

RESEARCH ARTICLE

High prevalence of Phasi Charoen-like virus from wild-caught *Aedes aegypti* in Grenada, W. I. as revealed by metagenomic analysis

Maria E. Ramos-Nino^{1☯‡*}, Daniel M. Fitzpatrick^{2☯}, Scott Tighe³, Korin M. Eckstrom^{1☯3}, Lindsey M. Hattaway², Andy N. Hsueh², Diana M. Stone², Julie Dragon³, Sonia Cheetham^{2‡*}

1 Department of Microbiology, School of Medicine, St. George's University, St. George's, Grenada, West Indies, **2** Department of Pathobiology, School of Veterinary Medicine, St. George's University, St. George's, Grenada, West Indies, **3** University of Vermont Massively Parallel Sequencing Facility, Burlington, Vermont, United States of America

☯ These authors contributed equally to this work.

‡ These authors are joint senior authors on this work.

* mramosni@sgu.edu (MERN); scheetha@sgu.edu (SC)



OPEN ACCESS

Citation: Ramos-Nino ME, Fitzpatrick DM, Tighe S, Eckstrom KM, Hattaway LM, Hsueh AN, et al. (2020) High prevalence of Phasi Charoen-like virus from wild-caught *Aedes aegypti* in Grenada, W.I. as revealed by metagenomic analysis. PLoS ONE 15 (1): e0227998. <https://doi.org/10.1371/journal.pone.0227998>

Editor: Guido Favia, Universita degli Studi di Camerino, ITALY

Received: October 11, 2019

Accepted: January 3, 2020

Published: January 31, 2020

Copyright: © 2020 Ramos-Nino et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The sequences for the PCLV Grenada isolate have been deposited in GenBank under the accession numbers MN109951 (for the S segment/nucleocapsid coding region), MN109952 (for the M segment/glycoprotein coding region), and MN109953 (for the L segment/RNA dependent RNA polymerase coding region). Raw data from RNA-Seq can be found at <https://www.ncbi.nlm.nih.gov/sra/PRJNA564787>

Abstract

Arboviruses cause diseases of significant global health concerns. Interactions between mosquitoes and their microbiota as well as the important role of this interaction in the mosquito's capacity to harbor and transmit pathogens have emerged as important fields of research. *Aedes aegypti* is one of the most abundant mosquitoes in many geographic locations, a vector capable of transmitting a number of arboviruses such as dengue and Zika. Currently, there are few studies on the metavirome of this mosquito particularly in the Americas. This study analyzes the metavirome of *A. aegypti* from Grenada, a Caribbean nation with tropical weather, abundant *A. aegypti*, and both endemic and arboviral pathogens transmitted by this mosquito. Between January and December 2018, 1152 mosquitoes were collected from six semi-rural locations near houses in St. George Parish, Grenada, by weekly trapping using BG-Sentinel traps. From these, 300 *A. aegypti* were selected for analysis. The metavirome was analyzed using the Illumina HiSeq 1500 for deep sequencing. The generation sequencing library construction protocol used was NuGEN Universal RNA with an average read length of 125 bp. Reads were mapped to the *A. aegypti* assembly. Non-mosquito reads were analyzed using the tools FastViromeExplorer. The NCBI total virus, RNA virus, and eukaryotic virus databases were used as references. The metagenomic comparison analysis showed that the most abundant virus-related reads among all databases and assemblies was Phasi Charoen-like virus. The Phasi Charoen-like virus results are in agreement to other studies in America, Asia and Australia. Further studies using wild-caught mosquitoes is needed to assess the impact of this insect-specific virus on the *A. aegypti* lifecycle and vector capacity.

Funding: This research was funded by The Windward Islands Research and Education Foundation's One Health Research Initiative Grant 4-11-10.

Competing interests: The authors have declared that no competing interests exist.

