Transmission of the Agent of Human Granulocytic Ehrlichiosis by *Ixodes spinipalpis* Ticks: Evidence of an Enzootic Cycle of Dual Infection with *Borrelia burgdorferi* in Northern Colorado


Previous work described an enzootic cycle of *Borrelia burgdorferi sensu lato* (hereafter referred to as *B. burgdorferi*) maintained by the rodent *Neotoma mexicana* and the tick *Ixodes spinipalpis* in northern Colorado. We investigated the incidence of coinfection among rodents with the agent of human granulocytic ehrlichiosis (aoHGE). aoHGE was detected in 23.5% of 119 rodent spleens examined. Biopsy results indicated that 78 (65.5%) of the 119 rodents were positive for *B. burgdorferi*, whereas 22 (78.5%) of the 28 animals that harbored aoHGE were also infected with *B. burgdorferi*. In 14 of 25 *I. spinipalpis* tick pools, aoHGE was detected by amplifying both the 16s rRNA and p44 gene of aoHGE. The ability of *I. spinipalpis* to transmit aoHGE was examined in C3H/HeJ mice. aoHGE was detected in their blood 5 days after *I. spinipalpis* infestation. This study confirms that both *B. burgdorferi* and aoHGE can be transmitted by *I. spinipalpis* ticks and that there is a high incidence of coinfection in rodents, predominantly *Peromyscus maniculatus* and *N. mexicana*, that inhabit the foothills of northern Colorado.

Since its first description in 1994 [1], human granulocytic ehrlichiosis (HGE) has been documented as an emerging tick-borne illness in the United States. There have been >400 cases, with the highest incidence in the upper northeastern and upper midwestern portions of the United States [2]. This disease incidence corresponds to the geographic distribution of the purported vector, *Ixodes scapularis* [3], whereas a small foci of human infection associated with *I. pacificus* has been reported in northern California [4].

Serosurveys indicate that *Peromyscus leucopus* is the main rodent reservoir for these agents in endemic areas [5–7]; however, in areas of the western United States other rodent species, predominantly *Neotoma fuscipes* and *N. mexicana*, are seropositive for the agent of HGE (aoHGE) and for *Borrelia burgdorferi sensu lato* (hereafter referred to as *B. burgdorferi*) [6]. Likewise, *I. spinipalpis* is a competent vector of Lyme disease in enzootic cycles that involve *Peromyscus* species and *N. fuscipes* in California [8] and *N. mexicana* in Colorado [9]. The incidence of coinfection with aoHGE has not been determined in these western habitats.

The recent discovery of an enzootic cycle of *B. burgdorferi* maintained by *N. mexicana* and *I. spinipalpis* and the speculation that an enzootic cycle of transmission of aoHGE exists in California [10] led us to investigate the possibility that a similar enzootic cycle exists that involves the transmission of aoHGE in Colorado.

Materials and Methods

**Collection of vertebrates and ticks.** Rodents were captured on the western edge of the Foothills Campus of Colorado State University, Fort Collins, Colorado, from February to October 1999. Captured animals were examined to identify individual species and then held over water so that replete ticks could be collected. En-gorged ticks were identified by use of standard taxonomic keys, were held at 22°C, and were allowed to molt to the next stage. Skin biopsy specimens were obtained from the rodents for *B. burgdorferi* analysis, and spleens were harvested for determination of aoHGE infection.

**Transmission of aoHGE by nymphal *I. spinipalpis*.** Eight *I. spinipalpis* nymphs, which were originally collected as larvae from *N. mexicana* in the spring of 1999, were placed on individual C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME). Nymphal ticks were allowed to feed to repletion, and blood was collected from mice into heparinized tubes 5 and 10 days after tick feeding, to test for transmission of aoHGE.

