Human Infections by Multiple Spotted Fever Group Rickettsiae in Tennessee

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Abstract. Rocky Mountain spotted fever is the most common tick-borne disease in Tennessee. However, *Rickettsia rickettsii* has rarely been isolated from endemic ticks, suggesting rickettsioses may be caused by other species. A total of 56 human serum samples that were serologically positive for exposure to *Rickettsia* were obtained from commercial laboratories in 2010 and 2011. In addition, 20 paired sera from patients with encephalitis and positive *Rickettsia* serology were obtained from the Tennessee Unexplained Encephalitis Surveillance (TUES) study. Using an immunofluorescence assay, reactivity of the sera to *R. rickettsii*, *Rickettsia montanensis*, *Rickettsia parkeri*, and *Rickettsia amblyommii* was tested, and a comparison of endpoint titers was used to determine the probable antigen that stimulated the antibody response. Cross-absorption was conducted for 94.8% ($N = 91$) of the samples due to serologic cross-reactivity. Of the commercial laboratory samples, 55.4% ($N = 31$) had specific reactivity to *R. amblyommii* and 44.6% ($N = 25$) were indeterminate. Of the paired TUES samples, 20% ($N = 4$) had specific reactivity to *R. amblyommii*, 5% ($N = 1$) to *R. montanensis*, and 5% ($N = 1$) to *R. parkeri*. Patients with specific reactivity to *R. amblyommii* experienced fever (75%), headache (68%) and myalgia (58%). Rash (36%) and thrombocytopenia (40%) were less common. To our knowledge, this is the first time *R. amblyommii* has been reported as a possible causative agent of rickettsioses in Tennessee.

INTRODUCTION

Spotted fever group (SFG) rickettsiosis is caused by obligate, intracellular, gram-negative bacteria of the genus *Rickettsia* with more than 20 different species that are globally distributed.¹ It is the most common tick-borne disease reported among residents of Tennessee, and Tennessee ranks second in the number of reported cases in 2012.² Most of these cases are classified as probable based on clinically compatible symptoms and a single serologic test with elevated antibodies.

The most serious of the SFG rickettsiosis is Rocky Mountain spotted fever (RMSF) caused by *Rickettsia rickettsii*. It occurs predominantly in the South Atlantic and South Central census regions, with the American dog tick (*Dermacentor variabilis*) serving as the primary vector in these regions.^{2–4} Despite the historical prevalence of RMSF in these regions, *R. rickettsii* is rarely found in ticks. Numerous tick surveys, including those conducted in Tennessee, have shown the absence of *R. rickettsii* but the presence of other SFG *Rickettsia* such as *Rickettsia amblyommii*, *Rickettsia parkeri*, and *Rickettsia montanensis*.^{5–10} The contradicting reports of increasing incidence of SFG rickettsiosis (including RMSF) and the rarity of *R. rickettsii* in ticks raise the likelihood that other SFG rickettsiae species are contributing to this increase in incidence. Previous studies have demonstrated evidence for infection with other less pathogenic SFG rickettsiae species that clinically resemble RMSF. *Rickettsia parkeri* was first confirmed pathogenic in humans in 2002, and although it results in an illness similar to RMSF, it exhibits a less severe clinical presentation of mild fever, multiple eschars, and a maculopapular eruption.^{11–13} *Rickettsia montanensis* is thought to be nonpathogenic in humans but has been reported to cause an afebrile rash illness in a patient after being bitten by a *R. montanensis*-positive tick.¹⁴ Finally, *R. amblyommii* has also been proposed as causing some

of the reported RMSF cases. In North Carolina, sera from probable cases of RMSF demonstrated greater titers for *R. amblyommii* than *R. rickettsii*, and *R. amblyommii* DNA has been sequenced from a tick that caused a rash in a patient.^{15,16}

Although seroprevalence studies of SFG rickettsial infection in the United States have been performed in the past,^{17,18} there have been limited attempts to determine if SFG rickettsiae other than *R. rickettsii* are contributing to the increase incidence of SFG rickettsiosis. In this study, we investigated serologic human exposure to four SFG rickettsiae in Tennessee: *R. rickettsii*, *R. montanensis*, *R. parkeri*, and *R. amblyommii*.

