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Starkey, M. Nelson, "Variations in Microbiota of Culex (melanoconion) Ocossa and Culex (culex) Declarator Across Peri-urban and Rural Environments" (2015). *Honors Theses*. 119.
https://dx.doi.org/10.32597/honors/119/
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HONS 497
Honors Thesis

Variations in Microbiota of *Culex (Melanoconion) ocossa* and *Culex (Culex) declarator* Across Peri-urban and Rural Environments

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March 30, 2015

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ABSTRACT

Mosquitoes serve as the primary mode of transmission for many tropical infections such as Venezuelan equine encephalitis virus, dengue virus, and malaria. Therefore, mosquitos are an important area of study for disease control. This study is part of a larger goal to examine the differences in gut microbiota of *Culex melanoconion* species across two different habitats: periurban and rural. Mosquitoes were collected from locations around Iquitos, Peru and their gut bacterial DNA was extracted and analyzed. Based on previous studies, it is believed that the bacterial flora will differ among mosquitoes reared in different locations.

INTRODUCTION

Mosquitoes serve as the primary mode of transmission for many tropical infections such as Venezuelan equine encephalitis virus (VEEV), dengue virus (DENV), and malaria (Turell et al, 2008). Importantly, however, not all mosquitoes are equally capable of transmitting every disease agent. Because of this, research into factors that affect vector competence is important to the medical community as it can lead to non-invasive ways of controlling transmission of tropical infectious diseases to humans. As has been seen in other studies, gut microbiota can vary greatly across species, as well as geographically distinct members of the same species (Osei-Poku et al, 2012), and the composition of the gut bacterial community has been shown to effect vector competence (Frentiu et al, 2014). In fact, *Aedes aegypti* engineered to harbor the endosymbiont, *Wolbachia*, has been shown to be less able to transmit DENV (Bian et al, 2013), and the release of *Wolbachia*-infected *Ae. aegypti* is the basis of a large-scale trial to limit DENV infections. It is now apparent that mosquito gut microbiota can play a role in human risk for vector-borne diseases. This study will examine the differences in the gut microbiota of *Culex (Melanoconion) ocossa and Culex (Culex) declarator* across two different habitats: peri-urban and rural. *Culex (Melanoconion)* spp are principle vectors for VEEV, and both the presence of *Culex* species and human risk for VEEV infection have been shown to vary geographically.

The goals of this study are to survey mosquito species in two habitats, peri-urban and rural, and to compare bacterial species in selected mosquitoes from each site. We will investigate how bacterial community composition within mosquitoes compares across both species and environment, examining bacteria between two different species that are reared in the same environment, and bacteria in a single mosquito species from two different environments. These

results will add to our understanding of how midgut flora is established and whether microbiota varies predictably by host.

This project involved fieldwork of collecting mosquitoes in CDC light traps in two different habitats in Iquitos, Peru. Following collection, laboratory testing will be carried out to identify bacterial DNA in selected mosquitoes. Procedures that will be used are DNA extraction, polymerase chain reaction (PCR), gel electrophoresis, gel extraction, and DNA sequencing. Fieldwork and most lab work will be conducted at the Naval Medical Research Unit Six (NAMRU-6) laboratories in Iquitos, Peru. DNA sequencing will be carried out by partners at the NAMRU-6 laboratory in Lima, Peru.

METHODOLOGY

Collection

Mosquitoes were obtained by trapping with CDC light traps. These traps work by first baiting the mosquitoes with CO_2. The CO_2 is obtained by either having a source of dry ice near the trap or using a mixture of yeast and sugar water that produces the gas. Once mosquitoes are attracted by the CO_2, a light at the top of the trap draws them in closer. They are then sucked down from the light by a fan and trapped in a chamber until the traps are collected. Traps were placed at two different locations. Two traps were placed in the backyard of a house in the San Juan district of Iquitos (this served as the peri-urban site) and two were placed in a forested area near the village of Zungaro Cocha (this served as the rural site). (Figure 1) Traps were set between 4:00 and 5:00 pm every day for three days, and collected the following morning between 7:00 and 8:00 am.

