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High prevalence of "*Candidatus* Rickettsia andeanae" and apparent exclusion of *Rickettsia parkeri* in adult *Amblyomma maculatum* (Acari: Ixodidae) from Kansas and Oklahoma

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Abstract

Amblyomma maculatum (the Gulf Coast tick), an aggressive, human-biting, Nearctic and Neotropical tick, is the principal vector of *Rickettsia parkeri* in the United States. This pathogenic spotted fever group *Rickettsia* species has been identified in 8–52% of questing adult Gulf Coast ticks in the southeastern United States. To our knowledge, *R. parkeri* has not been reported previously from adult specimens of *A. maculatum* collected in Kansas or Oklahoma. A total of 216 adult *A. maculatum* ticks were collected from 18 counties in Kansas and Oklahoma during 2011–2014 and evaluated by molecular methods for evidence of infection with *R. parkeri*. No infections with this agent were identified; however, 47% of 94 ticks collected from Kansas and 73% of 122 ticks from Oklahoma were infected with "*Candidatus* Rickettsia andeanae" a spotted fever group

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Rickettsia species of undetermined pathogenicity. These preliminary data suggest that "*Ca*. R. andeanae" is well-adapted to survival in populations of *A. maculatum* in Kansas and Oklahoma, and that its ubiquity in Gulf Coast ticks in these states may effectively exclude *R. parkeri* from their shared arthropod host, which could diminish markedly or preclude entirely the occurrence of *R. parkeri* rickettsiosis in this region of the United States.

INTRODUCTION

Amblyomma maculatum (the Gulf Coast tick) is an aggressive, human-biting, Nearctic and Neotropical tick that is distributed widely across many countries in the Western Hemisphere. In the United States, *A. maculatum* is the principal vector of *Rickettsia parkeri*, a bacterial pathogen that causes a febrile, eschar- and rash-associated illness that clinically resembles Rocky Mountain spotted fever (RMSF) (Paddock and Goddard, 2015). Populations of *A. maculatum* occur throughout the southeastern and south-central states and along much of the eastern seaboard. Molecular surveys of Gulf Coast ticks collected from several locations in multiple states within its southern and eastern range reveal estimated rates of infection with *R. parkeri* in 8–52% of questing adult ticks (Sumner et al., 2007, Paddock et al., 2010, Fornadel et al., 2011, Wright et al., 2011, Varela-Stokes et al., 2011, Jiang et al., 2012, Ferrari et al., 2012, Nadolny et al., 2014, Florin et al., 2013, Pagac et al., 2014 and Florin et al., 2014). More than 35 cases of *R. parkeri* rickettsiosis have been identified in patients from 9 states (Paddock and Goddard, 2015).

"*Candidatus* Rickettsiae andeanae" was first described from specimens of *A. maculatum* and *Ixodes boliviensis* collected in Peru (Blair et al., 2004), and subsequently from Gulf Coast ticks in the United States (Paddock et al., 2010) and other tick species in Argentina, Brazil, and Chile (Pacheco et al., 2007, Abaraca et al., 2012 and Nieri-Bastos et al., 2014). "*Ca* R. andeanae" has been isolated recently in culture, although some difficulties remain in establishing continuously infected cell lines (Luce-Fedrow et al., 2012 and Ferrari et al., 2013). To our knowledge, no cases of *R. parkeri* rickettsiosis have been described from Kansas or Oklahoma, despite well-established populations of *A. maculatum* in those states which have existed for more than 40 years. In this study we used molecular techniques to evaluate adult Gulf Coast ticks collected from multiple sites in Kansas and Oklahoma for infections with *R. parkeri* or "*Ca*. R. andeanae".

METHODS

Tick collection and processing

During 2011–2014, questing adult *A. maculatum* ticks were collected from vegetation by using cloth drags or flags at multiple sites in 9 counties of Kansas (Anderson, Butler, Crawford, Geary, Morris, Neosho, Osage, Riley, and Shawnee) and 9 counties of Oklahoma (Cleveland, Cotton, Kiowa, Lincoln, Payne, Osage, Tillman, Tulsa, and Washington). Field-collected specimens were placed in 70% ethanol and transported to the laboratory where these were air-dried, identified using a standard taxonomic key (Keirans and Litwak, 1989), transferred to individual 1.5 ml microcentrifuge tubes, and stored at −80 °C prior to DNA extraction.

