Infection Prevalences of Common Tick-borne Pathogens in Adult Lone Star Ticks (Amblyomma americanum) and American Dog Ticks (Dermacentor variabilis) in Kentucky

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Abstract. Rocky Mountain spotted fever, Lyme disease, and ehrlichiosis are tick-borne diseases that are reported annually in Kentucky. We conducted a survey to describe infection prevalence of tick-borne pathogens in Amblyomma americanum and Dermacentor variabilis ticks collected in Kentucky. During 2007–2008, we collected 287 ticks (179 D. variabilis and 108 A. americanum) from canine, feral hog, horse, raccoon, white-tailed deer, and human hosts in six counties in Kentucky. Ticks were screened for Rickettsia spp., Borrelia spp., and Ehrlichia spp. by using polymerase chain reaction. Forty-one (14.3%) ticks (31 A. americanum and 10 D. variabilis) were polymerase chain reaction–positive for a Rickettsia spp. Fourteen (4.9%) ticks (6 A. americanum and 8 D. variabilis) were positive for E. chaffeensis, and 4 A. americanum (1.4%) were positive for E. ewingii. One (0.4%) A. americanum was positive for Borrelia lonestari. Although Rocky Mountain spotted fever is diagnosed in Kentucky, no R. rickettsii was found in ticks in this study.

INTRODUCTION

Tick-borne rickettsioses are of public health importance because they are a substantial cause of morbidity and mortality worldwide.1 Ticks are responsible for transmitting most vector-borne diseases in the United States. Ticks are capable of transmitting a variety of pathogens, which cause diseases such as spotted fever rickettsiosis, Lyme disease, and human ehrlichiosis.2 In the United States, the most commonly reported rickettsial human pathogen is the obligate intracellular bacterium Rickettsia rickettsii, the causative agent of Rock Mountain spotted fever (RMSF).3 The vectors that are historically attributed in the transmission of R. rickettsii are the American dog tick (Dermacentor variabilis) in the eastern United States and the Rocky Mountain wood tick (Dermacentor andersoni) in the western United States.4 Rock Mountain spotted fever is characterized by a fever, rash, and other possible complications such as encephalitis, coagulopathy, and respiratory disorders.5 There were 15 cases of RMSF reported in Kentucky during 2004–2008.6

In addition to R. rickettsii, other rickettsial species may cause morbidity. There is serologic evidence that another spotted fever group Rickettsia (SFGR) (Rickettsia amblyomnii) may also be a human pathogen.7,8 In North Carolina, serum samples from patients that had probable cases of RMSF were tested and had higher end-point titer to R. amblyomnii than to R. rickettsii.9 In 2006, an adult Amblyomma americanum tick infected with R. amblyomnii was removed from a North Carolina patient with a macular rash at the tick attachment site.10 Amblyomma americanum ticks have been found to be infected with R. amblyomnii in the southeastern United States and in the lower midwestern and coastal New England regions.11–14 Rickettsia parkeri, another SFGR, has also been associated with human illness. The principle vector for R. parkeri is Amblyomma maculatum, the Gulf Coast tick.3 The first confirmed human case of spotted fever rickettsiosis caused by R. parkeri was reported in 2004.15 In 2007, Whitman and others detected and isolated R. parkeri from an eschar that developed after a tick bite from a patient in Virginia.16

The causative agent of Lyme disease is a gram-negative bacterial spirochete (Borrelia burgdorferi), which is spread by the tick Ixodes scapularis in the eastern United States and the tick Ixodes pacificus in the western United States. The most common characteristic of early stage Lyme disease is an erythema migrans rash accompanied by nonspecific symptoms such as fever, malaise, fatigue, headache, myalgia, and arthralgia.17 Other major symptoms include arthritis and regional lymphadenopathy.18 In Kentucky, 18 cases of Lyme disease were reported in 2006–2008,4 with an average incidence of 0.5 per 100,000 persons during 1992–2006.19 An additional Borrelia species (Borrelia lonestari), has been tentatively associated with a Lyme borreliosis–like disease in the United States, which is sometimes referred to as southern tick–associated rash illness (STARI).20–23 It is believed that the symptoms of STARI are less severe than those of Lyme disease.24 Borrelia lonestari was identified and characterized in A. americanum ticks in 1995 and was first isolated in 2004.20,22,23 Borrelia lonestari has additionally been identified in A. americanum ticks that were removed from humans, including patients from Kentucky.25

