Molecular Characterization of Anaplasma phagocytophilum and Borrelia burgdorferi in Ixodes scapularis Ticks from Pennsylvania

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Ixodes scapularis ticks were collected in 2000 and 2001 from two areas in Pennsylvania and tested for the presence of Anaplasma phagocytophilum and Borrelia burgdorferi by PCR and DNA sequencing. Of the ticks collected from northwestern and southeastern Pennsylvania, 162 of 263 (61.6%) and 25 of 191 (13.1%), respectively, were found to be positive for B. burgdorferi. DNA sequencing showed >99% identity with B. burgdorferi strains B31 and JD1. PCR testing for A. phagocytophilum revealed that 5 of 263 (1.9%) from northwestern Pennsylvania and 76 of 191 (39.8%) from southeastern Pennsylvania were positive. DNA sequencing revealed two genotypes of A. phagocytophilum, the human granulocytic ehrlichiosis (HGE) agent and a variant (AP-Variant 1) that has not been associated with human infection. Although only the HGE agent was present in northwestern Pennsylvania, both genotypes were found in southeastern Pennsylvania. These data add to a growing body of evidence showing that AP-Variant 1 is the predominant agent in areas where both genotypes coexist.

Lyme borreliosis and human granulocytic ehrlichiosis (HGE) have emerged as two of the most commonly recognized tick-borne illnesses in the northeastern and upper Midwestern regions of the United States. Lyme borreliosis is the most highly reported vector-borne infectious disease in the United States, with an estimated nationwide incidence of 5 per 100,000 population (5). Since the initiation of national surveillance in 1992, >20,000 cases of Lyme disease have been diagnosed in the state of Pennsylvania, with the vast majority of cases reported from the five-county region (Chester, Delaware, Montgomery, Philadelphia, and Bucks) surrounding the Philadelphia metropolitan area. In 1999, the Centers for Disease Control and Prevention (CDC) reported 2,623 Lyme disease cases in Pennsylvania, second only to New York’s 4,262 cases (5).

Although HGE has recently been made a reportable disease in Pennsylvania, it is still difficult to assess the county-specific incidence (22). To date, there have been <30 confirmed cases in the state, most occurring in the southeastern region. Although it is likely that failure to diagnose and differentiate chilblain infections from other illnesses presenting acute febrile symptoms contributes to the low number of HGE cases in Pennsylvania, the prevalence of the etiologic agent of HGE, Anaplasma phagocytophilum, in the state is unknown.

Since the agents of both Lyme disease and HGE are transmitted by the same tick vector (Ixodes scapularis) and have the same rodent reservoir (Peromyscus leucopus), A. phagocytophilum is likely to be found in areas where Lyme disease is endemic (4, 12, 16, 20, 23, 25). Most previous studies of the distribution of A. phagocytophilum and the agent of Lyme disease, Borrelia burgdorferi, in ticks have focused on these organisms in areas where the ticks are endemic and where there is a high prevalence of human disease. In this study, we identified and characterized A. phagocytophilum and B. burgdorferi in I. scapularis ticks collected from two counties in southeastern Pennsylvania having a high incidence of Lyme disease and few cases of HGE and from a county in northwestern Pennsylvania with a modest number of Lyme disease cases and no reported HGE cases.

MATERIALS AND METHODS

Sample collection and DNA preparation. Adult I. scapularis ticks were collected in 2000 and 2001 from Presque Isle State Park in Erie County (northwestern Pennsylvania) (Global Positioning System coordinates, 42°10′17″N, 80°05′40″W) and from sites in Delaware and Chester Counties (southeastern Pennsylvania). Questing ticks were collected by flagging, and engorged adult females were removed from 30 white-tailed deer (Odocoileus virginianus), freshly shot at a controlled deer hunt in Ridley Creek State Park in Delaware County (Global Positioning System coordinates, 39°57′03″N, 75°26′24″W). The tick specimens were preserved in ethanol until the DNA extraction procedure was initiated. DNA was extracted from the ticks using DNeasy tissue extraction kits (Qiagen Inc., Chatsworth, Calif.) following the manufacturer’s recommended protocol.