Molecular analyses

Genomic DNA was extracted from tick specimens by using a DNA Minikit (Qiagen, Valencia, CA) and eluted in a final volume of 100 μL. Extracted samples were evaluated for DNA of *R. parkeri* and "*Ca*. R. andeanae" using the QuantiTect Multiplex PCR Kit (Qiagen) and primers and probes targeting sequence of the *ompB* gene (Jiang et al., 2012). For each real-time PCR assay, 2.5 μL of tick extract was mixed with 0.4 μM of the forward and reverse primers (Rpa129F and Rpa224R for *R. parkeri*, or Rand957F and Rand1062R for "*Ca*. R. andeanae") and 0.2 μM of the FAM-labeled probe (Rpa188probe for *R. parkeri*, or Rand1003probe for "*Ca.* R. andeane"), in a final reaction volume of 25 μL. Cycling was performed on an Mx3005P thermal cycler (Agilent, Santa Clara, CA) and conditions consisted of 15 min at 95 °C, 45 cycles of 1 min at 95 °C, and 1 min at 60 °C. Ct values <40 were considered positive for the respective agent. Both assays were validated by testing a panel of DNA extracts of *A. maculatum* ticks naturally infected with *R. parkeri* or "*Ca*. R. andeanae", confirmed previously by using a conventional *ompA* PCR assay and sequence analysis (Sumner et al., 2007 and Paddock et al., 2010), or identified negative for DNA of *R. parkeri* and "*Ca*. R. andeanae" by using a broad-range, *Rickettsia* species real-time PCR assay targeting sequence of the *glt*A gene (Stenos et al., 2005 and Denison et al., 2014).

As additional confirmatory steps, a subset of 5 tick extracts from Kansas and 5 from Oklahoma that tested positive for "*Ca.* R andeanae" by the real-time assay were evaluated by using a hemi-nested PCR assay with primers RR190.70 and RR190.701 in the primary reaction and RR190.70 and RR190.602 in the secondary reaction (Sumner et al., 2007), followed by sequence analysis of the amplified segments of the rickettsial *ompA* gene. A subset of 10 *A. maculatum* extracts representing ticks collected from 5 counties in Kansas, and 10 extracts representing ticks collected from 5 counties in Oklahoma, were selected for further analysis to verify the morphological species identification by using a conventional PCR assay with primers T1B and T2A (Beati and Keirans, 2001) and sequencing of the amplified segments of the ixodid mitochondrial 12S ribosomal DNA gene.

RESULTS

A total of 216 adult Gulf Coast ticks were evaluated, comprising 53 male and 41 female specimens from Kansas collected during 2012–2013, and 52 male and 70 female specimens collected from Oklahoma during 2011–2014 (Table 1). Of the Kansas specimens, 51 (54%) were obtained from multiple sites in Geary County during May-July 2013, whereas 87 (71%) of the total Oklahoma specimens were collected from 3 sites in Payne county during 2011–2013 (Fig. 1). Of the 20 tick extracts assessed by PCR and sequencing of a 313-bp segment the ixodid mitochondrial 12S rDNA gene, 6 samples from Kansas and 8 from Oklahoma were identical with each other and revealed 100% identity with *A. maculatum* (GenBank accession number JX192922). An additional 4 tick extracts from Kansas and 2 from Oklahoma were identical with each other, but differed from the other 14 mitochondrial 12S rDNA sequences by 3 nucleotide substitutions and revealed 99% identity with the corresponding GenBank bank sequence for *A. maculatum.*

Of the Kansas ticks, 44 (47%) were infected with "*Ca.* R andeanae", including 29 (56%) of the male specimens and 15 (37%) of the female specimens. Of the ticks collected in

