamookensis* (Table 2).

Characterization of 'Tillamook-Like' Isolates

Four historical isolates, cultivated from pooled or individual *I. pacificus* ticks and serotyped previously as 'Tillamook-like' strains, were retrieved from liquid nitrogen storage at the Centers for Disease Control and Prevention (CDC) and Rocky Mountain Laboratories (RML), and propagated in Vero E6 cells (Table 3). These included strain Grants Pass, isolated from a pool of 68 adult ticks collected at the city dump of Grants Pass, Josephine County, OR in 1967 (Hughes et al. 1976); strain 66170B, isolated from a pool of 4 adults collected near Rowena in Wasco County, OR in 1976 (RML, unpublished data); strain CA 288, isolated from an adult tick collected from Hastings Natural History Reservation in Monterey County, CA in 1979 (Philip et al. 1981), and strain CA 277, isolated from an adult tick collected near Annapolis in Sonoma County, CA in 1980 (Lane et al. 1981). Each isolate was revived and subsequently passaged in Vero E6 cells at 32°C. The identity of each historical isolate was confirmed by PCR amplification and sequencing of segments of the 17 kDa antigen and *glt*A, genes, and loci Rtill_00381 and Rtill_01168, as described previously. Each historical isolate was evaluated for coinfection with a *Mycoplasma* species using the Universal Mycoplasma Detection Kit (ATCC, Manassas, VA), in accordance with the manufacturers' recommendations.

Electron Microscopy

Vero E6 cells infected with a fourth passage of the Cache Creek Canyon strain of *R. tillamookensis* were washed in 0.1 M phosphate buffer at pH 7.3 and fixed in buffered 2.5% glutaraldehyde for 2 h at 4°C. The pellet was post-fixed in 1% osmium tetroxide, stained in 4% uranyl acetate, dehydrated through a graded series of alcohols and acetone, and embedded in a mixture of Epon-substitute and Araldite. Thin sections were stained with 4% uranyl acetate and Reynold's lead citrate.

Statistical Analyses

Differences among proportions were evaluated by using χ-square. Wilson score 95% confidence intervals were calculated by using JMP Version 15.1 (SAS Inc).

Results

DNA extracts of 1,264 *I. pacificus* ticks, comprising 348 nymphs collected from 9 locations in five counties, and 916 adults (544 females and 372 males) collected from 24 locations in 13 counties were evaluated by a real-time PCR assay designed to detect *R. tillamookensis* (Table 1). The limit of detection of the assay was determined to be one 1 genomic copy/2 µl of sample, with a PCR efficiency of 95.2%, and an R^2 value of 0.99. The assay did not amplify DNA of any of 27 other *Rickettsia* species evaluated. DNA of *R. tillamookensis* was detected in 10 (2.9%) nymphs (95% CI: 1.6–5.2%) and 17 (1.9%) adults (95% CI: 1.2–3.0%). There

Table 3. Historical and contemporary strains of *Rickettsia tillamookensis* isolated from *Ixodes pacificus* ticks

Strain designation	Origin (year of tick collection)	Source[a]	Percent identity with Tillamook 23^T 17 kDa gene[b]	Current passage history at CDC[c]	Reference
Tillamook 23^T[d]	Tillamook County, OR (1967)	9 females	–	23E/12V	Hughes et al. 1976
Grants Pass[d]	Josephine County, OR (1967)	33 females, 35 males	100	2V[e]	Hughes et al. 1976
66107B[d]	Wasco County, OR (1976)	4 females	100	3E/5V	RML, unpublished
CA 288[d]	Monterey County, CA (1979)	1 adult	99.7	1E/8V	Philip et al. 1981
CA 277	Sonoma County, CA (1980)	1 adult	100	13V	Lane et al. 1981
Alameda	Alameda County, CA (2016)	1 female	99.7	5V	This study
CA16-124	Alameda County, CA (2016)	1 male	99.7	3V	This study
CA20-6	Nevada County, CA (2020)	1 female	100	5V	This study
Cache Creek Canyon	Yolo County, CA (2021)	1 female	100	4V	This study