PCR detection. A nested PCR assay targeting a 546-bp amplicon of the 16S rRNA gene was utilized to identify samples infected with A. phagocytophilum as described by Massung et al. (19). Nested PCR amplifying a 396-bp amplicon from the fla gene was performed as described by Johnson et al. (13) to identify specimens infected with B. burgdorferi. PCRs for both organisms were done with the Taq PCR Master Mix kit (Qiagen) and were performed in Perkin-Elmer (Applied Biosystems Division, Foster City, Calif.) 9600 or 2400 thermal cycler. Primary PCRs used the following parameters: a 30-s denaturation at 94°C, a 30-s annealing period at 55°C, and a 1-min extension at 72°C. The primary 40 cycles were preceded by a 2-min denaturation at 95°C and followed by a 5-min extension at 72°C. Nested PCRs used the same cycling conditions stated above but for only 30 cycles.

DNA sequencing of A. phagocytophilum 16S RNA and B. burgdorferi fla genes.

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DNA sequences were obtained using an Applied Biosystems 377 or 3100 automated DNA sequencer. Nested PCR products were purified using the Wizard DNA purification system (Promega, Madison, Wis.), and sequencing reactions were done with the ABI BigDye terminator kit (Perkin-Elmer, Applied Systems Division). DNA sequence alignment was performed using MegaAlign (DNASTAR, Inc., Madison, Wis.).

Nucleotide sequence accession numbers. Four novel sequences were determined for the B. burgdorferi fla gene, and each was submitted to GenBank (accession no. AY196690, AY196691, AY196692, and AY196693). The partial sequence of the 16S rRNA gene for AP-Variant 1 was also submitted to GenBank (accession no. AY193887).

RESULTS

PCR screening of DNA extracts from ticks. DNA extracts from a total of 454 adult I. scapularis ticks (240 males and 214 females) collected in 2000 and 2001 in Erie County (northwestern Pennsylvania) and from sites in Delaware and Chester Counties (southeastern Pennsylvania) were analyzed for the A. phagocytophilum 16S rRNA gene and the B. burgdorferi fla gene via nested PCR. Of 65 questing adult ticks (37 males and 28 females) collected from Erie County in 2000, none were found to be positive for A. phagocytophilum, whereas 28 (43.1%) were positive for B. burgdorferi. Of 198 additional questing adult I. scapularis ticks (102 females and 96 males) collected from the identical site in Erie County in 2001, 5 (2.5%) were found to be PCR positive for A. phagocytophilum and 134 (67.7%) were found to harbor B. burgdorferi. Four of the five ticks testing positive for A. phagocytophilum were also positive for B. burgdorferi.

PCR analysis of 73 partially engorged adult females collected in 2000 from 30 white-tailed deer in Delaware County revealed 62 (84.9%) to be positive for A. phagocytophilum, compared to eight (11.0%) testing positive for B. burgdorferi, with seven coinfections. Sixty-four questing adult males from the same area were also tested, and six (9.4%) and four (6.25%) were found to be positive for A. phagocytophilum and B. burgdorferi, respectively, with two coinfections. Furthermore, 8 of 54 (14.8%) adult ticks (11 partially engorged females and 43 questing males) collected from Chester County tested positive for A. phagocytophilum, and 13 of 54 (24.1%) were found to harbor B. burgdorferi, with 3 ticks infected with both organisms (Table 1). The percentage of coinfected ticks was not statistically significant by the chi-square test with one degree of freedom, and the coinfection rates are consistent with the two agents being acquired independently.

Identification of Borrelia and Anaplasma genotypes. DNA sequence analysis of 16S rRNA amplicons generated from the 81 A. phagocytophilum positives among 454 (17.8%) I. scapularis ticks that were tested revealed the presence of two distinct gene sequences (with a 2-base difference), one of which matched the 16S rRNA sequence described for the HGE agent while the other was identical to the sequence of A. phagocytophilum variant 1 (AP-Variant 1) (18). All A. phagocytophilum organisms in I. scapularis from Erie County (northwest) had 16S rRNA gene sequences identical to that of the HGE agent (GenBank accession no. U02521). In contrast, all sequences obtained from I. scapularis ticks collected from Chester County (southeast) were the same as that of AP-Variant 1. Of the 68 PCR-positive adult I. scapularis ticks from Delaware County (southeast), 11 (16.2%; 9 engorged females and 2 questing males) contained 16S ribosomal DNA sequences identical to that of the HGE agent, while the sequences of the remaining 57 (83.8%; 53 engorged females and 4 questing males) were identical to those of AP-Variant 1 (Table 2). Although the nested 16S rRNA PCR assay will amplify DNA from the white-tailed deer agent, which is closely related to A. phagocytophilum, DNA from this organism was not detected in any of our samples. DNA sequencing of the B. burgdorferi fla gene from 25 random PCR-positive samples demonstrated >99% identity to the human pathogenic strains JD1 and B31. The fla gene sequences determined from seven ticks from Delaware County were identical to those of B. burgdorferi strain B31 (accession no. AB035617), and the sequence from one tick from Erie County was identical to that of strain JD1 (accession no.