Oklahoma, 89 (73%) were infected with "*Ca*. R. andeanae", including 38 (73%) of male ticks and 51 (73%) of the female ticks. All 10 samples amplified by PCR for a segment the rickettsial *ompA* gene demonstrated 100% identity with the corresponding 597-bp segment of "*Ca.* R. andeanae" strain Agripino Enciso (GenBank accession number KF179352). No specimens from Kansas or Oklahoma demonstrated molecular evidence of infection with*R. parkeri.*

The performance of the real-time assays was assessed by evaluating 49 DNA extracts of *A. maculatum* determined by other molecular methods to be infected with one or neither of these *Rickettsia* species. Of 25 *gltA*-negative *A. maculatum* extracts, none were positive for *R. parkeri* or "*Ca. R. andeanae*" by the real-time assays. Of 14 ticks positive for "*Ca.* R. andeanae" by conventional *ompA* PCR and sequence analysis, all were positive for "*Ca*. R. andeanae", and negative for *R. parkeri* using the real-time PCR assays for these rickettsiae. Of 10 Gulf Coast tick extracts from which *R. parkeri* had been confirmed previously by*ompA* PCR and sequence analysis, all were negative for "*Ca.* R. andeanae" and positive for *R. parkeri* by the real-time assays.

DISCUSSION

In this investigation, we identified infections with "*Ca.* R. *andeanae*" in 62% of Gulf Coast ticks sampled from multiple locations throughout Kansas and Oklahoma during 2011–2014. Surprisingly, we found no evidence of infection with *R. parkeri*, despite evaluating more than 200 ticks collected from 18 counties in these states. These preliminary data suggest that "*Ca*. R. andeanae" is well-adapted to survival in populations of *A. maculatum* in Kansas and Oklahoma and that its ubiquity in Gulf Coast ticks in this region may effectively exclude *R. parkeri* from their shared arthropod host. From a public health perspective, this situation could diminish markedly or preclude entirely the occurrence of *R. parkeri* rickettsiosis in this region of the United States. It remains unknown whether "*Ca*. R. andeanae" elicits infection or disease in humans or animals. It has been suggested that other putative rickettsial symbionts, including *Rickettsia amblyommii* and *Rickettsia montanensis* cause abortive, transient, or subclinical infections in humans, dogs, and other animals (Apperson et al., 2008, Zanetti et al., 2008, McQuiston et al., 2012, Grasperge et al., 2014 and Barrett et al., 2014).

To our knowledge, *R. parkeri* has never been reported from adult *A. maculatum* specimens collected in Kansas or Oklahoma (Jiang et al., 2012 and Barrett et al., 2014; data herein). There is a single description of *R. parkeri* detected in an engorged nymph removed from a cotton rat (*Sigmodon hispidus*) in Pittsburgh County, Oklahoma (Sumner et al., 2007). Inasmuch as the sampling methods were designed only to survey for *R. parkeri* or "*Ca*. R. andeanae" at a point in time, the absence of *R. parkeri* among any adult Gulf Coast tick specimen from any location in these states is unexpected; in contrast, consistently high infection rates with *R. parkeri* have been documented in adult *A. maculatum* from at least 7 other U.S. states (Paddock and Goddard, 2015). For counties represented by larger sample sizes, such as Payne County in Oklahoma and Geary County in Kansas, specimens were collected from several widely separated locations over multiple points in time. Indeed, 77

tick specimens from Payne County were collected from multiple, widely separated sites over 3 years.

During the first half of the 20th century, the U.S. range of *A. maculatum* was defined as a relatively narrow band extending approximately 150 miles inland along the Gulf Coast and eastern seaboard, from Texas to South Carolina. Established populations of *A. maculatum* were subsequently identified in Oklahoma and Kansas during the 1970s (Semtner and Hair, 1973 and Goddard and Norment, 1983). It has been hypothesized that Gulf Coast ticks were introduced to these landscapes by tick-infested cattle transported by ranchers from the Gulf Coast for forage-rich pasturage of the upland prairies, or by migrating birds that follow the Central flyway, which extends through northeastern Kansas to the Gulf Coast of Texas (Ketchum et al., 2009). Although coastal and inland populations of *A. maculatum* are reproductively compatible (Ketchum et al., 2006), these populations differ in genetic haplotypes and seasonal phenology (Ketchum et al., 2009 and Teel et al., 2010) and, as suggested by this study, their predominant rickettsial associates. Our findings are also in agreement with a previous study which identified 2 distinct mitochondrial 12S rDNA haplotypes among Kansas and Oklahoma populations of *A. maculatum* (Ketchum et al., 2009).

