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An Insight Into the Microbiome of the *Amblyomma maculatum* (Acari: Ixodidae)

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Abstract

The aim of this study was to survey the bacterial diversity of *Amblyomma maculatum* Koch, 1844, and characterize its infection with *Rickettsia parkeri*. Pyrosequencing of the bacterial 16S rRNA was used to determine the total bacterial population in *A. maculatum*. Pyrosequencing analysis identified *Rickettsia* in *A. maculatum* midguts, salivary glands, and saliva, which indicates successful trafficking in the arthropod vector. The identity of *Rickettsia* spp. was determined based on sequencing the rickettsial outer membrane protein A (*rompA*) gene. The sequence homology search revealed the presence of *R. parkeri*, *Rickettsia amblyommii*, and *Rickettsia endosymbiont* of *A. maculatum* in midgut tissues, whereas the only rickettsia detected in salivary glands was *R. parkeri*, suggesting it is unique in its ability to migrate from midgut to salivary glands, and colonize this tissue before dissemination to the host. Owing to its importance as an emerging infectious disease, the *R. parkeri* pathogen burden was quantified by a *rompB*-based quantitative polymerase chain reaction (qPCR) assay and the diagnostic effectiveness of using *R. parkeri* polyclonal antibodies in tick tissues was tested. Together, these data indicate that field-collected *A. maculatum* had a *R. parkeri* infection rate of 12–32%. This study provides an insight into the *A. maculatum* microbiome and confirms the presence of *R. parkeri*, which will serve as the basis for future tick and microbiome interaction studies.

Keywords

Amblyomma maculatum; *Rickettsia parkeri*; microbiome

Ticks transmit a variety of pathogens and are second only to mosquitoes in human and veterinary health importance (Sonenshine 1991). *Amblyomma maculatum* Koch, 1844, has emerged as an important arthropod of public health significance because of its competence as a vector for *Rickettsia parkeri* and experimental vector of *Ehrlichia ruminantium. R. parkeri* is the causative agent of human rickettsiosis (Paddock et al. 2010), and *E.*

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ruminantium is the etiological agent of a fatal cattle disease in South America and Africa (Uilenberg 1982). *A. maculatum* is distributed along the Atlantic and Gulf Coast region of the United States and is also present in several Central and South American countries (Teel et al. 2010). Bird migration and livestock transportation are two important factors affecting the distribution of *A. maculatum* (Hasle et al. 2009) and represent a serious threat in importing tick-borne diseases into the United States (Uilenberg 1982).

Ticks also harbor various nonpathogenic microbial organisms; however, knowledge of these microbial communities associated with ticks remains largely unknown because of limitations in culture-based techniques. Bacterial ribosomal-based sequence analysis ("metagenomics") has revolutionized the exploration of microbial communities in complex environments (Dowd et al. 2008a,b). This method has been successfully used to characterize the metagenome of *Ixodes ricinus* L., *Rhipicephalus microplus* (Canestrini, 1888), and *Amblyomma americanum* (L.) (Andreotti et al. 2011, Carpi et al. 2011, Menchaca et al. 2013), and has revealed a rich bacterial diversity in ticks, but with limited understanding of the functional significance of the associated bacterial communities. The bacterial genera *Stenotrophomonas*, *Pseudomonas*, *Rhodococcus*, and *Propriobacterium* have consistently been identified in tick tissues. Ticks are also frequently associated with various pathogenic bacteria of the *Borrelia*, *Rickettsia*, *Ehrlichia*, and *Anaplasma* genera, various bacterial endosymbionts, or both, which can have commensal, mutualistic, or parasitic relationships with ticks (Noda et al. 1997, Sacchi et al. 2004, Scoles 2004).

Tick-borne rickettsial diseases are caused by two groups of intracellular bacterial species belonging to 1) the spotted fever group of the genus *Rickettsia* (spotted fever group *Rickettsia* (SFGR); Raoult and Roux 1997), and 2) species from the *Anaplasma* and *Ehrlichia* genera (Dumler et al. 2001). Rickettsiae are obligatory intracellular gram-negative α-proteobacteria that are disseminated by arthropod vectors and affect an estimated one billion people worldwide (Parola et al. 2005, Walker and Ismail 2008). Ticks are the important reservoir of the SFGR (Raoult and Roux 1997).

This metagenomics study begins to investigate the functional role of microbial communities in organismal biology. The microbial community plays important roles in pathogen transmission, vector competence (Burgdorfer et al. 1973, Clay et al. 2008, Vilcins et al. 2009), and tick reproductive fitness (Zhong et al. 2007), and likely has other undiscovered roles in vector ecological and physiological adaptation. In this study, we examined the microbiome associated with blood-fed *A. maculatum* and further screened for SFGR agents. This is the first report cataloging the microbial diversity associated with *A. maculatum*-isolated tissues during pathogen development. The identification of the *A. maculatum* microbiome and further detection of *R. parkeri* in tick tissues provides the basis for future tick–pathogen interaction studies.

Materials and Methods

Tick Rearing

Adult Gulf-Coast ticks were obtained from three different sources. Wild-caught *A. maculatum* were collected from the Sandhill National Wildlife Refuge (Gautier, MS) using the drag-cloth method as described previously (Falco and Fish 1988). These ticks were collected in late summer and early fall of 2011 and 2012. Questing adult ticks were collected and identified based on morphological characteristics (Keirans and Litwak 1989). Rickettsial identification within the wild-caught ticks is described below. *A. maculatum* ticks that contain *Rickettsia endosymbionts* (lab colony) were purchased from the tick rearing facility at Oklahoma State University. *Rickettsia*-free *A. maculatum* ticks were purchased from the tick rearing facility at Texas A&M (TAMU) and were used in the immunological study of *R.*

parkeri. All adult male and female ticks were partially blood fed on a New Zealand rabbit or sheep according to the approved Institutional Animal Care and Use Committee (IACUC) protocol #10042001.

