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Ehrlichia phagocytophila Genogroup Rickettsiae in Ixodid Ticks from California Collected in 1995 and 1996

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A total of 1,246 ixodid ticks collected in 1995 and 1996 from seven California counties were examined for the presence of *Ehrlichia phagocytophila* genogroup rickettsiae by using a nested PCR technique. Of 1,112 adult *Ixodes pacificus* Cooley and Kohls ticks tested, nine pools, each containing five ticks, were positive (minimum percentage of ticks harboring detectable ehrlichiae, 0.8%). Positive ticks were limited to four of the seven counties (Sonoma, El Dorado, Santa Cruz, and Orange). In Santa Cruz County, three positive pools were identified at the home of an individual with prior confirmed human granulocytic ehrlichiosis. In El Dorado County, positive ticks were found at sites where cases of granulocytic ehrlichiosis in a horse and a llama had recently occurred. Among 47 nymphal *I. pacificus* ticks collected in Sonoma County, one positive pool was identified. Fifty-seven adult *Dermacentor occidentalis* Marx and 30 adult *D. variabilis* Say ticks, collected chiefly in southern California, were negative. These data, although preliminary, suggest that the prevalence of *E. phagocytophila* genogroup rickettsiae in ixodid ticks of California may be lower than in cognate vector populations (i.e., *I. scapularis* Say = *I. dammini* Spielman, Clifford, Piesman, and Corwin) in the eastern and midwestern United States.

Granulocytic ehrlichiosis caused by rickettsiae of the *Ehrlichia phagocytophila* genogroup has been reported in a number of mammalian species, including equines, ruminants, dogs, a llama, and humans (1, 4, 6, 12–14, 18, 34). In California, equine granulocytic ehrlichiosis (EGE) caused by *Ehrlichia equi* has been a recognized disease of horses since the 1960s (13, 18, 19). Most cases occur during the late fall, winter, and early spring, corresponding to the season of peak activity of the adult stage of the vector, *Ixodes pacificus* Cooley and Kohls, the western black-legged tick (3, 18, 26, 31, 32). Clinical manifestations include fever, lethargy, anorexia, distal limb edema, thrombocytopenia, and petechiae. In New England, *I. scapularis* Say (= *I. dammini* Spielman, Clifford, Piesman, and Corwin) (22) is considered a likely vector, whereas *I. ricinus* (L.) may be the vector in parts of Europe (17, 30). The vector in South America (9) is unknown.

Human granulocytic ehrlichiosis (HGE) is a recently described disease characterized by headache, myalgia, chills, and various combinations of leukopenia, anemia, and thrombocytopenia (1, 34). The causative ehrlichia (HGE agent) is most likely transmitted by ixodid ticks, including *I. scapularis* (21, 23, 29) and possibly *I. pacificus*. The HGE agent is known to cause granulocytic ehrlichiosis in horses (2, 14, 17, 20) and dogs (12, 14) as well and may, in reality, be conspecific with *E. equi* (2, 20).

Two confirmed cases of HGE have been recognized in California, both in residents of Santa Cruz County (11, 33). Both case patients reported exposure to ticks and no travel outside California during the putative exposure period. Because *I. scapularis* does not occur in California, suspicion has fallen on *I. pacificus* as a vector of HGE in this state. To provide initial data regarding the presence of *E. phagocytophila* genogroup rickettsiae in ixodid ticks of California, we examined 1,112 unfed adult *I. pacificus* ticks collected from seven counties during the 1995 to 1996 season by using a nested PCR technique (2–4, 20, 26). A smaller number of unfed *I. pacificus* nymphs and *Dermacentor occidentalis* Marx and *D. variabilis* Say adults also were tested.

MATERIALS AND METHODS

Tick collection. The majority of ticks were unfed, actively questing *I. pacificus* adults, 528 males and 584 females, obtained by flagging (28) or hand removal from sites in seven California counties (Fig. 1). In addition, we collected a small number of unfed nymphs (47 total) from a single location in Sonoma County. Small numbers of questing adults of two other ixodid tick species, *D. occidentalis* (57 individuals from Orange and El Dorado Counties) and *D. variabilis* (30 individuals from Orange County), were collected as well.