MATERIALS AND METHODS

Human sera collection. Human sera from 56 patients from 2010 to 2011, which tested positive for exposure to *Rickettsia rickettsii* by either enzyme immunoassay or immunofluorescence assay (IFA) in a commercial laboratory, were collected under routine public health surveillance. In addition, paired sera of 20 patients with a clinical syndrome of encephalitis and positive *R. rickettsii* serology were provided by the Tennessee Unexplained Encephalitis Surveillance (TUES) study. Clinical and laboratory data for these patients were obtained from medical records and case report forms.

Indirect IFA. An IFA was performed on each sample to detect reaction to four *Rickettsia* spp. antigens: *R. rickettsii*, *R. montanensis*, *R. parkeri*, and *R. amblyommii*. An IFA procedure was used as described previously with slight modifications.¹⁹ In brief, antigen-coated 12-well slides, prepared as described below, were allowed to equilibrate at room temperature and then blocked in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide for 10 minutes at room temperature. Human sera were diluted 2-fold starting at 1:64 and extended to final titers in a solution of PBS containing 1% BSA, 0.1% Tween 20, and 0.01% sodium azide. Of each dilution, 10 μ L was added to an antigen-coated well on slides for all four *Rickettsia* species and incubated at 37°C for 30 minutes in a humidified

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chamber. Slides were rinsed with a stream of wash buffer containing PBS with 0.1% Tween 20 and washed twice in wash buffer for 10 minutes. Once dry, each well received 10 μ L of fluorescein isothiocyanate-conjugated sheep antihuman IgG (Rockland Inc., Gilbertsville, PA) diluted 1:100 in the diluent solution. Slides were covered to protect fluorescence and incubated for 30 minutes at 37°C in a humidified chamber. Slides were subsequently rinsed and stained by dipping three times in 1% Evans blue solution, washed in distilled water three times, and dried. Finally, a coverslip was mounted to each slide with 90% glycerol, and the slides were observed under a fluorescence microscope at $\times 400$ magnification (Carl Zeiss Inc., Göttingen, Germany).

Sera were considered to contain antibodies against rickettsiae if reactive at a dilution of 1:64 or greater to any species. Because of serologic cross-reactivity among SFG rickettsiae, the presumed infective species, or probable antigen that stimulated the antibody response, had to have an endpoint titer at least two serial dilutions higher than the endpoint titer of any other *Rickettsia* species tested. Each slide included both a positive control of reactive serum and a negative control of diluent solution. The positive control serum was from a patient with confirmed RMSF and with known titers against all four antigens $\geq 1:4,096$. A Giemsa stain was performed to confirm presence of bacteria on the IFA slides.

Antigen preparation. IFA testing conducted at the Tennessee Vector-borne Disease Laboratory used slides prespotted with rickettsiae grown in Vero cells (*R. rickettsii* Sheila Smith, *R. montanensis* M5/6, *R. parkeri* Portsmouth, and *R. amblyommii* WB-82-like North Texas) and corresponding rickettsial lysates for cross-absorption and western blotting, prepared by N. L. Mendell, Department of Pathology, University of Texas Medical Branch at Galveston, Galveston, TX.²⁰ Rickettsiae were cultivated in confluent Vero cells and monitored until the cells were determined to be approximately 70–80% infected. The infected monolayer was scraped, harvested, washed with 0.1 M PBS by centrifugation at $500 \times g$ for 5 minutes, and resuspended in PBS with 1% bovine calf serum. The cell suspension was pipetted onto poly-L-lysine-coated 12-well Teflon-masked IFA slides at 10 μ L per well, allowed to dry, and fixed for 10 minutes with acetone. Each group of rickettsial antigen lysate was generated by scraping an equivalent quantity of each species of uniformly infected (80–100%) Vero cell (2.0×10^7 cells/flask) monolayers and centrifugation at $17,000 \times g$ for 15 minutes to pellet the infected Vero cells. Cells were resuspended in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM L-glutamine, and 2 mM sucrose and lysed by sonication on ice for four 15-second pulses at 60% amplitude to release the rickettsiae. Host cell debris was removed by centrifugation at $1,000 \times g$ for 5 minutes. The supernatant was passed through a 0.22- μ m-pore syringe-driven filter, and cell-free rickettsiae were pelleted at $17,000 \times g$ for 15 minutes and resuspended in PBS with 0.05% sodium azide. Rickettsiae were sonicated on ice for eight 15-second pulses at 60% amplitude to lyse the bacteria.