Identification

Once the containers that held the mosquitoes were returned to the lab, they were first placed in a freezer for 30 minutes. Following this, mosquitoes were transferred to a petri dish and viewed under a stereoscope. Mosquitoes were first sorted by sex and then into species and subspecies. Following sorting, the identification was reviewed by an expert. After the species were identified, they were then placed into labeled tubes and frozen at -80°C until needed.

Preparation for DNA Extraction

Twelve female mosquitoes were selected for the pilot study analysis: Three *Culex (Melanoconion) ocossa* from Zungaro Cocha and three from San Juan, as well as three *Culex (Culex) declarator* from each location. Under a sterile hood mosquitoes were sterilized using a two-step bath: first, soaking for 1 minute in a pure ethanol solution, and then, soaking for 1 minute in a phosphate buffered saline (PBS) solution. Each mosquito was then homogenized in 500 µl of PBS solution using a Retch Mixer Mill, then pulse-centrifuged to remove drops from the lids.

DNA Extraction

Following trituration, DNA extraction was performed using a QIAamp DNA Micro Kit (Qiagen, Valencia, CA) according to the guidelines provided by the manufacturer. First, 500 µl of the homogenate was transferred to a 1.5 ml microcentrifuge tube. One hundred and eighty microliters of ATL buffer was then added to the solution along with 20 µl of proteinase K. This solution was pulse-vortexed for 15 seconds and then kept in a heated incubator overnight at 56°C to lyse the sample. The following morning samples were removed from the incubator, and 200 µl of AL buffer was added to the solution and pulse-vortexed for 15 seconds. Two hundred microliters of pure ethanol was then added to each sample and incubated at room temperature for

5 minutes after mixing by pulse-vortexing. All samples were then placed in the centrifuge for 10 seconds to remove any drops from the lids. Each sample were transferred to a QIAamp MinElute column, placed in a 2 ml collection tube, and centrifuged at 8000 rpm for 1 minute. This step was performed twice to allow the entire sample to pass through the column. The 2 ml collection tube was then discarded and the column placed in a new collection tube. Five hundred microliters of AW1 buffer was placed in the column and centrifuged at 8000 rpm for one minute. The 2 ml collection tube was then discarded and the column placed in a new collection tube. AW2 buffer (500 µl) was added to the column and vortexed at 8000 rpm for 1 minute. The 2 ml collection tube was discarded and the column placed in a new collection tube and centrifuged at 14,000 rpm for 3 minutes to ensure that the membrane was dry. The 2 ml collection tube was discarded and the column placed in a 1.5 ml microcentrifuge tube. Twenty microliters of AE buffer was then added to the column and incubated at room temperature for 1 minute. Following this, the column was centrifuged at 14,000 rpm for 1 minute and the flow through containing the DNA from each sample was collected in a 1.5 ml microcentrifuge tube.

PCR

Two sets of primers were used to amplify the DNA. Because of this, two different master mixes of PCR reagents were created. Two solutions containing 140 µl PCR buffer, 56 µl dNTPs, 3.5 µl AmpliTaq Gold polymerase, and 402.5 µl water were made. In one tube, 14 µl of each primer, the 27 forward (5' – GAGTTTGATCCTGGCTCA – 3') (Vincent et al, 2011) and 342 reverse (5' – GCCTTGCCAGCCCGCTCAGCTGCTGCSYCCCGTAG – 3') (Kunin et al, 2010), was added. In another tube, 14 µl of each primer, the 348 forward (5' – ACTCCTACGGGAGGCAGCAGT – 3') and 700 reverse (5' – CGMATTTCACCKCTACAC – 3'), were added (Osei-Poku, 2012). For each DNA sample obtained from a mosquito, two PCR

solutions were then made: One using 45 µl of the primer solution in the first tube and 5 µl of the DNA sample, the other using 45 µl of the primer solution in the second tube and 5 µl of the DNA sample. All samples were then placed in a thermocycler with the following programing: One cycle at 95°C for two minutes (initial denaturation), followed by 30 cycles of 95°C for thirty seconds (denaturation), 55°C for thirty seconds (annealing), 72°C for thirty seconds (extension). One final extension at 72°C for 5 minutes was followed by incubation at 4°C until removal from the thermocycler.

