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Short communication

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Rickettsia parkeri and "*Candidatus* Rickettsia andeanae" in Questing *Amblyomma maculatum* (Acari: Ixodidae) From Mississippi

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Abstract

Amblyomma maculatum Koch (Acari: Ixodidae), the primary vector for *Rickettsia parkeri*, may also be infected with a rickettsia of unknown pathogenicity, "*Candidatus* Rickettsia andeanae." Infection rates with these rickettsiae vary geographically, and coinfected ticks have been reported. In this study, infection rates of *R. parkeri* and "*Ca.* R. andeanae" were evaluated, and rickettsial DNA levels quantified, in 335 questing adult *A. maculatum* collected in 2013 ($n = 95$), 2014 ($n = 139$), and 2015 ($n = 101$) from Oktibbeha County, MS. Overall infection rates of *R. parkeri* and "*Ca.* R. andeanae" were 28.7% and 9.3%, respectively, with three additional *A. maculatum* (0.9%) coinfected. While *R. parkeri*-infected ticks were detected all three years (34.7% in 2013; 13.7% in 2014; 43.6% in 2015), "*Ca.* R. andeanae" was not detected in 2013, and was detected at rates of 10.8% in 2014, and 15.8% in 2015. Interestingly, rickettsial DNA levels in singly-infected ticks were significantly lower in "*Ca.* R. andeanae"-infected ticks compared to *R. parkeri*-infected ticks ($P < 0.0001$). Thus, both infection rates and rickettsial DNA levels were higher for *R. parkeri* than "*Ca.* R. andeanae." Infection rates of *R. parkeri* were also higher, and "*Ca.* R. andeanae" lower, here compared to *A. maculatum* reported previously in Kansas and Oklahoma. As we continue to monitor infection rates and levels, we anticipate that understanding temporal changes will improve our awareness of human risk for spotted fever rickettsioses. Further, these data may lead to additional studies to evaluate potential interactions among sympatric *Rickettsia* species in *A. maculatum* at the population level.

Key words: *Rickettsia parkeri*, "*Candidatus* Rickettsia andeanae", *Amblyomma maculatum* (Gulf Coast tick), Mississippi

The Gulf Coast tick, *Amblyomma maculatum* Koch (1844), is currently considered native throughout the Western Hemisphere, with North American populations mainly established along the Gulf and Atlantic Coasts of the United States (Teel et al. 2010). Of medical importance, *A. maculatum* is the major tick vector for *Rickettsia parkeri*, an agent of spotted fever group rickettsiosis, of which there are now at least 37 identified human cases in the United States (Paddock and Goddard 2015). Reported infection rates of *R. parkeri* in *A. maculatum* vary in the southern states and may reach up to 56% in some areas (Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Florin et al. 2013, Budachetri et al. 2014, Nadolny et al. 2014, Pagac et al. 2014, Paddock and Goddard 2015, Mays et al. 2016). In addition to *R. parkeri*, *A. maculatum* may be infected with a spotted fever group rickettsia of unknown pathogenicity, "*Candidatus* Rickettsia andeanae," first identified in *A. maculatum* and *Ixodes boliviensis* from Peru (Blair et al. 2004). In the southeastern United States, "*Ca.* R. andeanae"-infected questing *A. maculatum* were reported at infection rates ranging from 1–6.3% (Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Jiang et al. 2012, Leydet and Liang 2013, Budachetri et al. 2014, Paddock and Goddard 2015, Mays et al. 2016). A high prevalence of "*Ca.* R. andeanae" was also recently reported in *A. maculatum* from Kansas and Oklahoma, whereas *R. parkeri* was absent (Paddock et al. 2015). Coinfections of *A. maculatum* with *R. parkeri* and "*Ca.* R. andeanae" are not common, but have been reported (Varela-Stokes et al. 2011, Ferrari et al. 2012, Leydet and Liang 2013, Budachetri et al. 2014), with one report documenting coinfections at a rate higher than expected by random chance (Ferrari et al. 2012).

