ANTI-ARTHROPOD SALIVA ANTIBODIES AMONG RESIDENTS OF A COMMUNITY AT HIGH RISK FOR LYME DISEASE IN CALIFORNIA

ROBERT S. LANE, RICHARD B. MOSS, YAO-PI HSU, TAO WEI, MARY L. MESIROW, AND MAY M. KUO

Department of Environmental Science, Policy and Management, University of California, Berkeley, California; Department of Pediatrics, Stanford University School of Medicine, Stanford, California

Abstract. The role of the western black-legged tick (Ixodes pacificus) versus that of other potential arthropod vectors in the epidemiology of Lyme disease was evaluated by determining the prevalence of anti-arthropod saliva antibodies (AASA) among residents (n = 104) of a community at high-risk (CHR). Salivary gland extracts prepared from I. pacificus, the Pacific Coast tick (Dermacentor occidentalis), the western cone-nose bug (Triatoma protracta), and the western tree-hole mosquito (Aedes sierrensis) were used as antigens in an ELISA. Sera from 50 residents of the San Francisco Bay region in northern California and 51 residents of Imperial County in southern California served as comparison groups. The prevalence of AASA ranged from 2% for A. sierrensis to 79% for I. pacificus in study subjects, 0% for D. occidentalis to 36% for I. pacificus among residents of the San Francisco Bay region, and 6% for I. pacificus to 24% for A. sierrensis in residents of Imperial County. The associations between AASA and demographic factors, potential risk factors, probable Lyme disease, and seropositivity for Borrelia burgdorferi were assessed for 85 members of the CHR. Seropositivity for I. pacificus and B. burgdorferi were significantly correlated, the relative risk of seropositivity to B. burgdorferi was about 5 (31% versus 6%) for subjects who were seroreactive to I. pacificus, nearly every individual who was seropositive for B. burgdorferi had elevated levels of antibodies to I. pacificus, and the mean titer for antibodies to I. pacificus was significantly higher for subjects seropositive versus those seronegative for B. burgdorferi. Together, these findings support the widely held belief that I. pacificus is the primary vector of B. burgdorferi for humans in northern California, and they demonstrate the utility of the AASA method as an epidemiologic tool for studying emerging tick-borne infections.

Anti-tick saliva antibody has been used as a biologic marker of tick exposure that is a risk factor for seropositivity to the Lyme disease spirochete Borrelia burgdorferi.1–5 In New Jersey, antibodies to salivary gland proteins of the vector tick Ixodes scapularis have been associated with measures of self-reported tick exposure (e.g., tick bites in the past year) among outdoor workers. Although problems with the specificity of the assay for certain ticks may limit its usefulness in some settings,1 this technique holds considerable promise as an outcome measure in epidemiologic studies of the prevention of Lyme disease or other emerging tick-borne infections.4,6 and for identifying individuals or groups at high risk for such diseases.

In California, the western black-legged tick I. pacificus has been implicated as the primary vector of B. burgdorferi to humans largely on the basis of circumstantial evidence.7–10 In fact, since surveillance for Lyme disease was initiated by the California Department of Health Services in 1983, no confirmed human case (of a cumulative total of more than 2,000 cases through 1997) has been associated with a known spirochete-infected I. pacificus or any other tick species (Murray RA, unpublished data). In this state, B. burgdorferi sensu lato (s.l.) spirochetes reportedly have been detected in or isolated from I. pacificus and 5 additional ixodid ticks including 2 human-bites, Dermacentor occidentalis and D. variabilis.8,9,11–15

Although Ixodes spp. ticks are the primary vectors of B. burgdorferi, the bites of mosquitoes, deer flies, and horse flies were incriminated as potential risk factors for acquisition of Lyme disease in the northeastern United States.16–19 In northern California, bites by bloodsucking flies and seropositivity for B. burgdorferi were associated significantly in residents of a rural community at high risk (CHR).20 The investigators cautioned that this association probably represented either an indirect measure of outdoor exposure or it may have been due to chance alone. So far, only 3 species of arthropods (2 fleas and 1 mosquito) other than ticks have been examined for the presence of spirochetes in California.12,21,22 and none was found to contain spirochetes.

The present study was undertaken to determine the prevalence of anti-arthropod saliva antibodies (AASA) among members of the CHR.20 In particular, we evaluated the degree of association between seropositivity for B. burgdorferi and arthropodal saliva proteins by I. pacificus and 3 other bloodsucking arthropods, i.e., the Pacific Coast tick (D. occidentalis), the western cone-nose bug (Triatoma protracta), and the western tree hole mosquito (Aedes sierrensis), that bite people in the same community.

MATERIALS AND METHODS

Study population. The initial study population consisted of 119 current or former members of a small rural community (population approximately 150) located in the foothills near Ukiah in southern Mendocino County, California. Ninety-three of these subjects completed a detailed questionnaire at the time of entry.20 In the present study, sera from 104 of the original subjects, 85 of whom completed the entry questionnaire, were evaluated for AASA. Some of the serum specimens used in the initial20 and subsequent serosurveys had been used up and therefore were unavailable for further testing. The community inhabits a former cattle ranch that encompasses about 5,300 acres (2,145 hectares) of rolling hills interspersed with ravines and covered predominantly by woodland-grass, dense woodland, and grassland. This community was the subject of a 1-year prospective investigation of risk factors for seropositivity to and contraction of Lyme disease between 1988 and

850
The cumulative percentage of seropositivity for Lyme disease in the subject population was 24%, and the annual incidence was 1.7%. The median age of 85 subjects evaluated epidemiologically in the current investigation was 37 (range = 4–53) years, the sex ratio was 1.1:1 (44 males and 41 females), and the average length of residency was 10.7 (range = 1–16; n = 84) years. Other characteristics of the study subjects are summarized elsewhere. Sera from 50 residents of the San Francisco Bay region in Northern California who did not have antibodies against B. burgdorferi were tested for AASA as a comparison group. These specimens were selected randomly from the serum bank of a clinical virologic-testing laboratory and were unaccompanied by demographic data. Sera from 51 residents of Imperial County in southern California, a region where I. pacificus and D. occidentalis ticks apparently are uncommon, comprised a second comparison group. These samples were obtained from individuals who had sought medical attention at various healthcare agencies (Reisen WK, unpublished data). The median age of the Imperial County residents was 39 (range = 17–82) years, the sex ratio was 1:1.1 (25 males and 26 females), and the average length of residency was 28.5 (range = 0.25–65) years.

