IMPACT OF MIDGUT BACTERIA ON AEDES ALBOPICTUS VECTOR COMPETENCE FOR LA CROSSE VIRUS

by

Deepa Ramaswamy

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Deepa Ramaswamy

Greenville, NC

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Approved by:

Stephanie Richards, PhD

Department of Health Education and Promotion, College of Health and Human Performance

**Abstract**

 La Crosse encephalitis is a pediatric illness caused by mosquito-borne La Crosse virus (LACV) and is endemic in western North Carolina. Mosquito control is the only defense against this disease. Not all mosquitoes can transmit LACV. Hence, risk assessments must be improved to target control efforts and protect public health. Midgut bacteria may impact the ability of mosquitoes to become infected with and transmit viruses, i.e. vector competence. Therefore, our pilot study sought to characterize the extent to which mosquito midgut bacteria impacts infection and dissemination rates (proxy for transmission rate) for LACV. We developed methodology to determine the antibiotic dose necessary to suppress midgut bacteria growth using the following groups: 1) no antibiotic; 2) low dose antibiotic; 3) high dose antibiotic. Antibiotics were administered to mosquitoes via sugar meal three days prior to midgut dissection and exposure to LACV. A subset of mosquito midguts was dissected and bacteria cultured for each group prior to delivering a LACV-infected blood meal. Surviving mosquitoes were collected at 14 days post-blood meal. Bodies and legs were separated and stored at -70°C until further processing (i.e. viral RNA extraction and qRTPCR). RNA extractions have been completed and analysis of LACV presence/absence and quantification is underway. This research is part of a larger study examining the overall role of mosquito midgut bacteria in vector competence. This study will ultimately help facilitate future experiments to evaluate impacts of varying levels of midgut bacteria diversity on vector competence.

**Introduction**

 La Crosse encephalitis is a pediatric illness caused by mosquito-borne La Crosse virus (LACV). It is endemic in western North Carolina with 100-200 human cases recorded every year (Joyce, 2011, pp. 389-394). LACV was first identified as a human pathogen in 1960 after its isolation from a four year old girl from Minnesota (Bennett, 2008, pp. 5-25). She suffered from meningoencephalitis and later died in La Crosse, Wisconsin which gives us the origin of the virus’ name (Bennett, 2008, pp. 5-25). In humans, the majorities of infections are mild and attributed to the “flu” or “summer cold” symptoms (Bennett, 2008, pp. 5-25). Annually an estimated 300,000 infections are reported nationwide but only 70-130 are serious cases (Bennett, 2008, pp. 5-25). However, not all mosquitoes can transmit LACV. Hence, risk assessments must be improved to target control efforts and protect public health. Midgut bacteria may impact the ability of mosquitoes to become infected with and transmit viruses. Because vector-borne diseases usually vary in space and time and are related to the ecology of the vector species, we purpose that the ecology of its mosquito vectors, such as their immature stages play an important role in the incidence of La Crosse encephalitis in the Appalachian region (Leisnham, 2012, pp. 217-228). For example, a study conducted with field caught *Aedes albopictus* in West Virginia showed that half of midgut bacterial species significantly reduced infectivity of LACV (Joyce, 2011, pp. 389-394). Therefore, this current pilot study was conducted to improve methods for studying midgut bacteria in *Aedes albopictus* and their potential interactions with LACV.

 Findings show that > 20 arthropod-borne viruses (arboviruses) have been isolated from field-caught *Aedes albopictus*, e.g. LACV, dengue virus (DENV), chikungunya virus (CHIKV), and West Nile virus (Joyce, 2011, pp. 389-394). *Aedes albopictus* oviposit in artificial or natural water-holding containers located in suburban environments such as old tires, buckets, tarps, plant pot dishes, and tree holes (Joyce, 2011, pp. 389-394). The mosquito midgut is the first point of contact for the virus after a blood meal is imbibed. Midgut infection and escape barriers must be overcome for a mosquito to become infected and subsequently transmit pathogens (Joyce, 2011, pp. 389-394). A study showed that the diversity of gut flora in *Aedes albopictus* infected with CHIKV varies over time and with infection status (Cirimotich, 2011, pp. 307-310). The same study showed that infection with CHIKV promotes growth of some bacterial species and decreases growth of other species (Cirimotich, 2011, pp. 307-310). Consequently, the goal of this research is to promote risk assessments improvement to target control efforts in order to protect public health.

