

## Absence of *Rickettsia rickettsii* and Occurrence of Other Spotted Fever Group Rickettsiae in Ticks from Tennessee

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**Abstract.** Rocky Mountain spotted fever (RMSF) is the most common tick-borne illness in Tennessee. Little is known about the occurrence of *R. rickettsii*, the causative agent, in ticks in Tennessee. To better understand the prevalence and distribution of rickettsial agents in ticks, we tested 1,265 *Amblyomma*, *Dermacentor*, and *Ixodes* adult and nymphal ticks. Additionally, we tested 231 *Amblyomma americanum* larvae. Ticks were collected from 49 counties from humans, wild animals, domestic canines, and flannel drags. Spotted fever group rickettsiae (SFGR) DNA was detected by polymerase chain reaction (PCR) in 32% of adult and nymphal ticks. A total minimum infection rate of 85.63 was found in larval pools tested. Three rickettsial species, *Rickettsia montana*, *Rickettsia amblyommii*, and *Rickettsia cooleyi* were identified by molecular analysis. *Rickettsia rickettsii* was not detected. This study suggests that some RMSF cases reported in Tennessee may be caused by cross-reactivity with other SFGR antigenically related to *R. rickettsii*.

### INTRODUCTION

Rocky Mountain spotted fever (RMSF), a potentially fatal disease caused by *Rickettsia rickettsii*, is the most common tick-borne disease reported in Tennessee. The vector typically attributed with RMSF transmission and maintenance in the southeastern United States is *Dermacentor variabilis* (the American dog tick),<sup>1</sup> although the widely distributed *Rhipicephalus sanguineus* (the brown dog tick), is also found in Tennessee<sup>2</sup> and has been shown to transmit the pathogen in other parts of the country.<sup>3,4</sup> Tennessee historically reports one of the highest incidence rates for RMSF in the United States,<sup>5,6</sup> which have rapidly increased in recent years. In 2008, 224 cases were reported to the Tennessee Department of Health (TDH), a 20% increase over 2007 and 120% increase over 2004. Western Tennessee has been recognized for the highest level of RMSF activity, where six deaths were attributed to the disease from 2001 to 2005.<sup>7</sup> Despite frequent laboratory testing and reports of RMSF, the true incidence in Tennessee is unclear. The majority of RMSF cases reported to the TDH are classified as probable based on clinically compatible symptoms and a single serologic test with elevated immunoglobulin (Ig) G or IgM antibodies. For most cases, paired convalescent sera were not collected. In addition, it has been proposed that some RMSF cases reported in the United States may be caused by non-*R. rickettsii* spotted fever group rickettsiae (SFGR) as a result of cross-reactivity among SFGR antibodies.<sup>8,9</sup> *Rickettsia amblyommii*, in particular, has been suggested as a potential cause of high SFGR seroprevalence.<sup>10,11</sup>

Little is known about the diversity and prevalence of SFGR in ticks in Tennessee because of spatial and technical limitations of previous studies. In 1974 Burgdorfer and others<sup>12</sup> identified *R. rickettsii* in *D. variabilis* in the Land Between the Lakes area bordering Kentucky by examining hemolymph, which is not as reliable for species identification as modern molecular techniques. In 2001, a study by Kollars and Kengluocha<sup>13</sup>

in Shelby County, western Tennessee, used polymerase chain reaction (PCR) to identify SFGR in 1% of *D. variabilis* collected from raccoons and opossums, but SFGR were not identified to the species level. In this study, we used molecular assays to determine the prevalence and identity of rickettsiae in 49 of Tennessee's 95 counties from ticks collected from vegetation and a diverse array of host species.