Arthropodal collections and preparation of salivary gland extracts. Four arthropods that bite residents of the CHR were selected for investigation because they feed naturally on 1 or more species of vertebrates known to be infected with B. burgdorferi s.l. Besides I. pacificus, study subjects were assayed for antibodies to the Pacific Coast tick (D. occidentalis). The latter tick has a broad host range that overlaps that of I. pacificus and includes the dusky-footed wood rat (Neotoma fuscipes) and the California kangaroo rat (Dipodomys californicus). These 2 rodents are primary reservoirs of B. burgdorferi s.l. in northern California. We also assayed study subjects for antibodies to 2 bloodsucking insects, the western cone-nose bug (T. protracta) and the western tree hole mosquito (A. sierrensis). Triatoma protracta feeds abundantly on woodrats, and A. sierrensis bites various mammals including Columbian black-tailed deer (Odocoileus hemionus columbianus), a known host of B. burgdorferi s.l. Host, as used here, does not imply reservoir competence for such spirochetes.

Adults of both tick species were either the F1 progeny from a spirochete-free laboratory colony or they were collected by dragging low vegetation in Tilden Regional Park near the Berkeley campus of the University of California. The laboratory colonies were derived from adult ticks collected at the University of California Hopland Research and Extension Center in Mendocino County. Wild-caught material was used in addition to the laboratory colonies because of the large number of ticks needed for the assays. Moreover, tests of 1,470 specimens of both ticks from Tilden Regional Park yielded just 1 spirochete-infected adult I. pacificus. Tritatoma protracta nymphs and adults were collected mainly from the stick houses of woodrats and secondarily from human domiciles in Amador or Mendocino counties. Aedes sierrensis females were the F2 progeny of a laboratory colony that originated from specimens collected in Mendocino County or they were reared from larvae obtained from tree holes in Marin County.

Paired salivary glands excised from unfed females of I. pacificus (n = 170), D. occidentalis (n = 170), T. protracta (n = 69), and A. sierrensis (n = 393) were frozen in sterile saline at −74°C. To prepare antigens, the frozen glands were thawed and sonicated in a Virsonic 300 ultrasonic cell disrupter (Virtis, Gardiner, NY) or a Braun-Sonic 1510 sonicator (B. Braun, South San Francisco, CA) at 4°C for 10 sec, followed by centrifugation at 2000 rpm for 20 min. The supernatant was aspirated and the protein concentration was determined using a commercial kit (Coomassie Plus Protein Assay Reagent Kit; Pierce, Rockford IL) or a protein assay (Bio-Rad Laboratories, Hercules, CA).

To determine if the salivary gland extracts (SGEs) prepared from field-derived arthropods contained B. burgdorferi DNA, the extracts used in the initial trial were tested individually with a polymerase chain reaction assay. An attempt was made to amplify a 365-basepair, 16S rDNA target sequence of the B-31 type strain of B. burgdorferi using biotinylated primers. The D. occidentalis extract was not assayed similarly because of insufficient material upon completion of the serologic testing. However, spirochetes were not detected in 861 D. occidentalis adults collected from the same field site. In a follow-up trial, the SGE prepared from I. pacificus was assayed for the presence of B. burgdorferi s.l. DNA by amplification of the 16S ribosomal rrfl(5S)-rrrl(23S) intergenic spacer region according to previously described methods.

Serologic analyses. To ascertain the prevalence of AASA in the subject population, an ELISA was performed in flat-bottomed, 96-well Nunc Immunoplate Polysorp (E & K Scientific Products, Campbell, CA) or Dynatech Immunolon I (Dynatech Laboratories, Chantilly, VA) microtiter plates. The wells were coated with 100 µl of SGE solutions (protein concentration = 5 µg/ml) in phosphate-buffered saline. To generate a standard curve, wells were coated with 20 µg/ml of affinity-purified goat anti-human IgG F(ab)’2 fragment (Cappel/Cooper Biomedical, West Chester, PA) and incubated for 1 hr at 37°C. Next, the wells were aspirated and unbound sites were blocked with 200 µl of 1% bovine serum albumin for 1 hr at 37°C. The plates were then washed 3 times in wash buffer (phosphate-buffered saline, 0.05% Tween 20, 0.01% merthiolate) and incubated with 100 µl of various dilutions of test serum or purified human IgG (Miles Scientific, Naperville, IL or Sigma Chemicals, St. Louis, MO) overnight at 4°C. After 3 more washes, 100 µl of goat anti-human IgG-peroxidase conjugate (Sigma Chemicals) at a dilution of 1:1,000 for all 4 assays was added to each well and incubated for 1 hr at room temperature. Following 3 more washes, 100 µl of 0.5 mg/ml of o-phenylenediamine was added to each well. The reaction was terminated after 10 min by adding 50 µl of 5 N sulfuric acid. Results were determined calorimetrically by optical density reading at 490 or 492 nm using an automated ELISA reader (BioTek Instruments, Winoos, VT or Bio-Rad Laboratories, respectively).

Immunoglobulin G concentrations (µg/ml) were determined by construction of anti-isotype curves on each plate. Standard curves were constructed with serial dilutions of known quantities of purified human IgG fragments as described above. Results from the CHR and the San Francisco Bay region comparison group test samples were interpo-
lated to the IgG-anti-IgG standard curve using at least 2 test serum dilution points exhibiting linear parallelism with the standard curve. However, results from the Imperial County test samples were correlated with the previously standardized test samples from the CHR using 3 test serum dilution points.

To establish a cut-off titer for each antibody assay, the mean IgG concentration of AASA was determined for a group of 36 normal control sera. The controls consisted of 12 adults (7 women and 5 men) who were laboratory/medical personnel at Stanford University with no known history of tick bites, and 24 infants and young children less than 3 years of age who presumably had not been exposed to all 3 arthropods. The sera for the infants and children were obtained from the Allergy Laboratory Bank of stored sera at Stanford University. All 36 sera were selected on the basis of their having low levels of total IgE (i.e., nonatopy). For *A. sierrensis*, only the sera from the 24 infants or children were used to determine the cut-off titer because of the high probability that most if not all of the adults had been exposed to mosquitoes. Cut-off values were computed separately for each AASA as the mean optical density plus 3 SD. The cut-off value (µg/ml) for each arthropod was as follows: *I. pacificus* = 2.40; *D. occidentalis* = 5.09; *T. protracta* = 0.39; and *A. sierrensis* = 1.29.

