Development of a Mitochondrial 12S rDNA Analysis for Distinguishing Sciuridae Species With Potential to Transmit *Ehrlichia* and *Borrelia* Species to Feeding *Amblyomma americanum* (Acari: Ixodidae)

LISA S. GOESSLING,1 BRIAN F. ALLAN,2,3 RACHEL S. MANDELBAUM,3 AND ROBERT E. THACH3

J. Med. Entomol. 49(3): 772–776 (2012); DOI: http://dx.doi.org/10.1603/ME11203

**ABSTRACT** Unique oligonucleotide probes were synthesized to distinguish among closely related vertebrate mitochondrial rDNA sequences present in residual bloodmeals in emergent *Amblyomma americanum* (L.) (Acari: Ixodidae) nymph life-stage ticks. Use of these probes enabled the identification of the Eastern gray squirrel as an important bloodmeal source in nymphs harboring *Ehrlichia* and *Borrelia* species. These results were confirmed by identifying these same bacterial genera in Eastern gray squirrel tissues.

**KEY WORDS** *Ehrlichia*, *Borrelia*, Sciuridae, *Amblyomma*, tick

Identification of remnant host DNA in arthropod vectors of infectious disease provides an opportunity to obtain considerable insight into the transmission dynamics of pathogens, particularly zoonotic pathogens transmitted from wildlife to humans (Kent 2009). Recent efforts to identify remnant host DNA in *Ixodes* ticks have converged upon a two-step process, using polymerase chain reaction (PCR) to amplify and biotin-label any remnant vertebrate DNA isolated from a tick, followed by reverse line blot (RLB) hybridization whereby host-specific oligonucleotide probes are used to detect the biotin-labeled amplified host DNA (Pichon et al. 2003). Several researchers have successfully used this technology to identify the reservoir hosts for numerous pathogens transmitted by *Ixodes ricinus* (Acari: Ixodidae), a preeminent vector of tick-borne diseases in Europe (Pichon et al. 2003, 2005; Cadenas et al. 2007; Humair et al. 2007). Recently, we adapted this technology to identify pathogens and remnant host DNA in the lone star tick (*Amblyomma americanum* L.), an emerging vector of infectious diseases in the southeastern United States (Allan et al. 2010).

In our previous study using nuclear 18S rDNA sequences for host bloodmeal identification, several field-collected nymph life-stage ticks that were infected with *Ehrlichia chaffeensis* contained bloodmeals that were identified as coming from one or more species in the family Sciuridae (Allan et al. 2010). This result was unexpected, as two previous studies failed to detect *E. chaffeensis* in any rodent species, including both gray and fox squirrels (*Sciurus carolinensis* and *S. niger*, respectively) (Lockhart et al. 1997a, 1998). To determine more specifically which species of Sciuridae were the source of infected bloodmeals, the 12S mitochondrial rDNA system described by Humair et al. (2007), which was previously shown to be specific for many taxa at the species level, was developed for North American vertebrate species. This novel region of DNA and the corresponding host probes developed for it were then used to reanalyze DNA samples previously analyzed using the 18S nuclear rDNA system. Newly field-collected *A. americanum* nymphs were also tested.

**Materials and Methods**

**Field Collections.** In total, 867 questing *A. americanum* nymph life-stage ticks were collected from sites located in 12 natural areas spanning the urban-to-rural human land-use gradient in east-central Missouri (five of these sites had also been used in a previous study, described in Allan et al. 2010). Tick collections were performed in areas with a mature canopy of oak-hickory eastern deciduous forest, and a forest understory comprised of a mixture of native and invasive plant species. Ticks were collected using CO₂-baited traps and stored in 70% ethanol as described previously (Allan et al. 2010).

Squirrels were collected from two sites in the greater St. Louis area (University City and Kirkwood), using Kania model 2000 traps (Kania Industries, Nanaimo, British Columbia, Canada) baited with peanut butter. The trapping protocols were approved by the Washington University Animal Studies Committee (approval number: 20100035) and the Missouri Department of Conservation.