**Isolation of DNA from spleen and blood of rodents and from nymphal nymphs.** For isolation of rodent DNA, individual spleens were teased apart, and DNA was extracted using a QIAamp DNA mini kit (Qiagen, Valencia, CA). Heparinized blood (200 μL) was spun at 450 g for 3 min, and the whole-cell pellet was subjected to DNA extraction, using a QIAamp DNA blood mini kit (Qiagen). For isolation of tick DNA, 125 nymphal ticks were pooled in groups of 5 within individual plastic bags, were macerated between metal plates, and were homogenized by adding 1 mL of DNA.
STAT-60 (Tel-Test, Friendswood, TX). The tick homogenate was incubated with chloroform, and DNA was precipitated with 100% isopropanol. All tick DNA extracts were tested for the presence of tick mtDNA [11].

Polymerase chain reaction (PCR) to amplify aoHGE. All rodent spleens and tick pools were tested for aoHGE by PCR, using primers Ehr 521 and Ehr 747 [3]. Blood collected in experimental transmission studies, as well as spleen and tick DNA extracts, were also tested using primers MSP3F 5′-CCAGGTTAGCAAGATTAAGAG-3′ and MSP3R 5′-GCCCAGTAAACACATGATTGC-3′, which are specific for a 334-bp portion of the p44 gene of aoHGE. To amplify aoHGE-specific DNA by using the MSP3F and MSP3R primers, we added DNA (100 ng) separately to a 50-µL reaction volume containing 100 pmol each of primers MSP3F and MSP3R and a master mix described elsewhere [3]. DNA was amplified by use of a 3-step cycling program set for an initial 4-min denaturation at 94°C, 40 cycles of denaturation at 94°C for 30 s, a 30-s annealing at 55°C, and a 1-min extension at 72°C.

Amplification products were visualized on ethidium bromide-stained 3% agarose gels. All positive amplicons were sequenced by adding 50 ng of purified amplicon and 3.2 pmol of primer to a dye-terminator cycle sequencing reaction (Perkin-Elmer Applied Biosystems, Foster City, CA) with AmpliTaq FS DNA polymerase (Perkin-Elmer). All PCR reactions were set up in a dedicated PCR site where aoHGE had never been handled; templates and products were handled at second and third sites, respectively, where aoHGE had never been investigated.

Culture of B. burgdorferi. Rodent ear biopsies were obtained by sterile surgical technique and cultured in BSK II medium (Perkin-Elmer). All PCR reactions were set up in a dedicated PCR site where aoHGE had never been handled; templates and products were handled at second and third sites, respectively, where aoHGE had never been investigated.

Culture of B. burgdorferi. Rodent ear biopsies were obtained by sterile surgical technique and cultured in BSK II medium (Sigma, St. Louis) [9]. Cultures were examined weekly by dark-field microscopy for the presence of spirochetes.

Statistical analysis. Rates of single infection and coinfection were analyzed by use of Fisher’s exact test, with P < .05 considered statistically significant.

Results

Incidence of infection with aoHGE and B. burgdorferi in northern Colorado. A total of 119 rodents were captured between February and October 1999 (table 1). All spleens were examined by PCR for aoHGE, and the overall infection rate was 23.5% (28/119 were positive). In terms of B. burgdorferi infection, 65.5% of rodent biopsies were culture positive. Isolates of P. leucopus (28/119 were positive). In terms of infection in rodent species aoHGE and Borrelia burgdorferi sensu lato in infection in rodent species in northern Colorado from February through October 1999.

<table>
<thead>
<tr>
<th>Rodent species</th>
<th>aoHGE</th>
<th>B. burgdorferi</th>
<th>aoHGE + B. burgdorferi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neotoma mexicana</td>
<td>14/36 (38.8)</td>
<td>25/36 (69.4)</td>
<td>12/36 (33.3)</td>
</tr>
<tr>
<td>Peromyscus maniculatus</td>
<td>13/63 (20.6)</td>
<td>39/63 (61.2)</td>
<td>9/63 (14.2)</td>
</tr>
<tr>
<td>Peromyscus difficilis</td>
<td>0/5</td>
<td>1/5 (20.0)</td>
<td>0/5</td>
</tr>
<tr>
<td>Microtus ochrogaster</td>
<td>1/15 (6.6)</td>
<td>13/15 (86.6)</td>
<td>1/15 (6.6)</td>
</tr>
<tr>
<td>Total</td>
<td>28/119 (23.5)</td>
<td>78/119 (65.5)</td>
<td>22/119 (18.4)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. rodents positive/no. total (%). Infection with B. burgdorferi was determined by skin biopsy and culture. Infection with aoHGE was determined by polymerase chain reaction amplification of both the 16s rRNA and p44 genes of aoHGE from splenic DNA.

a Statistically significant increase in coinfection, Fisher’s exact test (P < .05).