Among the reports of rickettsiae in *A. maculatum*, rickettsial levels within infected ticks are not well-documented. In the present study, we evaluated infection rates of *R. parkeri* and "*Ca*. R. andeanae" and quantified rickettsial DNA levels in questing adult *A. maculatum* from central Mississippi, over a 3-yr period. By continuing to monitor infection rates and additionally document rickettsial levels of selected pathogenic and (presumably) nonpathogenic tick-associated rickettsiae in this region over time, we provide insight into natural maintenance of these organisms and potential changes in risk for pathogen exposure.

Materials and Methods

Amblyomma maculatum Collections and Assays

Adult questing *A. maculatum* were collected by flagging/dragging in May–September of 2013–2015 from four sites within Oktibbeha County, MS (Fig. 1). The 2013 *A. maculatum* samples were previously from another study (Lee et al. 2014). We identified *A. maculatum* in the laboratory based on a standard taxonomic key (Keirans and Litwak 1989) and kept them in a humidity chamber (saturated potassium nitrate, ~93% humidity) until processing. To reduce external contaminants, we washed *A. maculatum* by vortexing 3 min in each of the following: 0.17% sodium hypochlorite, 0.5% benzalkonium chloride, 70% ethyl alcohol, and sterile phosphate buffered saline (pH 7.4). Ticks were bisected sagittally and genomic DNA extracted from individual halves using a DNeasy Blood and Tissue Kit (Qiagen, Limburg, Netherlands). The other tick halves were archived (−80 °C) and extracted DNA samples stored (−20°C) until testing.

For quality control, DNA extracts were tested in a PCR assay to amplify a fragment of the tick mitochondrial 16S rRNA gene (Black and Piesman 1994). All tick extracts were positive by this assay, and subsequently screened for *R. parkeri* and "*Ca*. R. andeanae" DNA using a TaqMan multiplex quantitative (Q)PCR assay with *Rickettsia*-wide primers and species-specific probes. Primers and probes are listed in Table 1, with the exception that concentrations of both QrompB primers in initial screening were at 300 nM. Nontemplate and positive controls were included in each assay, and reactions tested on a Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA). Extracts that were initially positive by QPCR were re-assayed to quantify rickettsiae by modifying the TaqMan multiplex QPCR to include TaqMan primers and probe for the *A. maculatum* macrophage migration inhibitory factor (MIF) gene (sequences kindly provided by E. Harris and K. Macaluso, Louisiana State University). In each multiplex QPCR assay, 3 µl sample DNA was mixed with Brilliant Multiplex Master Mix 2X (Agilent Technologies), ROX reference dye (30 nM), and probes and primers (Table 1) in a 25 µl reaction volume. We performed QPCR on a