Tick Tissue Isolation

Blood-feeding ticks (n = 134) were removed 8 d postinfestation, weighed, and dissected to isolate midguts (MG) and salivary gland (SG) tissues from each female tick (Karim et al. 2002). The carcasses (whole tick without the midgut and salivary gland tissues) were used to determine the infection rate (2012 collection). Genomic DNA was extracted from a small piece of isolated midgut and one salivary gland to test for SFGR infection. Tick saliva was collected after injecting saliva extraction solution (Ribeiro et al. 1992). Briefly, dopamine and theophylline (1 mM each in 20 mM 3-(N-morpholino) propanesulfonic acid-buffered saline with 3% dimethyl sulfoxide, pH 7.0) were injected into the dorsum hindquarter as a stimulant for salivation (Needham and Sauer 1979). The collected saliva was used immediately after collection or stored at −80°C.

DNA Extraction

Genomic DNA was extracted from tick tissues using the DNeasy blood and tissue kit (Qiagen, Valencia, CA), following the manufacturer's protocol. From the 2011 field collection, genomic DNA was collected from midgut tissues, salivary glands, and male ticks. From the 2012 field collection tick carcasses were also used for the genomic DNA extraction following the same protocol. The extracted DNA samples were stored at −20°C until further use.

454-Pyrosequencing

DNA from field collected and laboratory colony-raised tick tissues was used for bacterial tag-encoded titanium amplicon pyrosequencing (bTETAP; Dowd et al. 2008a,b). The output used for analysis had an average read length of ≈450 bp, with sequencing extending across V1 and into the V3 ribosomal region (MRDNA, Shallowater, TX). This procedure used the forward primer 27F (5′-GAGTTTGATCNTGGCTCAG-3′) and the reverse primer 519R (5′-GTNTTACNGCGGCKGCTG-3′) in relation to *E. coli* 16S. Amplicon sequencing was performed as recommended by the manufacturer (Roche Applied Science, Indianapolis, IN) for titanium sequencing on the FLX-titanium platform.

Microbial 16S rDNA sequences were curated to obtain Q25 sequence data, which were processed using a proprietary analysis pipeline (MRDNA), which trimmed sequencing reads to remove barcodes, primers, and short sequences <200 bp. Furthermore, the sequences with ambiguous base calls and homopolymer runs exceeding 6 bp were deleted. The sequences were than denoised, and chimeras were removed before operational taxonomic units (OTUs) clustering was performed using USEARCH (Drive5, WA). OTUs were defined after removal of singleton sequences, clustering at 97% similarity (Dowd et al. 2008a,b, 2011; Edgar 2010; Capone et al. 2011; Eren et al. 2011; Swanson et al. 2011). The taxonomic level of classification of OTUs was performed using BLASTn against a curated GreenGenes database (DeSantis et al. 2006) and compiled into each taxonomic level into both "counts" and "percentage" files. GENE-E software was used to visualize the percentage of bacterial genera in tick tissues and saliva, relative to each sample (Fig. 1).

SFGR Detection

The presence of SFGR was detected by using outer membrane protein A (*ompA*) gene-specific primers in a nested polymerase chain reaction (PCR) reaction (Blair et al. 2004). The primers RR 190–70 and RR 190–701 (Table 1) were used for the primary reaction, and

190-FN1 and 190-RN1 (Table 1) for the nested PCR reaction. In the primary reaction, ≈150 ng of DNA template was added to 2× PCR Master Mix (Promega, Madison, WI) and the appropriate primers (400 nM). In the nested reaction, the same mixture was used except with the nested primers and 2.5 μl from the primary reaction. SFGR PCR was performed in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Richmond, CA) as follows: one cycle at 95°C for 3 min, 35 cycles of 95° for 20 s, 46°C for 30 s, and 63°C for 60 s, and one cycle at 72°C for 7 min. For each reaction, two negative controls (no-template and no-primer) and one positive control (50 ng of a known SFGR) were included. The amplicons were analyzed on a 2% agarose gel containing ethidium bromide and observed using a ultraviolet transilluminator. The PCR products were purified (Qiagen) and sequenced at Eurofins MWG Operon (Huntsville, AL). Partial sequences obtained were analyzed by BLASTn from National Center for Biotechnology Information (NCBI) for homology searches. All PCR reactions were set up in a PCR hood using sterile technique. The PCR amplicon sequences obtained from this study were assigned GenBank accession numbers: JQ914757-81 and JX134636-41.

Quantification of *R. parkeri*

The presence of *R. parkeri* in tick tissues detected from the outer membrane protein A (*rompA*) gene study were further validated and quantified using the rickettsial outer membrane protein B (*rompB*)-based qPCR assay (Jiang et al. 2012). The *R. parkeri ompB* gene from positive *ompA* assay samples and a *R. parkeri*-positive sample were first amplified by PCR using primers Rpa129 F and Rpa224R (Table 1). The amplified *rompB* PCR product was visualized on an agarose gel and purified. The purified PCR product was serially diluted 10-fold (2×10^8 to 2×10^1) and used for standard curve preparation. The qPCR reaction consists of 2× Master Mix (Promega, Madison, WI), 100 ng DNA template, 0.7 μM of each primer (Rpa129F and Rpa224R), 0.4 μM probe (Rpa188p; Table 1), and 8 mM $MgSO_4$. The qPCR reactions were performed in a Thermal Cycler (CFX96 Real time detection system, Bio-Rad Laboratories) subjected to one cycle each of 50°C for 2 min and 95°C for 2 min, and 45 cycles of 95°C for 15 s and 60°C for 30 s. A no-template control and a positive control were included in each qPCR run. Each sample was analyzed in triplicate, and the obtained threshold cycle (Ct) values were used to calculate the copy number based on the standard equation.

