Detection of Insect-Specific Viruses in Mosquitoes at the Colorado State University Center for Vector-Borne and Infectious Diseases Insectary

Honors Thesis

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By

Lila Domenico

Microbiology, Immunology, & Pathology

Gregory Ebel, Microbiology, Immunology, & Pathology

Brian Geiss, Microbiology, Immunology, & Pathology

Kaitlynn Williams, Microbiology, Immunology, & Pathology

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Abstract

Insect-specific viruses (ISVs) are viruses that can infect arthropod cells, like ticks and mosquitoes, but not vertebrate cells. It has also been shown that ISV infections decrease the growth of arboviruses, like West Nile virus and Dengue, during experiments where mosquitoes or mosquito cell lines were coinfected. Because of this unique ability to decrease arboviral growth, combined with their inability to replicate in vertebrate cells, research has started to expand on potential biocontrol applications of ISVs. However, since arbovirus research is conducted at facilities like the insectary at the Center for Vector-Borne and Infectious Diseases (CVID), the presence of ISVs in the mosquitoes used for these studies could influence experimental outcomes. For that reason, we determined which ISVs are the most commonly found in various mosquito populations and used RT-PCR to screen multiple colonies of mosquitoes reared in the insectary at CVID. We screened for the following ISVs: Culex flavivirus, Aedes flavivirus, Cell fusing agent virus, Phasi Charoen-like virus, Kamiti River Virus, Eilat Virus, and Calbertado virus. We collected mosquitoes from different colonies of *Aedes triseriatus*, *Aedes aegypti*, *Aedes albopictus*, *Culex quinquefasciatus*, and *Culex tarsalis* from the insectary to screen for these ISVs. With pooled mosquito homogenates from each of these species, we extracted RNA and analyzed the extractions using RT-PCR, using previously published primers for the ISVs of interest. Our results show that, of the colonies tested from the insectary at CVID, none of the ISVs screened for were present. These findings indicate that the colonies maintained at CVID are favorable control organisms for future arboviral research.

Introduction

Mosquitoes are hosts to a multitude of viruses that can be transmitted to humans and cause serious disease. Arboviruses such as West Nile virus, dengue fever, and Zika virus are all vector-borne diseases transmitted from mosquitoes to humans that cause worldwide outbreaks and are a consistent threat to public health (Weaver et al., 2018). In addition to arboviruses, mosquitoes can harbor a distinct group of

viruses known as Insect-specific viruses (ISVs). These viruses can replicate in insects but, in contrast to arboviruses, do not replicate in vertebrate cells and cannot be transmitted to humans. Several studies have shown that ISVs have the capability to decrease arboviral growth within the host mosquito, making them a promising form of biocontrol for multiple arboviruses (Chen et al., 2023). For example, cell fusing agent virus (CFAV), can negatively interfere with the growth of Dengue and Zika virus in an *Ae. aegypti* cell line (Baidaliuk et al., 2019). Additionally, this phenomenon was also observed during infection with Culex flavivirus (CxFV) which may also suppress the growth of West Nile virus in Culex pipiens (Bolling et al., 2010). These previous studies highlight the potential of ISVs to play an important role in vector-borne disease transmission to humans.

While ISVs have been found in numerous wild mosquito populations, little research has been focused on screening laboratory-maintained colonies for them. ISVs are maintained in wild mosquito populations with vertical transmission of ISVs from a female mosquito to its offspring (Bolling, 2010). This evidence suggests that ISVs could be maintained in laboratory-reared colonies of mosquitoes, passed from one generation raised in an insectary to another. The presence of ISVs in laboratory colonies could potentially influence experimental outcomes of arboviral research. We hypothesized that ISVs are present in the colonies of mosquitoes maintained in the insectary at the Center for Vector-Borne and Infectious Diseases (CVID). The knowledge of ISV presence and prevalence in these colonies could help increase confidence in results obtained from future arboviral research. If ISVs were present in the colonies, future research focuses could determine the mechanism causing specific ISVs to interfere with the growth of arboviruses.

Therefore, we used RT-PCR to screen for seven ISVs–Aedes flavivirus (AEFV), CxFV , Calbertado virus (CLBOV), CFAV, Eilat virus (EILV), Kamiti River virus (KRV), and Phasi Charoen-like virus (PCLV)–in six species of mosquitoes maintained in the insectary: *Ae. aegypti* (Chetumal, Poza Rica, Salvador), a *Aedes albopictus*, *Aedes triseriatus*, *Culex tarsalis*, *Culex quinquefasciatus*, and *Anopheles gambiae*.

Each mosquito species was screened for specific viruses based on prior research indicating the presence of particular ISVs in wild-caught individuals of that species. Our results indicate that there are no ISVs present in the mosquito colonies maintained at CVID.

