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Evaluation of Gulf Coast Ticks (Acari: Ixodidae) for *Ehrlichia* and *Anaplasma* Species

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Abstract

Amblyomma maculatum Koch (the Gulf Coast tick) is an aggressive, human-biting ixodid tick distributed throughout much of the southeastern United States and is the primary vector for *Rickettsia parkeri*, an emerging human pathogen. *Amblyomma maculatum* has diverse host preferences that include white-tailed deer, a known reservoir for *Ehrlichia* and *Anaplasma* species, including the human pathogens *E. ewingii* and *E. chaffeensis*. To examine more closely the potential role of *A. maculatum* in the maintenance of various pathogenic *Ehrlichia* and *Anaplasma* species, we screened DNA samples from 493 questing adult *A. maculatum* collected from six U.S. states using broad-range Anaplasmataceae and *Ehrlichia* genus-specific PCR assays. Of the samples tested, four (0.8%) were positive for DNA of *Ehrlichia ewingii*, one (0.2%) was positive for *Anaplasma platys*, and one (0.2%) was positive for a previously unreported *Ehrlichia* species closely related to *Ehrlichia muris* and an uncultivated *Ehrlichia* species from *Haemaphysalis longicornis* ticks in Japan. No ticks contained DNA of *Ehrlichia chaffeensis*, *Ehrlichia canis*, the Panola Mountain *Ehrlichia*, or *Anaplasma phagocytophilum*. This is the first identification of *E. ewingii*, *A. platys*, and the novel *Ehrlichia* in questing Gulf Coast ticks; nonetheless the low prevalence of these agents suggests that *A. maculatum* is not likely an important vector of these zoonotic pathogens.

Keywords

Amblyomma maculatum ; Gulf Coast tick; *Anaplasma* ; *Ehrlichia*

Amblyomma maculatum Koch (the Gulf Coast tick) is an aggressive human-biting tick found across much of the southern and mid-Atlantic region of the United States, though the recognized distribution of this medically important tick has increased markedly in the past 70 yr (Nadolny et al. 2015). In the United States *A. maculatum* is the primary vector of *Rickettsia parkeri*, a rickettsial human pathogen that causes an illness similar to Rocky Mountain spotted fever (RMSF), and is also infected variably with "*Candidatus* Rickettsia andeanae," a spotted fever group *Rickettsia* of unknown pathogenicity (Paddock and

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Goddard 2015, Paddock et al. 2015). A few surveys also reveal that *A. maculatum* is rarely infected with *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis, and the Panola Mountain *Ehrlichia* (PME), a suspected human pathogen (Reeves et al. 2008, Lee et al. 2014, Loftis et al. 2016).

The Gulf Coast tick has a wide host range, with immature stages typically feeding on ground-nesting birds and rodents, while adults tend to feed on larger mammals, including white-tailed deer (*Odocoileus virginianus*) (Teel et al. 2010), which serve as reservoirs for several species of Anaplasmataceae, including several pathogenic *Ehrlichia* species (Lockhart et al. 1997, Yabsley et al. 2002, Varela-Stokes 2007, Yabsley et al. 2008, Teel et al. 2010, Lobanov et al. 2012, Tate et al. 2013). Herein, we describe a survey of 493 questing adult *A. maculatum* samples collected from six U.S. states to evaluate for infections with *Ehrlichia* and *Anaplasma* species.

Materials and Methods

Tick Collecting and Processing

Questing adult *A. maculatum* were collected from vegetation by using flannel cloth flags during 1999–2015. Specimens collected prior to 2015 were obtained from multiple locations in Georgia, Florida, Kansas, Mississippi, North Carolina, and Oklahoma to survey for *R. parkeri* and "*Ca*. R. andeanae" (Sumner et al. 2007, Paddock et al. 2010, Varela-Stokes et al. 2011, Paddock et al. 2015; Table 1). Ticks collected in 2015 were frozen at −80 °C or preserved in 70% ethanol at ambient temperature until the time of DNA extraction. Species identification was determined by standard taxonomic keys.

DNA Extraction of Ticks and PCR Screening for Anaplasmataceae

DNA was extracted from ticks using a QIAamp DNA Mini kit or DNeasy extraction kit (QIAGEN, Valencia, CA) and eluted into a final volume of 200 µl. Samples collected in 2015 were stored at 4°C until PCR analyses were performed. DNA samples made during 1999–2014 were stored at −80°C until specific tests were performed.

