**Vertical Transmission of Insect Specific Viruses in *Aedes aegytpti***

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**Abstract**

*Aedes Aegypti* mosquitoes are a major vector of pathogenic arboviruses, including dengue and zika viruses. In this project, we quantified the vertical transmission efficiency of insect specific viruses that are common in wild mosquitoes. These viruses cannot be transmitted to vertebrates but may impact the biology of their host. Viruses with efficient vertical transmission have an inherent ability to spread through host populations and so could have important applications in biocontrol. We first reared mosquitoes from eggs, crossed them and sampled adults and offspring from those crosses. Then we performed RNA extraction, Reverse Transcription (RT) and qPCR to screen for viruses in each sample.  We found that three viruses, Verdadero virus, Rennavirus and *Aedes* Anphevirus, exhibited efficient biparental vertical transmission. This property likely underlies their success in the wild and their persistent infection of colonized mosquitoes and means these could be good candidates for gene delivery through mosquito populations.

**Background**

Mosquitos from the *Aedes aegytpi* genera are by far one of the biggest transmitters of arbovirus diseases in our world today. They are ubiquitous and mostly found in tropical and subtropical regions. *Aedes* also have high vectorial capacity and vector competence and actively seek out humans for a blood meal which makes them more likely to transmit arboviruses [1].

*Aedes* are persistently infected with a myriad of viruses, their virome. Most often, when people think of viruses within mosquitos, they typically think of very medically prevalent viruses such as Dengue, Zika, Yellow Fever, etc. But there are many more viruses at play than one may think [2]. The virome is composed of viruses associated with the host, viruses that infect the host itself and viruses associated with ingested blood meals. All these viruses can be detected through metagenomic sequencing.

The first Insect Specific Virus (ISV) that was described is a member of the Flaviviridae family and is named CFAV – Cell Fusing Agent Virus. It was first discovered in 1975 within an *Aedes aegypti* cell line and was further characterized in later years [6]. More recent research has shown that ISVs have the potential to affect the transmission of arboviruses. It has been found that infections by ISVs are much more abundant and prevalent than arbovirus infections in wild mosquitoes [3]. Viruses like Phasi Charoen-like virus and Humaita-Tubicanga virus are major ISVs that have been identified at high prevalence’s and have a wide distribution [3,4]. We have also seen major groups of ISVs belonging to Flaviviridae, Togaviridae, and Rhabdoviridae families but there is no current data showing the mechanisms of how ISVs establish an infection in the mosquito [5]. Current control methods of mosquitos include insecticides, repellents, and other personal protection methods. The downside to these methods is insecticides can be harmful to humans or have an impact on non-target species, also, mosquitos can and have been developing resistance to our current control methods [7]. Through increased knowledge of ISVs, we can start to develop a better understanding of the complex interactions that are occurring within the mosquito and can have a better understanding of how to develop new arbovirus control strategies.

The main goal of this study was to find ISVs that are biparentally vertically transmitted at high efficiency that could potentially be good candidates for biocontrol agents. A biocontrol agent is an organism which is used to control a “pest species”. This idea can be applied to viruses whereby infecting an organism with this biocontrol agent it will help control the spread of a potentially worse virus to a new host. See figure 1 for reference in mosquitos. Some of the most important factors of finding a good biocontrol agent are that they should be able to infect a high number of individuals, have a low fitness cost and replicate in tissues relevant to arbovirus transmission. All these factors are represented in a virus that is vertically transmitted at high efficiency by both parents. Once we find a virus that has those properties, we can then investigate how it already plays into the mosquito system or how we can integrate it into the system via reverse genetics. This study focuses on the natural route looking at viruses that already infect populations of *Aedes aegypti* at high rates.

**Diagram

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**Figure 1** – Biocontrol Agent Pathway: A biocontrol agent is in our case a virus of interest that is infecting *Aedes aegypti* mosquitos that will stop the transmission of other deadly arboviruses to humans when bitten by the mosquito.

**Materials and Methods**

Map

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**Figure 2** – Map of original collection locations of all colonies of *Aedes* used for this project.