Preparation of *R. parkeri* Tissue Culture

R. parkeri seed stock (1 ml) was diluted (1:10) in sterile brain heart infusion buffer or Snyder's 1 buffer and used for the inoculation of two T-162 cm^2 flasks containing Vero cells (~$2–3 \times 10^6$ cells/ml). Twenty milliliters of the old media from each flask was discarded. The diluted seed stock was inoculated into each flask, rocked at RT for 1 h, replenished with 20 ml of fresh Eagle's minimum essential medium containing 2.5% fetal bovine serum, and then placed back into the incubator. Culture flasks were incubated at 35°C and 5% CO_2 until 20–30% of the infected cells had sloughed off (6–7 d). Sterile 5-mm glass beads were used to disrupt cell adhesion. The pooled cell suspension was centrifuged at 10,000 × *g* for 30 min. The cell pellet was resuspended in Snyder's 1 buffer (0.22 M sucrose, 3.6 mM KH_2PO_4, 8.6 mM Na_2HPO_4, and 4.9 mM glutamic acid) at a ratio of one cell pellet to 4 ml of Snyder's 1 buffer. *R. parkeri* stock was aliquoted in 1 ml volumes, placed in cryogenic vials and stored at −80°C.

Preparation of Whole Cell Antigen for Mouse Antibody Generation

R. parkeri was propagated in several T-162 cm^2 flasks of Vero cells grown in Eagle's minimum essential medium supplemented with 2.5% fetal bovine serum in 5% CO_2 at 35°C. Heavily infected cells were harvested at 6–7 d postinfection, using sterile 5-mm-diameter

glass beads. Cells were spun at 7,600 × *g* for 40 min at 4°C. The pellet was resuspended in 5 ml of K36 buffer (0.1 M KCl, 0.015 M NaCl, 0.05 M KH_2PO_4, and pH 7.0) and homogenized with a glass homogenizer. The homogenate was centrifuged at 250 × *g* for 5 min at 4°C. The supernatant was collected and spun at 7,600 × *g* for another 40 min at 4°C. The pellet was resuspended with K36 buffer. The suspension was then used for mouse immunization. The sera from rickettsimic mouse blood were used for the detection of *R. parkeri* in tick tissues.

Immunodetection of *R. parkeri* in Tick Tissues

R. parkeri-infected female *A. maculatum* midgut and salivary glands tissues were suspended in 100 μl of extraction buffer (0.15 M Tris-HCl, pH 8.0, 0.3 M NaCl, 10% glycerol, and Protease inhibitor cocktail) followed by sonication (3 by 5 s). The sonicated samples were centrifuged at 20,000 × *g* for 10 min at 4°C. The resulting supernatants were removed, and the protein concentration was estimated using the Bradford assay (Bradford 1976). *R. parkeri*-infected Vero cells were subjected to the same procedure. The extracted supernatants were analyzed by electrophoresis on a 4–20% SDS-PAGE and transferred onto a nitrocellulose membrane in a Transblot Cell (Bio-Rad) following the manufacturers' instructions. A duplicate gel was stained with GelCode Blue (Pierce, IL) for visualization. *R. parkeri*-infected Vero cell supernatant was used as a positive control. *A. maculatum* obtained from the Texas A&M tick rearing facility have previously been shown to be *Rickettsia* free (Moraru et al. 2013) and, therefore, these samples were used as a negative control. Nonspecific binding was reduced by incubating the blot with 5% skim milk and mouse pre-immune sera (1:10,000). The nitrocellulose membranes were incubated with mouse *R. parkeri* polyclonal antibody (1:500 dilution). The antigen–antibody complex was visualized with horseradish peroxidase-conjugated anti-mouse IgG (KPL) at a dilution of 1:10,000 and detected with SuperSignal chemiluminescent substrate (Pierce) using Bio-Rad ChemiDox XRS. The same blot was reprobed with the monoclonal anti-β-Actin-peroxidase (1:25,000; Sigma).

Results

Microbiome of *A. maculatum*

From field collected *A. maculatum* tissues, we obtained 27,691 sequence reads for analysis after trimming and removing all low-quality sequences. In total, 12,330 sequence reads obtained from midgut tissues, 13,009 sequences reads from salivary glands, and 2,352 sequence reads from saliva were searched against the GreenGenes databases. Similarly, we obtained, in total, 13,927 sequence reads from lab colony *A. maculatum* midgut and salivary gland tissues together. This provided a reference for comparing field and lab colony *A. maculatum* tissues.

In field collected *A. maculatum*, the bacterial phyla Proteobacteria (83.39% MG, 95.90% SG, and 92.18% saliva), Actinobacteria (3.92% MG, 2.28% SG, and 5.23% saliva), and Firmicutes (11.0% MG, 1.73% SG, and 2.59% saliva) were found in all tick tissues, and these phyla account for >95% of the bacterial communities in all tested tick tissues. Minor bacterial phyla detected in tick midguts include Bacteroidetes (1.42%), Spirochaetes (0.17%), Cyanobacteria (0.07%), and Fusobacteria (0.02%) whereas salivary gland tissues had only a few reads from Bacteroidetes (0.05%), Spirochaetes (0.01%), and Chloroflexi (0.03%). In lab colony *A. maculatum*, we observed bacterial reads representing Proteobacteria (64.90% MG; 99.49% SG), Firmicutes (20.53% MG, 0.51% SG), Bacteroidetes (13.25% MG, 0% SG), and Actinobacteria (1.32% MG, 0% SG) phyla.