Ehrlichia chaffeensis is a gram-negative obligate intracellular bacterium and the etiologic agent of human monocytotropic ehrlichiosis.27–29 Ehrlichia chaffeensis is maintained in a zoonotic cycle involving its principal reservoir, the white-tailed deer (Odocoileus virginianus) and A. americanum ticks.30–33 This disease is characterized by fever, headache, myalgia, thrombocytopenia, leukopenia, and increased liver enzyme levels. Most cases cause only mild illness, although more serious complications, including death, can occur. Human monocytotropic ehrlichiosis is most commonly reported from the southeastern and south central United States.34 The A. americanum tick is also a vector for Ehrlichia ewingii,35,36 the cause of granulocytic ehrlichiosis in humans.37–39 Thirteen cases of ehrlichiosis were reported in Kentucky in 2008, a 325% increase over 2007.4 In this study, we used molecular methods to determine the infection prevalence of ehrlichial, rickettsial, and borrelian species in A. americanum and D. variabilis ticks.
collected from a variety of wildlife and domestic hosts from six counties in Kentucky.

MATERIALS AND METHODS

Tick collection and identification. Adult ticks were collected from May through August 2008 by the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services (USDA APHIS-WS) from six counties in Kentucky. Ticks were collected as a convenience sample in routine USDA APHIS-WS nuisance calls. Ticks were collected from personal pets, feral hogs, canines, horses, a raccoon, a white-tailed deer, and a human. All ticks were sent to the Tennessee Department of Health Vector-Borne Diseases Laboratory and were identified to species and life stage based on morphologic criteria.40

Isolation of DNA. Ticks were individually homogenized with metal beads and resuspended in 225 μL of phosphate-buffered saline. DNA was extracted from 200 μL of the homogenate by using a 5 PRIME Manual Ready PCR DNA Column Kit (5 PRIME Inc., Gaithersburg, MD) according to the manufacturers’ instructions.

Identification of rickettsiae. Extracted DNA was initially screened by using a real-time polymerase chain reaction (PCR) to amplify the 17-kDa gene of all SFGR.41 For species identification, a conventional PCR specific for the outer membrane protein A gene of SFGR was conducted on positive samples as described by using primers Rr190.602n and Rr190.70p.42 The PCR products were detected by electrophoresis on 10 μL of product on a 2% agarose gel containing ethidium bromide. Field isolates of R. amblyommii from A. americanum ticks and double-distilled water were used as positive and negative controls, respectively, for all real-time and conventional PCRs. The outer membrane protein A ampiclons from PCR-positive samples were further subjected to a restriction fragment length polymorphism assay by digestion with Rsal (Promega, Madison, WI) and PstI (Fermentas, Glen Burnie, MD) at 37°C for 1 hour.43 Digested fragments were subjected to electrophoresis on 10% polyacrylamide gels. A subset of restriction fragment length polymorphism samples were verified by sequence analysis with a 3130x1 Genetic Sequencer (Applied Biosystems, Foster City, CA) and BigDye Terminator (Applied Biosystems).

Identification of ehrlichiae. Extracted tick DNA was screened for Ehrlichia chaffeensis by using a nested PCR assay to amplify a segment of the variable length PCR target (VLPT gene).44 Extracted tick DNA was screened for Ehrlichia ewingii by using a nested, species-specific conventional PCR specific for the 16S ribosomal RNA gene.45 Deer blood specimens positive for E. chaffeensis and E. ewingii, as confirmed by sequence analysis, were used as positive controls, and double-distilled water was used as a negative control. The PCR products were detected by electrophoresis of 10 μL of product on 2% agarose E-gels (Invitrogen, Carlsbad, CA) and visualized under ultraviolet light. All positive samples were verified by sequence analysis.

Identification of Borrelia spp. Extracted tick DNA was screened for Borrelia spp. by using a nested PCR assay to amplify the 16S–23S ribosomal RNA gene intergenic spacer region as described by Bunikis and others.46 This more conserved region enables detection of other Borrelia species circulating such as B. lonestari. Ticks were also screened by using a PCR assay to detect Borrelia burgdorferi specifically. Forward primer SL1 and reverse primer SL2 were used as described.47 A standard laboratory strain B. burgdorferi isolate from Shelter Island, New York (strain B31) was used as a positive control, and double-distilled water was used as a negative control in all reactions. All positive samples were sequenced to determine and confirm species.

Statistical analysis. Pearson chi-square tests were used for statistical analyses.