The sensitivity of the assay was established by examining the standard curve for the interaction with net absorbance plus 2 SD in control wells (i.e., no antigen, sample, and anti-human IgG conjugate; or antigen, no sample, and secondary antibody). Also, serum from 1 researcher who had been fed upon repeatedly by *A. sierrensis* was assayed as a positive control to this mosquito.

The specificity of each arthropodal assay was examined by cross-inhibition ELISA, except for *A. sierrensis* due to insufficient antigen. Briefly, known positive serum samples for each antigen were preincubated with homologous or heterologous (control) antigen prior to the ELISA. Three or 4 concentrations (0.25, 2.5, 25.0, and for *D. occidentalis* only, 60.0 µg/ml as well) of each antigen were pre-incubated separately with each sample. The specificity was considered established if the homologous antigen, but not the control antigen, inhibited ELISA activity in a dose-dependent manner.

Intra-assay reproducibility was determined by running the same sample 4–6 times with each antigen on the same day. Inter-assay reproducibility was determined by running 3 samples per antigen at least 4 times on different dates.

To determine seroreactivity to *B. burgdorferi*, subject sera were tested by conventional indirect immunofluorescence in 2 laboratories and by anticomplement indirect immunofluorescence in a third laboratory as described previously. The type strain of *B. burgdorferi* (B31) from Shelter Island, New York was used as the primary antigen in all 3 laboratories. Immunoblotting confirmed 99% of the nondiscrepant seroreactivity in a third laboratory as described previously. The ELISA results from the first *I. pacificus* assay would have been different if the SGE had been made from partially fed instead of unfed female ticks. Therefore, the ELISA was repeated twice with sera from 18 residents of the CHR using each of 2 SGEs as antigen, i.e., the salivary glands from 140 *I. pacificus* females that had partially fed for 3 days on naive New Zealand white rabbits and the salivary glands from 110 unfed female ticks. These ticks were collected from low vegetation at Tilden Regional Park. The paired salivary glands from both groups of *I. pacificus* were excised, sonicated, and their protein concentrations were determined by the method of Bradford. The 18 serum specimens chosen for re-assay were those that yielded either low (n = 6), intermediate (n = 6), or high (n = 6) antibody titers against the SGE prepared from unfed *I. pacificus* adults in the initial assay.

Prior to running each assay, separate cut-off titers were determined for each type of *I. pacificus* SGE using 20 normal human sera. Serum specimens from 10 of the 24 infants/children used in the initial assay, and 10 specimens from young adults 19–22 years of age were tested by ELISA using 3 test serum dilution points. The young adults were students at the University of California at Berkeley who reported that they were unaware of having been bitten before by ticks. For each of the 2 SGE antigens, the cut-off titer was computed as the mean optical density plus 3 SD. These values correspond to 2.72 and 2.90 µg/ml for the SGEs prepared from unfed and partially fed ticks, respectively.

Informed consent was obtained from all human adult participants and from parents or legal guardians of minors, with an appropriate institutional review board having approved the project. The animal use procedures described in this research were approved by the Institutional Animal Care and Use Committee in compliance with the NIH “Guide for the Care and Use of Laboratory Animals.” The University of California, Berkeley, is American Association for Accreditation of Laboratory Animal Care accredited and has an Office for Protection from Research Risks Assurance Number: A3084–01.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).** To compare the composition and abundance of the salivary gland proteins from unfed versus partially fed *I. pacificus* females, the protein profiles of SGEs prepared from both groups of ticks were determined by SDS-PAGE. Aliquots of 11.7 µg of unfed and 8.2 µg of partially fed salivary gland proteins were run separately according to previously published protocols. Except that a 12% acrylamide separating gel and a 4% stacking gel were used. Proteins were electrophoresed for 2 hr at 40 mA on a vertical mini-gel system (C. B. S. Scientific, Del Mar, CA). Low molecular weight, high molecular weight, and prestained low molecular weight protein standards (Bio-Rad Laboratories) were run with each gel. Staining was accomplished overnight in 0.2% Coomassie brilliant blue R-250, 50% methanol, and 7% acetic acid; destaining was performed with 10% acetic acid and 30% methanol (v/v).

**Study variables.** In the previous 1-year prospective study of risk factors for Lyme disease, questionnaires were completed by 93 of 119 subjects at entry and 80 subjects 1 year later to assess the association of serologic status for antibodies to *B. burgdorferi* with 20 categorical and 47 continuous variables. In the present study, we evaluated the prevalence of AASA in 104 of the then current or former residents. Furthermore, we assessed the association between
AASA and self-reported arthropodal exposure histories, demographic factors (age, sex, length of residency), potential risk factors, probable Lyme disease, and seropositivity for *B. burgdorferi* among 85 of the 93 subjects who completed the entry questionnaire. Potential risk factors included hiking, woodcutting, pet ownership (dogs, cats), type of clothing worn in different seasons, and self-reported arthropodal exposure histories.

**Statistical analyses.** The coefficient of variation was used to measure the intra-assay and inter-assay reproducibility of the ELISA for each of the salivary antigens.\(^5\) The mean titers of antibodies to *I. pacificus* of seropositive subjects (n = 22) versus those seronegative (n = 63) for *B. burgdorferi* were compared with the 2-sample t-test (2-tailed); the antibody titer data were transformed first (natural logarithm) because their distributions were skewed.

The chi-square test, or Fisher’s exact test (2-tailed) when necessary by small expected frequencies, were used to compare pairs of dichotomous variables such as seropositivity for *B. burgdorferi* infection and evidence of exposure to specific arthropods. The Mann-Whitney test was used to compare 2 groups for a continuous outcome, and Pearson’s correlation coefficient (r) was used to describe and test associations between 2 continuous variables. No adjustments were made for multiple comparisons.