[a]All isolates obtained from adult, host-seeking *I. pacificus* ticks.
[b]All isolates demonstrated complete identity with the corresponding sequences of *gltA* and hypothetical protein genes Rtill_00381 and Rtill_01168 of *R. tillamookensis* strain Tillamook 23^T.
[c]E = passage in embryonated chicken eggs; V = passage in Vero cells.
[d]Isolate co-infected with a *Mycoplasma* species.
[e]Passage history not recorded prior to expansion for this study.

were no significant differences between aggregated infection rates in nymphs and adults ($p = 0.263668$) or between the percentages of total positive female (2.0%, 95% CI: 1.1–3.6%) and total positive male (1.6%, 95% CI: 0.7–3.5%) specimens ($p = 0.652269$). A segment of *omp*A with 99.8–100% identity with the corresponding 587-bp segment of the *omp*A of the *Rickettsia* endosymbiont of *I. pacificus* strain Humboldt (GenBank accession number GQ375161) was amplified from each of the 10 nymphs and 16 (94.1%) of adults that contained DNA of *R. tillamookensis*.

There were no significant differences among infection prevalence rates when aggregated by region ($p = 0.070735$); *R. tillamookensis* was detected in 0.6% (95% CI: 0.2–2.1%) of 341 specimens collected from five locations in the Outer Northern Coast Range (Humboldt and Marin counties), 3.4% (95% CI: 1.9–6.1%) of 295 specimens collected from four locations in the Inner Northern Coast Range (Sonoma, Napa, Solano, and Yolo counties), 2.8% (95% CI: 1.5–4.9%) of 399 specimens collected from 15 locations in the Southern Coast Range (Alameda, Contra Costa, Monterey, San Luis Obispo, Santa Barbara, Santa Clara, and San Mateo counties), and 1.7% (95% CI: 0.7–4.4%) of 229 specimens collected from eight locations in the Sierra Nevada foothills (El Dorado, Nevada, Placer, and Yuba counties). There were no significant differences in aggregated infection prevalence rates among woodland sites ($p = 0.502338$); *R. tillamookensis* was detected among 2.0% (95% CI: 1.1–3.6%) of 500 specimens collected from 14 predominantly evergreen woodlands, 3.5% (95% CI: 1.6–7.4%) of 171 total specimens collected from four predominantly deciduous woodlands, and 2.1% (95% CI: 1.2–3.8%) of 513 total specimens collected from 11 mixed woodlands. *Rickettsia tillamookensis* was not detected in any of 40 specimens collected from two coastal scrub sites or in any of 40 specimens from one savannah site.

Four novel isolates of *R. tillamookensis* were cultivated in Vero E6 cells from individual adult ticks collected from Alameda, Nevada, and Yolo counties (Table 3). Small, coccoid, coccobacillary, and rod-shaped bacteria were identified in the cytoplasm of Vero E6 cells within 5 d after primary inoculation (Fig. 1). Attempts to cultivate *R. tillamookensis* from two additional tick specimens identified by the real-time assay were unsuccessful, and one isolate became contaminated during a subsequent passage and was discarded. The isolates, designated Alameda, CA16-124, CA20-6, and Cache Creek Canyon were deposited in the CDC Rickettsial Isolate Reference Collection. Electron microscopy of the Cache Creek Canyon isolate revealed short rods free in the cytosol of Vero E6 cells measuring on average 0.93 × 0.29 µm. All rickettsiae possessed a trilaminar cell membrane surrounded by a halo zone and several were identified in the process of binary fission. A delicate fringe of fine minute projections was identified additionally on the outer leaflet of the cell membrane (Fig. 1).