### MATERIALS AND METHODS

**Tick collection and identification.** Adult and nymphal ticks were collected from April 2007 to September 2008 from 49 counties in Tennessee by the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services (USDA-APHIS-WS) and the TDH from humans, wild animals, and flannel drags. In addition, ticks were collected at or around the homes of some individuals that had previously been ill with RMSF. The wildlife tick hosts were raccoons, feral hogs, white-tailed deer, skunks, coyote, red foxes, groundhogs, horses, dogs, and opossum, a grey fox, and a feral cat, as previously described.<sup>14</sup> To collect additional numbers of dog ticks from high RMSF incidence counties in Western Tennessee, adult and nymphal ticks were collected by veterinarians from domestic canines during peak incidence months of July and August 2009. Larvae were collected by flannel drags and traps at locations of human infection, and from domestic canines. All tick samples were sent to the TDH Vector-Borne Diseases Laboratory and were identified to species and life stage based on morphological criteria.<sup>15</sup>

**DNA isolation.** Ticks were individually homogenized with metal beads and resuspended in 225  $\mu$ L phosphate buffered saline (PBS). DNA was extracted from 100  $\mu$ L of the homogenate using a Qiagen QiaAmp DNA Micro Kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions.

**Identification of rickettsiae.** Extracted DNA was initially screened using a real-time PCR to amplify the 17 kDa gene of all SFGR.<sup>16</sup> For species identification, a conventional PCR targeting the *ompA* gene of SFGR was conducted on positive samples as previously described using primers Rr190.602n and Rr190.70p.<sup>17</sup> Field isolates and dH<sub>2</sub>O were used as positive and negative controls, respectively, for all real-time and

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conventional PCRs. The *OmpA* amplicons from PCR-positive samples were further subjected to a restriction fragment length polymorphism assay (RFLP) by digestion with *RsaI* (Promega, Madison, WI) and *PstI* (Fermentas, Glen Burnie, MD) enzymes at 37°C for 2 hours.<sup>17</sup> Digested fragments were electrophoresed on 10% polyacrylamide gels. A subset of RFLP samples were verified by sequence analysis with a 3130x1 genetic sequencer (Applied Biosystems, Foster City, CA) using BigDye Terminator (Applied Biosystems).

**Ecoregions and statistical analysis.** Level III ecoregion classifications used in the spatial analysis were derived from the United States Environmental Protection Agency and the Tennessee Department of Environment and Conservation collaborative project map.<sup>18</sup> From east to west, the represented ecoregions include the Blue Ridge Mountains, the Ridge and Valley of eastern Tennessee, the Southwestern Appalachians, the Interior Plateau of middle Tennessee, the Southeastern Plains of west Tennessee, and the Mississippi Valley Loess Plains bordering Arkansas and Missouri. The vegetation among ecoregions varies greatly. The Blue Ridge Mountains has the most biodiversity with oak forests, northern hardwoods, spruce-fir forests, hemlock, and oak-pine forests. The Ridge and Valley region consists of oak forests with a multitude of springs and caves, and the Southwestern Appalachians have cropland, pasture, mixed oak, and shortleaf pine forests. The Interior Plateau consists of oak-hickory forest, bluestem prairie, and cedar glades. The Southeastern Plains in west Tennessee is made of cropland, pasture, and oak-hickory-pine forests. The Mississippi Valley Loess Plains is made of irregular plains and oak-hickory-pine forests.<sup>18</sup> Pearson  $\chi^2$  tests were used for statistical analyses.

RESULTS

A total of 1,265 adult and nymphal ticks, including 655 *Amblyomma americanum*, 555 *Dermacentor variabilis*, 38 *Ixodes texanus*, 11 *Ixodes cookei*, 4 *Ixodes scapularis*, and 2 *Amblyomma maculatum*, were tested for rickettsial DNA by real-time PCR. Of these, 401 (32%) ticks were positive for SFGR DNA by real-time and conventional PCR. Of the six tick species tested, only *D. variabilis*, *A. americanum*, and *I. scapularis* produced positive amplicons for rickettsial DNA (Table 1). The overall infection rate for *A. americanum* was significantly higher than that of *D. variabilis* ( $P < 0.001$ ). The sample size of *I. scapularis* was too small to conduct statistical analysis.