**Methodology and Results**

 The setting of the entire study took place in Dr. Stephanie Richard’s BSL-2 laboratory and teaching laboratory at East Carolina University’s Carol Belk Building. Mini-experiments were conducted along the way so that a protocol for the current study could be developed. The first mini-experiment that was conducted was to determine: 1) if viable and culturable bacteria were present within our colonized mosquitoes, 2) whether nutrient or dehydrated agar should be used to culture bacteria, and 3) what type of plating method (e.g. streaking, spreading, etc.) would maximize bacterial detection. The following is the protocol that was created from this mini-experiment:

1. Set a water bath for 37 °C
2. While the water bath is getting set to the temperature indicated in Step 1, aspirate 50 female mosquitoes from their cage and place them into a bin of wet ice to immobilize them.
3. Dissect five mosquitoes and isolate their midguts under a microscope in a sterile environment
	1. Place all five midguts into a 2 mL microcentrifuge tube with approximately 1 mL of distilled water and grind the midguts with a pestle by hand. Set aside.
4. Place five whole mosquitoes 1 mL microcentrifuge tube with approximately 1 mL of distilled water and grind the mosquitoes with a pestle by hand. Set aside.
5. Repeat steps three and four so that you now have four centrifuge tubes, i.e. two tubes with five midguts within each one, and two tubes with five whole mosquitoes each.
6. There will be two different types of agar used.
	1. Dehydrated
		1. Weigh out 2.35 g (on scale 2.40 g) of this type of agar and place in a 250 mL Erlenmeyer flask.
		2. Pipet out 100 mL of distilled water into the same 250 mL Erlenmeyer flask as in step 6ai.
		3. Gently swirl flask by hand and boil for 1 minute in a microwave until boiling and into a “thickish” liquid.
		4. Once complete, immediately pipet out with a sterile tip 50 mL of agar into another tube.
		5. Place the flask with the remaining agar into the water bath (which should be set at the proper temperature) for 20 minutes.
	2. Nutrient
		1. Weigh out 3.1 g of this type of agar and place in a new 250 mL Erlenmeyer flask.
		2. Pipet out 100 mL of distilled water into the same 250 mL Erlenmeyer flask as in the previous step.
		3. Gently swirl flask by hand and boil for 1 minute and 30 seconds in a microwave while keeping a close eye on the flask until boiling and into a “thickish” liquid.
		4. Once complete, immediately pipet out with a sterile 50 mL of agar into another tube.
		5. Place the flask with the remaining agar into the water bath which should be set at the proper temperature for 20 minutes beside the dehydrated agar flask.
7. Procure eight sterile petri dishes. Label them on the back outside over with the following:
	1. Type of Agar (Dehydrated or Nutrient)
	2. Type of Method (Streak or Mixture)
	3. Midguts or Whole
	4. Date
	5. Initials
8. Under the biosafety cabinet, pipet 15 ml of dehydrated agar each into two petri dishes while the agar is still hot. Partially cover with lid of plate to make sure there is no condensation and let it dry.
9. Do the same as in step eight but with pipetting 15 mL of nutrient agar each into two petri dishes.
10. While the dishes are cooling, start to heat up the loop “streaker” heater.
11. Once heater is hot and ready…
	1. Assemble the streak loop and sterilizing it for five seconds in the heater.
	2. Dip the loop into the midgut tube and dip the loop on one edge of the plate to cool before starting to spread the loop across 1/4th of the plate.
		1. Turn the plate 90 degrees and take a little bit of the previous 1/4th of the plate’s midgut dilution and spread individually and neatly in adjacent 1/4th of the plate.
		2. Repeat step 11b-ii until the entire plate is complete with three dilutions and one baseline dilution of the midgut bacteria tube.
	3. Repeat all the steps 11 mentioned above once again but with the same agar and in five whole mosquito solution.
	4. Repeat all the steps in 11 previously mentioned except this time in the nutrient agar plates.
12. Once this has been completed, you should have the following plates completed:
	1. one dehydrated streak dipped in five midguts solution
	2. one dehydrated streak dipped in five whole mosquitoes solution
	3. one nutrient streak dipped in five midguts solution
	4. one nutrient streak dipped in five whole mosquitoes solution
13. Now the flasks with the remaining agar in the water baths should be ready.
14. Remove flasks from water bath and bring into one after the other under the hood
15. Pipet 450 microliters of the five midguts solution into a new empty plate.
16. Add 15 mL of the dehydrated agar while still warm into the plate mentioned in step 15 and gently swirl the plate around until the entire plate is covered.
	1. Partially place its lid to eliminate condensation and set it aside to cool.
17. Repeat steps 15 and 16, but, instead of 15 mL of dehydrated agar, use 15 mL of nutrient agar.
18. Repeat all parts of steps 15, 16, and 17, but using five whole mosquito solutions instead of five midguts solution.
19. Now you should have eight petri dishes set aside in the biosafety cabinet cooling, in the meantime clean up the areas used for this experiment.
20. By the time all is cleaned up, the plates should be cooled down.
	1. Now invert the dishes so that the back, outside of the dishes are stacked with lid facing down and incubate at 30°C.
21. After two days you should examine the plates to see if there are any colonies of growth.