Serum cross-absorption. For samples whose endpoint titers were less than 4-fold difference, cross-absorptions with the *Rickettsia* antigens were performed to determine the probable antigen that stimulated the antibody response. In brief, sera were diluted 1:32 in diluent solution and mixed 1:1 with an excess of *Rickettsia* antigen lysate (1–1.5 mg/mL) for

20 hours at room temperature on a rotator. The mixture was then centrifuged at $10,000 \times g$ for 11 minutes, and the supernatant was retained for IFA and western blot. Rickettsial antigens of different species were standardized by deriving them from the same number of flasks that were harvested at the same level of infection. Furthermore, contaminated host cell was removed by zonal centrifugation and remaining rickettsial protein was adjusted to a concentration of 1.5 mg/mL.

After cross-absorption, species specificity was identified when incubation with the putative antigen resulted in removal of homologous and heterologous antibody and loss of serum reactivity in downstream IFA, while antigens from cross-reactive strains absorbed homologous antibody only and did not remove serum reactivity. Serologic species specificity in western blot analysis was determined by reactivity of species-specific antigens larger than 110 kDa. In this study, the presumptive causative agent was identified as the antigen that resulted in a ≥ 4 -fold decrease in titers relative to the other antigens after cross-absorption.

Protein gel electrophoresis and western blotting. For each purified *R. rickettsii*, *R. montanensis*, *R. parkeri*, and *R. amblyommii* antigens, 30 μ g were solubilized in RunBlue LDS Sample Buffer and RunBlue DTT Reducer (Expediton Inc., San Diego, CA) at 70°C for 10 minutes. Solubilized antigens were electrophoretically separated on a 4–12% RunBlue SDS gel (Expediton) using the RunBlue SDS Run Buffer (Expediton) for 1 hour at 180 V and then transferred onto a nitrocellulose membrane at 22 V for 40 minutes using the TE 70 Semi-Dry Transfer Unit (Amersham Biosciences Corp., Piscataway, NJ) and the RunBlue TG Blot Buffer (Expediton). Membrane was blocked in 1 $\times$ Tris-buffered saline (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) with 5% nonfat dry milk for 1 hour at room temperature and then incubated with serum diluted 1:500 in blocking buffer overnight at room temperature. Membrane was washed once in 1 $\times$ TBS with 0.05% Tween-20 for 10 minutes followed by another 10 minutes wash with 1 $\times$ TBS only. The membrane was then incubated with a phosphatase-labeled goat antihuman IgG antibody (KPL, Gaithersburg, MD) diluted 1:20,000 in blocking buffer for 1 hour at room temperature. Membrane was washed twice in 1 $\times$ TBS for 10 minutes each, and bands were visualized using the BCIP/NBT Phosphatase Substrate (KPL).

RESULTS

Cross-reactivity between species. A total of 56 single serum samples from commercial laboratories that were collected from a clinic or hospital visit were initially screened by IFA. All of these samples were cross-reactive to at least two species of SFG rickettsiae with titers $\geq 1:64$. Date of symptom onset was available for only 10 (21.7%) of the samples with nine acute sera and one convalescent serum. In addition, 20 unique patients with paired serum samples were also tested from the TUES cohort. Of these samples, only one pair had an acute serum that was reactive to a single species, *R. amblyommii*, with a titer of 1:128. The remaining TUES samples were reactive to at least two species with titers $\geq 1:64$. The majority (95%) of samples had titers less than 4-fold difference between species (Table 1) with the remaining 5% having specific reactivity or 4-fold or greater endpoint titer difference for *R. amblyommii* than the other species. Of those