A total of 327 *A. americanum* larval ticks were separated into 30 pools and tested for rickettsial DNA by real-time PCR. Twenty-eight pools tested positive for SFGR (Table 2). The

TABLE 1

Prevalence of *Rickettsia* spp. of each tick species by life stage

Tick species*	Males pos/ tested (%)	Females pos/ tested (%)	Nymphs pos/ tested (%)	Total pos/ tested (%)
<i>A. am</i>	57/120 (48)	73/132 (55)	179/403 (44)	309/655 (47)
<i>D. var</i>	47/316 (15)	39/232 (17)	4/7 (57)	90/555 (16)
<i>I. scap</i>	0/2	2/2 (100)	–	2/4 (50)
<i>I. tex</i>	0/1	0/24	0/13	0/38
<i>I. cook</i>	–	0/9	0/2	0/11
<i>A. mac</i>	–	0/2	–	0/2
Total	104/439 (24)	114/401 (28)	183/425 (43)	401/1265 (32)

\* *A. am* = *Amblyomma americanum*; *D. var* = *Dermacentor variabilis*; *I. scap* = *Ixodes scapularis*; *I. tex* = *Ixodes texanus*; *I. cook* = *Ixodes cookei*; *A. mac* = *Amblyomma maculatum*.

TABLE 2

Minimum infection rates (MIR/1000) of larval pools in selected counties in Tennessee\*

County	No. of larval pools	Total no. of larvae	No. positive pools	MIR/1,000
Henry	3	57	2	35.09
Henderson	19	158	19	120.25
Decatur	2	8	1	125.00
Carroll	2	13	2	153.85
Davidson	4	91	4	43.96
Total	30	327	28	85.63

\*MIR = minimum infection rate.

total minimum infection rate (MIR) of lone star ticks in the counties surveyed was 85.63. Higher MIRs were seen in counties with high human RMSF infection rates (120.25–153.85). Lower MIRs were seen in counties with lower RMSF incidence rates (35.09–43.96).

Three rickettsial species were identified by RFLP and sequence analyses. *Rickettsia montana* was identified in 53 (10%) *D. variabilis* from 16 counties and 5 ecoregions (Table 3). *Rickettsia montana* was identified in 2 (0.3%) *A. americanum* from the Southwestern Appalachians and Southeastern Plains ecoregions (Table 4). *Rickettsia amblyommii* was identified in 14 (3%) *D. variabilis* from 7 counties and 4 ecoregions (Table 3), and 259 (40%) *A. americanum* from 23 counties and 5 ecoregions (Table 4). All 28 of the SFGR positive larval pools were positive for *R. amblyommii*. *Rickettsia cooleyi* was identified in 2 (50%) *I. scapularis* from the Mississippi Valley Loess Plains ecoregion (Table 1). There was no observed difference in the prevalence of rickettsiae between male and female ticks of each species, although the prevalence of rickettsial DNA in *A. americanum* nymphs was significantly lower than in *A. americanum* adults ( $P < 0.001$ ).

*Dermacentor variabilis* were predominately collected from raccoons with a prevalence of 92%. *Amblyomma americanum* were predominately collected from white-tailed deer with a prevalence of 94%.<sup>14</sup> Ticks collected from raccoon and skunk wildlife hosts had the highest rate of infection (10% each) with *R. montana*. Ticks collected from white-tailed deer had an infection rate of 60% with *Rickettsia amblyommii*. In addition, ticks collected from cotton drags had an infection rate of 42% with *R. amblyommii* (Figure 1).

Prevalence of *R. amblyommii* in *A. americanum* ranged from 26% to 47% by ecoregion and was significantly lower in Ridge and Valley compared with other regions ( $P = 0.02$ ). *Amblyomma americanum* was not collected from the Blue Ridge Mountains, but *R. amblyommii* was detected in a single

TABLE 3

Prevalence of *Rickettsia amblyommii* and *Rickettsia montana* in *Dermacentor variabilis*—ecoregions listed from west Tennessee to east Tennessee

Ecoregion*	No. <i>D. var</i>	No. <i>R. amb</i> (%)	No. <i>R. mon</i> (%)
MVLP	52	0	2 (4)
SP	95	5 (6)	5 (7)
IP	64	3 (5)	3 (5)
SA	24	1 (4)	1 (4)
RV	301	4 (1)	40 (13)
BRM	19	1 (5)	2 (11)
Totals	555	14 (3)	53 (10)

\* MVLP = Mississippi Valley Loess Plains; SP = Southeastern Plains; IP = Interior Plateau; SA = Southwestern Appalachians; RV = Ridge and Valley; BRM = Blue Ridge Mountains.  
*D. var* = *Dermacentor variabilis*; *R. amb* = *Rickettsia amblyommii*; *R. mon* = *Rickettsia montana*.