We received *Aedes aegypti* Tapachulas mosquito eggs from Dr. Ken Olson’s lab as well as Poza Rica, New Orleans and Vergel eggs from Dr. Brian Foy’s lab at Colorado State University, Center for Vector Borne and Infectious Diseases, Fort Collins, CO.

**Rearing Mosquitos:**

The insect rearing room is set to conditions of 80% humidity and 80 degrees F. Hatch the oldest eggs first if you have multiple egg papers.

Day 1: Place egg paper in 5’’x 12’’ small plastic bin with ~800mL of autoclaved and cooled DI H2O. Add a small pinch of TetraMin fish food that has been ground into a powder. Cover container with plastic lid and label with species, strain, date, and the initials of who hatched. Day 2: Pour larvae from small plastic bin into a larger bin with ~2 inches of tap water in the bottom. Check for larval crowding, splitting the bin if necessary. Feed with ~1/4 teaspoon of fish food. Days 3 and 4: Check for larval crowding, splitting the bin if necessary. Feed with fish food if necessary. Days 6 and 7: Check bins for pupae. Pick out any pupae with a pipette or mesh scoop and place into a small cup for sorting. Be sure to change pipettes or clean the mesh scoop between strains. Keep different strains in clearly labeled sorting cups. Every day that you have pupae you will need to sex them and then place them into cartons for eclosion.

Diagram

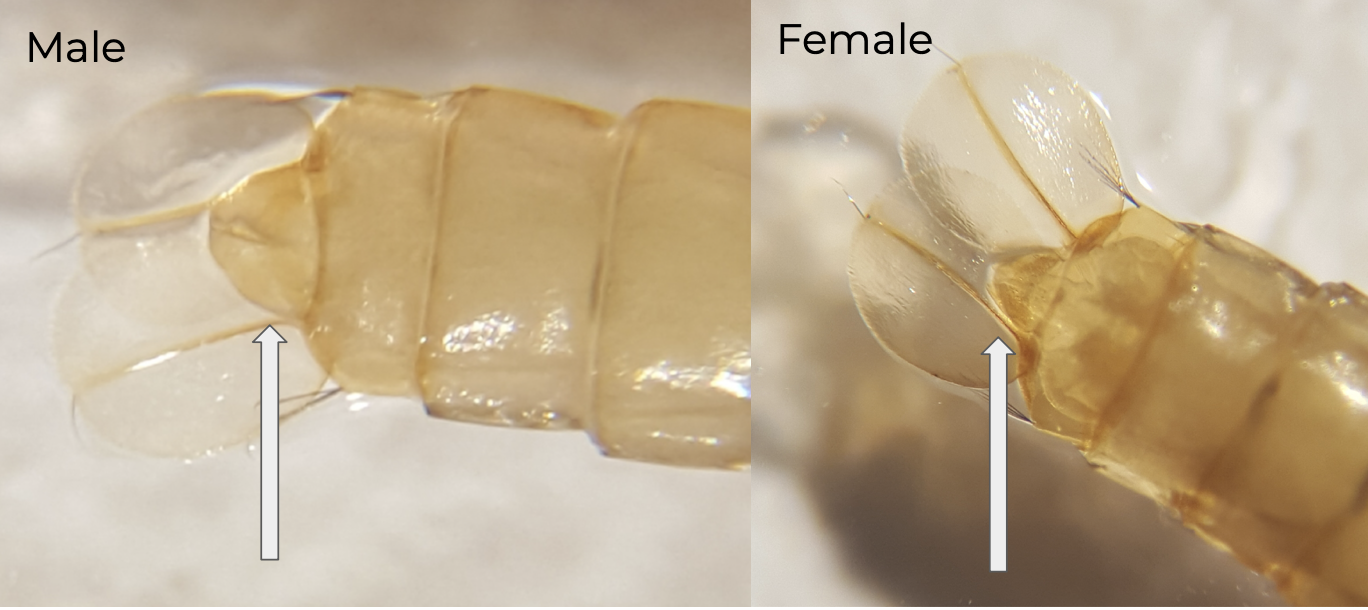
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**Figure 3** – *Aedes Aegypti* Lifecycle