**TABLE 1. Spatial and temporal distributions of A. phagocytophilum and B. burgdorferi in I. scapularis ticks collected from sites in northwestern and southeastern Pennsylvania**

<table>
<thead>
<tr>
<th>I. scapularis sex</th>
<th>Collection site</th>
<th>Yr</th>
<th>n</th>
<th>No. (%) PCR positive for A. phagocytophilum</th>
<th>No. (%) PCR positive for B. burgdorferi</th>
<th>No. (%) of coinfected samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Eric</td>
<td>2000</td>
<td>28</td>
<td>0 (0)</td>
<td>17 (60.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Male</td>
<td>Eric</td>
<td>2000</td>
<td>37</td>
<td>0 (0)</td>
<td>11 (29.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Female</td>
<td>Erie</td>
<td>2001</td>
<td>102</td>
<td>3 (2.9)</td>
<td>64 (62.7)</td>
<td>2 (2.0)</td>
</tr>
<tr>
<td>Male</td>
<td>Erie</td>
<td>2001</td>
<td>96</td>
<td>2 (2.1)</td>
<td>70 (72.9)</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td>Engorged Female</td>
<td>Delaware</td>
<td>2000</td>
<td>73</td>
<td>62 (84.9)</td>
<td>8 (11.0)</td>
<td>7 (9.6)</td>
</tr>
<tr>
<td>Males</td>
<td>Delaware</td>
<td>2000</td>
<td>64</td>
<td>6 (9.4)</td>
<td>4 (6.3)</td>
<td>2 (3.1)</td>
</tr>
<tr>
<td>Engorged Female</td>
<td>Chester</td>
<td>2001</td>
<td>11</td>
<td>3 (27.3)</td>
<td>3 (27.3)</td>
<td>1 (9.1)</td>
</tr>
<tr>
<td>Males</td>
<td>Chester</td>
<td>2001</td>
<td>43</td>
<td>5 (11.6)</td>
<td>10 (23.3)</td>
<td>2 (4.7)</td>
</tr>
</tbody>
</table>

* County in Pennsylvania.

**TABLE 2. DNA-sequencing results for samples testing positive for A. phagocytophilum by nested PCR amplification of 16S rRNA gene**

<table>
<thead>
<tr>
<th>I. scapularis sex</th>
<th>Collection site</th>
<th>Yr</th>
<th>No. sequenced</th>
<th>No. (%) with genotype of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HGE agent</td>
</tr>
<tr>
<td>Engorged female</td>
<td>Delaware</td>
<td>2000</td>
<td>62</td>
<td>9 (14.5)</td>
</tr>
<tr>
<td>Male</td>
<td>Delaware</td>
<td>2000</td>
<td>6</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>Engorged female</td>
<td>Chester</td>
<td>2001</td>
<td>3</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Male</td>
<td>Chester</td>
<td>2001</td>
<td>5</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Female</td>
<td>Erie</td>
<td>2001</td>
<td>3</td>
<td>3 (100.0)</td>
</tr>
<tr>
<td>Male</td>
<td>Erie</td>
<td>2001</td>
<td>2</td>
<td>2 (100.0)</td>
</tr>
</tbody>
</table>

* County in Pennsylvania.
from Pennsylvania for the presence of *A. phagocytophilum* infections residing in the areas sampled. This approach provides a rapid means for assessing the risk of Lyme disease and HGE to human populations residing in the areas sampled.