**RESULTS**

When assayed with SGES prepared from unfed arthropods, 79% of the residents of the CHR were seropositive for AASA against *I. pacificus* compared with 36% and 6% of residents of the San Francisco Bay region and Imperial County, respectively (Table 1). Residents of the CHR had a seroprevalence of 20% against *T. protracta*, which was twice that of residents from the San Francisco Bay region. In contrast, the prevalence of AASA to *D. occidentalis* and *A. sierrensis* was low for all 3 groups except for residents of Imperial County who had a seroprevalence of 24% to *A. sierrensis*.

The physiologic state of the *I. pacificus* salivary glands used to prepare the SGES did not affect the ELISA results. In a twice-repeated, follow-up trial, the mean human IgG antibody responses of the 3 groups of 6 subjects each from the CHR (i.e., who were either low, intermediate, or high antibody responders to *I. pacificus* in the trial in which the test antigen was prepared from unfed ticks) did not differ significantly when the SGE test antigens were prepared either from unfed or partially fed adult ticks (*P* = 0.500, 0.429, and 0.394 for the 3 pair-wise comparisons, respectively, by the Mann-Whitney test). *Borrelia burgdorferi* s.l. DNA was not detected in SGES prepared from field-derived *I. pacificus* with the polymerase chain reaction during either the initial or follow-up trial.

On the other hand, the protein profiles of the SGES prepared from unfed versus partially fed ticks exhibited some conspicuous differences when compared by SDS-PAGE (Table 2). The SGE from unfed ticks possessed 12 predominantly heavy protein bands that had approximate relative mobilities ranging from 28.9 to 94.1 kD. Only 4 proteins having relative mobilities of approximately 33.0, 71.0, 79.7, and 94.1 kD were shared by both SGES.

Levels (titers) of antibodies to *D. occidentalis* were positively correlated with those of antibodies to *B. burgdorferi* and to *I. pacificus* (Table 3). The correlation between levels of antibodies to *I. pacificus* and *B. burgdorferi* (*P* = 0.098) approached statistical significance, whereas AASA to *T. protracta* were not correlated with any of the other variables (Table 3). No attempt was made to statistically evaluate the

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th><em>Ixodes pacificus</em></th>
<th><em>Dermacentor occidentalis</em></th>
<th><em>Triatoma protracta</em></th>
<th><em>Aedes sierrensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rural community near Ukiah (n = 104)</td>
<td>82 (78.8)</td>
<td>4 (3.9)</td>
<td>21 (20.2)</td>
<td>2 (1.9)</td>
</tr>
<tr>
<td>San Francisco Bay area residents (n = 50)</td>
<td>18 (36.0)</td>
<td>0</td>
<td>5 (10.0)</td>
<td>1 (2.0)</td>
</tr>
<tr>
<td>Imperial County residents (n = 51)</td>
<td>3 (5.9)</td>
<td>4 (7.8)</td>
<td>NT*</td>
<td>12 (23.5)</td>
</tr>
</tbody>
</table>

* NT = not tested.

### Table 2

<table>
<thead>
<tr>
<th>Unfed ticks</th>
<th>Partially fed ticks</th>
</tr>
</thead>
<tbody>
<tr>
<td>M, Abundance</td>
<td>M, Abundance</td>
</tr>
<tr>
<td>26.0 H</td>
<td>28.9 M</td>
</tr>
<tr>
<td>26.6 H</td>
<td>30.7 L</td>
</tr>
<tr>
<td>33.0 H</td>
<td>33.0 L</td>
</tr>
<tr>
<td>34.3 H</td>
<td>42.2 L</td>
</tr>
<tr>
<td>35.3 M</td>
<td>50.4 L</td>
</tr>
<tr>
<td>36.3 M</td>
<td>54.6 M</td>
</tr>
<tr>
<td>39.0 M</td>
<td>57.0 H</td>
</tr>
<tr>
<td>41.5 H</td>
<td>71.0 L</td>
</tr>
<tr>
<td>44.3 H</td>
<td>79.7 L</td>
</tr>
<tr>
<td>71.0 H</td>
<td>94.1 L</td>
</tr>
<tr>
<td>79.7 H</td>
<td></td>
</tr>
<tr>
<td>94.1 L</td>
<td></td>
</tr>
</tbody>
</table>

* Determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. H = heavy; M = moderate; L = light.

### Table 3

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th><em>B. burgdorferi</em></th>
<th><em>Ixodes pacificus</em></th>
<th><em>Dermacentor occidentalis</em></th>
<th><em>Triatoma protracta</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. burgdorferi</em></td>
<td>1.000</td>
<td>0.142</td>
<td>0.463</td>
<td>0.125</td>
</tr>
<tr>
<td><em>I. pacificus</em></td>
<td>–</td>
<td>1.000</td>
<td>(P = 0.098)</td>
<td>(P = 0.001)</td>
</tr>
<tr>
<td><em>D. occidentalis</em></td>
<td>–</td>
<td>–</td>
<td>(P = 0.002)</td>
<td>(P = 0.422)</td>
</tr>
<tr>
<td><em>T. protracta</em></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
correlation or association between AASA to *A. sierrensis* and the other variables (Tables 3 and 4) because only 1.9% of the study subjects had antibodies to this mosquito (Table 1).

In the univariate analyses used to examine potential risk factors, seroreactivity to *I. pacificus* and *B. burgdorferi* were significantly associated (Table 4). Five times as many subjects (30.9%) who were seroreactive to *I. pacificus*, versus those who were seronegative to this tick (5.9%), also were seroreactive to *B. burgdorferi* (Table 5). The mean antibody titer for *I. pacificus* was significantly higher for subjects seropositive (12.7 μg/ml) versus those seronegative (9.2 μg/ml) for *B. burgdorferi* (t = 2.29, P = 0.025).

Seroreactivity for antibodies to *D. occidentalis* was positively associated with seropositivity for *B. burgdorferi* and with hiking in the univariate analyses (Table 4). Nearly 5 times as many subjects (100%) who were seroreactive to *D. occidentalis*, compared with those who were seronegative for this tick (22.2%), had significant antibody titers to *B. burgdorferi* (Table 5). However, these positive associations were based on just 4 seroreactive individuals, and all 4 individuals who had antibodies to *D. occidentalis* were seroreactive to both *B. burgdorferi* and *I. pacificus* (Table 5). Seroreactivity to *D. occidentalis* was not significantly associated with that for *I. pacificus* when tested using binary variables (i.e., seropositive versus seronegative) (Table 5), since it was using 2 continuous variables (i.e., titers) (Table 3).