Incidence of aoHGE in I. spinipalpis nymphal ticks. A total of 25 I. spinipalpis nymphal tick pools were collected and analyzed by PCR. All 25 samples contained tick mtDNA. Both the 16s rRNA and p44 genes of aoHGE were analyzed for each tick pool, and 14 (56%) of the 25 were positive by PCR for one or both of these genes (figure 1A). All tick pools that were positive by PCR for the 16s rRNA gene were confirmed positive by amplification of the p44 gene. All 16s rRNA amplicons were then sequenced and found to be identical to the sequence of aoHGE (GenBank accession number U02521) [1].

Transmission of aoHGE by I. spinipalpis nymphal ticks. To confirm that I. spinipalpis nymphs were competent vectors for aoHGE, we placed 8 nymphs on each specific pathogen-free mouse (n = 4) and monitored them for their ability to transmit aoHGE. Five days after tick feeding, the p44 gene could be amplified from the peripheral blood of 3 of the 4 mice (figure 1B, lanes 2–5). By day 10 after tick feeding, no aoHGE could be detected in the peripheral blood of these mice (figure 1B, lanes 7–10). Sequence analysis corroborated that aoHGE was transmitted and could be amplified from the peripheral blood of C3H/HeJ mice.

Discussion

To our knowledge, this is the first report of an enzootic cycle of dual infection involving B. burgdorferi and aoHGE in the western United States. Our results demonstrate that P. maniculatus and N. mexicana are major reservoirs for dual infection in Colorado. The incidence of infection with B. burgdorferi and aoHGE (69.2%) in P. maniculatus was similar to that reported in a highly endemic area of Connecticut, where rates of dual infection in B. leucopus were 53.8%–68.2% [7].

As reported here, the overall infection rate for aoHGE
appeared to harbor the highest incidence of B. burgdorferi tick pools, reflecting the difference in sampling techniques (peripheral blood was a true difference in California P. maniculatus was found among the fact that aoHGE could not be amplified from P. maniculatus and only 1 Colorado; however, in California, only N. mexicana and area of Minnesota (11.4%) [5]. Our data indicate that both Colorado (20.6%), in California (8%) [10], or in an endemic region of Connecticut [7]. Although the incidence of human infection with B. burgdorferi in the southwestern United States is minimal and no human cases of HGE have been confirmed in this geographic region, future research should be oriented toward surveillance studies that adequately assess the risk of I. spinipalpis ticks to transmit B. burgdorferi or aoHGE to humans.

Acknowledgments

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Figure 1. Polymerase chain reaction (PCR) amplification of agent of human granulocytic ehrlichiosis (aoHGE) in Ixodes spinipalpis ticks and transmission of aoHGE to specific pathogen±free mice by nymphal I. spinipalpis. A. PCR products amplified from DNA purified from I. spinipalpis tick pools (lanes 2, 4, 6, 8, 10, and 12), from positive culture controls (lanes 7 and 13), and from negative I. spinipalpis tick pools (lanes 3, 5, 9, and 11). Lane 1 is a 100-bp DNA ladder, and the expected size for the 16s rRNA and p44 gene amplicons are 247 bp and 334 bp, respectively. B. p44 PCR products amplified from DNA purified from individual C3H/HeJ mouse blood 5 days (lanes 2–5) and 10 days (lanes 7–10) after exposure to I. spinipalpis nymphal ticks, as described in Materials and Methods. Lane 6 represents blood from a negative control C3H/HeJ mouse exposed to uninfected I. scapularis ticks. Lane 11 is positive control DNA derived from cultured aoHGE. Lane 1 is a 100-bp DNA ladder, and the expected size of the p44 amplicon (334 bp) is indicated.