TABLE 1

Percentages of sera ($N = 96$) with endpoint titers $\geq 1:64$ for all four *Rickettsia* species as determined through initial screening by immunofluorescence assay

Difference in titers between species	%	Species	%
≥ 4 -fold	5	<i>Rickettsia amblyommii</i>	5
< 4 -fold	95	<i>Rickettsia rickettsii</i> , <i>Rickettsia montanensis</i> , <i>Rickettsia parkeri</i> , <i>R. amblyommii</i>	69
		<i>R. montanensis</i> , <i>R. parkeri</i> , <i>R. amblyommii</i>	9
		<i>R. parkeri</i> , <i>R. amblyommii</i>	7
		<i>R. rickettsii</i> , <i>R. montanensis</i> , <i>R. parkeri</i>	3
		<i>R. rickettsii</i> , <i>R. montanensis</i>	2
		<i>R. montanensis</i> , <i>R. amblyommii</i>	2
		<i>R. rickettsii</i> , <i>R. montanensis</i> , <i>R. amblyommii</i>	1
		<i>R. rickettsii</i> , <i>R. parkeri</i> , <i>R. amblyommii</i>	1

samples with cross-reactivity to multiple SFG species, cross-reactivity between all four species was common (69%).

Differential reactivities of commercial laboratory and TUES samples. After cross-absorption of the majority of the samples, 35 had specific reactivity to *R. amblyommii* antigens while one sample each had specific reactivities to *R. montanensis* and *R. parkeri*. However, even after cross-absorption, 39 samples had indeterminate specificities. None of the samples had specific reactivity to *R. rickettsii*. Among the commercial laboratory samples, more reactivity was observed for *R. parkeri* and *R. amblyommii* compared with *R. rickettsii* and *R. montanensis* (Table 2). In comparison, percent reactivity to *R. parkeri* and *R. amblyommii* were lower for the TUES samples. The mean titers of all four species were significantly lower in the commercial laboratory samples than those of the TUES samples. *Rickettsia amblyommii* had the highest mean titer (1:475) within the commercial laboratory samples while *R. rickettsii* (1:2,072) had the highest mean titer for the TUES samples. After cross-absorption, 55.4% of the commercial laboratory samples had specific reactivity to *R. amblyommii* while only 20% of the TUES samples had specific reactivity to *R. amblyommii* in addition to *R. montanensis* (5%) and *R. parkeri* (5%).

Western immunoblotting. One of the commercial laboratory samples with specific reactivity to *R. amblyommii*, based on a greater than 4-fold endpoint titer difference (1:2,048), was analyzed by western blot assay (Figure 1). This patient's serum showed reactivity to nonspecific antigens (bands < 50 kDa) for all four species. However, reactivity was observed with high molecular weight species-specific antigens for *R. amblyommii* and not with the other species. Without the need for cross-absorption, the IFA and western blot results indicate a strong and specific immune response to *R. amblyommii* antigens. This patient was classified as a non-case because the patient did not meet the clinical criteria of having fever. However, the patient did have rash, eschar, and elevation of liver enzymes in addition to a history of a tick bite.

Clinical characteristics of presumptive *R. amblyommii* cases. Of those samples with specific reactivity to *R. amblyommii*, signs and symptoms were queried through clinical records and case report forms (Table 3). Fever was the most commonly reported symptom (75%) among these patients, and the occurrence of rash was less common (36%). Headache and myalgia were also frequently noted ($> 50\%$). Laboratory findings, including anemia and thrombocytopenia were variably present. Eschar was rarely documented on physical exam. Of those patients with a record of tick exposure, 90% reported as having a history of tick bite.