Adult *I. pacificus*. In Shasta County, 66 adult *I. pacificus* ticks were collected in April 1996 at the Whiskeytown National Recreation Area. Ticks from Humboldt County were collected in December 1995 at Eel Rock (104 ticks), Harris (126 ticks), and in Garberville (103 ticks), all from hillside areas with limited public exposure. In Sonoma County, ticks were collected in February 1996 at Armstrong Woods State Park (100 ticks), at a residential location in Cloverdale (100 ticks), and at a hillside residential development in Sonoma (108 ticks). In Newtown, El Dorado County, 120 ticks were collected in March 1996 on a property where an ehrlichia had been identified in a diseased llama (4). In Diamond Springs, El Dorado County, 24 ticks were collected in January 1996 on a property where a case of EGE had recently been diagnosed.

In Santa Cruz County, ticks were collected in March 1996 from two properties where individuals with prior confirmed HGE were in residence, in Aptos (54 ticks) and Bonny Doon (76 ticks). In Los Angeles County, 15 ticks were collected in January 1996 from Monrovia Canyon. Ten ticks were collected in March 1996 from Solstice Canyon, Malibu, and eight ticks were collected in April 1996 from a second Malibu location. Ticks from Orange County were collected at Pico Canyon (5 ticks, February 1996) and Skeet Club Canyon (6 ticks, April 1996), San Clemente; Modjeska Canyon in the Santa Ana Mountains (10 ticks, March 1996); El Moro Canyon, Laguna Beach (2 ticks, April 1996); and Via Montoya and Via Cerro Rebal, San Juan Capistrano (75 ticks, March through May 1996).

Nymphal *I. pacificus*. Forty-seven *I. pacificus* nymphs were collected in May 1996 from a residential location in Cloverdale, Sonoma County.

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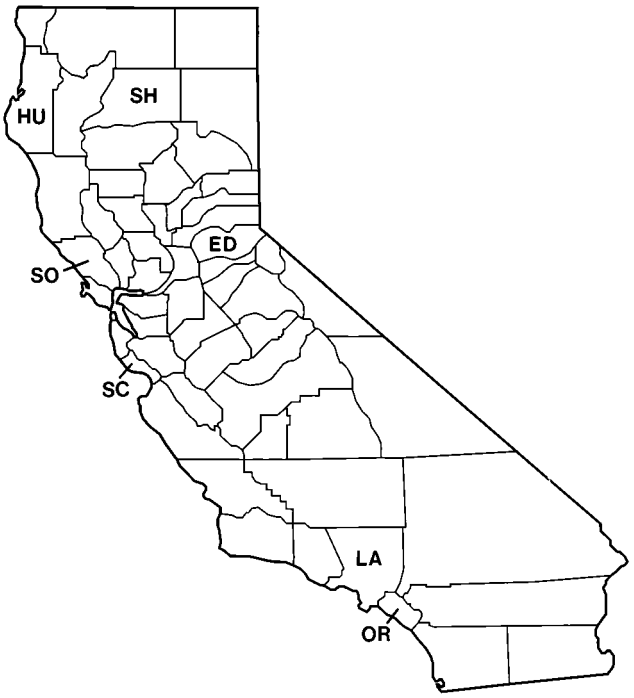

FIG. 1. Map of California showing counties where ixodid ticks were collected for PCR to detect *E. phagocytophila* genogroup rickettsiae. Abbreviations: SH, Shasta; HU, Humboldt; SO, Sonoma; ED, El Dorado; SC, Santa Cruz; LA, Los Angeles; OR, Orange. Pools from Sonoma, El Dorado, Santa Cruz, and Orange Counties yielded positive ticks.

Adult *D. occidentalis*. Fifty-four *D. occidentalis* adults were collected from the following sites in Orange County: Via Cerro Rebal and Via Montoya, San Juan Capistrano (24 ticks, April and May 1996); Skeet Club Canyon, San Clemente (15 ticks, April 1996), and El Moro Canyon, Laguna Beach (15 ticks in April 1996). Three ticks were collected at the llama site in Newtown, El Dorado County, in March 1996.

Adult *D. variabilis*. Thirty *D. variabilis* adults were collected in April 1996 at three sites in Orange County: Skeet Club Canyon, San Clemente (10 ticks); El Moro Canyon, Laguna Beach (4 ticks); and Via Montoya, San Juan Capistrano (16 ticks).