**Tick and Tissue DNA Extraction.** Tick DNA was prepared using the Chelex 100 method as described in Allan et al. (2010), which involves pulverization in a
bead mill in the presence of a 5% suspension of Chelex 100 (Bio-Rad Life Sciences, Hercules, CA) in water. For sequencing, DNA was extracted from small amounts of frozen (S. carolinensis) or ethanol preserved (Tamias striatus) muscle, using a QIAamp DNA Micro Kit (QIAGEN Inc., Valencia, CA), following the manufacturer’s instructions. For detection of pathogens in squirrel tissues, DNA was extracted from S. carolinensis organs (blood, spleen, liver, and heart) using either the Chelex 100 method or the QIAamp method. All DNA samples were stored at −20°C.

**PCR Amplification for RLB and Sequencing.** Approximately 50 ng of DNA was used in a 50 µl PCR...
reaction to amplify a portion of the 12S rDNA gene using primers and conditions as described in Humair et al. (2007). Primers 12S-6 F and B-12S-9R were used to amplify a 145 bp fragment for analysis by RLB. These same primers were used to sequence the T. striatus tissue DNA sample. S. carolinensis tissue DNA was sequenced using the 12S-12 F and 12S-13R primers.

**Probe Design.** Sequences of 12S rDNA for North American Sciuridae and other possible vertebrate host species were retrieved from Genbank. These sequences, along with those obtained for T. striatus and S. carolinensis, were aligned using MegAlign software (DNASTAR, Inc., Madison, WI). Areas of variability were used to design probes for 11 species, 2 genera, 1 family, and 1 class. In many cases, more than one probe was constructed for each. The probe names and sequences are shown in Table 1.

**Reverse Line Blot.** For RLB hybridization, probes were linked to an activated BioDyne C membrane, typically at concentrations of 400 or 1,000 pmol per lane. The RLB assay was done as previously described (Allan et al. 2010) except for the posthybridization washes where the membrane was washed twice for 10 min using 2× SSPE/0.5% SDS at 55°C.

**Results**

The ability of each new 12S rDNA probe to positively bind to its target DNA, but not to unrelated DNA, was tested using the RLB hybridization assay (Pichon et al. 2003, Humair et al. 2007, Allan et al. 2010). The successful development and implementation of these probes are shown in Fig. 1. It is evident that most probes react with acceptable specificity for their intended target DNAs. However, several probes (such as those for Sciurus spp., S. carolinensis, S. tridecemlineatus, and T. striatus) reacted with more than one DNA source. Nevertheless, the redundancy provided by using multiple probes for a given host in the RLB assay makes unequivocal decisions as to bloodmeal source possible. For example, the S. carolinensis DNA sample (lane 2) reacts only with the Sciurus spp. and S. carolinensis probes (second and third rows from the top). Similarly, although the S. niger DNA sample (lane 3) reacts faintly with the S. carolinensis and S. tridecemlineatus probes, its strong reactions with the Sciurus spp. and S. niger probes confirm its identity.

The distinctions among Sciuridae family members seen in Fig. 1 are far more detailed and precise than was possible using the nuclear 18S rDNA system (Pichon et al. 2003, Allan et al. 2010). Similar results have been obtained for distinguishing wild turkey (Meleagris gallopavo) from other bird species (not shown). This newly developed mitochondrial 12S rDNA system was then used to retest tick lysates

---

**Table 2. Tick Bloodmeal and Pathogen Identification**

<table>
<thead>
<tr>
<th>Bloodmeal source</th>
<th>No.</th>
<th>Pathogen</th>
<th>% infected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. virginianus</em></td>
<td>153</td>
<td>E. chaffeensis 5 E. ewingii 0 B. lonestari 0</td>
<td>5.2</td>
</tr>
<tr>
<td><em>S. carolinensis</em></td>
<td>143</td>
<td>2 3 3 5</td>
<td>5.6</td>
</tr>
<tr>
<td><em>S. niger</em></td>
<td>20</td>
<td>0 1 0 0</td>
<td>5.0</td>
</tr>
<tr>
<td>Procyon lotor</td>
<td>19</td>
<td>0 0 0 2</td>
<td>10.5</td>
</tr>
<tr>
<td><em>M. gallopavo</em></td>
<td>22</td>
<td>1 0 0 0</td>
<td>4.5</td>
</tr>
<tr>
<td>Aves species</td>
<td>27</td>
<td>1 0 0 0</td>
<td>3.7</td>
</tr>
<tr>
<td><em>D. virginiana</em></td>
<td>17</td>
<td>0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td>Sylvilagus or Lepus species</td>
<td>7</td>
<td>0 0 0 0</td>
<td>0</td>
</tr>
<tr>
<td><em>T. striatus</em></td>
<td>1</td>
<td>0 0 0 0</td>
<td>0</td>
</tr>
</tbody>
</table>

*No bloodmeals were detected from Spermophilus species, *G. volans*, *T. hudsonicus*, or *M. monax.*
previously analyzed using the 18S rDNA system (Allan et al. 2010). A typical result is shown in Fig. 2.