The sensitivities of the 4 antigen-specific ELISAs ranged from 1 to 2 ng/ml. In the cross-inhibition experiments, optimal inhibition occurred at 2.5 μg/ml of SGE for the 3 arthropods tested except for *D. occidentalis*, which required a much higher concentration of antigen (60 μg/ml). Binding of antibodies to *I. pacificus* to solid-phase–adsorbed *I. pacificus* antigen was inhibited 94% by pre-incubation with soluble homologous *I. pacificus* antigen, but was unaffected by pre-incubation with *T. protracta* or *D. occidentalis* antigens. Likewise, binding of antibodies to *T. protracta* to solid-phase—adsorbed *T. protracta* antigen was inhibited 100% by autologous antigen but was unaffected by pre-incubation with *I. pacificus* or *D. occidentalis* antigens. The binding of antibody to *D. occidentalis* to solid-phase *D. occidentalis* antigen was inhibited only 54% with a similar concentration of soluble antigen, but no inhibition was found with *I. pacificus* or *T. protracta*.

The serum specimen obtained from the mosquito researcher who had been bitten multiple times by *A. sierrensis* females had an elevated antibody level of 1.58 μg/ml against this mosquito.

Intra-assay reproducibility (i.e., as measured by the coefficient of variation) was 9.2% for *I. pacificus*, 10.7% for *D. occidentalis*, 10.9% for *A. sierrensis*, and 27.4% for *T. protracta*. Overall, the inter-assay reproducibility was 20.8%, which can be broken down by antigen as follows: 17.0% for *I. pacificus*, 15.3% for *D. occidentalis*, 26.7% for *T. protracta*, and 24.1% for *A. sierrensis*.

**DISCUSSION**

**Anti-tick saliva antibodies.** In California, the western blacklegged tick *I. pacificus* is the only species of human-biting tick that has been found routinely to contain *B. burgdorferi* in areas endemic for Lyme disease. However, direct evidence linking *I. pacificus* causally to confirmed cases of Lyme disease is meager and consists of just 2 patients who either saved the tick that bit them or implicated *I. pacificus* when shown representative specimens. In the present study, human exposure to the attachments of *I. pacificus* in northern California was found to be higher than anticipated. Residents of the CHR, who inhabit what might be described best as a nature preserve, are potentially exposed to the feeding activities of *I. pacificus* nymphs or adults for approximately 9 months of the year. In contrast, residents of the San Francisco Bay region most likely encounter *I. pacificus* or other human-biting ticks in semi-rural communities or while recreating or working in parklands or other recreational areas. In Imperial County, where *I. pacificus* apparently is uncommon, few of the residents tested had antibodies to *I. pacificus* as would be expected. Thus, the respective 79%, 36%, and 6% prevalences of seroreactivity to *I. pacificus* among residents of the CHR, the San Francisco Bay region, and Imperial County (Table 1), respectively, seem reasonable based on current knowledge of the geographic distribution, relative abundance, and expected degree of human exposure to this tick.

The high frequency of exposure to *I. pacificus* coupled with the high prevalence of *B. burgdorferi* s.l. infection in nymphal ticks at the CHR (Talleklint-Eisen L, Lane RS, unpublished data) presumably accounts for the observed association between antibodies to *I. pacificus* and Lyme disease seropositivity. Likewise, seropositivity to *B. burgdorferi* and antibodies in saliva to ticks (i.e., *I. scapularis*) were correlated significantly among outdoor workers in New Jersey, which was expected because exposure to and the
prevalence of *B. burgdorferi* infection in *I. scapularis* ticks are both high in the northeastern United States. At the CHR, the prevalence of spirochetal infection in *I. pacificus* nymphs in diverse leaf litter habitats on 12 separate properties averaged 12.4% (range = 3.9–41.3%) in spring/early summer of 1998 (Tallekli-Eisen L, Lane RS, unpublished data). The 10-fold variation in spirochetal prevalence detected in *I. pacificus* nymphs in that study demonstrates that the risk of being bitten by an infected nymph is unlikely to be uniform among properties. Other factors that affect the risk of encountering a spirochete-infected tick at a particular locality include the density of ticks, the total amount and spatial distribution of suitable tick-habitat, the species composition and abundance of wild vertebrates including reservoir hosts, and specific behaviors that put humans in direct contact with tick-infested habitats. In northwestern California, for instance, behaviors identified as risk factors include cutting wood and the use of wide maintained trails for more than 5 hr per week. Furthermore, the overall prevalence of infection in nymphal ticks at the CHR is comparable with that reported earlier for *I. pacificus* nymphs (13.6%) at another site in the same county, and it is similar to representative data for *I. scapularis* nymphs from areas endemic for Lyme disease in the northeastern United States.

Interestingly, the association between antibodies to *I. pacificus* and *B. burgdorferi* was statistically significant (Table 4), but the correlation between these same variables only approached statistical significance (Table 3). Although seemingly contradictory, these results actually are in general accord given the fundamental differences in the 2 statistical methods. In fact, one could not expect the *P* values to be any closer than those represented by our data because both were in the neighborhood of 0.05 (Tables 3 and 4). The Pearson correlation coefficient is based on raw antibody titers and therefore may be subject to perturbations from extreme values, whereas the chi-square test is based on dichotomized values of antibody titers.

The earlier observation that cutting wood is a risk factor for Lyme disease at the CHR may reflect the amount of time spent outdoors in *I. pacificus* nymphal habitats. The nymphal stage, which has been implicated as the predominant stage transmitting *B. burgdorferi* to humans in northwestern California, seeks its hosts from late winter through summer with a peak in May/June in hardwood forests carpeted with leaf litter. An active surveillance program revealed that in 1992 incident Lyme disease (physician-diagnosed erythema migrans) occurred mostly in spring and summer coincident with the nymphal activity period of *I. pacificus*. Thus, any activity that results in direct contact with the woodland biotope of *I. pacificus* nymphs may place humans at heightened risk of contracting *B. burgdorferi* infection. Although *I. pacificus* females occasionally transmit Lyme disease spirochetes to humans in California, the low prevalence of infection detected in female ticks (1.3%, *n* = 684) at the CHR indicates that this life stage may be of considerably less importance as a vector of *B. burgdorferi* there than the nymphal stage.