**Sexing pupae:**

Start by cutting the tip off one of your 3mL pipettes to ensure pupae can fit through the pipette. Label 2 small eclosion cups, one for females and the other for males. Then fill these about ½-¾ of the way full of clean water. Place a medium glass petri dish under a dissecting microscope and use the light on the underside of the petri dish when sorting. Suck up a group of pupae using the pipette with the tip cut off and squirt them out onto the glass petri dish. Remove any larvae using whichever pipette works best. Then remove all the excess water from around the pupae using a pipette that does NOT have it’s tip cut off (this makes it easier to suck up just the water and not the pupae). If any excess water is still around the pupae pile use a Kim Wipe to soak it up. Sort the pupae under the microscope using a micro paint brush, making two separate piles one for females and the other for males (you can start by separating them by size, typically females are larger than males). Figure 4 below shows you the difference between male and female pupae. When you have all the pupae sorted that are currently on the petri dish move them into their respective labeled female or male eclosion cups, you can use the micro paintbrush to do this or a larger one if easier. Repeat this until you have sorted all the pupae you picked that day. Label 2 64oz cartons with the respected strain and sex of the mosquitos you are working with. Then place each pupae cup into each carton (one carton for females and the other for males). Cover the carton with organdy, a paper lid, and a rubber band to secure the adults in. Place 1-2 raisins and a water cup on the top of the carton to sustain the mosquitos until they are ready to be crossed.

\*\*\* If you are new to sexing pupae, I recommend separating the female pupae out into smaller groups of about 5-15 depending on how many you have and rearing in smaller cartons to avoid any contamination by a male.



**Figure 4** – Male and Female Pupae (image courtesy of Dr. Adeline Williams)

**Making crosses:**

\*\* For each cross you will need 80-100 females and 15-30 males.

Start by anesthetizing the sexed mosquitos in a 4 °C fridge for about 5 minutes. During these 5 minutes place one glass petri dishes in each ice bucket filled with ice. After the mosquitos have been anesthetized, you can then count out the correct number of females and males for each cross as well as check that all females are virgin (no male in the female bins). For this I recommend that the unsorted cartons from the fridge can go into one petri dish and then you can sort and count them out into the second petri dish. Once they are sorted and you have the correct number of virgin males and females you can pour them from the petri dish into a large carton, cover it with organdy, put the lid around the top and put a rubber band on the top to make sure they are secure. Do this for each cross being made.

**Blood Feeding:**

**\*\*\*IMPORTANT – Remove all food (raisins, sugar) at least 12 hours prior to feeding. Remove all water cups 3-6 hours before feeding. If you don’t starve the mosquitos, they will not feed.**

First remove calf’s blood from the fridge and let it start to warm up. We order calf’s blood from Colorado Serum Company. While the blood is warming, cut hogs gut wide enough to fit the opening on the blood feeder with extra coming over the sides. Lay hog’s gut over the opening and stretch tightly, secure the hogs gut with a rubber band so that it is tight. It should bounce back and be rigid like skin. Place each feeder with hog’s gut onto a damp paper towel so that the hog’s gut doesn’t dry out. Once all the feeders are set up bring the feeders, the water bath, and the tubing into the room where you will do the feeding. Fill and plug in the water bath and set the temperature to around human body temp (37°C). Place one feeder on each bin that you will be feeding and connect them all to the water bath using tubing. Be sure to note which side of the feeder is the inlet and outlet. All the feeder’s flow should be the same direction. Start the water bath circulating and check to see if there are any water leaks in your set up. If there are no leaks, then you are good to move on. Add ~2mL of blood to each feeder and let the mosquitos feed for about 1 hour or until the hog’s gut is dried up. After feeding immediately set up each carton for egg collection. Also, take down the set up and clean all feeders and items with blood with bleach and throw away any blood contaminated trash in the biohazard trash.