RESULTS

During May–August 2008, 287 adult ticks were collected: 108 (37.6%) A. americanum and 179 (62.4%) D. variabilis. Of the A. americanum ticks collected, 75 (69.4%) were female and 33 (30.6%) were male. Of the D. variabilis ticks collected, 101 (56.4%) were female and 78 (43.6%) were male. Male ticks were not collected in this study because of the ease at which adult ticks can be identified and removed relative to immature ticks in a quick convenience sampling.

Forty-one (14.3%) ticks were infected with a Rickettsia spp. One A. americanum tick was infected with B. lonestari (0.35%). Fourteen (4.88%) ticks were infected with E. chaffeensis and 4 (1.39%) A. americanum ticks were infected with E. ewingii. None of the ticks were infected with B. burgdorferi (Table 1). The overall infection prevalence for A. americanum was significantly higher than that for D. variabilis (39% versus 10%; P < 0.0002). Among A. americanum ticks, male infection prevalence was significantly higher than female infection prevalence (45% versus 29%; P < 0.0336).

Thirty-two (11.1%) ticks were infected with R. amblyommii. Eight (2.8%) D. variabilis ticks were infected with R. montana, and 1 (0.4%) adult male D. variabilis was infected with R. parkeri. No A. americanum ticks were infected with R. montana or R. parkeri (Table 2). There were significantly more R. amblyommii infections than either R. montana or

<table>
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<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Prevalence of Rickettsia spp., Borrelia lonestari, Ehrlichia chaffeensis, and Ehrlichia ewingii in ticks from Kentucky, by species and sex</td>
</tr>
<tr>
<td>Tick</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td><strong>Amblyomma americanum</strong></td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td><strong>Dermacentor variabilis</strong></td>
</tr>
<tr>
<td>M</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
</tr>
<tr>
<td>31/108 (28.7)</td>
</tr>
<tr>
<td>10/179 (5.6)</td>
</tr>
<tr>
<td>6/78 (7.7)</td>
</tr>
<tr>
<td>41/287 (14.3)</td>
</tr>
</tbody>
</table>

*Ticks were co-infected with multiple species.
R. parkeri infections (P < 0.0002). Overall, A. americanum ticks were infected with Rickettsia spp. at a significantly higher prevalence than were D. variabilis ticks (28% versus 6.2%; P < 0.0002). Additionally, male A. americanum ticks were infected with Rickettsia spp. at a significantly higher prevalence than female A. americanum ticks (39% versus 23%; P < 0.0184) (Table 2).

Four A. americanum ticks were co-infected with two bacterial species. Two ticks were co-infected with R. amblyommii and E. ewingii. One tick was co-infected with R. amblyommii and E. chaffeensis and one tick was co-infected with R. amblyommii and B. lonestari.

Ticks were collected from 6 counties; most (231 ticks, 80.5%) were collected in Warren County. The remaining ticks were collected from five counties in central and western Kentucky. Ticks were removed from a variety of wildlife and domestic hosts; 80 from canines, 33 from feral hogs, 165 from horses, 4 from raccoons, 1 from a white-tailed deer, 1 from a human, and 3 from unknown hosts (Table 3). The ticks removed from the human, raccoons, and unknown hosts were not infected with any of the bacteria tested. The one tick removed from the white-tailed deer was infected with R. amblyommii. Ticks removed from feral hogs had the highest infection prevalence (11 of 33, 33%). Thirty-two (19%) ticks removed from horses and 16 (20%) ticks removed from canines were infected with one or more of the bacterial species tested (Figure 1).

### DISCUSSION

No R. rickettsii, the etiologic agent of RMSF, was found in this study. Cases of RMSF are reported annually across Kentucky. This finding is similar to recent tick surveys in Tennessee, which has one of the highest incidences of RMSF in the United States, where no R. rickettsii was found in more than 1,200 ticks tested.\(^{15}\) Cases reported as RMSF may be caused by other rickettsial species. Disease caused by different rickettsial species may have different presentations and severity. These findings could have important clinical implications in regions where multiple rickettsial species are found.

Forty-one (14.3%) ticks collected in this study were positive for Rickettsia spp. Similar results were found in Mississippi where 26% of A. americanum ticks were infected with Rickettsia spp.\(^{7}\) However, infection prevalence in ticks collected in nearby Tennessee was higher with 44% of A. americanum and 14% of D. variabilis collected infected with a Rickettsia spp. Concomitantly, there are significantly more human RMSF cases reported in Tennessee (224 cases reported in 2008) than in Kentucky.\(^{11}\)

