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Tick microbial communities within enriched extracts of *Amblyomma maculatum*

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ABSTRACT

Our objective of this study was to explore the bacterial microbiome in fresh or fresh-frozen adult *Amblyomma maculatum* (Gulf Coast ticks) using extracts enriched for microbial DNA. We collected 100 questing adult *A. maculatum,* surface disinfected them, and extracted DNA from individual ticks collected the same day or after storage at −80 °C. Because only extracts with microbial DNA concentrations above 2 ng/μL were considered suitable for individual analysis, we expected fewer samples to meet these requirements. Of individual ticks extracted, 48 extracts met this minimum concentration. We pooled 20 additional extracts that had lower concentrations to obtain seven additional pools that met the minimum DNA concentration. Libraries created from these 55 samples were sequenced using an Illumina MiSeq platform, and data sets were analyzed using QIIME to identify relative abundance of microorganisms by phylum down to genus levels. Proteobacteria were in greatest abundance, followed by Actinobacteria, Firmicutes, and Bacteroidetes, at levels between 1.9% and 6.4% average relative abundance. Consistent with the *Francisella*-like endosymbiont known to be present in *A. maculatum,* the genus *Francisella* was detected at highest relative abundance (72.9%; SE 0.02%) for all samples. Among the top ten genera identified (relative abundance ≥ 0.5%) were potential extraction kit contaminants, *Sphingomonas* and *Methylobacterium,* the soil bacterium *Actinomycetospora,* and the known *A. maculatum*-associated genus, *Rickettsia*. Four samples had *Rickettsia* at greater than 1% relative abundance, while nine additional samples had *Rickettsia* at low (0.01–0.04%) relative abundance. In this study, we used the entire microbe-enriched DNA extract for whole ticks for microbiome analysis. A direct comparison of the microbiome in microbe-enriched DNA and total genomic DNA extracts from halves of the same tick would be useful to determine the utility of this extraction method in this system. We anticipate that future tick microbiome studies will be valuable to explore the influence of microbial diversity on pathogen maintenance and transmission, and to evaluate niche-specific microbiomes within individual tick tissues.

1. Introduction

Like vertebrates, invertebrates carry a microscopic community of diverse organisms that may include bacteria, protozoa, and viruses. Arthropod vectors are unique among invertebrates because their microbial community may include pathogens that are transmissible to a host, most commonly via blood feeding. Interest in these microbial communities, or microbiomes, of tick vectors has recently gained momentum (Narasimhan and Fikrig, 2015). To begin to address current gaps in knowledge, investigators have characterized the microbiomes of ticks relative to rearing conditions (lab versus wild), sex, life stage, and geographical range, compared the microbiome within specific tissues, and evaluated tick microbial communities in relation to pathogen presence and after antibiotic treatment (Budachetri et al., 2014; Budachetri et al., 2016; Clayton et al., 2015; Trout Fryxell and DeBruyn, 2016; Van Treuren et al., 2015; Williams-Newkirk et al., 2014). While tick microbial communities may include a diverse array of microorganisms, studies in general have focused on bacteria, with next-generation sequencing tools utilizing the 16S rRNA gene to identify bacterial populations. Still, limitations exist, sample treatment varies,

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and the technology continues to evolve. Certainly the question of what is the optimal extraction method for microbiome studies is one that already has seen debate in the area of gut microbiomes in humans (Mackenzie et al., 2015). However, methods relied upon in the gut microbiome field generally use commercial kits for soil or stool samples, and specific protocols to enrich for microbial DNA, as opposed to using total genomic DNA extraction kits.

The Gulf Coast tick, *Amblyomma maculatum* (Acari:Ixodidae), is a vector of increasing medical and veterinary importance, with a geographic range that extends beyond the Gulf Coast to the Atlantic Coast and inland to states including Kansas and Kentucky (Paddock and Goddard, 2015; Teel et al., 2010). Budachetri et al. (2014) examined the microbiome of *A. maculatum* using genomic DNA extracts of salivary glands and midgut tissues collected from field and laboratory-reared tick populations after 8 days of vertebrate host infestation. Bacterial 16S rRNA sequences obtained using 454 pyrosequencing demonstrated that Proteobacteria constituted the majority of the bacterial communities from both tissues in the field and laboratory sources of ticks, and not surprisingly, *Francisella* and *Rickettsia* were the most common genera in salivary gland and midgut tissues, respectively, of field-collected ticks (Budachetri et al., 2014). Other approaches to studying the tick microbiome include the use of genomic DNA extracted from unfed field-collected *Amblyomma americanum* tick halves for bacterial 16S rRNA sequencing using an Illumina MiSeq platform (Trout Fryxell and DeBruyn, 2016) and using DNA extracts from surface-disinfected or untreated field-collected *A. americanum* with 454 pyrosequencing (Williams-Newkirk et al., 2014). Approaches may vary in part due to the question addressed, with additional variation possible due to differences in available technology or other resources.