**Egg Collection**

Immediately after blood feeding, set up the egg papers. It will take the mosquitoes three days to start laying eggs, but we put it in immediately after blood feeding to give the mosquito a water source as the paper towel acts as a wick. Line the small water cup with a thin strip of paper towel Wet the paper towel and fill the bottom of the cup slightly to give them a water bank to lay eggs on. Since *Aedes* are flood water mosquitos they will lay eggs next to water sources. To place the egg cup in the carton, anesthetize the bins and when all mosquitos are asleep quickly tape the newly made egg cup to the bottom of the carton. Wait 3 days for them to begin to lay eggs. Once you have enough eggs you can move onto drying. If you did not get a significant number of eggs after a 3-5 day’s post feeding, you will have to do another blood feed and egg collection. After you have sufficient eggs lay the papers egg side up out of water to dry. Let the papers dry fully and then they can be stored for up to 8 months. For best practices you should let the eggs dry and store them in the same conditions that you are growing mosquitos in.

\*\*Once you have collected pre-cross, post-cross, and offspring adults from each cross you can move onto performing RNA extraction, RT and qPCR.

**RNA Extraction**

For RNA extraction we have been using a bead-based RNA extraction method on the King Fisher machine.

(Link to published protocol)

**Reverse Transcription (RT)**

For reverse transcription for this project, we have been using a mashup RT protocol.

(Link to published protocol)

**Quantitative PCR (qPCR)**

For qPCR we have been doing 10uL reactions using NEB Luna Universal qPCR master mix and the primers listed in the table below.

NEB protocol: <https://www.neb.com/protocols/2016/11/08/luna-universal-qpcr-master-mix-protocol-m3003>

|  |  |  |
| --- | --- | --- |
| **Virus** | **Primer sequence** | **Amplicon size (bp)** |
| Anphevirus | FW 5’  GACAGAAGAAAGGGCTGTGC  RV 5’  ACTCGGGATACACAGCAACC | 353 |
| Verdadero virus | FW 5’ ATATGGGTCGTGTCGAAAGC  RV 5’ CACCCCGAAATTTTCTTCAA | 341 |
| Rennavirus | FW 5’  TTGTAAATCCCCCTGTGCTC  RV 5’  CGCAAAAATTGGGTTTGAGT | 497 |
| Phasi Charoen Virus | FW 5’  TGGAGTGCTCATTGAAAGAC  RV 5’  GAAGGGAACACTGGGAAAC | 92 |
| *Aedes Aegypti* Actin 1 Housekeeping Gene | FW 5’  CGTTCGTGACATCAAGGAAA  RV 5’  GAACGATGGCTGGAAGAGAG | 175 |

**Figure 5 –** Table of primers used for qPCR.

***Aedes Aegypti* Actin 1 Housekeeping Gene** - Dzaki, N., Ramli, K., Azlan, A. et al. Evaluation of reference genes at different developmental stages for quantitative real-time PCR in *Aedes aegypti*. Sci Rep 7, 43618 (2017). https://doi.org/10.1038/srep43618

Timeline

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**Figure 6** – Workflow of methods for making crosses and screening

**Results**

Calendar

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**Figure 7** – Vertical transmission of viruses from parent to offspring in Poza Rica and New Orleans crosses

Calendar

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**Figure 8** – Horizontal transmission of viruses during mating in Poza Rica and New Orleans crosses

Table, calendar

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**Figure 9** – Vertical transmission of viruses from parent to offspring in Poza Rica and Vergel crosses

Calendar

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**Figure 10** – Horizontal transmission of viruses during mating in Poza Rica and Vergel crosses



**Figure 11** – Vertical transmission of viruses from parent to offspring in Tapachula and New Orleans/ Vergel crosses



**Figure 12** – Horizontal transmission of viruses during mating in Tapachula and New Orleans/ Vergel crosses

**Discussion**

Preliminary screening of these crosses showed four viruses; Rennavirus, Verdadero virus and Anphevirus, and Phasi Charoen virus that are biparentally vertically transmitted at high efficiency. In our study we looked at adults before we mated them (pre-cross), after we mated them (post-cross) and their offspring. For the Poza Rica pre-cross adults, you see high levels of infection of three viruses, Rennavirus, Verdadero virus and Anphevirus. In Tapachula pre-cross adults you see high levels of Rennavirus infection and low levels of Phasi Charoen virus infection. New Orleans and Vergel pre-cross adults are all uninfected by any of these four viruses. Looking closer into the results we can see that when Poza Rica adults are crossed with New Orleans or Vergel adults you see some sort of horizontal sexual transmission with all three viruses infecting the Poza Rica strain. These viruses can also be successfully transmitted to offspring at high levels. When we crossed Tapachula adults with New Orleans or Vergel we see similar results for Rennavirus. Rennavirus can be horizontally transmitted post mating and vertically transmitted to offspring. Phasi Charoen virus was a low-level infection to start but was still transmitted horizontally post mating and vertically to offspring. We think our low-level of detection could be due to our relatively small sample size (n = 24).