Francisellaceae (0.22% MG, 82.34% SG), Enterobacteriaceae (30.58% MG, 0.1% SG, and 90.82% saliva), and Rickettsiaceae (51.47% MG, 11.40% SG, and 0.21% saliva) were abundant bacterial families detected in field-collected *A. maculatum* tick tissues and saliva, accounting for >80% of bacterial species detected in tick tissues. The majority of endosymbionts detected in this study belong to the Francisellaceae family. *A. maculatum* from the lab colony had abundant reads from Francisellaceae (34.44% MG, 99.27% SG), while most other bacterial families in midgut tissues were Enterobacteriaceae (27.81%), Veillonellaceae (9.93%), Ruminococcaceae (5.30%), Porphyromonadaceae (4.64%), Lactobaccillaceae (3.97%), Staphylococcaceae (1.32%), Propionibacteriaceae (1.32%), and Comamonadaceae (1.98%).

In wild-caught ticks, pyrosequencing revealed 54 different bacterial genera from midgut tissues, 23 different bacterial genera from the salivary gland tissues, and 16 bacterial genera from saliva (Figs. 1 and 2). We observed six bacterial genera in all tick tissues studied: *Francisella, Propionibacterium, Rickettsia, Pseudomonas, Corynebacterium,* and *Escherichia*. In addition, the Enterobacterial genera (*Raoultella, Ewingella, Escherichia,* and *Klebsiella*) account for ≈30% of the total microbial diversity in tick midgut tissues (Fig. 1). Reads from *Rickettsia* account for 46% of the total reads from midgut tissues but only 7% from salivary glands, confirming differential pathogen levels within tissues of blood-feeding wild caught *A. maculatum.*

Not surprisingly, the bacterial diversity is different in tick midguts and salivary glands (Fig. 2), which could be because of bacterial tissue specificity. The lab-maintained *A. maculatum* revealed *Francisella, Escherichia, Alistipes, Ruminococcus, Selenomonas, Staphylococcus, Tannerella, Trabulsiella, Lactobacillus, Propionibacterium,* and *Diaphorbacter* (Fig. 2). Surprisingly, *Francisella endosymbionts* were detected in the salivary glands of lab-maintained *A. maculatum. F. endosymbionts* have previously been identified in hard and soft ticks from different continents, but because of difficulty in culture-based techniques, little information is known about these endosymbionts (Ivanov et al 2011).

We determined the microbial diversity in field-collected tick saliva to evaluate potential microbial secretion. The saliva samples revealed sequences from *Shigella, Bacillus, Escherichia,* and *Micrococcus,* with most reads identified as originating from *Shigella* with a few from *Rickettsia* (Fig. 1). It is important to note that some of the detected bacteria could have resulted from environmental contamination, as many of these bacteria are commonly found in environmental samples. However, because *Rickettsiae* are obligate intracellular organisms, they were likely secreted from tick salivary glands. The saliva from laboratory-based ticks was not tested in this study.

Screening of SFGR in *A. maculatum*

The prevalence of SFGR infection in the collected ticks was confirmed using *ompA* gene-specific primers in nested PCR (Fig. 3). The PCR amplicon was sequenced, and the nucleotide homology was assessed by searching the nonredundant nucleotide collection at GenBank. Of the 11 male ticks examined, 54% (6 out of 11) were found to be SFGR positive with nearest homology (99–100%) to *R. parkeri, Rickettsia amblyommii,* or *R. endosymbiont* of *A. maculatum* (Table 2). Next, we examined SFGR infection in partially blood-fed female midguts, salivary glands, and saliva. Eighty percent of tick midguts (20 out of 25) contained SFGR with sequence homology to *R. parkeri, R. amblyommii,* or *R. endosymbiont* of *A. maculatum.* Interestingly, of the eight salivary glands tested for the presence of SFGR, 50% (4 out of 8) showed sequence homology with *R. parkeri* (Table 2). Although we identified rickettsial reads in tick saliva from pyrosequencing, no SFGR were detected from *rompA*-nested PCR (data not shown).

R. parkeri Quantification in *A. maculatum*

To further confirm the presence of *R. parkeri* in our field-collected ticks, we used a specific qPCR assay using *ompB* gene-specific primers and probe (Jiang et al. 2012). The male tick DNA samples were insufficient in concentration to test using the qPCR assay. The infection level of *R. parkeri* in the midgut samples ranged from 6 to 4,000 copies/μl, while the single pair of salivary gland that tested positive had an infection load of 1,794 copies/μl (Table 2). We observed some testing discrepancies in samples, in that some sequences with identity to *R. parkeri* based on *ompA* homology (Fig. 3) were not amplified in the more accurate qPCR assay. The apparent disparity could be because of the inherent difficulties in assigning Rickettsial species identity because of the small divergence between *ompA* sequences among rickettsial species (Fournier et al. 2003).

To study *R. parkeri* infection rate in field-collected *A. maculatum*, we expanded this assay to include more *A. maculatum* tick samples. The individual female tick carcasses (n = 83) (whole tick minus gut and salivary gland tissues) and male ticks (n = 5 groups; 3 per group) were screened for qPCR detection of *R. parkeri*. We observed a 32.5% infection rate in females samples (27 of 83), whereas three of five male tick samples tested positive for *R. parkeri* infection. Based on this assay, the *R. parkeri* infection level in field-collected *A. maculatum* showed 12–32% infection rate in field-collected female ticks.