 The results seen from this mini-experiment showed us that the nutrient agar worked better to maximize the bacteria on the plates and that the streak method was better than the mixture method when extracting the mosquito midguts and placing their bacterium solutions on the agar plates. Once the above mini-experiment showed us that in order to maximize the amount of midgut bacteria present nutrient agar should be used along with the streak method, we wanted to how long the female mosquitoes should be soaked in ethanol. This was a crucial mini-experiment to conduct because the ideal amount of time the mosquitoes are saturated in ethanol is used to sterilize the exterior surface of the mosquito without killing the bacteria inside the midgut. This would allow midgut bacteria to be isolated. The following protocol was created:

1. Aspirate 30 female *Aedes albopictus* mosquitoes.
2. Place them in a sealed container and on ice to immobilize them.
3. Place 10 mosquitoes in a centrifuge tube filled with ethanol for 10 minutes.
4. While the mosquitoes are soaking in ethanol, dissect 10 midguts and place it in a centrifuge tube labeled (“0 minutes”) that only has distilled water. Then set them aside.
5. By this time, the timer should have gone off for the mosquitoes already placed in ethanol, so carefully take the mosquitoes out of the ethanol tube and place them in water.
6. Place the last remaining 10 mosquitoes not used so far into a new tube filled with ethanol for five minutes.
7. During this time, dissect the 10 minute mosquitoes and place them in a tube of water labeled (“10 minutes”). Set aside.
8. Once the five minutes have run out, dissect them as well and place them in a tube labeled (“five minutes”).
9. Using a pestle, grind up midguts in each tube. Refrigerate these tubes for up to two days before streaking the bacteria on nutrient agar petri dishes using the protocol mentioned above.
10. Streak two petri dishes for each of the three midgut tubes created giving you a grand total of six dishes.
11. Incubate these dishes at 30°C for two days before looking for any colonies.

The results from this ethanol mini-experiment showed that placing the whole mosquitoes in ethanol for 10 minutes prior to dissecting them was most efficient in isolating them. It also showed the most diverse phenotypes of colonies as well when compared with the two other treatment groups.