Rickettsia amblyommii was the most common pathogen found, which is consistent with findings in other studies, which have found a high prevalence of R. amblyommii in A. americanum ticks in the southeastern and midwestern United States. In a survey conducted in nine states during 1998–2005, A. americanum ticks were infected with R. amblyommii at a prevalence ranging from 0% to 84% and an average infection prevalence of 41.2%.\(^{15}\) These findings were similar to those in a study showing that 40% of A. americanum ticks collected in Tennessee were infected with R. amblyommii.\(^{15}\) Amblyomma americanum ticks removed from humans in Kentucky were shown to be infected with R. amblyommii at a prevalence of 65%.\(^{48}\)

Although when originally isolated R. amblyommii was not known to be pathogenic to laboratory animals,\(^{49}\) there have since been several studies possibly linking the bacterial species to human illness. It has been speculated that bites from R. amblyommii infected ticks may be one cause of high sero-prevalence of antibodies to SFGR.\(^{12}\) Rickettsia amblyommii has also been temporally associated a macular rash when an engorged A. americanum tick infected with R. amblyommii was removed from a patient.\(^{10}\) In a 2008 study, three of six probable RMSF cases demonstrated a ≥ 4-fold increase in

### Table 2

<table>
<thead>
<tr>
<th>Tick Species</th>
<th>Sex</th>
<th>Rickettsia amblyommii, no. positive/ no. tested (%)</th>
<th>Rickettsia Montana, no. positive/ no. tested (%)</th>
<th>Rickettsia parkeri, no. positive/ no. tested (%)</th>
<th>Total Rickettsia spp, no. positive/no. tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amblyomma americanum</td>
<td>M</td>
<td>30/108 (27.8)</td>
<td>0/108</td>
<td>0/108</td>
<td>30/108 (27.8)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>13/33 (39.4)</td>
<td>0/33</td>
<td>0/33</td>
<td>13/33 (39.4)</td>
</tr>
<tr>
<td>Dermacentor variabilis</td>
<td>M</td>
<td>2/179 (1.1)</td>
<td>1/179 (0.6)</td>
<td>1/179 (0.6)</td>
<td>11/179 (6.2)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1/101 (1)</td>
<td>0/101</td>
<td>0/101</td>
<td>678 (7.7)</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td></td>
<td>32/287 (11.1)</td>
<td>8/287 (2.8)</td>
<td>1/287 (0.4)</td>
<td>41/287 (14.3)</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Host</th>
<th>Total ticks</th>
<th>Amblyomma americanum</th>
<th>Dermacentor variabilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine</td>
<td>80</td>
<td>19</td>
<td>61</td>
</tr>
<tr>
<td>Feral hog</td>
<td>33</td>
<td>31</td>
<td>2</td>
</tr>
<tr>
<td>Horse</td>
<td>166</td>
<td>57</td>
<td>109</td>
</tr>
<tr>
<td>Human</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Raccoon</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Not documented (unknown)</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

**Figure 1.** Infection prevalence of ticks collected from the three most common wildlife and domestic hosts in this study, Kentucky.
Mixson and others. 13 In Tennessee, 2.6% of infections were similar to those in a survey conducted by the University of Kentucky in 2006. 51 These studies suggest that E. ewingii has caused a confirmed human case of rickettsiosis in Mercer County in Kentucky in 2006. 51 These studies suggest that R. parkeri, similar to other Rickettsia spp., may be the cause of some disease that is reported as RMSF in Kentucky.

Rickettsia montana is a non-pathogenic rickettsia22 that was found in eight D. variabilis ticks in this study, which is consistent with results of previous studies.5, 54 In Tennessee, R. montana has been found at higher prevalences in D. variabilis ticks than in A. americanaum ticks (10% and 0.3%, respectively).11 This finding is consistent with the trend we found in Kentucky; 4.47% of D. variabilis and no A. americanaum being infected. Rickettsia montana has been shown to interfere with the transmission of pathogenic rickettsiae, including R. rickettsii, in D. variabilis ticks experimentally50 and may partly explain the lack of R. rickettsii found in Kentucky.

Borrelia lonestari has been tentatively associated with a Lyme-like illness referred to as STARI in the United States. In this study, one A. americanaum (0.9%) tick was infected with B. lonestari. Borrelia lonestari has been found in A. americanaum ticks parasitizing humans along the eastern coast and in southeastern and midwestern regions, including 13 ticks from Kentucky from 2001–2002.56 A study in Missouri found 5.6% of A. americanaum ticks to be infected with B. lonestari,56 and Mixson and others found infection prevalences ranging from 0% to 12.2% and an average infection prevalence of 2.5% in nine states.13 Borrelia burgdorferi was not found in any of the ticks we tested, although we did not collect its principal vector (I. scapularis).