Gel Electrophoresis

We then made a 2% agarose gel in TAE buffer. This was heated until dissolved and poured into a mold with two combs to form wells. After the gel had solidified it was removed from the mold and placed into an electrophoresis chamber and covered in TAE buffer. The samples were then loaded in wells 4-15 of both rows and the DNA ladder was added to well 3 for both rows. The gel ran at 80V for 42 minutes, after which the gel was removed from the chamber and pictures were taken using a Gel Doc EZ system imager. DNA was maintained at -20°C until sent for sequencing.

RESULTS

The totals of mosquitoes collected are recorded in Table 1. As can be seen in the table, *Culex melanoconion* spp and *Culex Culex* spp were the most abundantly captured, with *Cx. melanoconion* more abundant in rural environments and *Cx. Culex* more abundant in peri-urban environments. The data of our two species of interest, *Cx. (Mel.) ocossa* and *Cx. (Cx.) declarator*, were analyzed using a Fisher's Chi Square test to determine if there was a significant difference in abundance between the two locations. We found that there was a significant

difference in the two data points with *Cx. (Mel.) ocossa* collected more frequently at rural sites and *Cx. (Cx.) declarator* collected more frequently at peri-urban sites ($t(3) = 52.90$, $p < 0.001$) (Table 2).

Following extraction and PCR, we used gel electrophoresis to determine if DNA had been extracted from the mosquito. A picture of this gel is located in Figure 2. The gel was loaded with a ladder in lane 3 of both rows of the gel and DNA samples in lanes 4-15. With the exception of lanes 9 and 15 in row two, all of the odd numbered lanes in the gel show no DNA product. These odd lanes used the 348 forward/700 reverse primers and the even lanes used the 27 forward/342 reverse primers. Most of the even numbered lanes show that there was DNA isolated.

DISCUSSION

When examining the mosquito data we found that there was a significant difference in the locations of *Cx. (Mel.) ocossa* and *Cx. (Cx.) declarator*. *Cx. ocossa* was found primarily in the rural environment, while *Cx. declarator* was found mainly in the peri-urban environment. This correlates with where we would expect to find these species. From previous studies at this location and literature, we have seen that *Cx. (Mel.) ocossa* is a species that is found mostly in rural areas (Turell et al, 2005). This is because certain mosquito species preferentially live in certain areas, for example, *Culex quinquefasciatus* prefer to live in houses and reproduce in artificial water containers, explaining why they are found more abundantly in peri-urban environments than rural environments.

When examining the data from the gel we saw that some of the lanes showed no DNA in them and others had a DNA product. The majority of the lanes that did not have the DNA

product were the lanes that used the second primer that we mixed, the 348 forward and 700 reverse primer. The most probable explanation for this result is that either the annealing or extension temperature of the primers was incorrect. Upon further exploration into the melting temperatures of this primer set, we found that the best annealing temperature for these primers would have been a maximum of 50°C. Because we exceeded this temperature during the annealing phase, the primers might not have been able to anneal to the DNA that was present in the sample. This prevented the sample from being copied and resulted in no band appearing on the gel.