Two out of the six tick samples that had reacted with the E. chaffeensis probe in the 16S bacterial rDNA assay had also reacted with the 18S nuclear rDNA Sciurus probe, as previously reported (Allan et al. 2010). These same two samples now react strongly and specifically with the Eastern gray squirrel (S. carolinensis) and generic squirrel (Sciurus spp.) 12S mitochondrial rDNA probes (lanes 2 and 3 in Fig. 2). In contrast, there was no interaction between these DNA samples and the probes developed for fox squirrel (S. niger), ground squirrels (Spermophilus spp. and S. tridecimlineatus), chipmunk (T. striatus I and 2), flying squirrel (G. volans I and 2), woodchuck (M. monax I and 2), or red squirrel (T. hudsonicus).

Further insight was obtained by examining nymphal ticks that were sampled over a much wider area than in the earlier study (i.e., Allan et al. 2010). Of these 867 A. americanum nymphs examined, 409 provided identifiable bloodmeals using the new 12S RLB assay (Table 2). Bloodmeals were once again spread among a diverse array of vertebrate hosts, with a few species clearly playing an important role in pathogen transmission.

Recently, we have begun a study of pathogens carried by Eastern gray squirrels from the greater St. Louis area. Among 48 squirrels analyzed (Table 3), two contained DNA that reacted specifically with the probe for Borrelia lonestari, three with the probe for Rickettsia rickettsii, and one each with probes for E. chaffeensis, B. burgdorferi sensu stricto, and B. burgdorferi sensu lato. These results are consistent with the data shown in Table 2, and support a conclusion that Eastern gray squirrels may be a significant reservoir for transmitting B. lonestari and E. chaffeensis to A. americanum larvae.

Discussion

Several important findings emerge from this analysis. First, the number of tick bloodmeals that were taken from white-tailed deer, while still the largest cohort, was nearly equal to that from the two species in the genus Sciurus (Eastern gray squirrel and fox squirrel). Second, the proportion of bloodmeals from the two squirrel species and white-tailed deer that resulted in the acquisition of a pathogen is similar. Third, the amplified DNA from individual squirrel tissues occasionally reacts with specific probes for several of the same pathogens found in ticks. Taken together, these results provide support for our earlier conclusions regarding the potential importance of squirrel species as reservoirs for lone star tick-associated pathogens (Allan et al. 2010) and, in particular, establish in quantitative terms the prominence of the Eastern gray squirrel (S. carolinensis) as an important reservoir host. Given the widespread distribution of gray squirrels in both rural and urban environments, their potential as reservoirs for pathogens that cause human disease may be higher than has been previously recognized.

These findings raise immediately the question of whether infection rates are consistent across the human land-use gradient. In particular, the extent to which these rates may be influenced by the presence of other reservoir species, such as white-tailed deer (Lockhart et al. 1997b, Yabsley et al. 2002, Paddock and Yabsley 2007, Goddard and Varela-Stokes 2008), is of interest.

The high resolution among species within the Sciuridae family that was afforded by the technique used here bodes well for its extension to other systems. In anticipation of future applications we have begun a study of the large number of bird species that are common to eastern Missouri. Preliminary results suggest that a similarly high degree of species discrimination will be obtainable.

Acknowledgments

We thank the land managers of numerous private and public natural areas, including the Tyson Research Center, MO Department of Conservation, MO Department of Natural Resources, and St. Louis County Parks Department for logistical support of our study. We thank B. T. Thach and numerous volunteers who provided invaluable assistance in the field. This research was funded by grants from the Environmental Protection Agency (834495) and Time for Lyme, Inc.

References Cited


Received 16 September 2011; accepted 26 December 2011.