Processing of tick specimens. Ticks were frozen (−70°C), thawed, and segregated by sex into pools of ca. five ticks each. Ticks in each pool were cut in half with sterile scissors and placed into 2-ml microtubes containing 300 μl of zirconia-silica beads (0.5-mm diameter; Biospec Products, Bartlesville, Okla.) and 400 μl of DNA extraction buffer (10 mM Tris [pH 8.0], 2 mM EDTA, 0.1% sodium dodecyl sulfate, 500 μg of proteinase K per ml). Ticks were mechanically disrupted on a Mini-BeadBeater (Biospec) by using the high setting and three 1-min bursts. The tubes were spun at top speed in a microcentrifuge for 30 s and incubated at 56°C for 3 h and then at 97°C for 15 min. After pelleting, the supernatants were transferred to fresh, sterile tubes. The DNA was precipitated by adding 3 to 4 volumes of ice-cold absolute ethanol and 100 μl of 3 M sodium acetate and placing the tubes at −20°C for 24 to 48 h. The DNA was pelleted at top speed in a microcentrifuge for 15 min at 4°C and washed twice with ice-cold 70% ethanol. After drying, the DNA was resuspended in 100 μl of DNase-free water or Tris-EDTA (pH 8.0). The quality and quantity of the prepared DNA were assessed with tick mitochondrial DNA primers 16S+2 and 16S-1 (5) in a single-round PCR (35 cycles, 48°C annealing) prior to performance of the nested PCR.

Nested PCR. Components and conditions of the nested PCR for detecting *E. phagocytophila* genogroup rickettsiae have been described (3, 4, 20, 26). Cycling conditions per round were preheating to 94°C for 5 min; 40 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 1.5 min; and a final extension at 72°C for 7 min.

RESULTS

A total of 1,246 ixodid ticks were tested for *E. phagocytophila* genogroup rickettsiae. Results of the survey for adult *I. pacificus* are shown in Table 1. The data are expressed as the minimum number of ticks with detectable ehrlichiae (i.e., PCR positive), a positive result indicating that at least one tick in a pool of five contained ehrlichiae.

Of 1,112 *I. pacificus* adults tested, only nine pools were PCR positive (9/1,112 = 0.8% [minimum percentage of ticks harboring detectable ehrlichiae]). Seven of those pools originated in El Dorado and Santa Cruz Counties. In El Dorado County, two positive pools were identified at the llama site in Newtown while two others were found on the Diamond Springs property, where the EGE case had been diagnosed. The prevalence at the latter site was 2 of 24 (8.3%), the highest recorded for any location in our survey. The three positive pools from Santa Cruz County were collected at the Bonny Doon home of a former HGE patient (3/76 = 3.9%). Of the remaining two positive pools, the cohort from Orange County was collected along a bridle path in the vicinity of a riding stable. Identification of a pool of positive adults from Cloverdale, Sonoma County, prompted the subsequent collection of nymphal *I. pacificus* from that site. From the 47 nymphs collected, one positive pool was identified (1/47 = 2.1%). None of the 57 *D. occidentalis* adults or 30 *D. variabilis* adults was positive.

DISCUSSION

This study was undertaken to provide initial data regarding the prevalence of *E. phagocytophila* genogroup rickettsiae in ixodid ticks of California. This genogroup of closely related organisms is comprised of *E. phagocytophila*, *E. equi*, the HGE agent, and (tentatively) the llama ehrlichia (1, 4, 20, 34). All four agents are detectable with the nested PCR; however, *E. phagocytophila*, the cause of tick-borne fever of ruminants, is not known to occur in the United States (6, 30). Our findings are not intended to represent a comprehensive survey of the distribution of these rickettsiae in ticks throughout the state; rather, they represent exploratory data gathered from a number of key areas, including sites where cases of granulocytic ehrlichiosis have occurred. Thus, certain of the results are necessarily biased, in that some locations (e.g., in El Dorado and Santa Cruz counties) were examined on the basis of a known prior association with granulocytic ehrlichiosis. It is possible that with a more randomized sampling scheme, the overall prevalence among *I. pacificus* adults may be far less than 0.8%. Conversely, certain other areas of high prevalence may have been overlooked in the present survey.

Our results differ from PCR-derived data reported for *I. scapularis* in the northeastern and upper midwestern United States (21, 23, 29). For example, in 118 *I. scapularis* ticks collected from five sites in Connecticut, rates of positivity ranged from 10.4 to 90.9%, with an overall value of 50% (21).

