Title of research project: Viral metagenomics analysis of the field-caught *Aedes aegypti* from the dengue hotspot areas in Davao City, Mindanao Philippines

Names of Project Members
Von Carlo Dela Torre, MSc (University of the Philippines Mindanao)
Mae Responte, PhD (University of the Philippines Mindanao)
Yasutsugu Suzuki, PhD (Ehime University)
Kozo Watanabe, PhD (Ehime University)

Purposes

Mosquitoes are notorious vectors of diseases that pose significant threats to global public health. With urbanization and global warming altering ecosystems, the frequency of human-vector interactions and the risk of mosquito-borne disease transmission continue to rise [1,2]. Female mosquitoes, often referred to as "flying syringes," feed on diverse hosts, making them invaluable tools for environmental disease surveillance through their blood and nectar meals [3,4]

The specific aims of this study are to investigate the influence of mosquito feeding behavior, including preferences for humans, animals, and plants, on virome composition and to identify the contributions of different food sources to virome dynamics through metabarcoding and viral metagenomic techniques. We aim to address the limitations of previous studies by conducting year-round sampling across diverse spatiotemporal scales, assessing the availability, population size, and diversity of animal hosts, and examining the effects of these factors on mosquito blood-feeding patterns. Additionally, we seek to explore the ecological and epidemiological implications of mosquito viromes, particularly concerning arbovirus transmission potential, by focusing on the salivary glands and midgut virome composition and by examining viral species diversity in mosquitoes from varied habitats at the human-animal- environment interface.

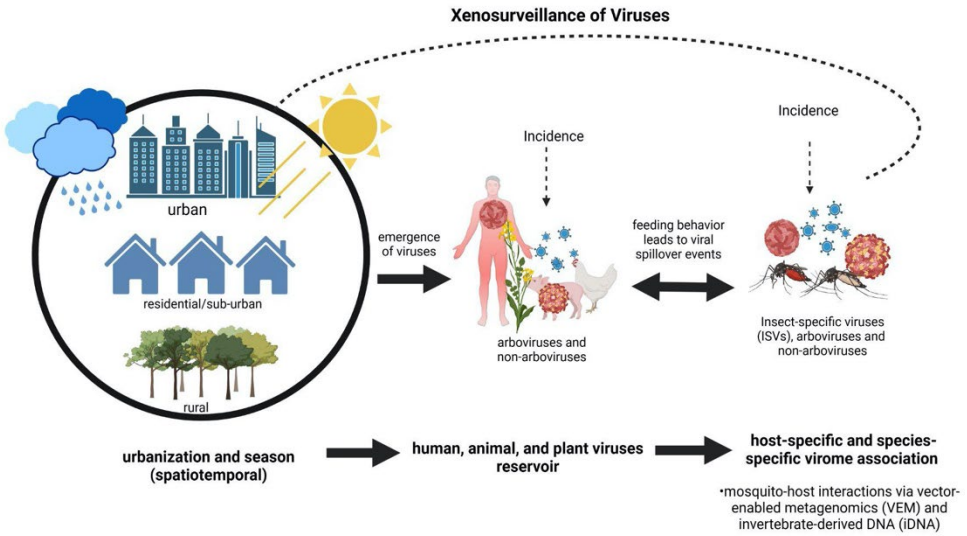

Figure 1. Conceptual Framework

Methods

Optimization of vertebrate 16s rRNA and 12s rRNA detection and RNA of mosquito heads concentration and quality

Laboratory-reared blood-fed female mosquitoes were collected using an insect aspirator. These female mosquitoes were kept individually in a 1.5 ml microcentrifuge tube containing 1.0 ml of 95% ethanol and stored at -80 Celcius until further analysis.

1. DNA was extracted using QIAGEN DNAEasy Blood and Tissue kit, whereas the total RNA was extracted using QIAGEN Viral RNA mini kit from the homogenized mosquito tissue samples.
2. DNA and RNA extracts were quantified using NanoDrop™ Spectrophotometer (Thermo Fisher Scientific; Massachusetts, United States), Qubit™ 4 Fluorometer (Thermo Fisher Scientific; Massachusetts, United States), and agarose gel electrophoresis (AGE).
3. DNA extracts were subjected to conventional PCR to amplify the 16s rRNA 12s rRNA (vertebrate).

