Title of Research Project

Capturing natural occurrences of CFAV-EVEs and probing its consequences on CFAV prevalence in wild-caught *Aedes aegypti* mosquitoes

Names of Project Members

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Purposes

The primary objective of this project was to recapitulate the interaction between the Cell Fusing Agent Virus (CFAV) and its corresponding endogenous viral elements (CFAV-EVEs) as observed in laboratory colonies of *Aedes aegypti.* More specifically, field-obtained *Ae. aegypti* mosquitoes were surveyed for CFAV infection and the presence of CFAV-EVEs. Differences in the degree of infection and the number of EVEs among individuals were employed to infer the said interaction in a more natural setup.

Methods

Mosquito collection and rearing

Mosquito eggs were collected via the ovitrap method from June to September 2024 from three cities in Metro Manila, Philippines – Valenzuela City, Pasig City, and Parañaque City. Eggs were reared in the laboratory until adulthood. Briefly, ovitrap paddles were soaked in tap water for at least 24 hours. Larvae and pupae were fed Brewer's yeast. Adults were allowed to emerge and were given 5% honey solution *ad libitum*. *Aedes aegypti* were identified via the characteristic lyre-like markings on its thorax. Meanwhile, sex determination was accomplished using morphological characterization of the antennae.

CFAV-EVE detection

DNA was extracted from dissected heads via several extraction

methods for optimization purposes. Nevertheless, all methods were derived from the Quick-DNA Tissue/Insect Miniprep Kit protocol (Zymo Research, USA).

To generate CFAV-specific EVE profiles per individual mosquito, PCR was done using primers targeting previously identified CFAV-EVEs[1][2]. Each reaction contained 10 µL PCR Master Mix (2X) (Thermo Fisher Scientific, USA), 1 µL each of the forward and reverse primer (5 µM to 10 µM), 7 µL sNFW, and 1 µL of the template DNA. All tubes were subjected to an initial denaturation step at 95°C for 3 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 60 seconds. A final extension step at 72°C for 10 minutes then followed. Visualization of the amplicons was done via AGE.

CFAV titer quantification

RNA was isolated from the remaining tissues post-head removal via a standard TRIzol-based method (Invitrogen, USA) with the reagent volumes scaled down by half. DNAse treatment was done using the TURBO DNA-free kit (Invitrogen, USA) following the manufacturer's protocols. cDNA was then synthesized with the ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs, USA) following the manufacturer's protocols for random primers.

Preliminary qPCR-based quantification of CFAV infection and the *rp49* housekeeping gene was carried out with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA) and a standard template solution composed of cDNA from ten samples representative of all three collection sites. Each 10 µL reaction is composed of 5 µL SsoAdvanced universal SYBR® Green supermix (2X), 1 µL of each primer (final concentration of 500 nM), 2 µL template, and 1 µL triple sterilized sNFW. All standards and no template controls were loaded in three technical replicates per primer pair. Amplification was then conducted as follows: acclimatization at 50°C for 10 minutes, initial denaturation at 95°C for 5 minutes, and 40 denaturation and annealing cycles at 95°C for 10 seconds and at 56°C for 30 secs, respectively. Quantification and melt curve analysis were done at 95°C

to 65°C in 0.5°C increments.

Results

A total of 57 individuals were subjected to CFAV-EVE profiling. Summarized frequencies for each EVE screened are shown in Figure 1.