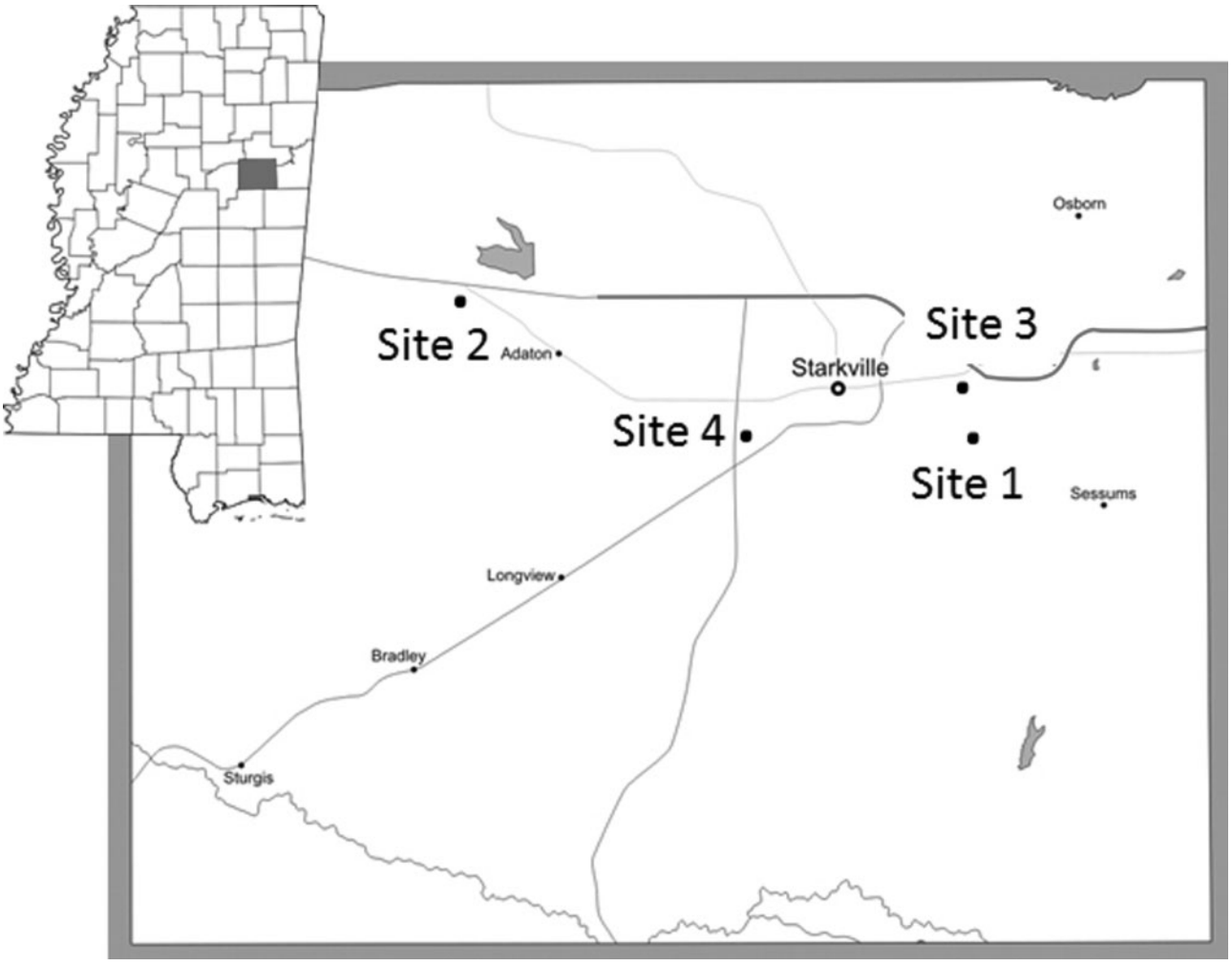

Figure. 1. Geographical location of *A. maculatum* collection site in Oktibbeha County, MS. Site points were based on geographical coordinates.

Table 1. Primers and probes, with final concentrations, used in TaqMan Multiplex QPCR assay for quantification of rickettsial levels

Primer/probe name	Sequence (5′ → 3′)	Final concentration
QrompB_F	AAGTGGTACTTCAACATGGG	400 nM
QrompB_R	GCACCACCTTGGATTAAAG	400 nM
CaRa_probe_FAM	ATCGCGGAAGGTGCTCAAGTTAATG	50 nM
Rp_probe_HEX	ATTTTGGAAGGTGCGCAAGTTAATGC	400 nM
Amac MIF.18F	CCAGGGCCTTCTCGATGT	300 nM
Amac MIF.99R	CCATGCATTGCAAACC	300 nM
Amac MIF.63_Cy5	TGTTCTCCTTTGGACTCAGGCAGC	200 nM

Rickettsial *ompB* sequences used for design of primers and probes were GenBank accession numbers GU131157 and AF123717.

Stratagene Mx3005P with a two-step cycling profile consisting of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All positive extracts were tested in duplicate and all assays included 10-fold dilutions (10^7 to 10^2) of plasmid template mixture combining plasmids constructed using *R. parkeri* GFPuv Oktibbeha strain, "*Ca.* R andeanae" and *A. maculatum*. Nontemplate (water) controls were included in each run for quality control. Only data from multiplex QPCR assays with efficiencies between 90% and 110% for all three targets, and R squared values above or equal to 0.985 were used for evaluating rickettsial levels. We calculated levels in each extract using the ratio of *rompB* copy number to tick MIF copy number, for each rickettsial species. An annual and overall index of coinfection was calculated (Ginsberg 2008) using numbers of *A. maculatum* determined positive for *R. parkeri*, "*Ca.* R. andeanae," or both, by QPCR.