TABLE 4

Prevalence of *Rickettsia amblyommii* and *Rickettsia montana* in *Amblyomma americanum*—ecoregions listed from west Tennessee to east Tennessee

Ecoregion*	No. <i>A. am</i>	No. <i>R. amb</i> (%)	No. <i>R. mon</i> (%)
MVLP	15	7 (47)	0
SP	291	110 (38)	1 (0.5)
IP	196	82 (42)	0
SA	100	46 (46)	1 (1)
RV	53	14 (26)	0
BRM	—	—	—
Totals	655	259 (40)	2 (0.3)

\*MVLP = Mississippi Valley Loess Plains; SP = Southeastern Plains; IP = Interior Plateau; SA = Southwestern Appalachians; RV = Ridge and Valley; BRM = Blue Ridge Mountains.

*A. am* = *Amblyomma americanum*; *R. amb* = *Rickettsia amblyommii*; *R. mon* = *Rickettsia montana*.

*D. variabilis* from that ecoregion (Table 3). The prevalence of *R. montana* in *D. variabilis* was significantly higher in the two easternmost ecoregions (8%) compared with the four western ecoregions (2%) ( $P = 0.01$ ).

## DISCUSSION

Despite frequent reports of human RMSF infection in Tennessee, *R. rickettsii*, the etiologic agent of RMSF, was not identified from the 1,265 adult and nymphal ticks and 327 larval ticks collected from 49 counties throughout the state (Figure 2). Previous studies have isolated *R. rickettsii* from *D. variabilis* ticks both in the east coast and the Midwest.<sup>19,20</sup> In 1981 Gordon and others found *R. rickettsii* infection rates in *D. variabilis* ticks to be only 1.3% in Ohio.<sup>19</sup> In Connecticut *R. rickettsii* infection rates in *D. variabilis* ticks were found to be 5.4% in a 1986 study.<sup>20</sup> However, more recent studies are showing *R. rickettsii* isolated from several other tick species. In southern California, Wikswo and others found<sup>8</sup> *R. rickettsii* in *Dermacentor occidentalis* ticks at a low rate of 0.3%. *Rickettsia rickettsii* has also been found in *Rhipicephalus sanguineus* ticks associated with an RMSF outbreak in Arizona.<sup>4</sup>

Additionally, *R. montana* is a non-pathogenic rickettsiae<sup>21</sup> that has previously been found in naturally infected *D. variabilis*.<sup>20,22</sup> *Rickettsia montana* infection in *D. variabilis* has been shown to be as high as 4.2%. It has been shown to interfere with vertical transmission of pathogenic rickettsiae in *D. variabilis* and *R. rickettsii* models,<sup>23</sup> which may partially explain the infrequent detection of *R. rickettsii* in nature. For this reason,

Infection rates of ticks collected by cotton drags and a variety of host species

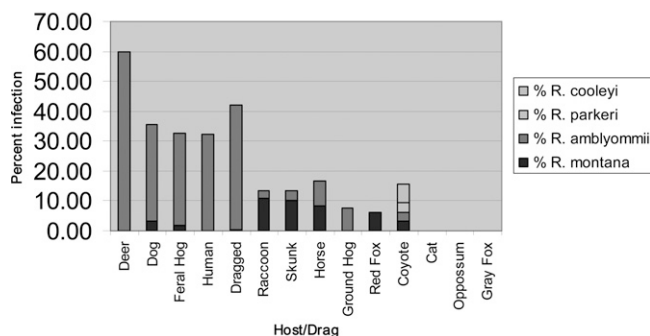


FIGURE 1. Tick infection rates of *Rickettsia* spp. by drag, human, or wildlife host collection.