The next stage of the project will be Next-Generation Sequencing (NGS) of the DNA recovered from the mosquito specimens. NGS is a type of sequencing that will allow for rapid and accurate identification of the entire community of bacteria in a mosquito. This method will allow for the entire gut community to be analyzed from a single sample where previously this could not be done accurately with Sanger sequencing. While Sanger sequencing provides a consensus sequence, an average that may suggest the dominant bacterial species that are present, NGS allows for "deep" sequencing, or the sequencing of many individual members of the community. Following sequencing, the output data can be analyzed using software that matches sequences with know bacterial species giving us a view at the entire gut community in the mosquitoes. This data about gut microbial communities can then be analyzed in a variety of ways.

One way to analyze the bacterial communities between species would be too compare the proportions of the bacteria genera in the gut of various species of mosquito. This would show if there is a large difference in the bacterial community composition between the two species of mosquitos. Results from this type of test could be shown in a variety of different ways. They

could be shown in a bar graph that highlights the species that are most abundant or they could be shown in a different figure that highlights the proportions of each bacterial species in each mosquito species.

Based on the findings of previous research, we expect that the bacterial species found in these two mosquito species will be more closely related when the mosquitos come from the same environment than from the same species of mosquito. This is because previous studies have shown that the dominant effect on the mosquito gut flora is the environment in which the mosquito is raised (Wang et al, 2011). This factor has been shown to be more important in determining gut flora than either mosquito species or a parent-offspring relationship (Coon et al, 2014). As seen by Wang et al, the effect of the environmental bacteria to the adult species gut bacteria seems to wane over time. At juvenile stages the gut bacteria proportions will very closely resemble the water in which they were raised. However, as they mature the gut bacteria proportions will change with certain bacterial species becoming more prominent. This implies that the mosquito acquires bacteria from the environment and incorporates this into the gut. Also, based on research by Osei-Poku et. al., we expect the most abundant bacterial genera to be *Aeromonas*, *Asaia*, and *Chyseobacterium*. If further study indicates that these bacterial species are in fact the dominant species of bacteria in these mosquitos, then further research into these bacterial genera should be carried out. Potential studies could look to see if bacteria in these genera could lower the vector competence of the mosquito. This would lead to an effective method of vector control for these species as the bacteria are already naturally found in the mosquitos.

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Figure 1: This figure shows the collection sites of mosquitos. A indicates an aerial view of the city of Iquitos. B indicates Zungaro Cocha with the red dot marking the location of the trap and C indicates San Juan with the red dot marking the location of the trap.

Species	Zungaro Cocha	San Juan
Culex (Melanoconion) ocossa	46	0
Culex (Melanoconion) gnomatus	4	0
Culex (Melanoconion) vormerifer	2	0
Culex (Melanoconion) atratus group	4	0
Culex (Melanoconion) adanuci	2	1
Culex (Melanoconion) theobali	4	0
Culex (Melanoconion) pedroi	2	1
Culex (Melanoconion) partesi	0	0
Culex (Melanoconion) spissipes	1	0
Culex (Melanoconion) spp	98	1
Coquillettidia hermanoi	92	6
Coquillettidia arribalzagae	1	0
Coquillettidia nigricans	16	0
Coquillettidia venezuelenis	10	1
Culex (Culex) amazonensis	1	1
Culex (Culex) coronator	22	1
Culex (Culex) declarator	33	61
Culex (Culex) quinquefasciatus	12	69
Mansonia indubutans/titillans	2	0
Ochlerotatus serratus	1	0
Uranotania apicalis	6	0
Uranotania hystera	0	1
Uranotania pallidoventer	1	0
Uranotania lowii	0	1
Uranotania grometrica	1	0
Psorophora ferox	1	0
Aedes squamipennis	3	0
Culex (Culex) corninger	2	0

Table 1: This table shows the number of each species of mosquito collected and the location where they were caught.

Species	Zungaro Cocha	San Juan	
Cx. (mel) occassa	46	0	X^2=52.90
Cx. Declarator	33	61	p<0.001

Table 2: This table shows the Fisher's Chi Square test results.