We examined *I. scapularis* ticks collected in 2000 and 2001 from Pennsylvania for the presence of *A. phagocytophilum* and *B. burgdorferi*. Ticks were collected from three forested areas having high tick densities and categorized by the CDC as being at high risk for Lyme disease: Erie County in the northwestern corner of the state and Chester and Delaware Counties in southeastern Pennsylvania (6). Lyme borreliosis is very common in southeastern Pennsylvania, and the five-county region surrounding Philadelphia has reported the vast majority of Lyme disease cases documented from 1988 to 2001 for the state. Chester County had the highest number of cases, with 4,832 reports during this period, followed by bordering Delaware County, where 2,965 cases were documented (http://webserver.health.state.pa.us/health/site/). In fact, only Windham (296.5 cases per 100,000 population) and Litchfield (244.7 cases per 100,000 population) Counties in Connecticut had a higher Lyme disease incidence than Chester County (221.6 cases per 100,000 population) in 1999 (http://www.dph.state.ct.us; http://webserver.health.state.pa.us/health/site/). Chester County is also one of three counties in Pennsylvania where more than five cases of HGE have been confirmed. Hence, we were not surprised to find both *A. phagocytophilum* and *B. burgdorferi* in deer ticks collected from the Delaware and Chester County areas. However, the number of engorged ticks containing *A. phagocytophilum* that were collected from white-tailed deer were extremely high in Delaware (84.9%) and Chester (27.3%) Counties. Interestingly, the DNA sequences amplified from these ticks revealed that a large percentage (>86%) harbored an *A. phagocytophilum* variant (AP-Variant 1). To date, AP-Variant 1 has never been isolated or PCR amplified from a confirmed HGE infection. The Rickettsial Laboratory at the CDC has PCR amplified and sequenced the 16S rRNA genes from >50 confirmed HGE cases, and the AP-Variant 1 sequence has never been detected. These data suggest that AP-Variant 1 is not a human pathogen. However, additional studies will be needed to confirm that AP-Variant 1 is not capable of causing HGE. AP-Variant 1 is the predominant *A. phagocytophilum* genotype found in *I. scapularis* ticks collected from Rhode Island and has also been identified in Wisconsin, Maryland, and Connecticut (3, 18, 19). Questing ticks from Delaware and Chester Counties were less frequently infected than the engorged ticks, but again, they contained a high percentage of AP-Variant 1 (81.8%). The percentages of *B. burgdorferi*-positive ticks in Delaware and Chester Counties ranged from 6.3 to 27.3%, rates that are relatively low for an area where Lyme disease is endemic. Previous studies from the northeastern and mid-Atlantic regions have shown that infection rates in adult tick populations in areas where Lyme disease is endemic generally range from 20 to 80% (21, 24, 26; L. Schwartz, D. Fish, and T. J. Daniels, Letter, N. Engl. J. Med. 337:49, 1997). The percentage of ticks positive for *B. burgdorferi* was higher in the engorged females than in questing males (11 versus 6.3% in Delaware County; 27.3 versus 23.3% in Chester County). White-tailed deer are not recognized as efficient reservoirs for harboring *B. burgdorferi*, and therefore, it is unlikely that the higher prevalence in engorged females was due to acquisition of the agent from deer (17). Using a murine model, tick feeding has been shown to stimulate *B. burgdorferi* growth in the tick midgut and dissemination to the salivary glands, with the spirochete load peaking 72 h postattachment (9). Our results suggest that the apparent higher prevalence we found in engorged females may have been due to an increase in spirochete replication stimulated by the ticks feeding on deer. Thus, in ticks with low-level infections that would be undetectable by PCR, the blood meal activated growth of the agent to a level detectable by PCR.

Although Erie County in northwestern Pennsylvania has been designated as a high-risk area for Lyme disease by the CDC based on vector distribution and human exposure factors, surveillance records show that <15 cases of Lyme borreliosis per year have been reported from 1993 through 2000, and there has never been a case of HGE reported in Erie County (6). Our results from Erie County suggest that a significant risk exists for acquiring Lyme borreliosis, as 61.6% of ticks were PCR positive for *B. burgdorferi*, and the high rate of *B. burgdorferi* positives was consistent in both 2000 and 2001. While no ticks were positive for *A. phagocytophilum* in Erie County in the year 2000, five positive ticks were identified in 2001, and the sequence found in each of the five matched the DNA sequence of the human pathogenic form of *A. phagocytophilum*. Although the overall percentage of positives was low (<2%), these data provide the first evidence that *A. phagocytophilum* is present in northwestern Pennsylvania and suggest the possible emergence of the organism and HGE in that area.