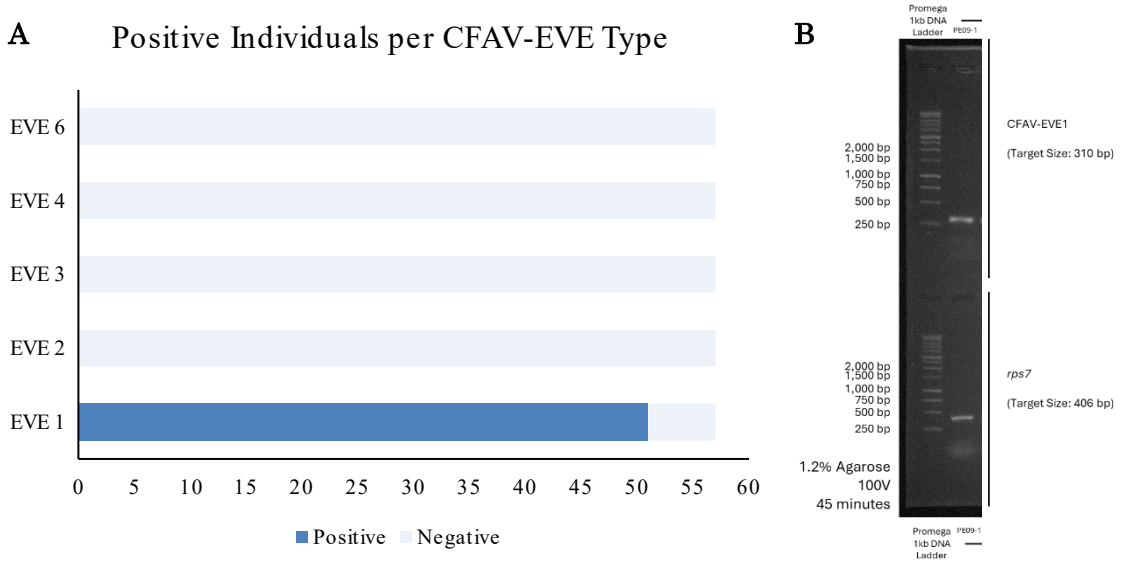


Figure 1. (A) Summarized frequencies for each of the analyzed CFAV-EVEs across the 57 individuals screened and (B) a sample CFAV-EVE 1 positive lane as visualized in AGE. The total count of EVE-positive individuals is represented by a darker blue bar over the more transparent bar, representing the total number of individuals.

CFAV-EVE 1 was detected in 89.47% (51 out of 57) of individuals studied. The obtained percentage is higher than expected for a locus likely inherited in a Mendelian manner. This may be indicative of the presence of EVE1 homozygous-positive individuals. Moreover, the absence of the other four EVE types screened across all samples, irrespective of collection site, was also intriguing.

Preliminary qPCR-based detection and quantification of CFAV infection was done on a pool of ten samples covering all three egg collection sites. The *rp49* housekeeping gene was successfully amplified at four different standard dilutions, but not the target CFAV NS3 transcript (Figure 2). This suggests the samples were either not infected with CFAV or have titers below the qPCR detection threshold.

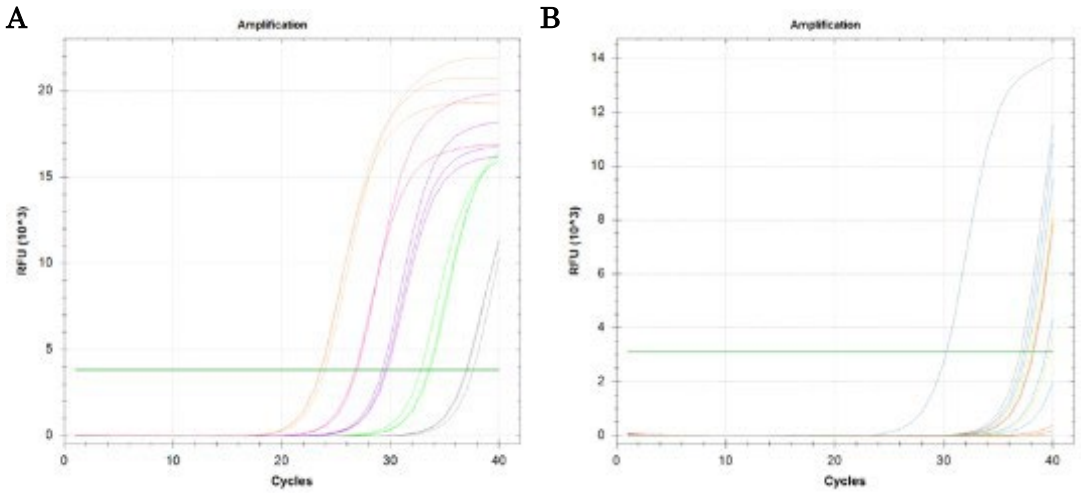


Figure 2. Amplification curves for the (A) housekeeping *rp49* and (B) CFAV. Each curve of the same color represents one technical replicate at a specific concentration. Meanwhile, gray lines represent no template control replicates.