Plaque assays were performed to determine virus titer (concentration) of our LACV stock sample. Two different types of plaque assay protocols were used (Neutral Red and Crystal Violet). The first half of the protocol used was the same for both and then after incubation in 35°C the different plaques (Neutral Red and Crystal Violet) were performed. To perform the plaque assay, 10-fold dilutions of a virus stock were prepared and 0.1 mL aliquots were inoculated onto susceptible (Vero) cell monolayers. After the incubation period was completed to allow the virus to attach to the cells, the monolayers were covered with nutrient agar to form the gel for the plates. This process was done with the following reagents:

* 1. SeaPlaque Agarose, low melting. FMC Biotechnology. Cat: 50113
	2. 2x EMEM (includes L-Glut). Cambrex. Cat: 12-668E
	3. Neutral Red Solution. Sigma. Cat: N2889
	4. Complete media (use to dilute samples to be tested): EMEM, 10% FBS and penicillin/streptomycin.
	5. Fungizone (100x) solution (any manufacturer).

The step-by-step procedure used to perform the plaque assays in this study was the following:

1. Preparation of cell cultures.
	1. This should be done a few days before the plaque assay itself.
	2. Split VERO cells from their culture flasks as usually done (by using Trypsin-EDTA). Wash cells appropriately.
	3. Count calls using Trypan Blue exclusion.
	4. Seed cells in 6-well plates using 5x104 cells per well (three ml total volume per well). Using this number of cells you can expect confluent wells within 3 to 4 days. Use more cells for seeding if cultures are needed earlier.
	5. Incubate at **35oC**. Monitor confluence of cultures. For plaque assays cells must reach at least 90% confluence.
	6. One 6-well plate will be needed per sample to be tested. Seed accordingly.
2. Preparation of samples (to be done when VERO cells are confluent)
	1. For **each sample** to be tested, label six 15-ml conical tubes. These tubes will be used for serial dilution of samples.
	2. Add 1.8 ml of complete media to each of the tubes.
	3. For **each sample** add 0.2 ml to its correspondent tube No.1. Mix briefly by pipeting up and down.
	4. From tube No.1 take 0.2 ml and add it to tube No.2. Mix briefly.
	5. Continue in this way until all six tubes are mixed. There is no need to remove 0.2 ml from last tube.
3. Infection of VERO cells.
	1. Remove VERO cell cultures from incubator and aspirate media, leaving about 0.2 ml in each well.
	2. Add 0.2 ml from dilutions (as prepared on D) to corresponding wells. The small volumes are to optimize infection of VERO cells by virus.
	3. Make sure VERO cell layers are totally covered with media by gently rocking the plates front-to-back and left-to-right. Do not swirl the plates as this may cause media to pool on the edges, leaving the center uncovered.
	4. Return plates to incubator (at **35oC**) and leave for 1 hour. Once or twice during that hour briefly rock the plates again.
	5. While cells incubate, prepare 1st overlay.
4. Preparation of 1st overlay reagents (for up to ten plates).
	1. In sterile flask mix 1.8 gm of **Seaplaque Low Melting Agarose** and 100 ml of ddH2O
	2. Microwave until completely melted (solution must be totally translucent).
	3. Place in 40oC water bath for at least 30 minutes.
	4. In a separate conical tube mix: 10 ml of heat-inactivated **FBS**, 2 ml of **non-essential amino acid solution** and 0.2 ml **penicillin/streptomycin** (100x) solution (or your favorite antibiotic).
	5. Check the **2xEMEM** for **L-Glutamine** content. If there is no **L-Glut** in the media, add 1 ml to it. Add 2 ml of **Fungizone** (this is to prevent fungi contamination)
5. 1st overlay [to be done when VERO cells have incubated for 1 hr (as in E) and agarose drops in temperature].
	1. Take Vero cells out of incubator.
	2. Mix 1st overlay reagent in the following order: Add 100mL of 2x EMEM to the agarose and swirl. Add FBS solution to flask and swirl.
	3. Gently add 3 ml of agarose solution to each plate well.
	4. Leave plates uncovered inside flow hood for about 15 minutes to dry.
	5. When plates appear to have dried, incubate them at **35oC for 48 hours**.