Three of our other laboratory findings lend additional support to the growing body of circumstantial evidence that *I. pacificus* is the primary vector of *B. burgdorferi* to humans in California. First, the relative risk of seropositivity to *B. burgdorferi* is 5 times greater (30.9% versus 5.9%) for subjects who were seroreactive to *I. pacificus* (Table 5). Second, 21 (95.5%) of 22 study subjects from the CHR who were seropositive for *B. burgdorferi* tested positive for antibodies to *I. pacificus*. Third, subjects seroreactive to *B. burgdorferi* antigen had a significantly higher mean antibody titer to *I. pacificus* than did subjects who were seronegative for this spirochete.

It is our considered opinion that the positive associations of antibodies to *D. occidentalis* with Lyme disease seropositivity and hiking are spurious and of no epidemiologic significance for several reasons. The assay for antibodies to *D. occidentalis* itself was of low specificity compared with the other arthropodal assays. Moreover, these findings are based on only 4 of 85 subjects who possessed antibodies to *D. occidentalis*, and who also happened to have antibodies to *B. burgdorferi*. A much more plausible explanation for these confounding associations is that people having antibodies to *D. occidentalis* have greater tick exposure in general, and therefore are more likely to have been bitten by a spirochete-infected *I. pacificus* tick. This is underscored by the fact that titers of antibodies to *D. occidentalis* were significantly correlated with titers of antibodies to *I. pacificus* (Table 3). Furthermore, all 4 subjects who were seroreactive to *D. occidentalis* likewise contained elevated levels of antibodies to *I. pacificus*. The lack of association of antibodies to *D. occidentalis* and *I. pacificus* in the univariate analysis is doubtless attributable to the 20-fold difference in seroprevalence to these 2 tick species (Table 1). Finally, the results of extensive tick/spirochete surveys carried out in many localities in California in combination with vector competence studies demonstrate conclusively that *D. occidentalis* is an incompetent experimental vector of *B. burgdorferi* s.s.

**Anti-insect saliva antibodies.** The western cone-nose bug (*T. protracta*) is an intermittent, bloodsucking parasite of woodrats (*Neotoma* spp). These bugs normally reside in the lodges of woodrats except in late spring and summer when they engage in dispersal flights. Dispersing bugs sometimes invade homes following their attraction to artificial light. Persons typically are attacked by *T. protracta* while sleeping indoors, and those bitten repetitively may develop severe and sometimes life-threatening allergic reactions.

At the CHR, 9 (10.6%) of the 85 study subjects reported exposure to this bug (Lane RS, unpublished data), and therefore the 20% prevalence of AASA to *T. protracta* appears reasonable because many people bitten nocturnally would be unaware of their exposure unless they subsequently developed an allergic reaction. Similarly, 6.7% of residents inhabiting a community in a coastal chaparral-oak woodland area in southern California were found to be at risk for developing serious immediate hypersensitivity reactions to the bites of *T. protracta*. The anthropophilic feeding habits of *T. protracta* and its close association with the dusky-footed woodrat, a proven reservoir host of *B. burgdorferi* prompted us to evaluate the possible association of antibodies to *T. protracta* with Lyme disease. The fact that elevated levels of antibodies to this bug were not correlated or associated with either Lyme disease seropositivity or probable
Lyme disease suggests that it does not transmit *B. burgdorferi* to humans.

The 10% prevalence of antibodies to *T. protracta* among residents of the San Francisco Bay region seems somewhat high. Although the woodrat host of *T. protracta* is present in parklands throughout this region, this rodent is absent in metropolitan areas where much of the human population resides. The lower intra-assay and inter-assay reproducibilities of the *T. protracta*-specific ELISAs (i.e., as shown by their higher coefficients of variation) versus the other 3 antigen-specific ELISAs suggest that the higher than expected seroprevalence rate may be artifactual in part. In the absence of histories for each of the 5 seroreactive individuals, however, we are unable to conclude whether the specificity of the assay is good or if their sera may have contained nonspecific, cross-reacting antibodies to *T. protracta* SGE.

In California, the western tree hole mosquito (*A. sierrensis*) can be a nuisance to humans who live in urban or suburban areas having old ornamental trees or who visit mature orchards or forested recreational lands. The lack of association between antibodies to *A. sierrensis* and seropositivity for *B. burgdorferi* was not surprising because an earlier attempt to detect siphocytes in several hundred field-derived females of this mosquito was fruitless. On the other hand, the low seroreactivity to *A. sierrensis* among residents of the CHR was unanticipated because this mosquito can be quite abundant in certain wooded areas there. Two explanations may be offered for this apparent paradox. First, this mosquito may not bite people as often as assumed because of its focal distribution in woodlands and its restricted flight range. Second, *A. sierrensis* females may induce short-lived antibodies if small quantities of salivary gland proteins are injected while it feeds. In contradistinction to ixodid ticks such as *I. pacificus* and *D. occidentalis*, which require up to a week or longer to feed fully, *A. sierrensis* normally completes a blood meal within 1–2 min. Moreover, the mosquito researcher exposed repeatedly to the bites of *A. sierrensis* had a minimally elevated antibody titer of 1.58 µg/ml even though he had been fed upon by approximately 300 *A. sierrensis* within 1 month before his blood sample was taken. In contrast, 11% of the 82 study subjects from the CHR who were seroreactive against *I. pacificus* antigen had serum titers an order of magnitude or more above the established cut-off titer.

Curiously, 24% of the residents from Imperial County were seroreactive to *A. sierrensis* antigen even though this mosquito has not been recorded there. We speculate that these seropositive residents may have had cross-reactive anti-salivary antibodies as a result of exposure to other *Aedes* spp. Several species of human-biting *Aedes* occur in Imperial County, including *A. dorsalis, A. taeniorynchus*, and *A. vexans*. Of these, *A. dorsalis* breeds in intertidal marshes and along the margins of lakes and bays in inland areas; *A. taeniorynchus* inhabits salt marshes, although it also breeds adjacent to salt marshes in brackish or fresh water habitats like ditches or borrow pits; and the floodwater species *A. vexans* can be abundant along wooded water courses and in agricultural areas. We conclude that further studies are needed to determine the extent of cross-reactive antibodies present in persons who are exposed to salivary antigens from *A. sierrensis* and related species of *Aedes*.