Introduction

The most common mosquito-borne arbovirus diseases are transmitted by two genera of mosquitoes: *Aedes* and *Culex* [1–6]. The public health impact of these arboviruses has increased dramatically over the past years, spreading to new geographic locations and increasing in incidence within their range [7]. *Aedes* mosquitoes together with *Culex quinquefasciatus* are important vector mosquitoes in the Americas, and are the most abundant mosquitoes found in populated areas in Grenada, West Indies [8]. From the genera *Aedes*, two specific species, *Aedes aegypti* and *Aedes albopictus* have been shown to be important vectors of globally important arboviruses including the dengue viruses (DENV) [9], yellow fever virus [10], Zika virus (ZIKV) [11], and chikungunya virus [12].

Preventive treatments against mosquito-borne diseases are mostly limited to personal protection and mosquito population control using insecticides [13]. However, intensive and repeated insecticide use leads to the development of mosquito resistance, which has been reported to have a significant impact on transmission dynamics of microorganisms. For instance, a study on the effects of insecticide resistance mechanisms on vector competence of the mosquito *C. quinquefasciatus* demonstrated dissemination of West Nile Virus (WNV) in the mosquito body, leading to an increase in transmission efficiency by resistant mosquitoes [14]. Similarly, current research continues to elucidate interactions among mosquitoes, their endogenous microbiota, and other pathogens they transmit. In *A. aegypti*, the microbiota in the mosquito has been reported to influence the susceptibility to infection to arboviruses [13,15,16]. For example, DENV replication has been reported to be affected by gut bacteria [17,18] which exert antiviral activity through mechanisms not completely understood [13,19–21], but that may be indirectly associated to innate antiviral responses and antimicrobial peptides by the gut microbiota [17]. Studies on the microbiome reinforce the great potential for the development of microbial-based strategies to control vector-borne pathogens [13].

In recent years, metagenomics has emerged as a powerful tool to study microbial diversity in a culture independent manner [22], an approach that is helping define the microbiome of mosquitoes [23–26]. Part of the uncovered microbiome is the insect-specific viruses (ISVs), viruses restricted to arthropods that are unable to replicate in vertebrate cells [27]. Insect-specific viruses are highly prevalent in wild mosquito populations [25,28–31] and have been reported to suppress [32–36], enhance [37,38], or have no effect [39] on replication of medically important arboviruses, potentially affecting vector competence [40–42].

There is a potential use of ISVs as biocontrol agents. The close genetic similarity between ISVs and arboviruses have a potential for interference in their replication either through upregulation of antiviral immune responses in the vector or via superinfection exclusion (i.e., homologous interference), where similar viruses can block each other through competition [42,43].

The present study sheds some light on the virome of *A. aegypti* mosquito in Grenada, an endemic region for several medically important arboviruses in the Caribbean.

Material and methods

Mosquito collection

Three hundred female *A. aegypti* mosquitoes were randomly selected out of 1,152 mosquitoes collected between January 2018 and December 2018, twice each week from six semi-rural locations in St. George Parish, Grenada (12° 15' 46" N 61° 36' 15" W) (Fig 1). Biogents Sentinel (Biogents, Regensburg, Germany) traps baited with octenol and yeast-based carbon dioxide attractants [44] were placed within 3 m of houses to attract mosquitoes. After 24 h, traps were