Ehrlichia chaffeensis and E. ewingii infection prevalences in this study were similar to those in a survey conducted by Mixson and others.13 In Tennessee, 2.6% of A. americanaum ticks were found to be infected with E. chaffeensis, and 0.8% were infected with E. ewingii, and no other Amblyomma, Ixodes, or Dermacentor ticks tested were positive for any Ehrlichia spp.13 A study conducted during 1996–2001, white-tailed deer from eight states were tested for E. ewingii by PCR. It was found that 5.5% were positive for E. ewingii infection, including one deer from Kentucky. White-tailed deer are an important reservoir for E. chaffeensis and E. ewingii in the southeastern and south central United States and are an abundant wildlife species in Kentucky.37, 55

Four (1.4%) ticks were co-infected with two species of bacteria. All of these ticks were A. americanaum and were infected with R. amblyomnii and one additional bacterial species. Although the pathogenicity of R. amblyomnii has not been confirmed, persons bitten by co-infected A. americanaum ticks may be at risk of disease complications because of multiple pathogens, although they may not all be causing symptoms. Identifying pathogens in a tick removed from a patient does not identify which organism is causing disease. Amblyomnii americanaum ticks are aggressive biters89 and are a vector for multiple pathogens, which increases the risk of patients with co-infection. This risk increases as the populations of A. americanaum ticks rise and spread throughout the United States. Amblyomnii americanaum tick populations have been expanding from the southeastern United States into the northeastern and midwestern United States partly because of the increased density of their common host, the white-tailed deer57, 59, 61 and as such are an emerging threat to public health.

Amblyomnii americanaum ticks had higher infection prevalence than D. variabilis ticks, potentially because of their aggressive biting behavior. In addition, A. americanaum ticks have a broader host range than D. variabilis, which may also contribute to the higher infection prevalence. Also, A. americanaum ticks are the vector for the most abundant pathogen found in this study (R. amblyomnii) (Tables 1 and 2). Additionally, males had a higher Rickettsia spp. infection prevalence in A. americanaum and D. variabilis ticks (Tables 1 and 2). This prevalence is potentially caused by the life cycle of male and female ticks. Female ticks take large blood meals to support egg production. After all eggs are deposited, the female dies. Males may live longer and take several blood meals from multiple hosts, which increases the potential for contracting and transmitting bacterial pathogens.42

Most (80.5%) of the ticks were collected in Warren County in Kentucky. There are 120 counties in Kentucky, of which 12 counties, dispersed throughout the state, where 16 RMSF cases have been diagnosed during 2006–2010. Although there were no cases of RMSF diagnosed in Warren County in this five-year period, bordering counties have had diagnosed RMSF cases. There were 27 cases of Lyme disease reported in Kentucky during 2005–2010. These cases, similar to reported cases of RMSF, were distributed throughout the state. There were 16 counties that reported Lyme disease during 2005–2010; we collected ticks from 3 of these counties and from bordering counties. There was no significant difference between tick densities found on the variety of hosts among all six counties. Because ticks were collected as a convenience sampling on USDA APHIS-WS nuisance calls, no host-seeking ticks were collected. Ticks removed from feral hogs had the highest infection prevalences. This finding may be potentially caused by their lifestyle because it has been documented that they migrate through many different types of habitats and spend approximately half of their time grazing in shrub lands that may be heavily tick infested.63

The absence of R. rickettsii in this study should be investigated further. If ticks are co-infected with multiple rickettsial species, R. rickettsii may not be amplified by our molecular methods. Rhipicephalus sanguineus ticks have recently been found to be a vector for R. rickettsii in Arizona, but no R. sanguineus ticks were collected in this study.64 Serologic studies should be conducted in RMSF patients to determine whether other SFGR, such as R. amblyomnii and R. parkeri, are causing disease in humans. There should be regular surveys to monitor Ehrlichia infection prevalences in ticks over time to determine if increases in human ehrlichiosis cases in Kentucky are related to increased infection in the tick populations. Lastly, additional studies should be conducted to determine what species of Borrelia are causing the Lyme disease cases reported annually in Kentucky.

In this study, we found multiple pathogens that are known or suspected causes of human illness. Physicians should be aware of the common tickborne diseases in their area of
practice and include them in the differential diagnosis for patients with a febrile illness. It is important for physicians to be aware that multiple species of Rickettsiae, Borrelia and Ehrlichia can cause tick-borne diseases.

Received October 14, 2010. Accepted for publication April 6, 2011.

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REFERENCES