**Sensitivities and specificities of the antigen-specific ELISAs.** Our experimental design did not enable us to determine the applied sensitivities or the specificities of the 4 antigen-specific ELISAs for members of the CHR. That is, study subjects were asked at entry to report their general exposures to undetermined species of ticks, mosquitoes, and bugs during their last 2 years of residency at the CHR. Except for the researcher who had been bitten repeatedly by *A. sierrensis* and who had an elevated antibody titer against the SGE prepared from this mosquito, similar data concerning recent exposure to the feeding activities of the 2 tick species and to *T. protracta* were unavailable. Furthermore, people often do not recognize tick bites very well, particularly nymphal bites, and therefore self-reported measures of tick exposure may be misleading. Nonetheless, the variable seroprevalences to the different arthropodal antigens in all 3 subject populations (Table 1) generally agree with what is currently known about the geographic distribution and local abundance of these arthropods. For instance, the 13-fold difference in seroprevalence between residents of the CHR compared with those of Imperial County mirror the anticipated degree of human contact with *I. pacificus* in these regions, and it strongly suggests that our assay is a reasonably sensitive indicator of exposure to this tick.

The specificities of our laboratory assays, as measured by cross-inhibition ELISA, were high for *I. pacificus* (94%) and *T. protracta* (100%), but considerably lower for *D. occidentalis* (54%). The latter finding may have been due to antibody excess, nonspecific binding, or cross-reactivity with salivary gland antigens of other arthropods. Cross-reactive antibodies, if indeed they were involved, might help to explain the observed positive correlation between seroreactivities to *D. occidentalis* and *I. pacificus* (Table 3), as well as the positive association between seroreactivity to *D. occidentalis* and Lyme disease seropositivity (Table 4).

Schwartz and others, who pioneered research into the use of anti-tick saliva antibodies as a biologic marker of tick exposure, expressed similar concerns with the specificity of their AASA assay involving the ticks *Ixodes dammini* (now *I. scapularis*) and *D. variabilis* plus the mosquito *A. aegypti*. In their initial assay, all 3 antibodies to arthropods were intercorrelated and each of them was correlated with antibody titers to *B. burgdorferi* as well. In a subsequent study, however, Schwartz and others reported that their assay had a specificity of 0.56 and a sensitivity of 0.81 for a bite by *I. dammini* that had fed completely on subjects. They determined specificity by measuring anti-tick saliva antibody levels in subjects having a recent and confirmed *I. dammini* or *D. variabilis* tick bite. Similar efforts to evaluate the sensitivities and specificities of AASA assays among subjects known to have been fed fully upon *I. pacificus*, *D. occidentalis*, and the insects we studied are needed to determine their validity and thus utility as epidemiologic tools.

To develop a potentially more sensitive and specific AASA, it might be profitable to use as antigen one or more immunodominant recombinant proteins present in the sali-
vary glands of vector arthropods.\textsuperscript{65–57} For example, the antibody responses of rabbits against the saliva of Amblyomma americanum and D. variabilis ticks were found to possess unique and shared components.\textsuperscript{55} Antibodies directed against low molecular mass (< 20 kD) salivary gland antigens present in Amblyomma americanum may be specific for this tick, whereas antibodies against high molecular mass (85, 86.3, and 111 kD) salivary gland antigens in D. variabilis may be specific for the latter. Moreover, the antibody responses of humans against recombinant tick calreticulin (rTC), a cDNA-derived protein isolated from the salivary glands of Amblyomma americanum, can be used as a biologic marker of previous tick exposure.\textsuperscript{56,57} Because tick calreticulin cannot be detected in the saliva of feeding ticks until the third day of attachment, it may be particularly useful as a marker for tick bites of longer duration. Also, a cDNA-derived protein is more convenient to use than whole salivary glands as antigen in an assay, and it may confer improved specificity because the antibody measured is directed against an individual protein.\textsuperscript{56} The test for antibody to rTC was found to have a sensitivity of 0.50 and a specificity of 0.86 for subjects who had been fed upon fully by an I. scapularis tick, and tick engorgement indices were positively correlated with antibody levels to rTC.\textsuperscript{57}

Although the protein profiles of the SGEs prepared from unfed versus partially fed I. pacificus adults in the present study exhibited some notable differences in composition (i.e., presence or absence of certain bands, relative abundance of different proteins) when compared by SDS-PAGE, the physiologic state of the I. pacificus salivary glands used to prepare the SGEs did not affect the ELISA results. This suggests that antigenic proteins present in the salivary glands of I. pacificus are unaffected during feeding. New kinds of salivary gland proteins also are synthesized after the attachment of the hard ticks Amblyomma americanum\textsuperscript{58,59} and I. dammini (now I. scapularis).\textsuperscript{60} whereas the composition of the SGEs of either unfed or variously fed ticks do not undergo significant changes in the rapidly feeding soft ticks Ornithodoros erraticus and O. moubata.\textsuperscript{61} We repeated in part our initial AASA assay for I. pacificus, which used an SGE prepared from unfed adult ticks, with an SGE derived from partially fed adult ticks because other researchers used partially fed ticks in their assays.\textsuperscript{1,5} Accordingly, we were concerned about possible discrepancies in ELISA results when 2 different types of SGEs are used as antigen. Since the test results for antibodies to I. pacificus for members of the CHR were similar irrespective of the type of SGE, we recommend that unfed ticks be used to prepare SGEs in any subsequent AASA studies using whole salivary glands. A considerable savings in time, cost, and research animals’ lives result when it is unnecessary to prepfeed ticks on vertebrate hosts.

In conclusion, the AASA method is a useful tool for evaluating the risk of human exposure to Lyme disease and other emerging tick-borne diseases. Collectively, serologic data presented here and previously published biologic evidence support the widely held belief that I. pacificus is the primary vector of B. burgdorferi to humans in the far western United States. In contrast, 3 other arthropods that occasionally bite humans in this region, i.e., the Pacific Coast tick (D. occidentalis), the western cone-nose bug (T. protracta), and the western tree-hole mosquito (A. sierrensis) apparently are not involved in the epidemiology of Lyme disease.

