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A snapshot of the microbiome of *Amblyomma tuberculatum* ticks infesting the gopher tortoise, an endangered species

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Abstract

The gopher tortoise tick, *Amblyomma tuberculatum*, has a unique relationship with the gopher tortoise, *Gopherus polyphemus*, found in sandy habitats across the southeastern United States. We aimed to understand the overall bacterial community associated with *A. tuberculatum* while also focusing on spotted fever group *Rickettsia*. These tortoises in the Southern Mississippi region are a federally threatened species; therefore, we have carefully trapped the tortoises and removed the species-specific ticks attached to them. Genomic DNA was extracted from individual ticks and used to explore overall bacterial load using pyrosequencing of bacterial *16S rRNA* on 454-sequencing platform. The spotted fever group of *Rickettsia* was explored by amplifying rickettsial outer membrane protein A (*ompA*) gene by nested PCR. Sequencing results revealed 330 bacterial operational taxonomic units (OTUs) after all the necessary curation of sequences. Four whole *A. tuberculatum* ticks showed Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes as the most dominant phyla with a total of 74 different bacterial genera detected. Together Rickettsiae and Francisella showed >85% abundance, thus dominating the bacterial community structure. Partial sequences obtained from *ompA* amplicons revealed the presence of an uncharacterized *Rickettsia* similar to the Rickettsial endosymbiont of *A. tuberculatum*. This is the first preliminary profile of a complete bacterial community from gopher tortoise ticks and warrants further investigation regarding the functional role of Rickettsial and Francisella-like endosymbionts in tick physiology.

Keywords

Amblyomma tuberculatum; gopher tortoise; ticks; endosymbionts; microbiome; *Rickettsia*

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INTRODUCTION

The *Amblyomma tuberculatum* (Marx) is commonly known as gopher tortoise tick because it specifically parasitizes the gopher tortoise (*Gopherus polyphemus*), a threatened species according to Endangered Species Conservation Act of 1973 (Figure 1A-1D). The gopher tortoise is found across the southeastern United States in a unique habitat of deep sandy soils and frequently observed with infestation of both nymphal and adult *A. tuberculatum* (Ennen and Qualls, 2011), and has been noted that both the tortoise's and the tick's geographical distributions are intricately related with the other (Cooney and Hays, 1972; Ennen and Qualls, 2011).

The microbiome has been linked to several human diseases such as obesity and inflammatory bowel diseases (Turnbaugh and Gordon, 2009). In invertebrates, studies suggests that the microbiome or a particular bacteria may be responsible for regulating animal behavior and vice versa (Ezenwa et al., 2012). Such investigations of the microbiome and metagenomics have revealed previously unanswered biological questions, like discovering cellulose digesting microbes in pandas that are responsible for helping in digestion (Zhu et al., 2011). Likewise, there could be a possible role of the symbiotic bacteria present in blood sucking arthropods to aid in innate immunity development or blood digestion (Eleftherianos et al., 2013). Development of next-generation sequencing platforms have made it easier to investigate bacterial communities associated with different environmental samples and differing physiological conditions which were previously unamenable with only culture-based techniques (Mardis, 2008). Here, we have studied the bacterial community of *A. tuberculatum*, an obligate hematophagous arachnid, and have reported that its bacterial community is predominated by two bacterial endosymbionts of the *Rickettsia* and *Francisella* genera. Though functional significance of these tick endosymbionts is not known, these two genera were also abundantly present in *Amblyomma maculatum* ticks found within the same geographical region (Budachetri et al., 2014; Noda et al., 1997). This study provides baseline information about bacterial associations in this unique tick species and emphasizes the consideration of studying of *Rickettsia* and *Francisella* endosymbionts in future studies.