We confirmed all 2015 extracts that were positive by *rompB* multiplex QPCR for one *Rickettsia* sp. ($n = 60$) or both ($n = 1$) by sequencing *rompA* gene amplicons from species-specific PCR assays (Paddock et al. 2010, Varela-Stokes et al. 2011). PCR amplicons were purified (DNA Clean and Concentrator, Zymo Research, CA), bidirectionally sequenced (Eurofins MWG Operon, Huntsville, AL), and sequences aligned (ClustalX2) (Larkin et al. 2007). Consensus sequences were identified using BLAST (Basic Local Alignment Search Tool) analysis in the National Center for Biotechnology Information (NCBI) database.

Statistical Analyses

The occurrence of *R. parkeri* and "Ca. *R. andeanae*" infection in *A. maculatum* was assessed in separate logistic regression using PROC LOGISTIC in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). Gender, year, and gender by year interactions were initially included as explanatory variables. The gender by year interaction was not significant for either outcome and was removed, with the models refit. Penalized maximum likelihood estimation was used for the "Ca. *R. andeanae*" models due to quasi-complete separation of data points because no "Ca. *R. andeanae*" positive ticks were collected in 2013. The effect of year and *Rickettsia* species on rickettsial levels was assessed by ANOVA using PROC MIXED in SAS for Windows 9.4. The two main effects and their interaction were initially included as explanatory variables in the model. The year by *Rickettsia* species interaction was not significant and was removed, with the model refit. Pair-wise comparisons of years were made with Tukey correction of p-values. Due to low sample size for coinfected ticks, a Wilocoxon Signed Rank test using PROC UNIVARIATE in SAS for Windows 9.4 was used to compare "Ca. *R. andeanae*" to *R. parkeri* levels. An alpha level of 0.05 was used to determine statistical significance for all analyses.

Results and Discussion

A total of 335 *A. maculatum* were collected between 2013 and 2015; no significant sex bias was observed in our population. Infection rates of *R. parkeri* and "*Ca.* R. andeanae" in *A. maculatum* varied annually, with overall rates of single infections at 28.7% and 9.3%, respectively (Table 2). The odds of detecting *R. parkeri* in 2014 were significantly lower compared to 2013 (OR 0.3; CI 0.156–0.567) and 2015 (OR 0.2; CI 0.110–0.383). In 2013, 33/95 (34/7%) of *A. maculatum* were positive for *R. parkeri* but "*Ca.* R. andeanae" was not detected. Not surprisingly, the odds of detecting "*Ca.* R. andeanae" in 2014 were significantly higher than in 2013 (OR 24.1; CI 1.435–404.8) and for 2015 compared to 2013 (OR 36.9; CI 2.196–618.649). There was no significant difference in infection rates based on *A. maculatum* gender (*R. parkeri* infection $P = 0.9474$; "*Ca.* R. andeanae" infection $P = 0.6572$). The overall coinfection rate was 0.9% (3/335 coinfected *A. maculatum*). The index of coinfection (IC) was calculated (Ginsberg 2008), with the IC between 0 and -11.9 among study years and an overall IC of -5.42, indicating fewer coinfected ticks than expected by chance alone. Rickettsial levels (calculated as the ratio of rickettsial *ompB* copy number to tick MIF copy number) in positive ticks, varied in singly infected ticks. The level of "*Ca.* R. andeanae" (6.76; SE 1.066) was significantly lower than *R. parkeri* (14.26; SE 0.584; $P < 0.0001$). Rickettsial levels were also significantly different between 2014 and 2013 (Adj $P < 0.0001$), and between 2015 and 2013 (Adj $P < 0.0001$), likely because no "*Ca.* R. andeanae"-infected tick was detected in 2013. However, there was no significant difference in rickettsial levels between 2014 and 2015 (Adj $P = 0.9081$). Finally, there was no significant difference in rickettsial levels between *R. parkeri* and "*Ca.* R. andeanae" in coinfected ticks ($P = 1.0$); however, there were only three coinfected ticks to evaluate.