Four historical isolates (Grants Pass, CA 277, CA 288, and 66107B), established during 1978–1981 and designated 'Tillamook-like' strains of *Rickettsia* by mouse serotyping (Philip et al. 1978a, b, 1981; RML unpublished data), were revived in cell culture. Coinfections with *Mycoplasma* were detected in the Grants Pass, CA 288, and 66107B isolates. The four novel and four archival isolates were confirmed as *R. tillamookensis* by PCR amplification and sequence analyses of four genetic loci. The 358-bp sequences of the 17 kDa antigen gene of two novel isolates (Alameda and CA16-124), and one historical isolate (CA 288) were identical with each other and differed from the corresponding sequence of *R. tillamookensis* strain Tillamook 23^T at one nucleotide position; the sequences of each of the other isolates possessed 100% identity with each other and with that of the type strain. Among each of the novel and historical isolates, there was complete identity with the 341-bp sequence of *glt*A, the 460-bp sequence of the hypothetical protein gene Till_00381, and the 462-bp sequence of the hypothetical protein gene Till_01168 of *R. tillamookensis* Tillamook 23^T. Neither *Rickettsia* sp. G021 nor *Rickettsia* sp. G022 were detected by *ompA* PCR in established passages of the historical or novel isolates.

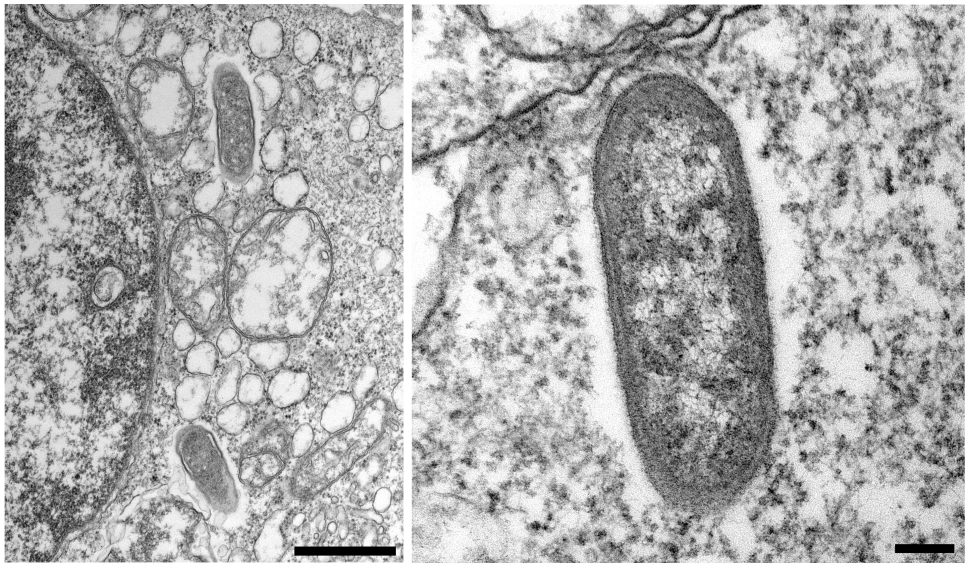

Fig. 1. Electron microscopical images of *Rickettsia tillamookensis* (strain Cache Creek Canyon) following 9 d of growth in Vero E6 cells. Short, rod-shaped bacteria free in the cytosol (left), each surrounded by a halo zone and possessing a trilaminar cell membrane with a delicate fringe of minute projections on the outermost leaflet (right). Four percent uranyl acetate and Reynold's lead citrate stain. Bars = 800 nm (left), 100 nm (right).

Discussion

We identified DNA of *R. tillamookensis* in 2.9% (95% CI: 1.6–5.2%) of nymphs and 1.9% (95% CI: 1.2–3.0%) of adults from an aggregate sample of 1,264 host-seeking *I. pacificus* ticks collected from predominantly woodland environments within the Pacific Coast Ranges and the Sierra Nevada foothills of California during 2016–2021. The prevalence of infection among adult ticks was similar to the 1–2% rates of infection described approximately 40 yr ago by investigators using culture-based methods of detection (Philip et al. 1981, Lane et al. 1981). Although site-specific infection rates varied greatly, frequencies of infection remained consistently low (i.e., <4%) when aggregated by stage, sex, habitat type, or geographical region. The four isolates of *R. tillamookensis* cultivated from individual tick specimens represent the first novel strains of this *Rickettsia* species obtained in 40 yr. Four historical isolates, characterized previously as 'Tillamook-like' by a mouse serotyping technique and maintained as frozen stocks for several decades, were revived and confirmed as *R. tillamookensis* by contemporary molecular methods.