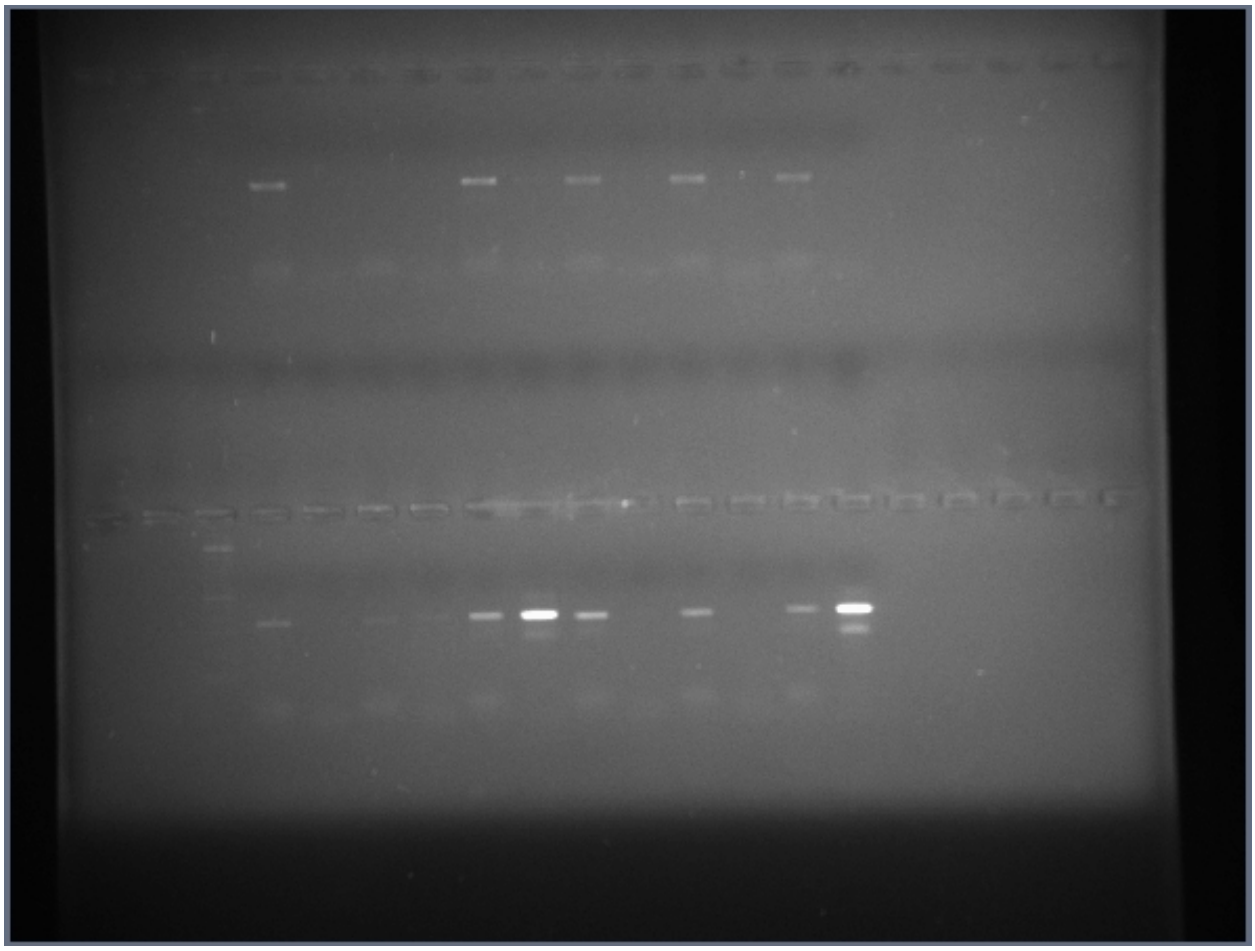

Figure 2: This picture shows the gel electrophoresis that was performed on the DNA results.