FIGURE 2. Human Rocky Mountain spotted fever (RMSF) incidence rates by county and tick collection by county.

the observation of increased infection rates of *R. montana* in *D. variabilis* in the two easternmost ecoregions of Tennessee is notable. The average human RMSF incidence rate for those two regions is 0.83 per 100,000, which is significantly lower than the Southeastern Plains of western Tennessee at 4.2 ( $P < 0.001$ ). Ticks are capable of maintaining naturally occurring mixed rickettsial infections as was shown in Ohio in 2006.<sup>24</sup> Although a causal relationship cannot be determined between high incidence of *R. montana* and low incidence of RMSF from this study, ecological factors such as interference may potentially impact the rickettsial transmission cycle.

*Rickettsia cooleyi* was first characterized as a novel rickettsiae from *I. scapularis* in 1998.<sup>24</sup> The pathogenicity of this species remains unclear and it is possible that it causes clinical symptoms distinct from typical RMSF. It may also cause a mild to asymptomatic immune response leading to seroconversion without disease.<sup>25</sup> However, Noda and others<sup>26</sup> suggest that it is an endosymbiont restricted to the ovaries of the tick, thus inhibiting its transmission to host species.

*Rickettsia amblyommii* has been found to frequently infect *A. americanum* throughout the southeastern states, lower Midwest, and coastal New England of the United States.<sup>10,27,28</sup> Since a 1993 study identified *R. amblyommii* as a possible agent of illness among members of a military unit,<sup>29</sup> researchers have begun examining the organism as a possible cause of human illness potentially misdiagnosed as RMSF. In 2007 it was temporally associated with a macular rash when it was detected in an *A. americanum* removed from a patient.<sup>30</sup> More recently, a group in North Carolina obtained serum samples from patients considered probable cases of RMSF and found that some of the patients had higher end-point titers against *R. amblyommii* antigen than *R. rickettsii*.<sup>11</sup>

In our study, the most common rickettsial species in all sexes and stages of ticks was *R. amblyommii*. The finding of *R. amblyommii* in the *A. americanum* larval pools indicates that transovarial transmission of *R. amblyommii* may be occurring. This may be occurring more often in counties of the state where higher incidence of human RMSF has been reported, as indicated by MIR values in high incidence counties (Henderson, Decatur, and Carroll) versus counties of lower relative incidence (Henry and Davidson). In addition, several questing nymphal *A. americanum* and *D. variabilis* ticks were collected that were infected with SFGR, implying that transstadial transmission of *Rickettsia* spp. is also occurring. Thus, some *R. amblyommii* may be maintained in the tick populations of Tennessee by feeding on infected hosts and transovarial and transstadial transmission. A high percentage (38–47%) of

*A. americanum* ticks were infected with *R. amblyommii* in our study in all ecoregions with the exception of the Ridge and Valley ecoregion where 26% of *A. americanum* were infected with this rickettsial species. Interestingly, the lowest incidence of human RMSF is also in the Ridge and Valley ecoregion in eastern Tennessee (Figure 2).

We expected to find *R. rickettsii* in western Tennessee because of the high prevalence of RMSF infection in the region and were surprised not to find any throughout the entire state. Other pathogenic rickettsiae exist in nature and may cause human illness. For example, *R. parkeri* was identified as a human pathogen in 2004<sup>31–33</sup> and was detected in an *A. americanum* from Knox County, Tennessee in 2007.<sup>34</sup> It is possible that the current human surveillance system over-reports RMSF cases based on antibodies, which cross-react against other SFGR, including *R. parkeri* and *R. amblyommii*. Because *R. amblyommii* was found in nearly half of all *A. americanum*, which frequently feeds on humans,<sup>35</sup> and is antigenically related to *R. rickettsii*, a large proportion of patients may test seropositive as a result of exposure to this organism.

Studies have not been able to produce pathology consistent with RMSF infection in animals following artificial infection with *R. amblyommii*.<sup>1,12</sup> If *R. amblyommii* truly is non-pathogenic or only mildly pathogenic, the severe disease observed in western Tennessee remains unexplained by rickettsial species other than *R. rickettsii*. Additionally, active surveillance is currently underway in western Tennessee to determine the etiologic agent of severe disease attributed to RMSF.

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