Acknowledgments: We thank K. Graham, D. E. Kain, J. E. Kleinjan, X. Li, K. A. Padgett, and J. A. Pascolello for excellent technical assistance. J. R. Clover, E. Hartmann, and P. Mervine for providing insects for dissection, R. E. Chiles, P. S. Dietrich, E. T. Lennette, W. K. Reisen, and J. O. Washburn for some of the human sera used in this study, and W. C. Reeves and L. Tallekli-Eisen for constructive comments on the manuscript. We also thank all of the study subjects at the CHR for participation and enthusiastic support of our research.

Financial support: This research was supported in part by a grant from the National Institutes of Health (AI-22501) and by a Cooperative Agreement (U50/CCU906594) from the Centers for Disease Control and Prevention to Robert S. Lane.

Authors’ addresses: Robert S. Lane, Mary L. Mesrow and May M. Kuo, Department of Environmental Science, Policy and Management, University of California, Berkeley, CA 94720. Richard B. Moss, Yao-Pi Hsu and Tao Wei, Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305-5786.

Reprint requests: Robert S. Lane, Division of Insect Biology, 201 Wellman Hall, University of California, Berkeley, CA 94720.

REFERENCES

14. Brown RN, Lane RS, 1996. Reservoir competence of four chap-


**Statement of Ownership, Management, and Circulation**

(Required by 39 USC 3689)

<table>
<thead>
<tr>
<th>Publication Title</th>
<th>2. Publication Number</th>
<th>3. Filing Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>The American Journal of Tropical Medicine &amp; Hygiene</td>
<td>0 0 0 0 0 0 0 0</td>
<td>10/11/99</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4. Issue Frequency</th>
<th>5. Number of Issues Published Annually</th>
<th>6. Annual Subscription Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monthly</td>
<td>12</td>
<td>$ 330.00</td>
</tr>
</tbody>
</table>

7. Complete Mailing Address of Known Office of Publication (Net Price) (Street, City, State, and Zip Code):
The American Society of Tropical Medicine and Hygiene, 60 Revere Drive, Suite 500, Northbrook, IL 60062

<table>
<thead>
<tr>
<th>8. Complete Mailing Address of Headquarters or General Business Office of Publisher (Net Price)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The American Society of Tropical Medicine and Hygiene, 60 Revere Drive, Suite 500, Northbrook, IL 60062</td>
</tr>
</tbody>
</table>

9. Full names and Complete mailing Addresses of Publisher, Editor, and Managing Editor (or leave blank):

**Publisher (Name and complete mailing address):**
The American Society of Tropical Medicine and Hygiene, 60 Revere Drive, Suite 500, Northbrook, IL 60062

**Editor (Name and complete mailing address):**
James W. Katzir, MD, Case Western Reserve University School of Medicine, Division of Geographic Medicine, 10990 Eastlind Avenue, Cleveland, OH 44106-4983

**Managing Editor (Name and complete mailing address):**
James W. Katzir, The American Journal of Tropical Medicine & Hygiene, 3000 Blericliffe Road, Suite A-11, Atlanta, GA 30329

10. Owner (do not leave blank): If the publication is owned by a corporation, give the name and address of all shareholders owning or holding 1 percent or more of the total amount of stock. If not owned by a corporation, give the names and addresses of the individual owners. If owned by a partnership or other unincorporated group, give its name and address as well as those of each individual owner. If the publication is published by a nonprofit organization, give its name and address:

**Full Name**
American Society of Tropical Medicine & Hygiene

**Complete Mailing Address**
60 Revere Drive, Suite 500, Northbrook, IL 60062

11. Known Bondholders, Mortgagees, and Other Security Holders Owning or Holding 1 Percent or More of Total Amount of Bonds, Mortgages, or Other Securities, Promissory Notes, check box:

**Full Name**

**Complete Mailing Address**

12. Tax Issues (Check box to indicate if nonprofit organization authorized to mail at special rates) [Check one]

- [ ] No Change during Previous 12 Months
- [ ] Increased During Previous 12 Months (Publisher must submit explanation of change with this statement)

13. Publication Title
The American Journal of Tropical Medicine Hygiene

14. Issue Date for Circulation Data (Month and Year)
September 60

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of Copies (less than 20,000)</td>
<td>4555</td>
<td>3750</td>
</tr>
<tr>
<td>Paid/Requested outside: Country Mail Subscriptions (Indicate Form 3541, include advertisement proof and exchange copies)</td>
<td>3568</td>
<td>3463</td>
</tr>
<tr>
<td>Paid/Requested outside: Prepaid Mail Subscriptions (Indicate Form 3541, include advertisement proof and exchange copies)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Paid/Requested outside: Prepaid Mail Subscriptions (Indicate Form 3541, include advertisement proof and exchange copies)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Paid/Requested outside: Prepaid Mail Subscriptions (Indicate Form 3541, include advertisement proof and exchange copies)</td>
<td>1068</td>
<td>1042</td>
</tr>
<tr>
<td>Paid/Requested outside: Prepaid Mail Subscriptions (Indicate Form 3541, include advertisement proof and exchange copies)</td>
<td>3620</td>
<td>3528</td>
</tr>
<tr>
<td>Free Distribution included in paid subscription</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Free Distribution included in paid subscription</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Free Distribution included in paid subscription</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Free Distribution included in paid subscription</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>Free Distribution included in paid subscription</td>
<td>3040</td>
<td>3544</td>
</tr>
<tr>
<td>Free Distribution included in paid subscription</td>
<td>367</td>
<td>266</td>
</tr>
<tr>
<td>Free Distribution included in paid subscription</td>
<td>4070</td>
<td>3790</td>
</tr>
<tr>
<td>Free Distribution included in paid subscription</td>
<td>98.6</td>
<td>98.8</td>
</tr>
</tbody>
</table>

16. Publication of Statement of Ownership
Publication required. filed or printed in this issue of this publication.

**Signature and Title of Editor, Publisher, Business Manager, or Owner**

**Date**
10/11/99
ERRATUM

In the Am J Trop Med Hyg 61: 399, 1999, the title of the article “Increase of Leptospirosis in Dengue-Negative Patients after a Hurricane in Puerto Rico in 1966” by Sanders and others should have read “Increase of Leptospirosis in Dengue-Negative Patients after a Hurricane in Puerto Rico in 1996.” The journal staff regrets this error.