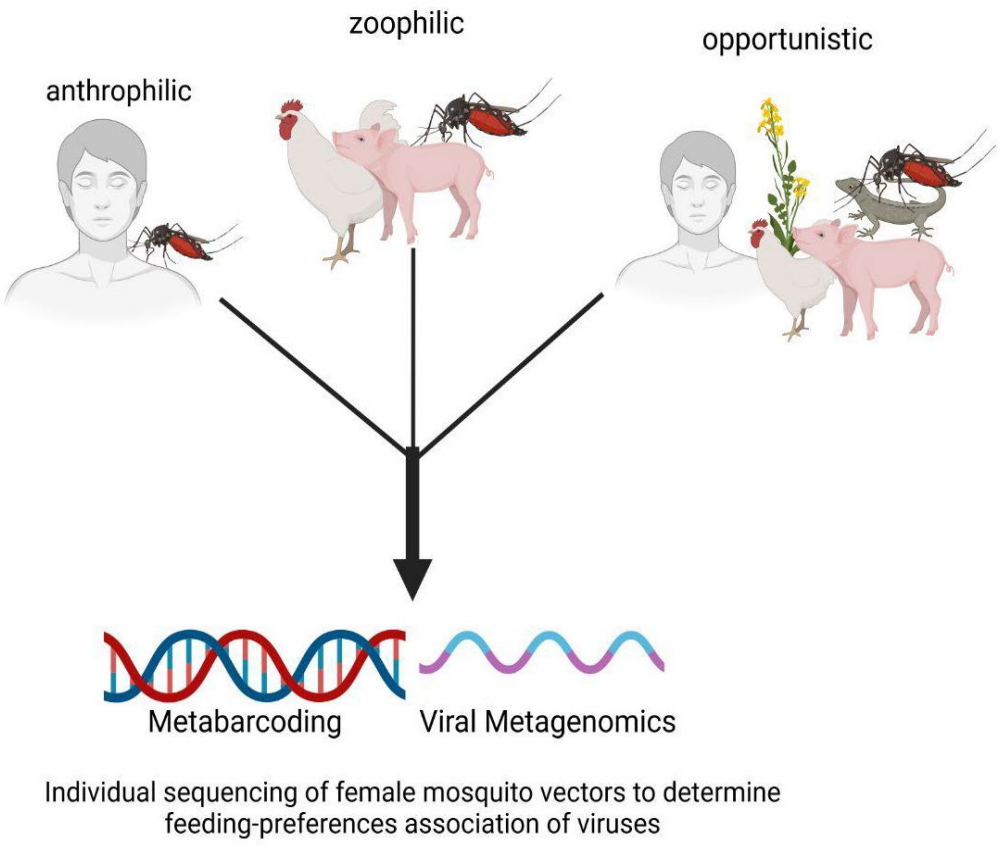

Figure 2. Methodological Framework

Results

Optimization of DNA extraction

- Extracted 135 mosquitoes. Post-mortem (0–24 hours) and post-blood fed treatment (0–48 hrs) using QIAGEN DNEasy Blood and Tissue kit
- RNAse A was used and elution was decreased in 30–48 hrs to improve DNA concentration and purity

Table 1. DNA concentration and purity of blood-fed mosquitoes

Sample (triplicate)	Nucleic Acid Concentration[#] (ng/μl)	260/280 (DNA purity)*
0–24 hrs 200 μl elution	1.82–31.2	2.02–3.31
30–48 hrs 100 μl elution with RNAse A	1.34–53.4	1.37–2.14

[#]target ng/μl: at least 5ng/μl for metabarcoding
*ideal 260/280 ratio: 1.8–2.0

Optimization of mosquito's head RNA extraction

- Used ATL buffer and proteinase K to homogenize the tissue overnight
- Extracted the RNA using QIAMP Viral RNA Minikit
- Ethanol precipitation of RNA was employed to increase the concentration

Table 2. RNA concentration and purity before ethanol precipitation

Sample (60 μl elution)	Nucleic Acid Concentration[#] (ng/μl)	260/280 (RNA purity)*
Before ethanol precipitation		
Sample 1	12.7	2.82
Sample 2	7.58	3.00
After ethanol precipitation		
Sample 1	29.8	2.88
Sample 2	21.2	2.90

[#]target ng/ul: at least 20 ng/μl in 20 μl for shotgun metagenomics (Macrogen South Korea)
*ideal 260/280 ratio: 2.0–2.2

Amplification of 16S and 12S rRNA

- Touchdown PCR was done to amplify the 16S and 12S rRNA of the vertebrate's blood in mosquitoes
- Used three DNA polymerases: Hot-start: Amplitaq Gold, Phusion Plus; Non hot-start: Phusion.
- Phusion Plus showed best results in giving distinct and clearest bands (200–210 bp).