Tick extracts were screened using an Anaplasmataceae-specific real-time PCR assay (Li et al. 2002, Allerdice et al. 2016) and 4 µl of DNA extract. Primers ECHSYBR-F and ECHSYBR-R were used to amplify a 155-bp product of the 16S ribosomal RNA gene. All PCR reactions were conducted in duplicate on a BioRad CFX 96 thermal cycler using the BioRad SsoFast EvaGreen Supermix kit (Life Science, Hercules, CA). Each set of reactions included two negative controls, and *Ehrlichia muris* AS145^{T} extracted from cell culture was used consistently as a positive control. To verify DNA quality, all tick samples were screened using conserved primers T1B and T2A to amplify a 360 bp-portion of the tick mitochondrial 12S rRNA gene as previously reported (Beati and Keirans 2001).

Samples that produced amplicons with the SYBR Green real-time assay were visualized in 1.5% agarose gels containing 0.1 µg/ml ethidium bromide. Amplicons were extracted and purified using the Promega Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). Products were sequenced in both directions and assembled using Sequencher 5.1

(Gene Codes, Ann Arbor, MI). Resultant sequences were compared to GenBank data using BLASTn analysis.

12S rRNA PCR products were also visualized in agarose gels to verify that DNA quality was sufficiently high to be used for PCR. The 12S rRNA gene amplicon was sequenced as described above for those samples that were positive for an Anaplasmataceae species in order to verify species identification of the tick.

Results and Discussion

Of the 493 DNA extracts obtained from adult Gulf Coast ticks collected from six states, only six (1.2%) contained DNA of an Anaplasmataceae species. Four (0.8%) specimens, collected in Copiah County, MS, in 2004 (1), Craven County, NC, in 2010 (1), and Geary County, KS, in 2013 (2) revealed an identical Anaplasmataceae sequence showing >99% identity to the corresponding segment of the 16S rRNA gene of *Ehrlichia ewingii* strain Stillwater (NR_044747). A single tick collected in Franklin County, FL, in 2007 produced an amplicon with >99% identity to the corresponding segment of the 16S rRNA gene of *Anaplasma platys* strain Okinawa (AF536828). One tick sample collected in Neosho County, KS, in 2013 produced a 16S rRNA amplicon (KX365750) showing 95% identity to a noncultivated *Ehrlichia* species detected in *Haemaphysalis longicornis* ticks from Okinawa, Japan (HQ697589) (Matsumoto et al. 2011). No tick samples were found to contain *E. chaffeensis*, PME, *Ehrlichia canis*, or *Anaplasma phagocytophilum*. All ticks included in this study produced amplicons of appropriate size when screened with the 12S rRNA PCR assay. Additionally, all Anaplasmataceae-positive ticks were confirmed as *A. maculatum* by amplification and sequencing of a 360-bp segment of the 12S rRNA gene

To further characterize the previously unreported *Ehrlichia* species detected in the tick from Kansas, analysis was performed using a nested PCR assay targeting a 595-bp segment of the heat shock operon (*gro*EL) (Telford Iii et al. 2011). The amplified portion of the *gro*EL gene (KX365751) exhibited 95% identity to *E. muris* AS145^{T}. A maximum likelihood phylogenetic tree based on this portion of *gro*EL indicates that this sample clusters most closely with *E. muris* and the recently named *E. muris* subspecies *eauclairensis*, formerly referred to as the *E. muris*-like agent (EMLA; Fig. 1) (Pritt et al. 2016).

Ehrlichia ewingii causes granulocytic ehrlichiosis in humans and dogs (Buller et al. 1999, Goodman et al. 2003, Little et al. 2010, Harris et al. 2016, Nichols Heitman et al. 2016) and is transmitted predominantly by *Amblyomma americanum* (Harmon et al. 2015, Sayler et al. 2016). Infection rates in this species range from 0.0–7.6% dependent on location (Wolf et al. 2000, Steiert and Gilfoy 2002, Long et al. 2004, Mixson et al. 2006, Cohen et al. 2010, Gleim et al. 2016, Maegli et al. 2016, Pompo et al. 2016). *Anaplasma platys* is a recognized cause of disease in dogs and less commonly in humans (Arraga-Alvarado et al. 2014, Breitschwerdt et al. 2014) and is believed to be transmitted primarily by ticks in the *Rhipicephalus sanguineus* group (Ramos et al. 2014).