MATERIALS AND METHODS

Ticks

All ticks were collected directly from gopher tortoises that were trapped using a collapsible Tomahawk® Model 18 Live Trap (81.28 × 25.4 × 30.48) covered with burlap and pine needles to provide shade. The site of collection was from Wiggins Airport (Wiggins, MS) and Crossroads site within the DeSoto National Forest, MS. Ticks were manually pulled from the tortoise using forceps and placed directly into a 50 mL centrifuge tube with a moistened paper towel until preservation. Traps were checked two to three times daily, ticks collected, and tortoises placed directly back into their burrow of capture. A total of 18 partially-fed female ticks were collected from 12 gopher tortoises. The partially-fed ticks were brought into lab and cleaned by alternating distilled water and 70% ethanol. Ticks were identified using standard morphological keys. Tick dissection was performed using routine procedure under a dissection microscope with a scalpel. Institutional Animal Care and Use

Committee (IACUC) at the University of Southern Mississippi approved all animal capture protocols.

Tick DNA extraction

Genomic DNA was extracted from individual whole ticks (n=18) and tick midguts (n=2) using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), following the manufacturer's protocol.

454-pyrosequencing

Out of eighteen female and two female tick midguts, only four whole female ticks were chosen to observe representative microbiome in whole ticks pulled out from different tortoises. The female tick midguts were chosen to get idea of differential bacterial profile within tick. Tick DNAs were individually processed for the bacterial tag-encoded titanium amplicon pyrosequencing (bTETAP) approach (Dowd *et al.*, 2008a). In a modified version of this process, *16S* universal Eubacterial primers, 530F (5'-GTG CCA GCM GCN GCG G) and 1100R (5'-GGG TTN CGN TCG TTG), were used for amplifying the 600 bp region of *16S rRNA* genes. A single step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used under the following conditions: 94°C for 3 minutes, followed by 32 cycles of 94°C for 30 seconds, 60°C for 40 seconds, and 72°C for 1 minute, followed by a final elongation step of 72°C for 5 minutes. Following PCR, all amplicon products from each individual sample were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents according manufacturer's guidelines.

Sequences processing

The sequences obtained were curated on a proprietary analysis pipeline (www.mrdnalab.com, MR DNA, Shallowater, TX). Sequences were depleted of barcodes and primers, followed by the removal of short sequences (<200 bp), sequences with ambiguous base calls, and sequences with homopolymer runs exceeding 6 bp. Sequences were then de-noised and chimeras removed. Operational taxonomic units were defined after removal of singleton sequences showing clustering at 3% divergence, or 97% similarity (Dowd *et al.*, 2008a, 2008b, 2011; Edgar, 2010; Eren *et al.*, 2011; Swanson *et al.*, 2011). The remaining sequences were analyzed using BLASTn against GreenGenes databases (DeSantis *et al.*, 2006). The obtained similarities were assigned to taxonomic classification for bacteria. Taxonomic levels were assigned based on identity of sequences to reference databases: >97% identity to define species, between 95-97% for genus, 90-95% for family, 80-85% for order, 77-80% for phyla and <77% considered as unclassified. All the raw reads were submitted to GenBank under Bio-Project PRJNA288043.

Spotted Fever Group *Rickettsia* Detection (SFGR)

Using the *rompA* gene (Rickettsial outer membrane protein A), identification of SFGR was analyzed by nested PCR reaction. The method for this PCR assay was previously described (Budachetri *et al.*, 2014). Briefly, in the primary reaction, 150 ng of DNA template was

added to 2X PCR Master mix (Promega, Madison, WI) and 400 nM of each the forward primer 5'-ATGGCGAATATTTCTCCAAAA-3' and the reverse primer 5'-GTTCCGTTAATGGCAGCATCT-3' were added. The second nested PCR used the same materials except for the primary reaction being used as a template and the primers used: forward 5'-AAGCAATACAACAAGGTC-3' and reverse 5'-TGACAGTTATTATACCTC-3'. All PCR reactions were performed in MyCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA) using the following thermal cycler parameters: one cycle 95°C for 3 min, followed by 35 cycles of 95°C for 20s, 46°C for 30s, and 63°C for 60s, and one last cycle of 72° for 7 min. Two negative controls (non-template and no primer) and one positive control (50ng of a known SFGR) were included for each reaction run. The amplicons (540 bp) were analyzed on a 2% agarose gel containing SybrSafe DNA gel stain (Invitrogen, Eugene, OR) and observed using an ultraviolet transilluminator. The PCR products were purified (Qiagen, Valencia, CA) and sequenced at Eurofins MWG Operon (Huntsville, AL). The sequences obtained were compared using NCBI BLAST program against the non-redundant database. The nucleotide homology results were assigned for each sequence and the partial *rompA* sequences were deposited to GenBank with the following accession number, JQ695844-54.