In this study, we used extracts enriched for microbial DNA, and bacterial 16S rRNA sequencing using an Illumina MiSeq platform, to evaluate the microbiome of unfed, field-collected *A. maculatum*. Sequencing based on MiSeq technology has been used in a variety of applications and provides comparable coverage to other commercial sequencing systems (Metzker, 2010; Glenn, 2011; Quail et al., 2012). Because the ability to use archived ticks is desirable for long-term field studies and allows flexibility in general, we extracted samples after storage at −80 °C, as well as fresh samples extracted on the day of collection. Of 100 ticks collected, we analyzed 48 individual tick extracts and seven pools (20 ticks) by Illumina MiSeq. Based on identified taxa, we confirmed presence of some of the previously reported genera, absence of others, and additional genera associated with other ixodid ticks. This study differed from previous microbiome studies of *A. maculatum* in its use of whole ticks and DNA extracts enriched for microbial DNA. However, due to the concentration of microbe-enriched DNA extracted, and the requirements for performing Illumina MiSeq, we were unable to extract genomic DNA from half of the same tick for comparison. Thus, we did not assess whether the use of enriched extracts provides a more comprehensive snapshot of bacterial abundance. Still, the utility of this approach in future studies may be of value when exploring interactions among pathogens and other tick-associated bacteria in tick populations.

2. Materials and methods

2.1. Collection of A. maculatum

Adult ticks were collected by flagging or dragging vegetation with a 1 m^2 piece of muslin cloth on six different dates from May 4, 2016 to June 22, 2016 from sites in Starkville, a city in Oktibbeha County, Mississippi. Once collected, ticks were morphologically identified (Goddard and Layton, 2006) and *A. maculatum* were surface disinfected with 5% household bleach then rinsed in water prior to placement in individual 1.5 mL microfuge tubes. Ticks were then placed in a −80 °C freezer (within 4 h of collection) until DNA extraction 8–16 days later ("frozen") or immediately extracted that day ("fresh").

2.2. DNA extraction of whole A. maculatum

To enrich for microbial DNA in our extracts, we used the protocol for isolating DNA from stool for pathogen detection in the QIAamp Fast DNA Stool Mini Kit (found in kit handbook, page 20–22; Qiagen Inc., Valencia, CA). This protocol includes an additional heating step (70 °C) for lysis of microorganisms including bacteria and other parasites. We additionally modified the protocol to use whole tick bodies, rather than stool material. Previously cleaned ticks were placed in 250 µL InhibitEX Buffer and cut multiple times with a scalpel blade (no. 11) prior to vortexing for 1 min and heating at 95 °C for 10 min to lyse microorganisms, then the remainder of the manufacturer's protocol followed. In addition, 30 µL of molecular biology grade water was used for initial elution and then transferred back onto the column for a final centrifugation at 20,000 × *g* to increase DNA concentration.

We used a Qubit® 3.0 Fluorometer and Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA) to measure DNA concentration in our extracts. We selected this approach to more accurately measure DNA as a low concentration was expected using the selected extraction protocol for microbial DNA. The Nanodrop 1000 and 2000 Spectrophotometers, also available to us, have a claimed detection limit of 2 ng/µL, and are unreliable at low concentrations. A concentration at or above 2 ng/µL was required for sequencing library preparation. Extracts with concentrations below the minimum were pooled with like samples of the same sex, storage method, and collection date to meet minimum concentration, then subjected to clean-up and concentration using a Zymo Research DNA Clean and Concentrator (Zymo Research, Irvine CA) and concentrations re-assayed using the Qubit 3.0 Fluorometer as above.

2.3. Library preparation and sequencing using Illumina MiSeq platform

Sequencing libraries were constructed based on the V4 region of the bacterial 16S rRNA gene using 10 ng total DNA from individual or pooled extracts. Amplification conditions and sequencing from were performed as previously described by Kozich et al., specifically in the supplementary files for that publication (Kozich et al., 2013).

2.4. Sequence data processing

Raw sequencing data were processed using a Quantitative Insights into Microbial Ecology (QIIME) pipeline (1.9.0) (Caporaso et al., 2010). De-multiplexed sequences were assembled (multiple_join_paired_ends.py) for operational taxonomic units (OTUs) table construction. The OTUs with 97% identity were assigned taxonomy based on the Greengene database (13_5). After removing chimera OTUs and low reads number samples from table, we calculated relative abundances across increasing taxonomic levels from phylum to genus, and alpha and beta diversity were generated.

3. Results

We visited four sites in Starkville, MS from May 4, 2016 to June 22, 2016, and collected *A. maculatum* ticks in two sites. One of these sites yielded 96 ticks over six visits, and the other site yielded four ticks on a single visit, June 2 (Table 1). Sixty-eight ticks were used for sequencing. Of 25 ticks that were extracted on the same day as collection (fresh), six individual extracts (24%) had sufficient DNA to be used for library preparation and microbiome analysis and two (females) had sufficient DNA after pooling (1 pool). All of the four ticks collected on June 2 were extracted fresh but none had sufficient DNA for sequencing, thus all analyzed ticks were from one location. Of the 75 ticks that were extracted after a period of 8–16 days at −80 °C storage (frozen), 42 individual extracts (56%) had sufficient DNA for library preparation and 18 had sufficient DNA after pooling in groups of 2–4 (6 pools). Samples with insufficient DNA concentrations (n = 19; average