For 2015 extracts positive only for *R. parkeri* by QPCR, 39/44 had consensus *rompA* sequences 100% identical to available *R. parkeri* (e.g., KF782320.1 and KC003476.1). Rickettsial *ompA* amplicons from all extracts positive for "*Ca.* R. andeanae" by initial QPCR (16/16) were also 100% identical to "*Ca.* R. andeanae" (e.g., KF179352.1 and KF030932.1). Of the five *R. parkeri* samples where a consensus sequence could not be resolved, one extract had one unambiguous sequence which demonstrated 99% identity with *R. parkeri* (e.g., KF782320.1 and KC003476.1). Two samples could not be confirmed by sequencing. For the remaining two samples, consensus sequences were 100% identical to multiple rickettsiae including *R. parkeri* (e.g. KP861344.1), an endosymbiont of *A. maculatum* (KP172268.1), and uncultured *Rickettsia* (e.g., JQ914775.1). We considered all 44 extracts positive for *R. parkeri* based on the two QPCR and specific *rompA* PCR assay results prior to sequencing. For *R. parkeri* and "*Ca.* R. andeanae"-specific *rompA* amplicons in the 2015 coinfected tick, a consensus sequence for the

Table 2. Number of *A. macluatum* [male:female] positive for rickettsial DNA [male:female] out of total *A. maculatum* collected per site in Oktibbeha County, MS, over the 3-yr collection period

Site no.	2013			2014			2015		
	R. parkeri	"*Ca.* R. andeanae"	Coinfected	*R. parkeri*	"*Ca.* R. andeanae"	Coinfected	*R. parkeri*	"*Ca.* R. andeanae"	Coinfected
1	16 [6:10]/45 [18:27]	0 [0:0]/45 [18:27]	0 [0:0]/45 [18:27]	15 [9:6]/66 [35:31]	12 [4:8]/66 [35:31]	2 [1:1]/66 [35:31]	13 [7:6]/42[22:20]	5 [4:1]/42[22:20]	0 [0:0]/42[22:20]
2	17 [8:9]/50 [23:27]	0 [0:0]/50 [23:27]	0 [0:0]/50 [23:27]	2 [0:2]/10 [1:9]	0 [0:0]/10 [1:9]	0 [0:0]/10 [1:9]	0 [0:0]/1[0:1]	0 [0:0]/1[0:1]	0 [0:0]/1[0:1]
3	NS[a]	NS	NS	NS	NS	NS	31[13:18]/58[24:34]	11[4:7]/58[24:34]	1[0:1]/58[24:34]
4	NS	NS	NS	2 [1:1]/63 [37:26]	3 [2:1]/63 [37:26]	0 [0:0]/63 [37:26]	NS	NS	NS
Total	33 [14:19]/95 [41:54]	0 [0:0]/95 [41:54]	0 [0:0]/95 [41:54]	19 [10:9]/139 [73:66]	15 [6:9]/139 [73:66]	2 [1:1]/139 [73:66]	44 [20:24]/101[46:55]	16 [8:8]/101[46:55]	1 [0:1]/101[46:55]

Tick extracts initially positive by *rompB* QPCR were confirmed by *rompA* amplicon sequencing (2015 samples) and final QPCR analysis for rickettsial levels (2013–2015).

[a]Not sampled.

"*Ca.* R. andeanae" *rompA* amplicon was 100% identical to "*Ca.* R. andeanae", whereas one unambiguous sequence direction for the *R. parkeri rompA* amplicon was 99% identical to *R. parkeri.*