Materials and Methods

Mosquito collection and homogenization

All mosquitoes were long-established colonies collected from the insectary at CVID. The species collected were *Aedes triseriatus*, *Aedes aegypti* (Poza Rica), *Aedes aegypti* (Chetumal), *Aedes aegypti* (Salvador), *Aedes albopictus* (Salvador), *Anopheles gambiae* (Gambia), *Culex quinquefasciatus*, and *Culex tarsalis*. They were each reared in standard rearing conditions at 75% humidity with 16:8 (day:night) cycles. The *Aedes* and *Anopheles* strains were all reared at 28 °C while *Culex* were reared at 26-27°C.

For sample collection, round-bottom tubes were prepped with a stainless steal ball bearing (bb) and mosquito diluent made with 20% FBS, 50 µg/ml gentamicin, 2.5 µg/ml amphotericin, 100 IU/mL penicillin, 100µg/ml streptomycin in 1X PBS. Mosquitoes were aspirated from large bug dorms or collected as pupae and placed in smaller cartons. From the aspirator or the cartons, the mosquitoes were placed in the freezer for 1-2 minutes to knock them down. The mosquitoes were then placed into a petri dish on ice and sorted into 4 groups with 25 mosquitoes each, not distinguishing between male and female. Each group of 25 mosquitoes was placed into one of the prepped, round-bottom tubes. The mosquitoes were then placed into the QIAGEN Tissue Lyser II. Homogenized mosquitoes were then vortexed and spun down in a centrifuge at 12000 RPM for 1 minute. All tubes were stored in a -80°C freezer.

ISVs screened in each species

Each mosquito species was only screened for ISVs that were previously found in wild-caught mosquitoes of the same species. For each combination of ISV and mosquito species, three previous studies were referenced to confirm that the ISV was present in wild-caught mosquitoes in some regions.

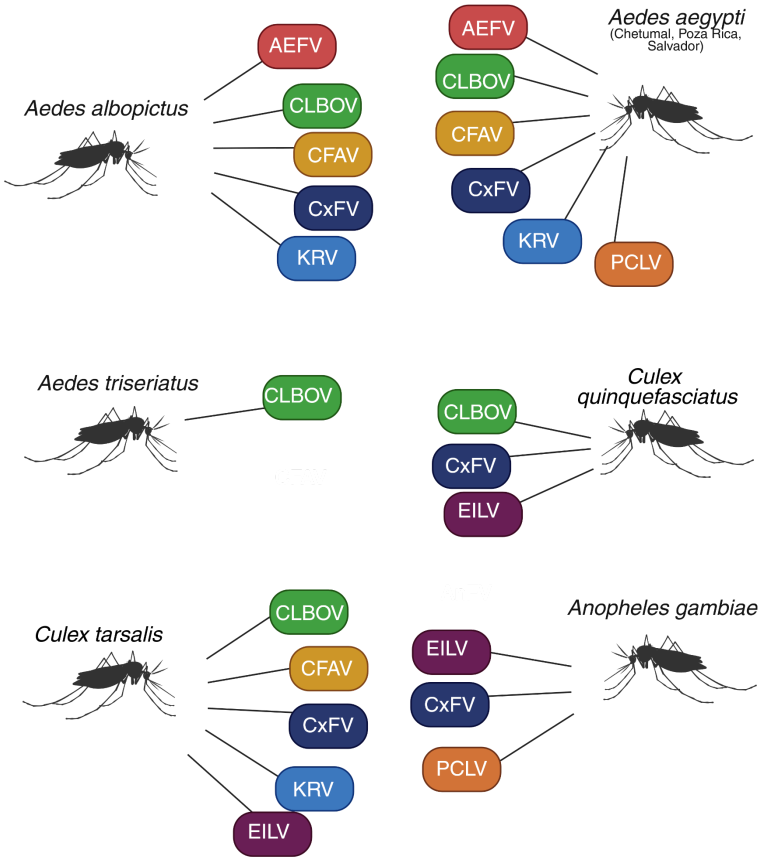

Figure 1. Each ISV was tested in specific mosquito strains based on presence in wild mosquito populations

RNA extraction

RNA extractions were carried out using the Direct-zol RNA Miniprep kit from Zymo for all mosquito samples and Aag2 cells following the manufacturer's instructions. RNA extracted from the samples was stored in a -80°C freezer.

RT-PCR

Primers for RT-PCR were found in the literature. Several reviews were used to determine which viruses we should screen for (Moonenet et al 2023; Öhlund et al., 2019). The primers that are being used are listed in Table 1. The thermocycler was programmed with an initial cycle of 30 minutes at 50°C for cDNA synthesis and 94°C for 2 min for pre-denaturation. This was followed by 35 cycles of 15 seconds at 94°C for denaturation, 30 seconds at 50°C to anneal, and 1 minute at 70°C for extension. A final cycle for 10 minutes at 72°C was added for extension. These conditions were used for all RT-PCR reactions.