DISCUSSION

This study is the first to demonstrate that SFG rickettsiosis cases reported in Tennessee may be caused by SFG species other than *R. rickettsii*. In addition to *R. rickettsii*, this serosurvey was conducted against other SFG species that are commonly found in endemic ticks, some with known pathogenicity. Among the four species tested, only specific reactivities to *R. amblyommii*, *R. montanensis*, and *R. parkeri* were observed while the rest of the samples were indeterminate. Of all the species evaluated in this study, *R. amblyommii* had the highest percentage of specific reactivity compared with the other species. Even though *R. amblyommii* has never been directly detected in human clinical samples, this finding is not unexpected especially in the context of previously published studies in humans, ticks, and canines. A recent seroepidemiologic study in North Carolina found that species other than *R. rickettsii* are causing seroconversions in paired sera, with six patient samples seroconverting to *R. amblyommii* only and another patient sample exhibiting specific reactivity to *R. amblyommii* after cross-absorption.²¹ In another study from North Carolina, a 4-fold increase in antibody titer to *R. amblyommii* antigens was observed in three of six probable RMSF cases.¹⁵ Thus, these studies indicate that humans are being exposed to *R. amblyommii*, and

TABLE 2

Differential reactivities of commercial laboratory ($N = 56$) and TUES ($N = 20$ paired sera) samples to all four SFG *Rickettsia* species antigens

		<i>Rickettsia rickettsii</i>	<i>Rickettsia montanensis</i>	<i>Rickettsia parkeri</i>	<i>Rickettsia amblyommii</i>
Commercial	Reactivity (%)	82.1	92.9	98.2	98.2
	Mean titer	361.7	262.2	375.9	474.8
	% specific reactivity	0	0	0	55.4
TUES	Reactivity (%)	92.5	97.5	90.0	90.0
	Mean titer	2,072	1,234	1,264	1,340
	% specific reactivity	0	5	5	20.0

SFG = spotted fever group; TUES = Tennessee Unexplained Encephalitis Surveillance. Percent reactivity and mean titer of the sera were based on the initial immunofluorescence assay (IFA) screening while specific reactivity was based on cross-absorption of sera followed by IFA.

FIGURE 1. Western immunoblotting of an immunofluorescence assay (IFA)-positive commercial laboratory sample without cross-absorption. Lanes: a, *Rickettsia amblyommii* antigen; b, *Rickettsia parkeri* antigen; c, *Rickettsia montanensis* antigen; d, *Rickettsia rickettsii* antigen; e, molecular weight marker. Bands < 50 kDa represent nonspecific spotted fever group antigens.

this species might be responsible for cases classified as RMSF. In addition, *R. amblyommii* was detected in a tick that subsequently caused rash at the bite site in a patient, although no other symptoms developed.¹⁶ Furthermore, tick surveys conducted in Tennessee have not detected *R. rickettsii* but *R. amblyommii*, *R. parkeri*, and *R. montanensis* were commonly found.^{5,10} Similarly, tick surveys conducted in neighboring states in the southeastern region revealed the same pattern of the predominance of *R. amblyommii* in *Amblyomma americanum* ticks, the most ubiquitous and aggressive tick species in this region.^{8,15,22,23} Finally, an Oklahoma study in canines used as sentinels for rickettsial diseases, also showed similar results. Dogs were naturally infected with *R. amblyommii* and *R. montanensis* after tick exposure.²⁴

TABLE 3

Symptoms of *Rickettsia amblyommii*-positive cases (N = 35)

Symptoms	%
Fever	75 (24/32)
Headache	68 (15/22)
Myalgia	58 (14/24)
Eschar	43 (6/14)
Anemia	40 (12/30)
Thrombocytopenia	40 (12/30)
Rash	36 (10/28)

Denominators are based on the presence of information on these symptoms from clinical records and case report forms.