(38.8%) was similar to that reported in the dusky-footed woodrat (N. fuscipes; 34%) in California [10], but it was higher than the rate of infection reported among Peromyscus species in Colorado (20.6%), in California (8%) [10], or in an endemic area of Minnesota (11.4%) [5]. Our data indicate that both N. mexicana and P. maniculatus are reservoirs for this infection in Colorado; however, in California, only N. fuscipes was infected, and only 1 P. maniculatus was seropositive [10]. Of interest is the fact that aoHGE could not be amplified from P. maniculatus in these California studies [10], yet a significant rate of infection was found among P. maniculatus in Colorado. Whether this was a true difference in Colorado P. maniculatus or just a reflection of the difference in sampling techniques (peripheral blood vs. spleen) remains to be determined.

Although the prairie vole (M. ochrogaster) in Colorado appeared to harbor the highest incidence of B. burgdorferi infection (86.6%), this was not statistically significant (P > .05). Only 1 of 15 animals tested was also infected with aoHGE, which possibly indicates that this species may be refractory to aoHGE infection, unless previously or simultaneously infected with B. burgdorferi. Although M. pennsylvanicus has been shown to be a competent reservoir for B. burgdorferi [12], to our knowledge this is the first report of M. ochrogaster as a reservoir species for B. burgdorferi infection. Previous studies have suggested that I. spinipalpis ticks may be a western vector of aoHGE [10]; however, the studies presented here are the first documentation that I. spinipalpis ticks harbor and can transmit aoHGE effectively. The only tick species recovered from rodents captured in the foothills of Colorado was I. spinipalpis. Our results indicate that ticks derived from N. mexicana were positive by PCR for both the 16s rRNA and p44 genes of aoHGE. Likewise, sequence analysis of 16s rRNA amplicons indicated a 100% sequence homology to aoHGE [1]. Moreover, this is the first study to demonstrate that I. spinipalpis can transmit aoHGE to specific pathogen±free mice in the laboratory. We successfully amplified aoHGE from the peripheral blood of 3 of 4 mice exposed to I. spinipalpis nymphs 5 days after tick feeding. The likelihood that coinfection in this part of Colorado is maintained by a second tick species, Dermacentor andersoni, is unlikely, because these ticks have not been shown to transmit B. burgdorferi. Similarly, D. variabilis was incapable of transmitting aoHGE in the laboratory [13].

Because I. spinipalpis has been described as nidicolous, the significance to human health of I. spinipalpis transmitting and maintaining cycles of B. burgdorferi and aoHGE infections in rodent reservoirs is unclear. There are several reports of I. spinipalpis found on humans in geographic regions as diverse as New Mexico (Centers for Disease Control and Prevention, unpublished data) and Oregon and Canada [14]. Likewise, the low infection rate of I. pacificus in areas of California with reported equine ehrlichiosis and a high prevalence of infection in N. fuscipes [10] suggests that I. spinipalpis may be a bridging vector of human ehrlichiosis in California. Moreover, it has been hypothesized that I. spinipalpis, which has a greater infection rate than does I. pacificus for B. burgdorferi, may pose a greater risk to humans in the Pacific Northwest [15], where questing behavior of these ticks outside of rodent nests has been recorded [14].

This study confirms that N. mexicana, P. maniculatus, and M. ochrogaster are the principal rodent reservoirs for B. burgdorferi in northeastern Colorado. Likewise, both the deer mouse (P. maniculatus) and the Mexican woodrat (N. mexicana) displayed rates of infection with aoHGE and B. burgdorferi similar to those demonstrated in endemic areas of Connecticut [7]. Although the incidence of human infection with B. burgdorferi in the southwestern United States is minimal and no human cases of HGE have been confirmed in this geographic region, future research should be oriented toward surveillance studies that adequately assess the risk of I. spinipalpis ticks to transmit B. burgdorferi or aoHGE to humans.
Branch, CDC) for helpful discussions regarding PCR and sequencing techniques.

References