Immunodetection of *R. parkeri* in *A. maculatum*

Western blotting was performed to detect the *R. parkeri* expressed proteins in tick tissues and evaluate the efficacy of this antibody in future studies. *R. parkeri* polyclonal antibodies cross-reacted with 30, 75, and 100 kDa proteins in *R. parkeri*-infected Vero cells and the cell supernatant. These samples served as the positive control (Fig. 4). *Rickettsia*-free *A. maculatum* tick midgut and salivary gland supernatants (originating from a tick colony maintained at Texas A&M University) had no notable cross-reactivity to *R. parkeri* antibody with respect to positive controls. The *R. parkeri* polyclonal antibody cross-reacted with a ≈70 kDa protein (Fig. 4B, Lanes 4 and 5) in field-collected tick midgut tissues but cross-reacted with a ≈75 kDa protein (Fig. 4B, Lanes 11 and 12) in salivary glands tissues. The differences could be because of posttranslational modification of the immunogenic protein or they could represent entirely different proteins. The field-collected *A. maculatum* midgut and salivary gland tissues were infected with *R. parkeri* or *R. endosymbionts*, based on their carcass. The *R. parkeri* polyclonal antibody cross-reacted with protein species presumably associated with rickettsial infection in midguts (Fig. 4B, Lane 6; 70 kDa) and salivary glands (Fig. 4B, Lane 13; 70 kDa). The same blot was reprobed with monoclonal anti β-Actin labeled with peroxidase and showed cross-reactivity of a 42 kDa band in Vero cells and tick tissues. β-Actin was used to show the reference protein level in both the tick tissues and Vero cells (Fig. 4C). The SDS-gel stained with GelCode Blue was also included as a reference (Fig. 4A).

Discussion

This study revealed the microbial diversity in field-collected and lab-maintained *A. maculatum* tick midguts, salivary glands, and saliva. Importantly, *F. endosymbionts* were identified in all samples tested. The bacterial community of *A. maculatum* maintained in the laboratory showed an average relative abundance of the *Francisella* genus to be 35 and 98% in midgut and salivary gland tissues, respectively. As obligatory blood feeders, ticks are required to maintain a relatively simple and restrictive microbial community (Lalzar et al. 2012). The uniform presence of the *Francisella* genus represents a systemic association between arthropods and these bacteria. A more diverse bacterial community in *A. maculatum* was expected because of its interactions with different animal hosts while

feeding in wild. In contrast, the bacterial community had abundant sequences assigned to the *Francisella* genus (80%), followed by *Rickettsia* (6%) and *Wolbachia* (4%) in field-collected *A. maculatum* salivary glands (Fig. 2). These findings are in agreement with previous reports for tick species. The abundance of *Francisella* species in tick tissues suggests that *Francisella* sustains an obligatory association with its tick hosts, and outcompetes other bacterial genera. The findings of *Francisella, Rickettsia,* and *Wolbachia* in field-collected ticks reported in this study support this assertion. Determining the extent to which field-collected ticks maintain endosymbionts (*Francisella, Rickettsia,* and *Wolbachia*), pathogens, or both, was outside the scope of this investigation. Bacteria from the *Rickettsia* and *Wolbachia* genus are known endosymbionts for several arthropod systems (Gottlieb et al. 2011). In this study, we detected bacteria from the genus *Rickettsia* and *Wolbachia* in tick salivary glands, but the bacterial density was significantly lower compared with *Francisella.* These findings support a facultative association between *Rickettsia, Wolbachia,* and ticks. The prevalence of obligatory or facultative endosymbionts in ticks has been proposed to be influenced by several factors including competition, increased virulence, and problems in vertical transmission (Mira and Moran 2002). Vector regulation of *Francisella* multiplication might explain the lower reads of *Rickettsia* and *Wolbachia* in *A. maculatum* salivary glands. Alternatively, interspecies competition of *Rickettsia* populations found (*R. parkeri* and *R. endosymbiont*) might result in decreased prevalence. In *Dermacentor andersoni* Stiles, the abundance of *Rickettsia peacockii* in tick ovaries was suggested to block transovarial transmission of *R. rickettsii* (Burgdorfer et al. 1981, Niebylski et al. 1997).

The survival or dominance of Enterobacteria in tick gut tissues (Fig. 2) could be under the control of the same mechanism that operates in mosquito guts, in that bloodmeal-induced oxidative stress results in a oxidative killing of many bacteria (Wang et al. 2011). The redox capacity of enteric bacteria may be important adaptation within blood-feeding arthropod guts, owing to high oxidative stress during blood metabolism. *Francisella* and *Wolbachia,* found in both midguts and salivary glands (Fig. 2), and *Candidatus Devosia* found predominantly in salivary glands (Fig. 2) are known endosymbionts (Scoles 2004, Vannini et al. 2004, Zhang et al. 2011). *Wolbachia* species have been proposed for use in insect pest control, importantly by *Wolbachia*-induced cytoplasmic incompatibility (Zabalou et al. 2004), and this methodology has been shown to block or reduce *Plasmodium falciparum* (malaria parasite) transmission from *Anopheles gambiae* Giles, 1902 (Hughes et al. 2011). Only the *Francisella*-like endosymbionts were found in lab-colony salivary glands while both the *Francisella*-like endosymbiont and *Rickettsia* (Figs. 1 and 2) were detected in field-collected salivary glands, suggesting further study is warranted to understand their interaction with their host.

Symbionts are classified as obligate, providing nutrient supplements to their arthropod host, or facultative, aiding in immunity (Moran et al. 2008). The presence of the facultative symbiont, *Wolbachia,* has been shown to result in upregulated immune genes on pathogen infection in *Drosophila melanogastor* and *Aedes aegypti* L. (Rances et al. 2012, Eleftherianos et al. 2013). Clearly, the presence of *F. endosymbionts* in *A. maculatum* is of fundamental significance, given their occurrence in numerous other tick species including *Dermacentor variabilis* (Say, 1821), *D. andersoni, Dermacentor hunteri* Bishopp, 1912, *Dermacentor nitens* Neumann, 1897, *Dermacentor occidentalis* Marx, 1892, and *Dermacentor albipictus* (Packard) (Niebylski et al. 1997, Sun et al. 2000, Scoles 2004), but the nature of this symbiotic relationship has not yet been determined.