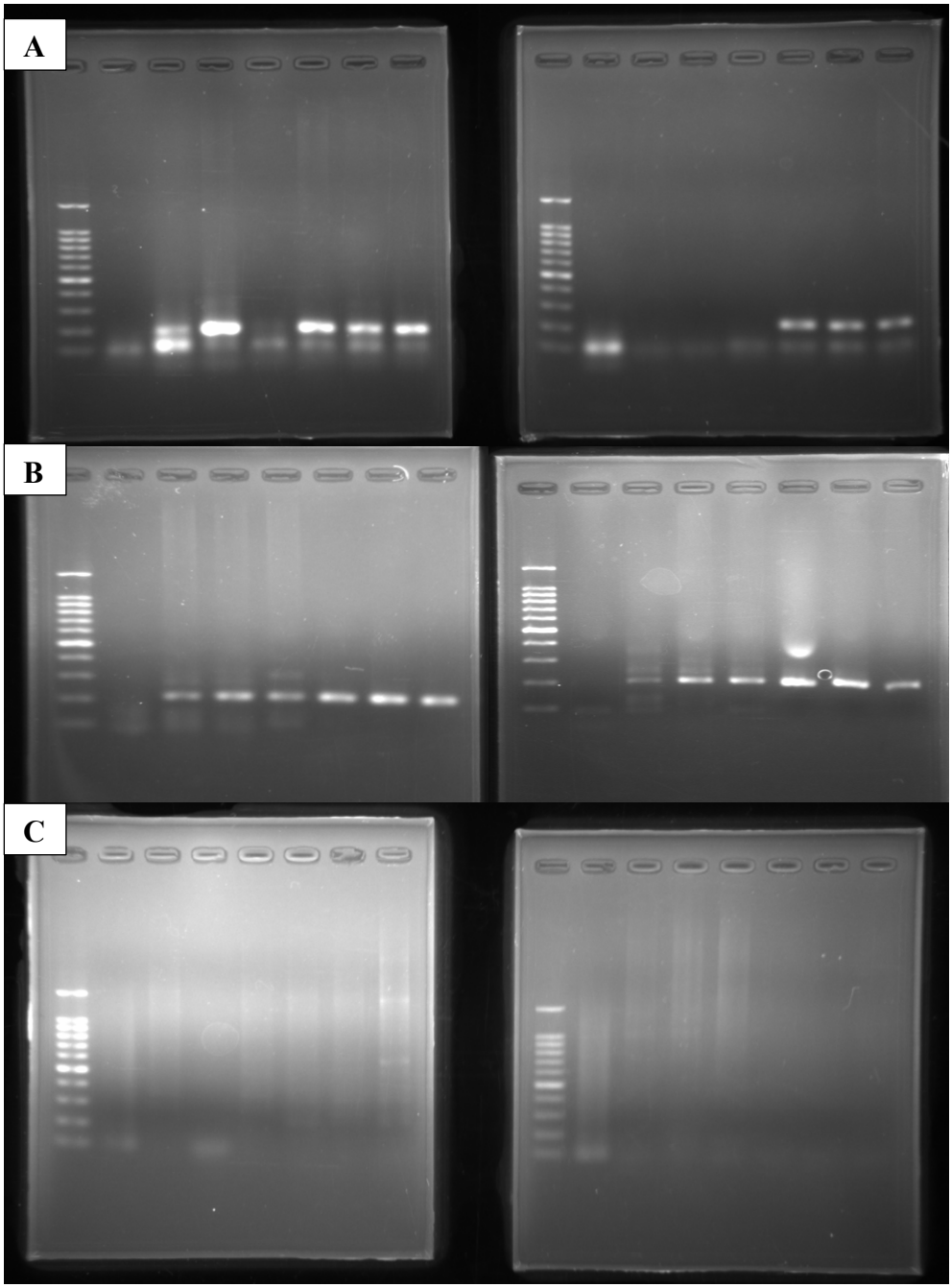

Figure 3. 12s rRNA (left) and 16s rRNA (right). A: Amplitaq Gold; B: Phusion Plus; C. Phusion.

For population-level analysis (e.g., SNPs, haplotype diversity):
Phusion Plus is best due to high fidelity and proofreading activity.
AmpliTaq Gold may be used if working with degraded DNA, but errors may affect results.

For community-level analysis (e.g., species richness, OTU/ASV estimation):
Phusion Plus is ideal for accurate species resolution.
AmpliTaq Gold may introduce artificial diversity due to its error-prone nature.

Future Challenges

- Although we have successfully amplified the 16S and 12S rRNA in laboratory reared mosquitoes, amplifying them in field-collected mosquitoes might be challenging due to many abiotic and biotic factors that are beyond our control. Thus, further optimization will be conducted once the fieldwork commenced.

References

[1] de Almeida J.P., Aguiar E.R., Armache J.N., Olmo R.P., Marques J.T. The virome of vector mosquitoes. 2021. Curr. Opin. Virol. 2021;49:7–12. doi: 10.1016/j.coviro.04.002

[2] Wu Q, Guo C, Li XK, Yi BY, Li QL, Guo ZM, Lu JH. A meta-transcriptomic study of mosquito virome and blood feeding patterns at the human-animal-environment interface in Guangdong Province, China. One Health. 2023 Feb 1;16:100493. doi: 10.1016/j.onehlt.2023.100493. PMID: 36817976; PMCID: PMC9932184.

[3] Brinkmann A, Nitsche A, Kohl C. 2016. Viral metagenomics on blood- feeding arthropods as a tool for human disease surveillance. Int J Mol Sci 17:1743. https://doi.org/10.3390/ijms17101743.

[4] Molaei G, Andreadis TA, Armstrong PM, Anderson JF, Vossbrinck CR. 2006. Host feeding patterns of *Culex* mosquitoes and West Nile virus transmission,northeastern United States. Emerging Infectious Diseases 12: 468–474.