Among *R. tillamookensis*-positive ticks, we found a surprisingly high rate of co-infection with the endosymbiont *Rickettsia* sp. G021, as DNA of this species was detected in 96.3% of the adults and nymphs containing DNA of *R. tillamookensis*. *Rickettsia* sp. G021, a spotted fever group *Rickettsia* species similar with or identical to *Rickettsia monacensis* (Alowaysi et al. 2019), has been detected nearly ubiquitously among host-seeking *I. pacificus* ticks collected in California (Phan et al. 2011, Cheng et al. 2013a, Stephenson et al. 2017, Couper et al. 2019, Osborne et al. 2020, Socarras et al. 2021), comprising approximately 60 and 80% of the total bacterial reads from the microbiomes of western blacklegged nymphs and adults, respectively (Kwan et al. 2017, Chicana et al. 2019, Couper et al. 2019). In female *I. pacificus* this bacterium is identified within oocytes (Bagheri et al. 2017). Despite the frequency of coinfection with *Rickettsia* sp. G021, cultivation of this agent requires highly specific conditions of temperature, cell line selection, and media requirements (Alowaysi et al. 2019) that we excluded during this investigation to select for pure isolates of *R. tillamookensis*. A third, and as-yet uncharacterized spotted fever group *Rickettsia* species, *Rickettsia* sp. G022, also has been detected in *I. pacificus* at levels similar with those we observed for *R. tillamookensis*. Among 247 western blacklegged ticks collected from 6 California counties, infections with *Rickettsia* sp. G022 were detected in only 2.0% (95% CI: 0.3–3.8%); notably, all specimens that harbored *Rickettsia* sp. G022 were also infected by *Rickettsia* sp. G021 (Cheng et al. 2013a, b).

The overall abundance and frequency of *Rickettsia* sp. G021 in *I. pacificus* could explain, in part, the relative rarity of *R. tillamookensis* and possibly *Rickettsia* sp. G022. Molecular evaluations of several species of host-seeking, field-collected ticks document simultaneous infections with more than one *Rickettsia* species in a small subset of specimens (Wikswo et al. 2008, Carmichael and Fuerst 2010, Berrada et al. 2011, Paddock et al. 2018, Hecht et al. 2019). Nonetheless, multiple laboratory assessments suggest that larval ticks inherit only one *Rickettsia* species from a superinfected maternal tick, through a process termed rickettsial interference (Burgdorfer et al. 1981). For example, this phenomenon could be responsible for the exclusion of transovarial transmission of *R. rickettsii* by *Rickettsia peacockii* in *Dermacentor andersoni* (Burgdorfer et al. 1981), *R. rhipicephali* by *Rickettsia montanensis* in *Dermacentor variabilis* (Macaluso et al. 2002), and *R. rickettsii* by *R. bellii* in *Amblyomma dubitatum* (Sakai et al. 2014). A more recent study has challenged the concept of rickettsial interference by suggesting that interspecific competition between *Rickettsia* species is neither identical among all species, nor is it necessarily unidirectional (Levin et al. 2018). The rate of transstadial and transovarial transmission of *Rickettsia* sp. G021 is estimated at 100% (Cheng et al. 2013b,) and rickettsial exclusion of *R. tillamookenesis* by the highly prevalent *Rickettsia* sp. G021 could conceptually account for relative scarcity of *R. tillamookensis* detected among adult and immature *I. pacificus*. Other studies have determined that the composition and richness of the *I. pacificus* microbiome are influenced considerably by host species (Swei and Kwan 2017). In this context, it is likely that infections with *R. tillamookensis* in *I. pacificus* could be acquired predominantly, or possibly exclusively, during hematophagy by the larval and nymphal stages, and influenced considerably by one or more reservoir host species.