Interestingly, *Rickettsia* reads were present in saliva (Fig. 1), suggesting rickettsial secretion from salivary glands. The detection of *Rickettsia* in midgut, salivary glands, and saliva is important with respect to the possible pathogen development cycle. The putative trafficking

route in ticks begins with midgut tissues acquiring or harboring the pathogen from an infected vertebrate host, the development and replication, trafficking to the salivary glands, and finally transmission to a mammalian host via salivation (De Silva and Fikrig 1995). Interestingly, the only *Rickettsia* species we identified in *A. maculatum* salivary glands was *R. parkeri,* supporting its unique ability to migrate from midgut to salivary gland (Table 2).

We detected *R. parkeri, R. amblyommii,* and *R. endosymbiont* of *A. maculatum* in field-collected *A. maculatum. R. parkeri* has been frequently reported from different field-collected *A. maculatum* (Cohen et al. 2009, Paddock et al. 2010, Trout et al. 2010, Varela–Stokes et al. 2011, Wright et al. 2011), with an estimated infection rate of 28.1–41%, similar to this study. Similarly, *R. amblyommii* has been identified in many different *Amblyomma* species (Labruna et al. 2004; Apperson et al. 2008; Ogrzewalska et al. 2008, 2009, 2010, 2011; Jiang et al. 2010; Bermudez et al. 2011). Culturing rickettsial endosymbionts from the soft tick, *Carios capanensis* (Neumann, 1901), has been attempted (Mattila et al. 2007), but complete characterization remains incomplete. The public health significance of *R. parkeri* was recognized more than 60 yr after its discovery in *A. maculatum* (Parker et al. 1939, Paddock et al. 2004). In addition, a human rickettsiosis originating from *R. amblyommii* infection has been recently reported (Apperson et al. 2008). In fact, the pathogenicity of many rickettsial agents (which may include tick endosymbionts) remains unknown because of the lack of specific diagnostic approaches, minor differences in clinical symptoms, and the fact the same antibiotic regimen is prescribed for all rickettsial infections.

Stenotrophomonas, Pseudomonas, Rhodococcus, and *Propriobacterium* in *A. maculatum* were reported in *Ixodes ricinus* L. and were proposed to be part of the core microbiome of Ixodid ticks (Carpi et al. 2011); however, specific or functional classification has not yet been achieved (Moran et al. 2008). The detection of *Mycobacterium, Bacillus, Streptococcus, Clostridium, Streptomyces, Pseudomonas, Streptococcus, Corynebacterium, Staphylococcus, Papilibacter, Coprococcus, Eubacterium, Roseburia, Pantoea, Ruminococcus,* and many other environmental and soil bacterial genera identified in this study have previously been reported in ticks (Andreotti et al. 2011, Carpi et al. 2011).

In this study, we attempted the detection of *R. parkeri* in tick tissues using a mouse-generated polyclonal *R. parkeri* antibody. However, rickettsial polyclonal antibodies extensively cross-react with antigens from many different Rickettsial species (Anderson and Tzianabos 1989), and, currently, this prevents the use of Rickettsial antibodies as a means of specific detection of Rickettsial species in tick tissues. The use of a species-specific rickettsial antibody with cross-adsorption could provide a specific determination of *R. parkeri* (Raoult and Paddock 2005). The differences in antibody reactivity in Vero cells and the tick tissues (Fig. 4) could be because of different antigen expression profiling of *R. parkeri* in mammalian (Vero cell) and arthropod systems (tick). Differences in antigenic profiling was reported in *Ehrlichia chaffeensis*-infected tick and mammalian cells (Kuriakose et al. 2011). Overall, these data underscore the difficulty in serological differentiation among *Rickettsia.*

In conclusion, we described the *A. maculatum* microbiome and further confirmed *R. parkeri* infection, which could be the basis of future studies examining the interactions between *R. parkeri* and the tick microbiome. The known pathogenic and nonpathogenic microbes likely interact with the tick vector, and a better understanding of these interactions could open new avenues for vector and disease control. The manipulation of the microbial communities by altering or inhibiting the growth of a particular bacterial strain could alter pathogen transmission (Hughes et al. 2011). The mechanism by which the tick midgut microbiome influences pathogenic rickettsial development could be used for tick-borne disease control strategies. Moreover, elucidating the precise role of the endosymbionts on the regulation of

the immune response and the corresponding pathogenic response of *R. parkeri*, will continue to be of considerable research interest. Further studies will focus on the influence the microbiome has on pathogen survivability, virulence, and development within the tick vector and the relationship between various symbionts and tick immunity with emphasis to *F. endosymbionts* and numerous *Rickettsiae.*