Clinical evaluations of patients with specific reactivities to *R. amblyommii* indicated that these patients commonly experienced fever, headache, and myalgia, while anemia, thrombocytopenia, rash, and the presence of eschar occurred < 50% of the time. Previously thought to be nonpathogenic, there is increasing evidence that *R. amblyommii* infection in humans may be symptomatic or may cause a mild illness after a tick bite. In a study by Apperson and others, none of the three patients that seroconverted to *R. amblyommii* experienced rash due to the infection, developed eschars at the site of the tick bite, or required hospitalization.¹⁵ However, a North Carolina woman did develop rash at the site of the bite from an *R. amblyommii*-positive tick.¹⁶ Similarly, in this study, we observed a sample with strong reactivity to *R. amblyommii* as confirmed by IFA and western blotting obtained from a patient who did not have fever but experienced rash and the development of an eschar. In comparison with *R. rickettsii* infection, where fever, headache, and rash are common, infection with *R. amblyommii* does not necessarily lead to the development of fever and the occurrence of rash is less likely. In addition, *R. amblyommii* infection does not lead to a high rate of eschar development unlike *R. parkeri*, where ~90% of patients develop an eschar.¹³ It is also interesting to note that more than half of the clinical samples from commercial laboratories had specific reactivities to *R. amblyommii*, and these samples had lower mean titers and experienced a milder form of illness than the TUES samples, all of which were from patients hospitalized with encephalitis. Although it is important to determine the clinical manifestations of *R. amblyommii* infection, the variability in the completeness of medical records in this study may underestimate the true rates of clinical findings.

As mentioned above, patient samples with specific reactivities to *R. amblyommii* did not all experience fever. Because the national surveillance case definition for SFG rickettsiosis requires the presence of fever for a case to be classified as probable or confirmed, many SFG rickettsiosis infections may be missed. In a case report by McQuiston and others, an afebrile patient bitten by an *R. montanensis*-positive tick did not meet the clinical criteria for probable or confirmed SFG rickettsiosis.¹⁴ Similarly, in this study, we found a patient with a strong specific serologic response to *R. amblyommii*, who was not classified as a case because the patient did not have fever. Therefore, this study further supports the need to reevaluate the national surveillance case definition for SFG rickettsiosis as the true burden of disease may be underestimated.

The IFA is the gold standard for serodiagnosis of SFG rickettsiosis. However, serologic cross-reactivity among the different SFG antigens makes it difficult to infer the specific SFG species causing the immune response. In this study, the majority of samples were cross-reactive to at least two different antigens. To address this issue, cross-absorption was performed to eliminate nonspecific, cross-reacting antibodies. Cross-absorption has been previously used to determine the causative agent of various rickettsial diseases.²⁵⁻²⁷ However, the high number of indeterminate samples even after cross-absorption was unexpected. Of 91 samples that were cross-absorbed, 31 had less than 4-fold difference in titers even after cross-absorbing with all four species and in 20 samples, all of the titers disappeared after cross-absorption. It is possible that these serologic responses may be due to exposure

from another SFG rickettsiae species that was not evaluated in this study. Considering that there are more than 20 different known SFG species worldwide and several of them occurring in North America, some of these immune responses may be caused by known species not tested in this study or by unknown species.¹ In addition, the majority of commercial laboratory samples had missing information on disease onset, and therefore it is not known whether the serum collected was acute or convalescent. Nevertheless, most patients seek medical care during the acute stage of disease when they are still experiencing clinical illness and during which laboratory testing is performed. Therefore, these commercial laboratory sera are most likely acute. And since patients lack specific antibody titers during the acute phase of illness, it is possible that the elimination of all titers after cross-absorption is due to the presence of only nonspecific, cross-reacting antibodies. Finally, one of the drawbacks of cross-absorption is the amount of antigens needed, especially, for sera with significantly high titers. This is true for the TUES samples where very high titers were observed for all four species, and even after cross-absorption, the titers remained high and no 4-fold differences were observed. Although no specific reactivity to *R. rickettsii* was observed, we cannot rule out the possibility that the titers for some of these indeterminate samples may be due to *R. rickettsii* infection.

In this study, we demonstrated that almost half of the samples tested had specific reactivity to *R. amblyommii*, suggesting that *R. amblyommii* rickettsiosis is occurring in the state of Tennessee and is associated with clinical illness. On the basis of our results, infection with *R. parkeri* and *R. montanensis* is also occurring among residents of Tennessee, and further studies are needed to fully understand the burden of other SFG rickettsiosis. Furthermore, because cross-reactivity is very common among the SFG, this study confirms the need for developing species-specific diagnostic methods. Identification of the specific SFG rickettsiae is critical to advancing the understanding of the burden of these infections and improving surveillance for rickettsial illness.

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