Fig. 2. Left, central erythematous lesion at the tick bite site with satellite vesiculopapular rash on the flank of a 56-yr-old man several days following the bite of an adult *Ixodes pacificus* tick in northern California (image courtesy of D. B. Steger). Right, eschar surrounded by erythematous halo on the flank of a 67-yr-old woman, six days following the bite from an adult *I. pacificus* tick in northern California (image courtesy of Ervic Aquino, California Department of Public Health).

An alternate hypothesis proposes that physiological conditions within *I. pacificus* could select for and ultimately determine the relative prevalence and abundance of tick-associated microbial species by creating internal conditions that favor growth of microbes that provide strong fitness benefits to the tick, perhaps to the detriment and exclusion of other species (Couper et al. 2019). Interestingly, *Rickettsia* sp. G021 possesses each of the genes necessary for de novo folate biosynthesis, whereas hard ticks lack such genes, thereby implying a crucial role for *Rickettsia* sp. G021 in the survival of *I. pacificus* (Hunter et al. 2015).

Experimental and historical observations suggest that *R. tillamookensis* could be pathogenic in human hosts. The most compelling data are those from male guinea pigs (*Cavia porcellus*) following intraperitoneal inoculation with a 10% yolk sac suspension of the type strain (Hughes et al. 1976). In that study, half of 80 infected animals developed 1–2 d of low-grade fever, and approximately 25% developed mild scrotal swelling. Historical reports also document the occurrence of a slow-healing ulcer seen occasionally at the site of attachment by *I. pacificus* ticks (Fig. 2), accompanied occasionally by fever, a sparse rash, and headache (Gregson 1942, Arthur and Snow 1968). Notably, 12% of 141 persons from Humboldt County, CA, who identified signs or symptoms after a recent tick bite also reported an eschar at the tick bite site (Pascoe et al. 2019).

Ixodes pacificus is distributed extensively across the western United States, where established populations are documented from 55 (94.8%), 18 (50%), 16 (41.3%), 4 (13.8%), and 1 (6.7%) of the counties of California, Oregon, Washington, Utah, and Arizona, respectively; isolated collection records exist for an additional 14 counties within these states, as well as two in Nevada (Eisen et al. 2016, Beck et al. 1963). Ensemble modeling revealed an additional 11 counties, predominantly in the Pacific Northwest, that contain suitable habitat for western black-legged ticks, representing a potential 12% increase in the number of United States counties with established populations of *I. pacificus* (Hahn et al. 2016). Outside the country, established populations of western black-legged ticks also exist in the Canadian provinces of British Columbia and Alberta (Lindquist et al. 2016, Kanji et al. 2022), as well as the state of Baja California, Mexico (Guzmán-Cornejo and Robbins 2010). In California, climatic changes predicted to occur by the end of the 21st Century indicate that that suitable habitats for *I. pacificus* could expand by 23%, with more than a third of the increase encompassing open-access lands where human encounters with ticks are frequent (Hahn et al. 2021).

Approximately 60–70% of tick bites in the western coastal states are attributable to *I. pacificus* (Clover and Lane 1995, Xu et al. 2019, Salkeld et al. 2019, Morshed et al. 2021) and serosurveys conducted among otherwise healthy persons in northern California and the Pacific Northwest document 3–6.3% seroprevalence to spotted fever group *Rickettsia* species (Stephenson et al. 2017, Straily et al. 2020), which implies that humans are exposed frequently to one or more *Rickettsia* species that could include *R. tillamookensis*. Almost all ticks collected for the present study quested along or near hiking trails at locations with public access. Aggregated infection rates with *R. tillamookensis* among nymphs and adults of *I. pacificus* are similar to those described for *B. burgdorferi* and *Borrelia miyamotoi* in northern California (Padgett et al. 2014, Salkeld et al. 2021, Xu et al. 2021), suggesting that the level of transmission of *R. tillmookensis* to humans could approach those estimated for pathogenic *Borrelia* spp. in this region. Collectively these data indicate that the potential public health impact of *R. tillamookensis* requires further investigation.

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