ISV	Primer Sequences	Size (bp)	Reference
AEFV	F- TGACCCTGGCCGCTTTAATA R- ATGTCGCGCTGTTTTCTTGT	174	Peinado et al., 2022
CxFV	F- TGAATTGCTCGC TGATTGTC R- TTATACCCCTCTCCGCAATG	207	Saiyasombat et al., 2011
CFAV	F- AATGAGACCTGTTCGCTTAG R- CGTTTGTCAATCAAGGCAG	344	Contreras-Gutierrez et al., 2017
PCLV	F- TCTCGCCATTCTTGGTCAAC R- AACCCAATGTGTCCTGGATT	257	Weger-Lucarelli et al., 2018
KRV	F- AAATGTCTCTGTGCTCGTT R- GCTATGCGGTCTGTTGTGA	164	Tree et al., 2016
EILV	F- CGACGATGACCGGAGAAGAG R- AAGACTCGGTCTGCCTGC	604	Joseph et al., 2023
CLBOV	F- ACCTTGAGTTCGAAGCG R- CTCCGCAACCTCAGTT	90	Tyler et al., 2011

Table 1. Primers for each ISV were identified from previous literature.

Positive controls

RNA from Aag2 cells was used as the positive controls for PCLV and CFAV. Aag2 cells from a stock maintained at CVID in liquid nitrogen were cultured at 28°C with 0% CO_2 in Schneiders insect cell medium (10% FBS, amphotericin, penicillin and streptomycin). RNA was extracted from the cultured cells (Weger-Lucarelli et al., 2018).

Positive controls for the other ISVs were made by downloading the genomes for each ISV from NCBI GenBank database. The accession numbers for each genome can be found in Table 2. Genomes were uploaded into Geneious Prime to isolate the primer binding sites and ensure they were amplifying the target portion of the genome. The region of the genome amplified by the primers, plus ~50 bp upstream and downstream of that section, were copied into Integrated DNA Technologies (IDT). A T7 promoter (5' – TAATACGACTCACTATAG – 3') was inserted on the 5' end of the sequence prior to ordering synthesized DNA fragments (gBlocks). Each of T7-tagged DNA amplicon was resuspended with nuclease-free water to a final concentration of ~5 ng/ul.

To generate RNA templates for RT-PCR, 1 ul of the DNA amplicons was amplified using the AmpliScribe T7 High Yield Transcription Kit. The RNA products were then quantified using a NanoDrop Spectrophotometer.

RT-PCR was performed using the SuperScriptTM One-Step RT-PCR System with PlatinumTM Taq DNA Polymerase kit following manufacturer's instructions. All products were visualized using agarose gel electrophoresis. Every mosquito pool collected was run with each combination of primers for every ISV indicated in the list of primers table above. The positive controls, both the T7 amplicons and Aag2 cells were amplified using their respective primers.

ISV	GCF/ Accession number
AEFV	KJ741266.1
CxFV	GCF_000869605.1
CFAV	GCF_000862225.3
PCLV	GCF_002814835.1
KRV	GCF_000851165.1
EILV	GCF_000898655.1
CLBOV	EU569288.1

Table 2. All ISVs and their corresponding GCF or accession numbers.

Gels

All RT-PCR products were visualized using a 3% agarose gel. The gel was stained with SYBR-safe and each sample loaded was dyed with Purple (6x) gel loading dye. The ladder used to visualize band sizes was New England Biolabs Quick-Load® Purple Low Molecular Weight DNA Ladder. They were imaged using a Bio-Rad ChemiDocTM MP Imaging System.

Statistical analysis

Excel was used for all statistical analysis. A z-test for proportions and confidence interval (CI) estimation was used to analyze the infection rate of each ISV in each mosquito colony. The one sample z-test conducted compared the observed portion of infected mosquitoes from the pools collected (0/100) to hypothesize infection rate. For the total infection rate estimation we used the assumption of a non-zero infection rate. We calculated a 95% CI for the infection rate using the Rule of Three, which assumes a binomial distribution (infected/ not infected) for each pool of 25 mosquitos and is more accurate given a 0% detection rate. For the power analysis, we used the estimate of <3% obtained from the CI and the whole sample size of 100 mosquitoes to estimate the probability that the study captured low-prevelence infections in the mosquito population.