**WHEN DOING NEUTRAL RED ASSAY**

1. Preparation and addition of 2nd overlay (to be done after 48 h).
	1. In flask mix 1.8 gm **Seaplaque Low Melting agarose**, 2.0 gm **Sodium Chloride** and 200 ml ddH2O.
	2. Microwave as done in F and place in 40oC water bath for cooling (for at least 30 minutes).
	3. When agarose reaches 40oC, add 9 ml **Neutral Red** solution and swirl thoroughly.
	4. Add 3 ml of agarose solution to each well. Allow plates to dry for about 15 minutes and place them in incubator (35oC) for 24 hours.

**WHEN DOING CRYSTAL VIOLET ASSAY**

1. Preparation and addition of 2nd overlay (to be done after 48 h).
	1. Place plates under microscope and count plaques or you can stain by crystal violet.
	2. Crystal violet (stock solution) = 1 g CV + 99mL 95% ethanol
	3. Mix: 10 mL CV stock, 5 mL ethanol (70% works fine), 9.5 mL ddH2O
	4. Remove agar by tilting plate over biohazard garbage (let it slide off)
	5. Apply 2 drops (plastic disposable pipette) of CV stain solution to each well. Apply more, if necessary. You need the stain to cover the entire well.
	6. Apply drops of water, rinse, and dump. Apply more drops of water, rinse, and dump.
	7. Gaps in stain = plaques
	8. Put plates in 35C incubator
	9. Count plaques 48 h later under microscope or on light box.

Overall, between the two different plaque assays (Neutral Red and Crystal Violet) completed using the same reagents mentioned above, more colonies with diverse phenotypes were seen through the Neutral Red plaque assay. At this point, it was also important to be aware that the diameter of the plaque changes depending on the LACV strain of the sample we used. Our virus stocks were determined to have 6.5 plaque-forming units LACV/mL.

Mosquito colonies were sugar fed with the antibiotic penicillin/streptomycin with the following doses in three different treatment groups:

* + - 1. High Dose (10 mL/kg) of antibiotics
			2. Low Dose (2 mL/kg) of antibiotics
			3. No antibiotics (Control Group)

Then a subset of mosquitoes from each of the three treatment groups mentioned above were surface sterilized using the ethanol mini-experiment protocol listed above 72 hours after they underwent antibiotic treatments. And the dissected mosquito midguts were cultured to determine differences in bacterial colony growth between groups. From this mini-experiment of the study the mosquitoes receiving high dose (A) of antibodies showed approximately 80% reduction in midgut bacteria colony growth compared to mosquitoes receiving either a low dose (B) or no dose (C). The following pictures present the colony growth results mentioned above.

  

 A B C

Following the above steps, mosquitoes in all groups were fed infectious blood meals containing LACV. Surviving mosquitoes in each of the groups were collected at 14 days post-LACV infected blood meals. At the 14 days post-infection time point, we ended up having N=11 mosquitoes alive in the no antibiotic group, N=8 in the low dose antibiotic group, and N=6 in high dose antibiotic groups. The bodies and legs were triturated separately and stored at -70 degree Celsius in 0.900 mL M199E (media) giving us a total of 55 samples for further analysis.

The 55 samples underwent viral RNA extractions to further determine infection and dissemination rates via a qRT-PCR analyses. The viral RNA extraction protocol we used for this study was through the QIAamp Viral RNA Third Edition Mini Handbook. The qRTPCR analyses are still underway to determine the extent to which reductions in midgut bacteria impacted mosquito infection and dissemination rates. This concludes all the methodology used so far in this study.

**Discussion**

 As there is little published data about the impact of *Ae. albopictus* midgut bacteria on LACV vector competence, any information gained here is useful for further studies. We expect the qRT-PCR results to be available in summer 2015. Once these results are obtained, we will report whether or not the presence/absence of midgut bacteria impact infection and dissemination of LACV. Once this is done, I hope to see future students expand this study to include another LACV vector, *Aedes triseriatus*. Ultimately, the goal of this research is to improve risk assessments to target control efforts to potentially dangerous mosquito populations in order to protect public health.

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