Several species of birds that migrate from wintering areas in Central and South America to breeding areas in the United States using the Central and Mississippi flyways are infected with *Rickettsia* sp. Argentina (Mukherjee et al., 2013), which is now recognized as "*Ca*. R. andeanae" (Paddock et al., 2010). Importantly, many of these passerine species, including the indigo bunting (*Passerina cyanea*), the painted bunting (*Passerina ciris*), the common yellowthroat (*Geothlypis trichas*), the rose-breasted grosbeak (*Pheucticus ludovicianus*) and the wood thrush (*Hylocichla mustelina*) also serve as hosts for immature *A. maculatum* ticks (Teel et al., 2010 and Florin et al., 2014). In this context, it is possible that migratory birds deposit large numbers of "*Ca*. R. andeanae"-infected *A. maculatum* ticks across upland prairie habitats of Oklahoma and Kansas. It is also intriguing to speculate that specimens of *A. maculatum* from Kansas and Oklahoma represent ancestral remnants of larger Gulf Coast tick populations that existed throughout the expansive grasslands of the Great Plains prior to European settlement of the United States (Teel et al., 2010). In this context, the remarkably high prevalence of "*Ca*. R. andeanae" observed among these inland populations could reflect an ancient association, rather than a recent introduction of ticks infected predominantly or exclusively with "*Ca*. R. andeanae".

The high rate of infection of *A. maculatum* with "*Ca*. R. andeanae" suggests its close association with Gulf Coast ticks as a facultative or secondary symbiont (Perlman et al., 2006). Similarly high rates of infection with "*Ca*. R. andeanae" (from 64–69%) have been reported in specimens of *Amblyomma parvum* collected in Argentina and Brazil (Pacheco et al., 2007 and Nieri-Bastos et al., 2014). These percentages are also consistent with the frequency of *R. amblyommii*, a vertically-transmitted rickettsial symbiont (Ponnusamy et al., 2014) detected in 37–57% of *Amblyomma americanum* ticks collected in Florida (Mixson et al., 2006 and Sayler et al., 2014), 45–60% in Georgia (Mixson et al., 2006 and Clay et al., 2008), 60–65% in Kentucky (Clay et al., 2008 and Jiang et al., 2010), 66–71% in Maryland (Jiang et al., 2010 and Zhang et al., 2012), 55–60% in North Carolina (Mixson et al., 2006,

Clay et al., 2008 and Smith et al., 2010) and 70–82% in Virginia (Jiang et al., 2010 and Nadolny et al., 2014).

The importance of "*Ca.* R. andeanae" to the epidemiology of tick-borne rickettsioses in the Americas remains to be determined. Cultivation of "*Ca*. R. andeanae" from naturally infected embryonic cells of *A. maculatum* implies that "*Ca*. R. andeanae" is vertically transmitted in Gulf Coast ticks (Ferrari et al., 2013). Multiple studies suggest that hard ticks cannot maintain simultaneously separate *Rickettsia* species by vertical transmission, as demonstrated by the exclusion of transovarial transmission of *Rickettsia rickettsii*by *Rickettsia peacockii* in *Dermacentor andersoni* (Burgdorfer et al., 1981), *Rickettsia rhipicephali* by *R. montanensis* in *Dermacentor variabilis* (Macaluso et al., 2002), and *R. rickettsii* by *Rickettsia bellii* in*Amblyomma dubitatum* (Sakai et al., 2014). The process whereby primary infection by one *Rickettsia* species excludes ovarian infection by another species has been termed rickettsial interference and this microbiological interaction can profoundly affect the distribution of tick-borne diseases. In 1981, Burgdorfer and colleagues identified a nonvirulent *Rickettsia* species in as many as 80% of *D. andersoni* ticks collected from the slopes of the Sapphire Mountains on the eastern side of the Bitterroot Valley (Burgdorfer et al., 1981). This *Rickettsia* species, initially designated as the East Side agent and later named *R. peacockii*, was detected at considerably lower frequencies (8–16%) on the western side of the valley. Importantly, historical data revealed that nearly all of the tick-derived isolates of *R. rickettsii*, and the great majority of human cases of RMSF, originated from the western slopes of the Bitterroot Valley (Niebylski et al., 1997). On a larger geographical scale, rickettsial interference between "*Ca*. R. andeanae" and *R. parkeri* is suggested by the observations in our study, and by surveys from other areas of the United States that demonstrate infrequency of "*Ca.* R. andeanae" among populations of *A. maculatum* where *R. parkeri* infections are common (Table 2). The relative distributions of these *Rickettsia* species among populations of *A. maculatum* from the Great Plains versus *A. maculatum* from southern coastal states may explain the disparate distribution of *R. parkeri* rickettsiosis between these two regions.