RESULTS AND DISCUSSION

In this study, we defined the microbiota associated with the adult female tick *A. tuberculatum* via *16S rRNA* gene hypervariable region V1-V3 sequencing via 454-pyrosequencing (Roche, USA). A total of 22,947 sequence reads from four *A. tuberculatum* were obtained; our data revealed a total of 330 bacterial OTUs comprising 330 taxonomical classifiable species. Additionally, we sequenced tick midguts (n=2) and obtained 9058 sequences. The bacterial diversity associated with *A. tuberculatum* is presented here at taxonomical levels as dominant bacterial phyla, families, and genera.

Of the twelve total bacterial phyla reported present in the whole *A. tuberculatum* tick samples, the Proteobacteria, Actinobacteria and Firmicutes were found to be the most dominant. The percent abundance varied with 89% Proteobacteria, 9% Actinobacteria, and only 1% Firmicutes, which is similar to the pattern of phyla prevalence observed in the *microbiome* of *A. maculatum* ticks obtained from the same geographical region (Budachetri et al., 2014). The presence of dominant species belonging to the phylum *Proteobacteria* in the microbiota of invertebrates has been widely reported (Arias-Cordero et al., 2012; Vicente et al., 2013) and is further supported by our observation of only Proteobacteria in the tested tick midgut samples.

There were a total of 55 bacterial families observed in the *A. tuberculatum* whole tick microbiome. Only eight bacterial families showed more than 1% abundance (Figure 2), with the most dominant being Rickettsiaceae (31 OTUs) and Francisellaceae (24 OTUs). The heatmap in Figure 2 shows the relative prevalence of these dominant tick bacterial families across the tick samples. Only Rickettsiaceae was observed in individual tick midgut samples (~98%). Overall, there were 74 bacterial genera were present in the whole *A. tuberculatum* tick samples. Of these, Rickettsia (55.8%), Francisella (35.2%), Dietzia (2.4%), Arthrobacter (1.1%), and Acinetobacter (1.1%) were the dominant genera observed based on number of reads, while the remainder of reported bacterial genera were less than 1%

(Supplementary Table 1). However, in tick midgut tissue samples, only *Rickettsia* (~99%) were present. The dominant presence of *Rickettsia* and *Francisella* in whole tick microbiome and only *Rickettsia* detected in tick midguts is an interesting observation. We believe that the dominance of *Francisella* reads found in whole ticks were likely due to colonization of *A. tuberculatum* salivary glands, as detected in *A. maculatum* microbiome (Budachetri *et al.*, 2014). Additionally, the presence of *Francisella* have been reported in high abundance from *Dermacentor* tick species, including *Dermacentor variabilis*, *D. andersoni*, *D. hunter*, *D. nitens*, *D. occidentalis* and *D. albipictus* (Niebylski *et al.*, 1997; Sun *et al.*, 2000; Scoles, 2004). The high abundance of *Rickettsial* and *Francisella*-like endosymbionts in tick species with unique hosts was similar to that of *A. maculatum* (Budachetri *et al.*, 2014), but a combination of *Coxiella*-like and *Rickettsial* endosymbionts were most abundant in *Amblyomma americanum* ticks (Clay *et al.*, 2008).