TABLE 1. Results of nested PCR testing for *E. phagocytophila* genogroup rickettsiae in *I. pacificus* adults collected in seven California counties in 1995 and 1996

County	Minimum no. of PCR positive ticks/total (%)		
	Males	Females	Total
Shasta	0/35 (0)	0/31 (0)	0/66 (0)
Humboldt	0/145 (0)	0/188 (0)	0/333 (0)
Sonoma	1/154 (0.6)	0/154 (0)	1/308 (0.3)
El Dorado	4/78 (5.1)	0/66 (0)	4/144 (2.8)
Santa Cruz	1/70 (1.4)	2/60 (3.3)	3/130 (2.3)
Los Angeles	0/7 (0)	0/26 (0)	0/33 (0)
Orange	0/39 (0)	1/59 (1.7)	1/98 (1.0)
Total	6/528 (1.1)	3/584 (0.5)	9/1,112 (0.8)

In Wisconsin (23), 7 (7.9%) of 89 *I. scapularis* ticks collected from three counties were PCR positive; all 7 positive specimens, however, originated in a single county (7/68 = 10.3%). By comparison, the highest prevalence recorded for any of our California counties was 2.8% (El Dorado). Whether such differences are due to variability in sampling methods, tick species, reservoir hosts, ehrlichial biology, or limits of PCR sensitivity is unknown. In our study, it was not possible to quantify the number of organisms in individual ticks or tick pools; thus, an endpoint titration to calculate the sensitivity of the nested PCR in ticks per se could not be performed. However, this type of study has been carried out with equine blood buffy-coat samples, where the detection limit is between 3 and 7.6 visibly infected neutrophils (3). This level of sensitivity, using two rounds of PCR with a total of 80 cycles, should have been sufficient to identify most positive pools (24), as the nested PCR has been shown to be capable of detecting ehrlichiae in individual ticks (3). Alternatively, it is possible that the numbers of ticks examined and the areas sampled in the *I. scapularis* studies was not large enough to assess the true overall prevalence of detectable ehrlichiae in these ticks.

Pooling of ticks for PCR might have falsely reduced our percentage of PCR-positive ticks; i.e., in some pools, more than a single positive tick may have been included. However, even if every tick in every positive pool had been harboring ehrlichiae—a stochastically improbable result—the overall prevalence would have risen to only 4%. The phenomenon of reactivation, whereby the efficiency of detecting tick-borne pathogens can be enhanced by prefeeding ticks on an uninfected host (25, 29), if it had been employed in our study, could conceivably have increased the number of positive pools. While this variable could have affected our data for California ticks, it would have affected the data for Connecticut and Wisconsin as well, as prefeeding of ticks was not performed in those studies either (21, 23).

Prior demographic (31), tick transmission (26, 32), and PCR (3, 4, 26) data have identified *I. pacificus* as a likely vector for *E. phagocytophila* genogroup rickettsiae in California. Attempts to detect these ehrlichiae in other ixodid ticks of California have been unsuccessful. Assuming *I. pacificus* to be a major vector, the search for potential reservoirs should focus on the known hosts for this tick. *I. pacificus* adults are the most likely vectors of ehrlichiae to certain large mammalian hosts, such as horses, based on observations of tick attachment (10, 31) and the seasonal predilection of EGE for late fall, winter, and early spring, when adult ticks quest (8, 15, 16, 18). Transstadial (but not transovarial) transmission of ehrlichiae in *I. pacificus* has been demonstrated (26, 32), indicating that reservoirs are more likely to be found among host species on which two life stages of *I. pacificus* feed. In California, such hosts include lizards (*Sceloporus occidentalis* and *Gerrhonotus* spp.), black-tailed deer (*Odocoileus hemionus columbianus*), certain diurnal rodents, and perhaps ground-inhabiting birds (10, 16, 27, 35). Until sufficient data concerning the seasonality of HGE in California are available, inferences cannot be drawn with regard to the life stage(s) most likely to infect humans.

The observed differences between *I. scapularis* and *I. pacificus* mirror the situation with Lyme disease, where the frequency of *Borrelia burgdorferi* infection in *I. pacificus* is markedly lower overall, although it may be higher in nymphs than in adults (7, 8). Although our study was not designed specifically to detect ehrlichiae in nymphs, results from the small sample of nymphs tested suggest that the prevalence in this life stage could be higher than in adults.

In conclusion, we have presented data describing an apparently lower prevalence of *E. phagocytophila* genogroup rickettsiae in *I. pacificus* from California than in *I. scapularis* from the eastern and midwestern United States. These data, although preliminary, are encouraging from a public health standpoint, in that the risk of exposure to granulocytotropic ehrlichiae in California (and perhaps in other western states devoid of *I. scapularis*) may be lower as well. We acknowledge the caveat, however, that additional vectors of these ehrlichiae may exist that have not been identified.

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