Virulent spotted fever group *Rickettsia* species may adversely affect the survival and fecundity of the tick host (Burgdorfer and Brinton, 1975, Niebylski et al., 1999, Labruna et al., 2011 and Soares et al., 2012). *Rickettsia parkeri* negatively impacts the survival of infected *Amblyomma triste* nymphs (Nieri-Bastros et al., 2013). At present there are no data regarding the impact of *R. parkeri* or "*Ca*. R. andeanae" on the fitness of *A. maculatum*; however, it is recognized that vertical transmission places selective pressure on pathogens for low virulence (Fine, 1975 and Yamamura, 1993). Models that incorporate the epidemiology of vertical and horizontal transmission and host demography demonstrate that increasing levels of vertical transmission favor the evolution of lower virulence in pathogens, i.e., when strains of high vertical transmission and low virulence organisms exist in a host, these strains generally out-compete other strains (Lipsitch et al., 1996). Further study of the microbiological interactions between *R. parkeri* and "*Ca*. R. andeanae" that occur within *A. maculatum* could provide valuable insights into the natural history of spotted fever group rickettsioses in the United States.

Our study was limited by small sample sizes from several of the collection sites, with <5 specimens from 5 (56%) of the surveyed counties in Kansas, and 7 (78%) of the locations in Oklahoma (Fig. 1). This investigation focused on adult specimens and it is possible that directed sampling of immature Gulf Coast ticks in Kansas and Oklahoma could produce evidence of *R. parkeri* infecting *A. maculatum* in these states. It is also possible that sampling of *A. maculatum* ticks from counties not represented in our survey might reveal infections with *R. parkeri*, particularly in eastern Oklahoma, as *R. parkeri*-infected Gulf Coast ticks have been described recently from the adjacent state of Arkansas (Trout et al., 2010). Finally, it is possible that a genetic variant of *R. parkeri* exists among Kansas and Oklahoma tick populations that is not detected by the primers or probe used in the real-time assay; nonetheless, this assay has correctly identified strains of *R. parkeri* in tick and human hosts from widely separated locations in the United States, including Arizona, Florida, Mississippi, and North Carolina, and in multiple provinces of Argentina (Romer et al., 2014), suggesting strong conservation of the molecular target. Despite these limitations, these results demonstrate a need for future investigations that explore the dynamics among various spotted fever group *Rickettsia* species within their tick hosts, and how these interactions affect the epidemiology of rickettsioses in human and animal hosts.