Thus, the three-year infection rate for *R. parkeri* (28.7%) was within the range previously reported for southeastern *A. maculatum* (Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Florin et al. 2013, Budachetri et al. 2014, Nadolny et al. 2014, Pagac et al. 2014, Paddock and Goddard 2015, Mays et al. 2016). However, it was higher than that previously reported for similar sites sampled in Mississippi; 19.1% of *A. maculatum* were singly infected with *R. parkeri* in the location labeled "North" reported by Ferrari et al. (2012). While infection rates for "*Ca.* R. andeanae" varied among study years, the overall infection rate of "*Ca.* R. andeanae" here (9.3%), was higher than the previously reported infection rates in the Southeast, and higher compared to the three-year rate (0%) reported for the "North" location by Ferrari et al. (Paddock et al. 2010, Varela-Stokes et al. 2011, Ferrari et al. 2012, Nadolny et al. 2014). Thus, both "*Ca.* R. andeanae" and *R. parkeri* rates were increased in the current study. This may reflect a combination of temporal changes in the natural maintenance of both rickettsiae due to abiotic and biotic factors, and random fluctuation in rates. Still, "*Ca.* R. andeanae" infection in our sampled *A. maculatum* was lower, and *R. parkeri* higher, to rates recently reported from populations in Kansas and Oklahoma (Paddock et al. 2015). While uncommon, *A. maculatum* coinfected with *R. parkeri* and "*Ca.* R. andeanae" have been reported (Varela-Stokes et al. 2011, Ferrari et al. 2012). In the current study, we detected 0.9% coinfected questing *A. maculatum* (3/335). The overall index of coinfection (IC) was −5.42, demonstrating that the coinfection rate was lower than expected by chance alone, in contrast to a previous study from Mississippi (Ferrari et al. 2012). In our study, infection rates of both *R. parkeri* and "*Ca.* R. andeanae" varied, although only two of the four sites within the county were sampled consistently over the three year period; some sites could not be resampled due to human alterations (e.g., construction). The most notable annual fluctuations were with infection rates of "*Ca.* R. andeanae," which did not appear to be negatively correlated to *R. parkeri* infection rates.

Geographical differences in infection rates, particularly the absence of *R. parkeri* and overwhelming presence of *Ca.* R. andeanae" in *A. maculatum* from Kansas and Oklahoma suggest that rickettsial exclusion by transovarial interference may be occurring on a broader population scale (Paddock et al. 2015). Infrequent evidence of "*Ca.* R. andeanae" in *A. maculatum* has been reported where *R. parkeri* is frequently found in this tick vector (Florin et al. 2013, Nadolny et al. 2014, Pagac et al. 2014). We found that the mean rickettsial level for *A. maculatum* singly infected with *R. parkeri* was significantly higher than for *A. maculatum* singly infected with "*Ca.* R. andeanae." This finding suggests that *R. parkeri* are maintained at a higher bacterial load than "*Ca.* R. andeanae" in questing *A. maculatum*. In contrast, the mean rickettsial levels for *R. parkeri* and "Ca. R. andeanae" in coinfected ticks were similar to each other and both low, compared to singly infected ticks. Using a nonparametric test for statistical analysis of these three ticks, we found no difference in rickettsial levels between the two rickettsial species. Considering the presence of "*Ca.* R. andeanae" and observed exclusion of *R. parkeri* in *A. maculatum* populations from Kansas and Oklahoma (Paddock et al. 2015), determining whether rickettsial levels in these populations vary from those detected here would contribute to a better understanding of geographical differences in *A. maculatum*-rickettsial maintenance. Of note, after completion of this study we were made aware that ticks from a laboratory-reared

colony (Oklahoma State University Tick Rearing Facility; OSU) were released on part of Site 2 for an unrelated study by another group. Over the three years, 61 adult *A. maculatum* were collected from Site 2. No tick from this site was positive for "*Ca.* R. andeanae," while 31.1% overall were positive for *R parkeri*. Given that we detected "*Ca.* R. andeanae" but not *R. parkeri* infection in PCR tests of OSU colony ticks in the past, we do not suspect that this release significantly impacted our study. The *A. maculatum* collected were more likely from the endemic population.

In summary, the current study demonstrated disparate mean rickettsial levels and infection rates for *R. parkeri* and "*Ca.* R. andeanae" in *A. maculatum* from Oktibbeha Co., MS. This is significant considering *A. maculatum* is a known and primary vector for the pathogen, *R. parkeri*, and is known to occasionally bite humans (Goddard 2002). Human cases have increased since the first case described in 2004, in part due to increased awareness and reporting (Goddard 2004, Paddock et al. 2004, Goddard and Varela-Stokes 2009, Paddock and Goddard 2015). Currently, the pathogenic potential for "*Ca.* R. andeanae" in *A. maculatum* and human or other vertebrate hosts is unknown. Understanding the relationship between *R. parkeri* and "*Ca.* R. andeanae" in the ticks, and monitoring infection rates and levels, will provide practical information for evaluating changes in human risk for *R. parkeri* rickettsiosis and the role of "*Ca.* R. andeanae" in affecting risk of spotted fever rickettsiosis.

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