Throughout the data we have also seen multiple phenotypes of infection, highly infected (ct 5-19), uninfected (ct 0), and low-level infections (ct 20-35). Rennavirus exhibited this quality of multiple phenotypes the best and can clearly be seen in the results. We see that typically a high positive female or male when mated with an uninfected individual can transmit the infection but more at a low-level. This could be some sort of low-level horizontal transmission or contamination from being in the same location as a high positive individual. We also see both high and low positive phenotypes in the offspring indicating that the virus can be vertically transmitted to offspring.

Our results also show that both maternal and paternal horizontal and vertical transmission of these viruses occurs however maternal transmission is more efficient for Rennavirus, Anphevirus and Verdadero virus. Looking at Phasi Charoen virus we see that horizontal transmission is very low and vertical transmission also occurs at low levels but is much more efficient through maternal transmission over paternal.

Overall, our results show that a highly infected male or female can successfully transmit Rennavirus, Anphevirus or Verdadero virus to their partner and subsequently have a large percentage of their offspring being infected. With this we can see good candidates that have the potential for being used as a biocontrol agent.

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**Citations**

[1] Gómez, M., Martinez, D., Muñoz, M. *et al.* *Aedes aegypti* and *Ae. Albopictus* microbiome/virome: new strategies for controlling arboviral transmission?. *Parasites Vectors* **15**, 287 (2022). https://doi.org/10.1186/s13071-022-05401-9

[2] Thannesberger J, Rascovan N, Eisenmann A, Klymiuk I, Zittra C, Fuehrer HP, Scantlebury-Manning T, Gittens-St Hilaire M, Austin S, Landis RC, Steininger C. Highly Sensitive Virome Characterization of *Aedes aegypti* and *Culex pipiens* Complex from Central Europe and the Caribbean Reveals Potential for Interspecies Viral Transmission. Pathogens. 2020 Aug 21;9(9):686. doi: 10.3390/pathogens9090686. PMID: 32839419; PMCID: PMC7559857.

[3] Olmo, Roenick P., et al. “Insect-Specific Viruses Regulate Vector Competence in Aedes Aegypti Mosquitoes via Expression of Histone H4” *BioRxiv,* Cold Spring Harbor Laboratory, 1 Jan. 2021, bioRxiv 2021.06.05.447047; doi: <https://doi.org/10.1101/2021.06.05.447047>

[4] Zakrzewski M, Rašić G, Darbro J, Krause L, Poo YS, Filipović I, Parry R, Asgari S, Devine G, Suhrbier A. Mapping the virome in wild-caught Aedes aegypti from Cairns and Bangkok. Sci Rep. 2018 Mar 16;8(1):4690. doi: 10.1038/s41598-018-22945-y. PMID: 29549363; PMCID: PMC5856816.

[5] Öhlund P, Lundén H, Blomström AL. Insect-specific virus evolution and potential effects on vector competence. Virus Genes. 2019 Apr;55(2):127-137. doi: 10.1007/s11262-018-01629-9. Epub 2019 Jan 10. PMID: 30632016; PMCID: PMC6458977.

[6] Stollar V, Thomas VL. An agent in the Aedes aegypti cell line (Peleg) which causes fusion of Aedes albopictus cells. Virology. 1975 Apr;64(2):367-77. doi: 10.1016/0042-6822(75)90113-0. PMID: 806166.

[7] Gabrieli P, Caccia S, Varotto-Boccazzi I, Arnoldi I, Barbieri G, Comandatore F, Epis S. Mosquito Trilogy: Microbiota, Immunity and Pathogens, and Their Implications for the Control of Disease Transmission. Front Microbiol. 2021 Apr 6;12:630438. doi: 10.3389/fmicb.2021.630438. PMID: 33889137; PMCID: PMC8056039.