Results

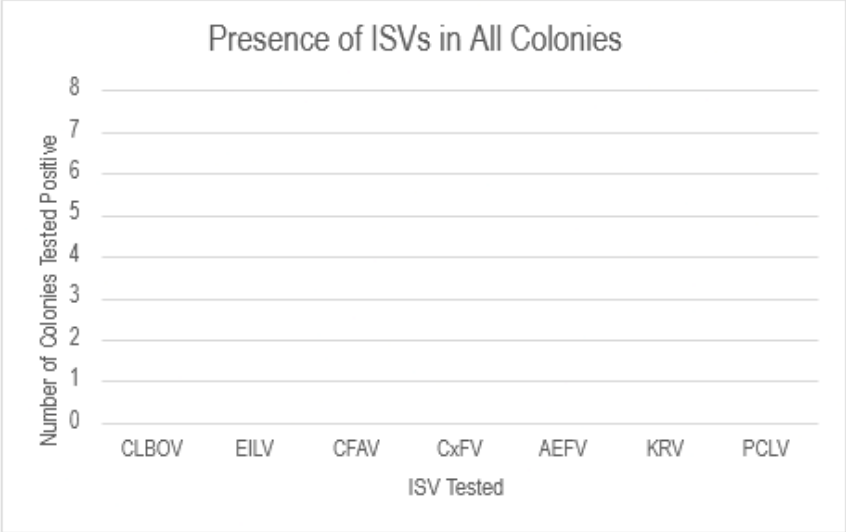

Figure 2. No ISVs were present in any of the mosquito colonies tested.

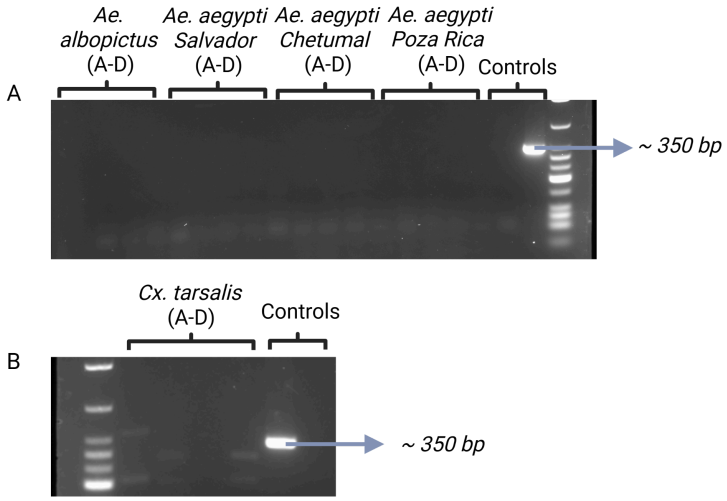

Figure 3. Example agarose gels showing the presence of CFAV with positive and negative controls appearing at the expected band sizes. A) Lack of bands appearing on the gel indicates that *Ae. albopictus*, and *Ae. aegypti* (Salvador, Chetumal, and Poza Rica) colonies are negative for CFAV infection. B) Lack of bands appearing on the gel indicates that the *Cx. tarsalis* colony is negative for infection with CFAV.

For each pool of 25 mosquitoes tested in every combination ISV and mosquito species, no band was present on the gel. The results show a 0% prevalence of the ISVs in every group of 100 total mosquitoes.

Using the Rule of Three with a 95% CI, our results show that the true infection rate of each ISV and mosquito species combination is between 0%-3% for the total mosquito colony in the insectary. The power analysis indicated that the population size collected of 100 mosquitoes has a 42% chance of detecting a 3% infection rate.

Conclusion

In this study, we aimed to identify which ISVs are present in the mosquito colonies maintained at the insectary at CVID. After screening for seven common ISVs, we found no current evidence of ISVs in the six mosquito colonies tested. This evidence suggests that the laboratory colonies of mosquitoes offer a controlled environment, limiting ISV interference, and supporting conclusions drawn from previous and future arboviral research in that facility.

Additional ISVs have been identified in wild mosquito populations that were not included in this study. There is potential that ISVs not included in this study may be present in the laboratory-reared mosquitoes. Some ISVs or fragments of viral genomes may be undetectable by RT-PCR due to genomic integration into the mosquito host. Whole genome sequencing of the laboratory colonies of mosquitoes could lead to additional ISV detection. Our power of 42% also indicates that larger samples should be collected from mosquito colonies in the future to ensure accurate detection of viruses for low prevalence infections.

The lack of ISVs in the mosquito colonies may support arboviral replication in the colonies from the insectary, however, many wild-caught mosquitoes naturally carry ISVs. Host-viral interactions exhibited in wild mosquito populations are absent in the laboratory-reared colonies due to the lack of ISVs. The controlled environment of the insectary could lead to the elimination of ISV due to selective pressures for mosquitoes that are not infected with ISVs.

Future research on the pathogenicity of ISVs in mosquitoes and the persistence of the viruses over successive generations of wild-caught mosquito colonies moved into controlled insectary conditions. The long-term stability of ISV infections may increase the understanding of the ecological roles of ISVs and their potential in biocontrol strategies.

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