DNA of *E. ewingii* has been detected previously in partially engorged adult *A. maculatum* collected from white-tailed deer (Mays et al. 2016); however, to our knowledge, this is

the first report of *E. ewingii* and *A. platys* detected in questing adult *A. maculatum*. Additionally, a novel *Ehrlichia* species was detected in an adult tick collected in Kansas. While the pathogenic EMLA identified in the upper Midwestern United States has recently been described as a subspecies of *E. muris* (Pritt et al. 2016), it could be possible that other *E. muris*-like agents exist in the United States outside of EMLA's limited range of Wisconsin and Minnesota.

The low percentages of *A. maculatum* adults infected by *Ehrlichia* and *Anaplasma* species are not unexpected based on previous reports and are consonant with the ecology and life history of the Gulf Coast tick. Unlike pathogenic tick-borne SFG *Rickettsia* species, *Ehrlichia* and *Anaplasma* species are not transmitted transovarially (Stich et al. 1989, Long et al. 2003). However, these bacteria are transmitted transstadially between the feeding stages of their tick vectors. This aspect of their ecology suggests that transmission of Anaplasmataceae from *A. maculatum* to humans can occur only when larval or nymphal stage ticks acquire the infection from a bacteremic reservoir host. The immature stages of *A. maculatum* feed most commonly on various species of ground-nesting birds and cotton rats (*Sigmodon hispidus*) (Teel et al. 2010, Paddock and Goddard 2015), none of which are recognized as reservoirs or amplifying hosts for *Ehrlichia* or *Anaplasma* species. In this context the risk of transmission of pathogenic Anaplasmataceae to humans by this tick is likely to be low.

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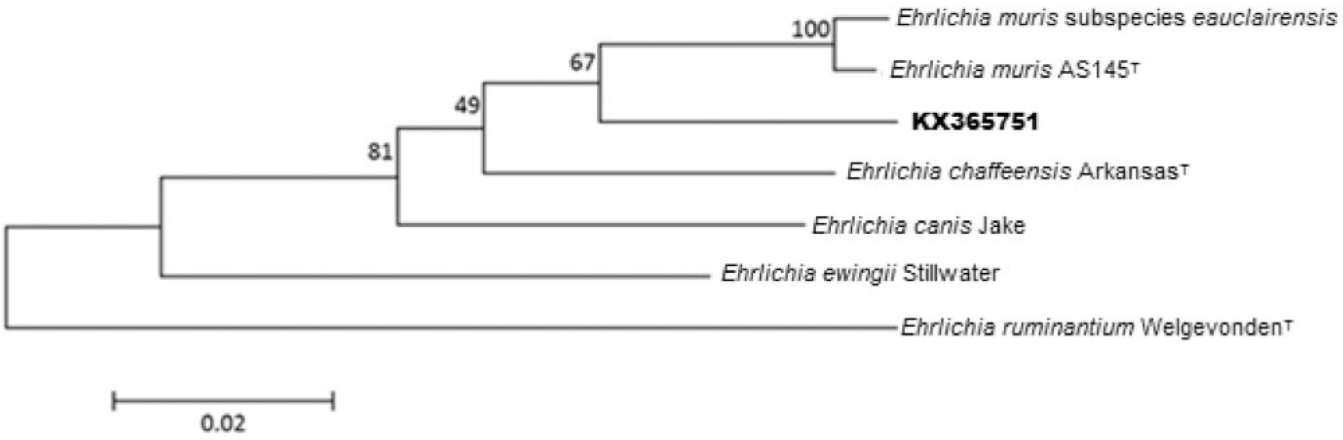

Fig. 1.

Molecular phylogenetic analysis was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (−1631.2822) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved seven nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 594 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

Table 1.

Collection locations for ticks used in this study

State	Year collected	Females	Males	Total
Georgia	1999	3	9	147
	2003	7	4	
	2005	3	4	
	2015	66	51	
Florida	2004	13	9	130
	2005	16	10	
	2007	40	42	
Mississippi	2003	7	3	77
	2004	5	4	
	2007	34	24	
Kansas	2012	1	5	70
	2013	32	32	
North Carolina	2009	23	18	41
Oklahoma	2013	18	10	28