The actual number of Lyme disease and HGE infections that are acquired in Erie County is likely to be higher than the number of cases reported for several reasons. Presque Isle State Park, where the ticks were collected, is a recreational site that is frequented by many individuals who live in surrounding counties, so the diagnosis may be made in the county where the individual resides rather than in Erie County. In the absence of erythema migrans in cases of Lyme disease, the other symptoms of both Lyme borreliosis and HGE are nonspecific and may include fever, chills, headache, myalgia, and malaise, leading to misdiagnosis that may also contribute to the low number of confirmed case reports from the area (22). Although physi-
cians practicing in areas throughout Pennsylvania where ticks are endemic are trained in the clinical assessment of Lyme disease, they also need to be aware of the symptoms of HGE. HGE and Lyme disease should be considered in the differential diagnosis of an acute febrile illness, particularly if there is a history of tick bite and the individual resides in, or has recently visited, an area of the state where I. scapularis is endemic.

A. phagocytophilum is present in Pennsylvania I. scapularis ticks in two forms, the HGE agent and AP-Variant 1, which differ by 2 bases within the amplified region of the 16S rRNA gene. Because standard PCR assays used to amplify A. phagocytophilum cannot distinguish between these two strains, studies based on PCR results without subsequent DNA sequencing may be misleading. For example, based on PCR data alone, we would have concluded that 8 (3 engorged females and 5 questing males) of 54 (14.8%) adult female deer ticks collected from Chester County in 2001 were infected with the HGE agent. Subsequent DNA sequencing revealed, however, that none of the samples were infected with the HGE agent. Rather, all eight of the ticks harbored AP-Variant 1, whose biological properties have not been characterized because no isolates of the organism have been obtained. Our data reinforce the suggestion that PCR assays alone are not adequate to distinguish the HGE agent from AP-Variant 1 (18). Either DNA sequencing or a single-nucleotide polymorphism assay designed to characterize the PCR product is required to differentiate these agents.

In this study, deer blood or tissue samples were not tested for the presence of either the HGE agent or AP-Variant 1, as the focus of the study was the prevalence of these agents in ticks. Likewise, the engorged ticks were randomly collected from 30 deer, and we were unable to identify which ticks were collected from each deer. However, the high prevalence of AP-Variant 1 in these ticks (62 positive of 73 tested in Delaware County) adds to a growing body of data which suggests that white-tailed deer may have an essential role in the natural maintenance of this agent (18). Even if one were to assume that each of the 11 AP-Variant 1-negative engorged ticks was the only tick collected from 11 different deer, then infected ticks must have been collected from each of the remaining 19 deer. Therefore, AP-Variant 1-positive ticks were, at a minimum, collected from approximately two-thirds of all the deer. These data suggest that deer may be a reservoir for AP-Variant 1 and that ticks may acquire the agent during feeding. Although previous studies have reported that white-tailed deer are a reservoir of the HGE agent, the role of white-tailed deer remains questionable, given that these studies were based on serological and PCR-based assays that did not examine the variable region of the 16S rRNA gene (17, 27, 28). Additional studies are needed to address the reservoir competency of white-tailed deer for both the HGE agent and AP-Variant 1.

Other questions exist regarding the high prevalence of the AP-Variant 1 strain in engorged ticks removed from deer. A. phagocytophilum is not transmitted transovarially, and because engorged females will not feed again, they will not be factors in either maintaining the strain in nature or transmission to other hosts, including humans. Therefore, additional studies are needed to determine the prevalence of the HGE agent and AP-Variant 1 in immature ticks, including both questing ticks and ticks feeding on deer or other potential reservoir species.

Cointected I. scapularis ticks have been shown to be capable of transferring both A. phagocytophilum and B. burgdorferi in a single feeding (1, 8, 14, 15). This phenomenon may further complicate the successful diagnosis and treatment of patients suffering from concomitant infections. Although the effects of dual infections on human susceptibility and clinical disease are uncertain, recent evidence obtained from in vivo laboratory experiments suggests that infection with either B. burgdorferi or A. phagocytophilum does not affect the acquisition of the other pathogen during subsequent or simultaneous feedings (15). Other studies have also demonstrated the simultaneous presence of A. phagocytophilum and B. burgdorferi in I. scapularis ticks (7, 26; Schwartz et al., letter). Factors affecting tick coinfection and the risk to humans posed by such dual infections will need further study.

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