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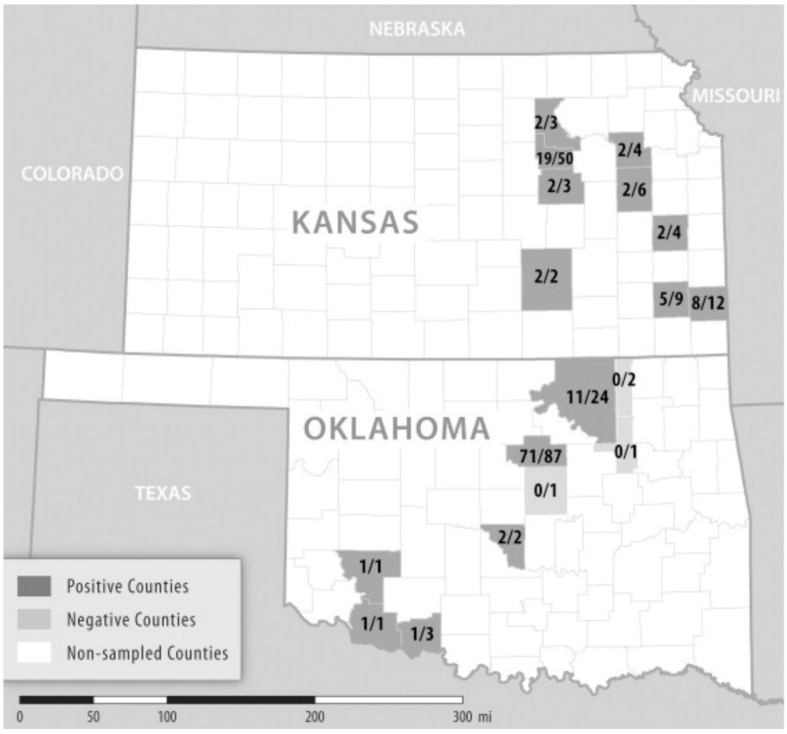

Fig. 1.

Frequency of infection with "*Candidatus* Rickettsia andeanae" among 216 questing adult *Amblyomma maculatum* ticks collected from Kansas (Anderson, Butler, Crawford, Geary, Morris, Neosho, Osage, Riley, and Shawnee counties) and Oklahoma (Cleveland, Cotton, Kiowa, Lincoln, Osage, Payne, Tillman, Tulsa, and Washington counties) during 2011–2014. Fractions represent the numbers of ticks infected with "*Ca*. R. andeanae" over the number of ticks that were evaluated by the real-time PCR assay. No molecular evidence of infection with *Rickettsia parkeri* was identified in any specimen of *A. maculatum* in either state.

Table 1

Molecular evaluation for infection with "*Ca.* Rickettsia andeanae "and *Rickettsia parkeri* in questing adult Gulf Coast ticks (*Amblyomma maculatum*), Kansas and Oklahoma, 2011–2014.

State (No. of counties)	Year	Number of ticks evaluated	Number (%) positive for	
			"*Ca.* Rickettsia andeanae"	***Rickettsia parkeri***
Kansas				
(1)	2012	6	2 (33)	0
(8)	2013	88	42 (48)	0
Oklahoma				
(1)	2011	18	14 (78)	0
(1)	2012	26	20 (77)	0
(2)	2013	44	37 (84)	0
(7)	2014	34	18 (53)	0
Total		216	133 (62)	0

Table 2

Molecular evaluation of adult Gulf Coast ticks (*Amblyomma maculatum*) in the United States for infection with "*Candidatus* Rickettsia andeanae" and *Rickettsia parkeri*, 2000–2014.

State(s)	Year(s)	Number of ticks evaluated	Number (%) positive for			Reference
			"Ca. R. andeanae"	*R. parkeri*	both *Rickettsia* spp.	
Florida	2005–2007	128	2 (2)	28 (22)	0	Paddock et al. 2010
Mississippi	2007	70	3 (4)	27 (39)	0	Paddock et al. 2010
	2008–2010	698	22 (3)	118 (17)	12 (2)	Ferrari et al. 2012
North Carolina	2009–2010	234	9 (4)	68 (29)	1 (0.4)	Varela-Stokes et al. 2011
Virginia	2010–2012	293	1 (0.3)	154 (52)	0	Naldony et al. 2013
Kentucky	2000–2009	10	0	3 (30)	0	Jiang et al. 2012
Kentucky and Tennessee	2012	105	0	15 (14)	0	Pagac et al. 2014
Delaware	2012–2013	26	0	2 (8)	0	Florin et al. 2013, Florin et al. 2014
Kansas	2000–2012	5	4 (80)	0	0	Jiang et al. 2012 E.Y. Stromdahl, pers. comm
	2012–2013	94	44 (47)	0	0	Data herein
Oklahoma	2000–2009	1	1 (100)	0	0	Jiang et al. 2012
	2011–2014	122	89 (73)	0	0	Data herein