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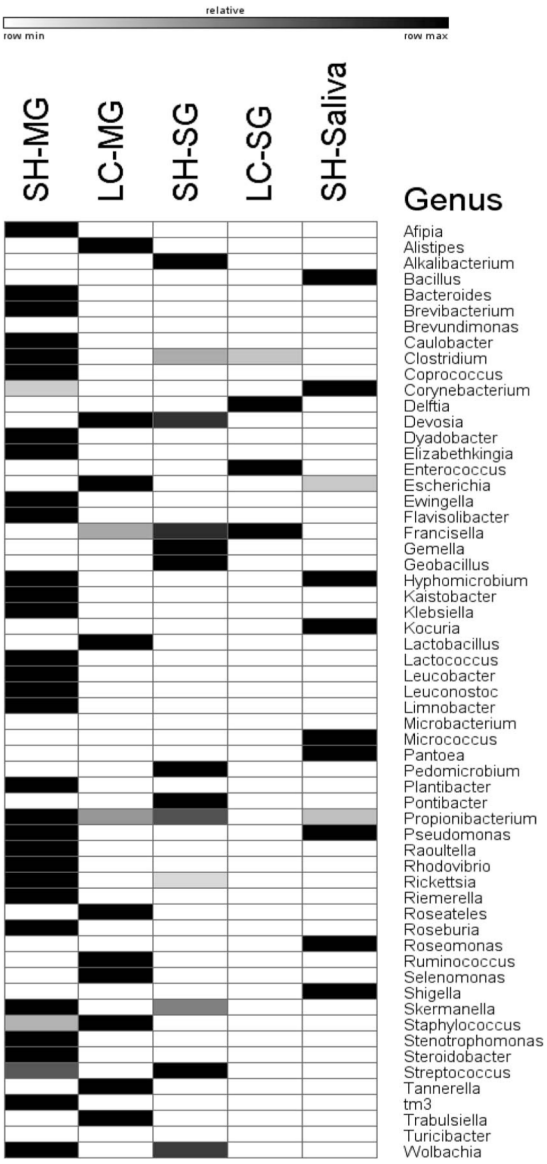

Fig. 1.

The relative abundance of bacterial genera in *A. maculatum* tissues. The field-collected ticks (SH) and lab colony (LC) ticks: midgut (MG), salivary glands (SG), and saliva.

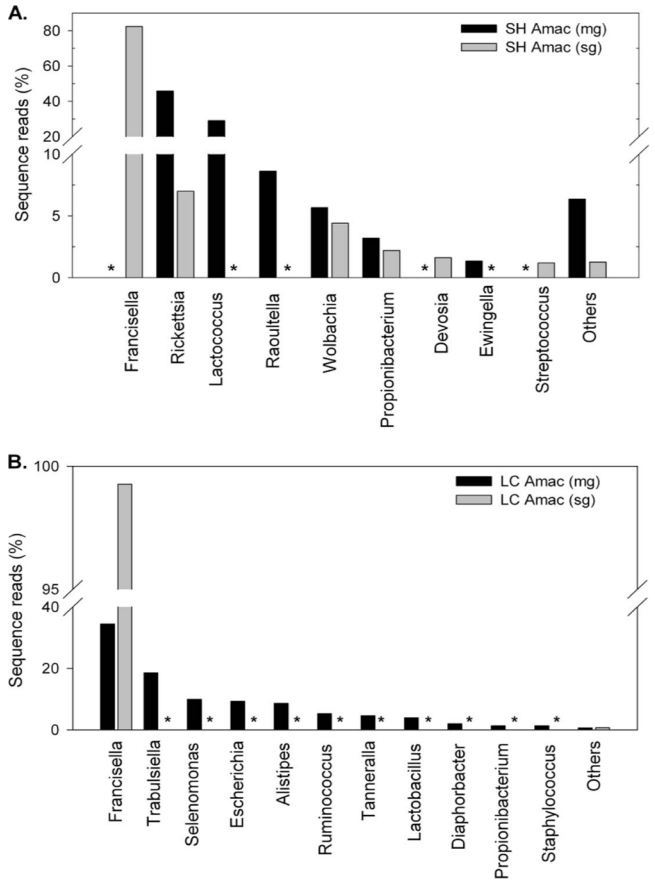

Fig. 2.

Microbiome of the partially blood-fed *A. maculatum* tissues. The bacterial diversity in the tissues from field-collected (A) and lab-based (B) female *A. maculatum* tissues based on 454-pyrosequencing approach. The asterisk sign (*) denotes no or <1% reads for that genera. Values below 1% were grouped as “Others.”

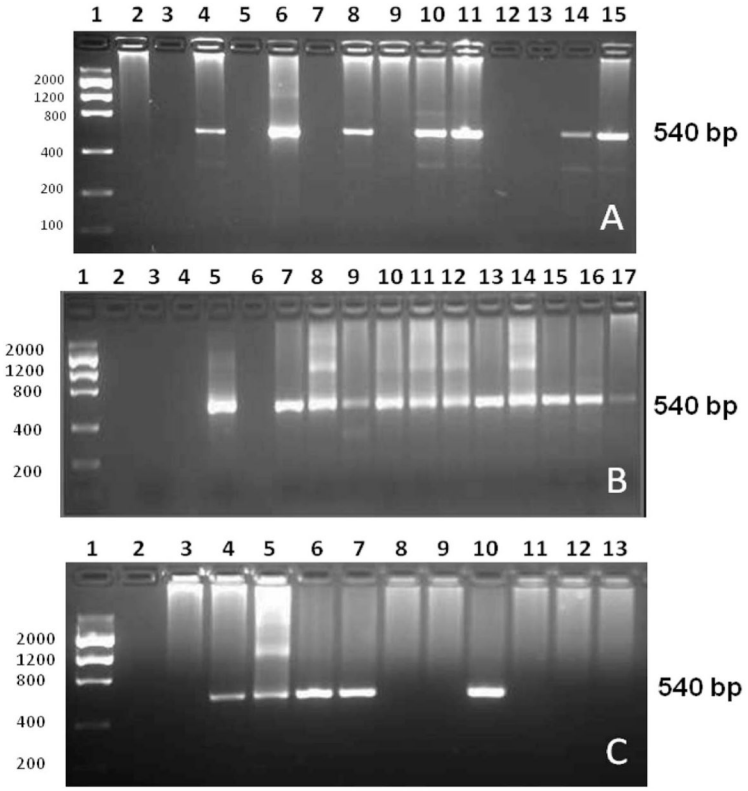

Fig. 3.