Rickettsia and *Francisella* endosymbionts are transovarially transmitted as well as transstadially maintained across the tick developmental cycles (Baldrige *et al.*, 2009; Wright *et al.*, 2015). The *Rickettsia* and *Francisella* endosymbionts may not act like pathogenic species from these genera. *Rickettsia rickettsii* and many other *Rickettsia* and *Francisella tularensis* found in ticks are pathogenic to humans (Parola *et al.*, 2005; Petersen *et al.*, 2009). The *Rickettsia rickettsii* was known to be lethal to its tick vector *Dermacentor andersoni* (Niebylski *et al.*, 1999). At the same time, however, *Rickettsial* endosymbiont from *Ixodes pacificus* and *Ixodes scapularis* appear to help its host in *de novo* biosynthesis of folic acid required for growth and development (Hunter *et al.*, 2015; Kurtti *et al.*, 2015). The necessity of symbiont for nutritional supplemental has been described in many hematophagous arthropods including ticks (Rio *et al.*, 2016). In an interesting study, *Rickettsia*-infected whiteflies developed high survival rates and faster, higher fecundity when compared to whiteflies lacking *Rickettsia* infection, suggesting the potential role of these endosymbionts in the survival in particular genetic line (Cass *et al.*, 2016; Himler *et al.*, 2011).

On the other hand, the sequencing of partial *ompA* amplicons obtained with nested PCR showed the presence of an uncharacterized *Rickettsia* similar to the *Rickettsial* endosymbiont of *A. tuberculatum*. Of the 18 ticks' DNAs subjected to PCR, 11 ticks' DNAs (>60% of total tested ticks) showed amplification of the *Rickettsia ompA* gene. The discovery of an uncharacterized *Rickettsia* warrants further investigation as there are increasing numbers of new *Rickettsia* species, many of which, upon characterization, are being found to be pathogenic to humans and animals following transmission by ticks (Walker and Ismail, 2008). Zemtsova *et al.* (2012) reported the discovery of an uncharacterized *Rickettsia* to be similar to *Rickettsia parkeri* by sequence study using four genes *ompA*, *ompB*, (*gltA*), and (*sca4*). *R. parkeri* is a disease-causing agent that creates a mild infection similar to Rocky Mountain spotted fever in humans (Paddock *et al.*, 2008, 2004). However, the pathogenicity of identified *Rickettsial* endosymbionts cannot be assessed without culture and further assays, which at this time is not capable by our lab.

Lastly, we must consider the prevalence of soil-associated bacteria found in the *A. tuberculatum* microbiome, which includes the genera *Dietzia*, *Arthrobacter*, *Acinetobacter* and *Gordonia*. Due to the thorough washing of the ticks prior to DNA extraction, we cannot

directly state that these soil bacteria are present due to surface contamination of the tick. Similarly, the prevalence of soil bacterial genera in the tick samples cannot be ruled out due to the sandy soil habitat of the gopher tortoise (Arenskotter et al., 2004; Koerner et al., 2009; Lal and Khanna, 1996). As with the uncharacterized *Rickettsia* identified in this study, our identification of soil bacteria leaves more questions of the microbial community and interactions within ticks can only be explained with future studies.

While the mere existence of bacteria in the tick microbiome does not explain their functional significance in the tick's life cycle and hematophagy, the depletion of microbiome in ticks using antibiotics suggested their possible role in reproductive fitness (Zhong et al., 2007). This is the first report providing evidence of baseline microbiome of *A. tuberculatum* infesting on the gopher tortoise. Our results showed highly dominant Rickettsial and Francisella endosymbionts which were also detected in *A. maculatum* (Budachetri et al., 2014). The identification of an uncharacterized *Rickettsia* in *A. tuberculatum* warrants further study to determine if its presence could be pathogenic to the ticks or host or serves as a primary endosymbiont that aids in this tick's survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1. *Amblyomma tuberculatum* adults parasitizing gopher tortoises

Collection of adult *A. tuberculatum* ticks from gopher tortoises collection was from located at Wiggins Airport (Wiggins, MS) and the Crossroads site within the Mississippi DeSoto National Forest (A-C). Pictures were taken prior to the removal of *A. tuberculatum* ticks from the tortoise. Distribution of the gopher tortoise and their decline in number has them listed as Federally Threatened across multiple states in the southeastern United States (D).

Figure 2. Bacterial family level profile from gopher tortoise ticks

Bacterial sequences representing bacterial families were transformed into percentages and those representing less than 1% were eliminated. The bacterial families were clustered based on Bray Curtis distance matrix and the heatmap was produced using method in R-statistical package.