Future Challenges

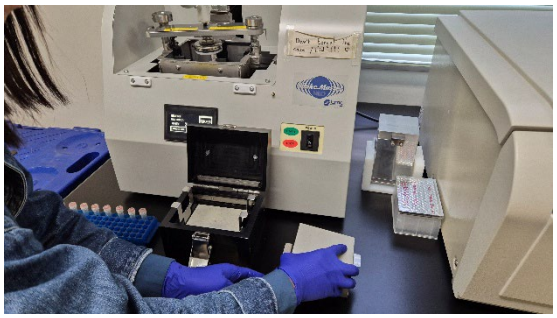
Achieving the originally proposed primary objective is highly contingent on obtaining sufficient quantities of mosquitoes with specific demographics. In this case, at least twenty individuals must be CFAV-EVE (+) and CFAV (+). Likewise, a similar number of CFAV-EVE (-) and CFAV (+) will be necessary for statistically meaningful comparisons to evaluate the consequences of CFAV-EVE on CFAV in the wild. Continuation of this project will, therefore, have to focus on expanding the number of individuals screened and collection sites.

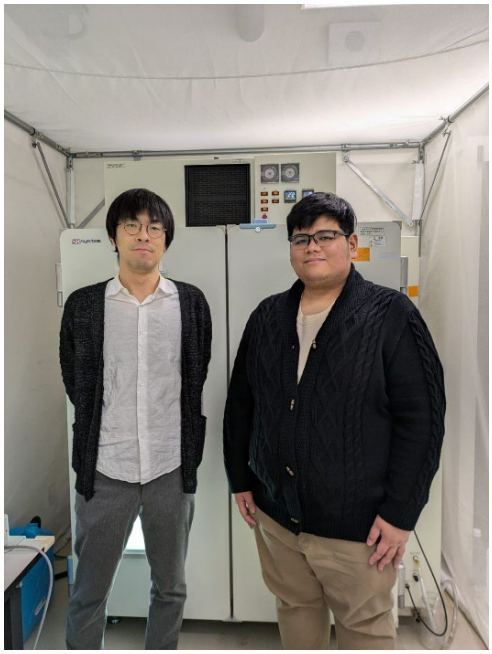
The ubiquity of CFAV-EVE 1 in this study highlights the need to elucidate better the various factors that may exert selective pressures that favor CFAV-EVE 1 (+) individuals. Alternatively, additional experiments should be explored to confirm that the observed high frequency of CFAV-EVE 1 (+) individuals is linked to heredity and not external selective pressures.

Other Activities

To better map out the future directions of this collaboration, AJC Feliciano visited Ehime University in February 2025. Regular discussions were held between AJC Feliciano and Dr. Suzuki during the trip. The former also shadowed Ms. Yuu Sekii, the laboratory technician, throughout the visit to learn new methods applicable to the current project. During one-on-one conversations,

the Molecular Ecology and Health (MECOH) lab members also presented their research interests and previous works.





(Left) Ms. Yuu Sekii demonstrated a technique that will be applied in future experiments in the Philippines. (Right) Picture of Dr. Yasutsugu Suzuki and AJC Feliciano after one of the discussions.

References

[1] Suzuki, Y., Baidaliuk, A., Miesen, P., Frangeul, L., Crist, A. B., Merkling, S. H., Fontaine, A., Lequime, S., Moltini-Conclois, I., Blanc, H., van Rij, R. P., Lambrechts, L., & Saleh, M. C. (2020). Non-retroviral Endogenous Viral Element Limits Cognate Virus Replication in Aedes aegypti Ovaries. *Current biology: CB*, *30*(18), 3495–3506.e6. https://doi.org/10.1016/j.cub.2020.06.057

[2] Crava, C. M., Varghese, F. S., Pischedda, E., Halbach, R., Palatini, U., Marconcini, M., Gasmi, L., Redmond, S., Afrane, Y., Ayala, D., Paupy, C., Carballar-Lejarazu, R., Miesen, P., van Rij, R. P., & Bonizzoni, M. (2021). Population genomics in the arboviral vector Aedes aegypti reveals the genomic architecture and evolution of endogenous viral elements. *Molecular Ecology*, *30*(7), 1594–1611. https://doi.org/10.1111/mec.15798