Molecular detection of SFGR in field-collected *A. maculatum*. Tick tissues were tested for the presence of SFGR using the *ompA*-nested PCR assay. (A) 1: DNA ladder; 2: no-template control; 3: no-primer control; 4: positive control; lanes 5–15: male tick DNA. (B) Lane 1: DNA ladder; 2, 4, and 6: Blank; 3: no-template control; 5: positive control; lanes 7–17: female midgut DNA. (C) 1: DNA ladder; 2: no-template control; 3: no-primer control; 4: positive control; lanes 5–13: female salivary gland DNA.

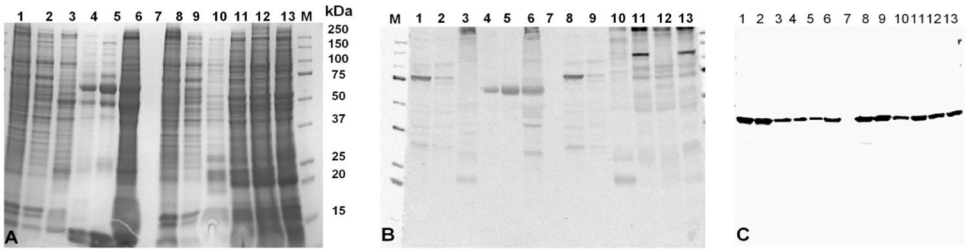

Fig. 4.

A 4–20% SDS-PAGE stained with GelCode Blue (A) and its corresponding immunoblot demonstrating cross-reactivity to the *R. parkeri* antibody (B) and *β*-actin (C). Standard protein marker adjoining molecular size lane (M); Lanes 1 and 2 were *R. parkeri* grown in Vero cells and the corresponding cell supernatant (Lanes 8 and 9). Lane 7 was empty. Lanes 3 and 10 were *A. maculatum* (Texas A&M) midgut and salivary gland tissues, respectively (*Rickettsia*-free tissues); Lanes 4 and 5 (midgut tissues) and lanes 11 and 12 (corresponding salivary glands), respectively, from field-collected *A. maculatum*; Lane 6 (midgut) and lane 13 (salivary gland) of lab colony *A. maculatum* (infected with *R. endosymbiont*).

Table 1

Primers and probe used in this study

Gene	Sequences	Amplicon size (bp)	Reference
ompA (primary)	(F) 5′-ATGGCGAATATTTCTCCAAAA-3′ (R) 5′-GTTCCGTTAATGGCAGCATCT-3′	590	(Blair et al. 2004)
ompA (nested)	(F) 5′-AAGCAATACAACAAGGTC-3′ (R) 5′-TGACAGTTATTATACCTC-3′	540	
ompB (qPCR)	(F) 5′-CAAATGTTGCAGTTCCTCTAAATG-3′ (R) 5′-AAAACAAACCGTTAAAACTACCG-3′ (Probe) 5′-6-FAM-CGCGAAATTAATACCCTTATGAGCAGCAGTCGCG-BHQ1-3′	96	(Jiang et al. 2012)

Table 2

SFGR identification in *A. maculatum* tissues

A. maculatum	Sample ID	SFGR species	Nucleotide identity (%)	GenBank	qPCR detection
Female midgut tissues	SH_Mg1	*R. parkeri*	100	JQ914757	—
	SH_Mg2	*R. parkeri*	96	JQ914758	—
	SH_Mg3	*R. parkeri*	92	JQ914759	—
	SH_Mg4	*R. parkeri*	98	JQ914760	—
	SH_Mg5	*R. endosymbiont* of *A. maculatum*	99	JQ914761	—
	SH_Mg7	*R. amblyommii*	99	JQ914762	—
	SH_B1	*R. parkeri*	100	JQ914763	—
	SH_B2	*R. parkeri*	100	JX134636	4,000 ± 1,106
	SH_B3	*R. parkeri*	100	JX134637	6 ± 2
	SH_B4	*R. endosymbiont* of *A. maculatum*	100	JX134638	—
	SH_B5	*R. endosymbiont* of *A. maculatum*	98	JX134639	—
	SH_B6	*R. endosymbiont* of *A. maculatum*	98	JX134640	—
	SH_B7	*R. parkeri*	100	JQ914764	—
	SH_B8	*R. parkeri*	99	JQ914765	755 ± 88
	SH_B9	*R. parkeri*	99	JQ914766	—
	SH_B10	*R. endosymbiont* of *A. maculatum*	100	JQ914767	—
	SH_D1	*R. endosymbiont* of *A. maculatum*	99	JQ914768	—
	SH_D2	*R. endosymbiont* of *A. maculatum*	100	JQ914769	—
	SH_D4	*R. endosymbiont* of *A. maculatum*	100	JQ914770	—
	SH_D5	*R. parkeri*	94	JQ914771	—
Female salivary gland tissues	SH_SG1	*R. parkeri*	100	JQ914772	1,794 ± 177
	SH_SG2	*R. parkeri*	100	JQ914773	—
	SH_SG3	*R. parkeri*	100	JQ914774	—
	SH_SG6	*R. parkeri*	100	JQ914775	—
Male tissues	SH_M2	*R. parkeri*	99	JX134641	ND
	SH_M4	*R. amblyommii*	100	JQ914776	ND
	SH_M6	*R. endosymbiont* of *A. maculatum*	99	JQ914777	ND
	SH_M7	*R. amblyommii*	99	JQ914778	ND
	SH_M10	*R. amblyommii*	100	JQ914779	ND

A. maculatum	Sample ID	SFGR species	Nucleotide identity (%)	GenBank	qPCR detection
	SH_M11	*R. amblyommii*	100	JQ914780	ND

ND, not determined.