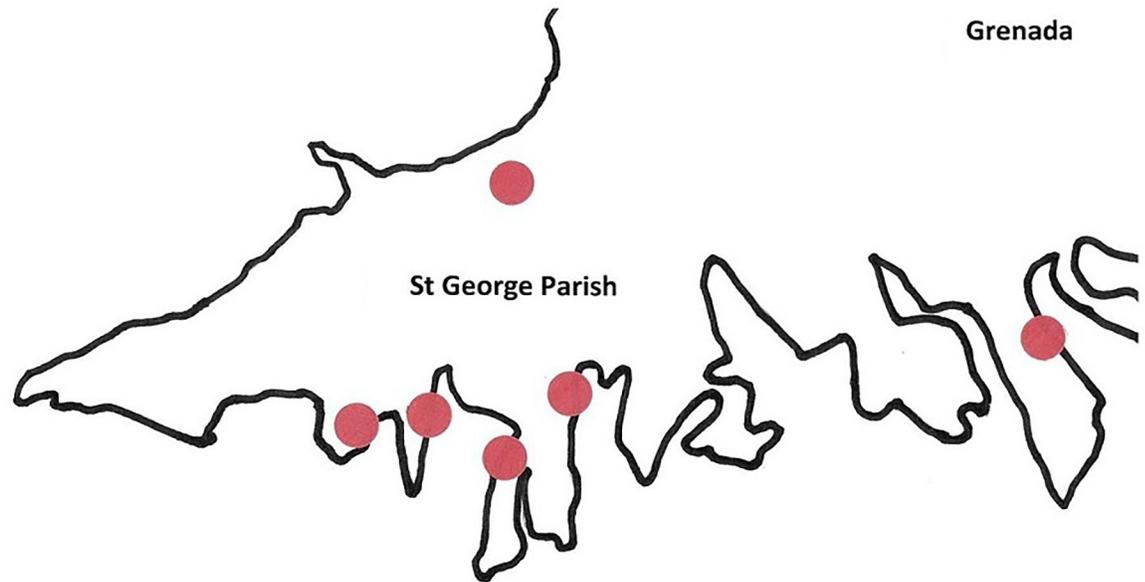


Fig 1. Map of collection sites in St. George Parish, Grenada.

<https://doi.org/10.1371/journal.pone.0227998.g001>

collected, and mosquitoes were stored at -80°C . Subsequently, mosquitoes were identified to species by morphological analysis. Because morphological keys that include recently introduced invasive taxa are not available for Caribbean islands, identification keys in Darsie and Ward [45] and in Rueda [46] were used to discriminate between species known to occur in Grenada based on the Walter Reed Biosystematics Unit [47]. Mosquitoes were then placed in RNAlater® (Sigma Aldrich, St. Louis, Missouri, USA) until further processing. Before RNA extraction, mosquito heads were removed using a sterile scalpel blade to prevent PCR inhibition [48]; wings and legs were also removed to reduce host RNA. No IACUC was required for the use of mosquitoes in this study.

Total RNA extraction and RNA-Seq

RNA extraction was performed in batches of 30 mosquitoes at a time (ten pools) using TRIzol (ThermoFisher, Carlsbad, California, USA). Invitrogen™ Phasemaker™ Tubes (ThermoFisher) were used for the phase separation. RNA was DNase-treated using TURBO DNA-free™ (ThermoFisher) and RNA quality was evaluated utilizing an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California, USA) as previously described [49].

All samples were pooled for library construction. Library construction was performed using the NuGEN Tecan universal RNA sequencing reagents custom ribosomal depletion probes (AnyDeplete™) against *A. aegypti* rRNA including 5.8S, 18S, 28S, ITS1, ITS2. The specific accession number used for the depletion probes included: AAEL028668-RA AAEL028668-RA-E1_exon:rRNA; NIGP01001771 dna:scaffold scaffold:AAegL5:NIGP01001771:6654:6806:1; AAEL028730-RA AAEL028730-RA-E1_exon:rRNA; NIGP01001771 dna:scaffold scaffold:AAegL5:NIGP01001771:12876:14043:1; AAEL028771-RA AAEL028771-RA-E1_exon:rRNA; NIGP01001771 dna:scaffold scaffold:AAegL5:NIGP01001771:7002:11112:1.

A metagenomic analysis flow chart can be found in Fig 2. Briefly, shotgun metagenomic sequencing was run using the Illumina HiSeq 1500 for deep sequencing. Raw fastq files were assessed for quality using Illumina FastQC version 1.0.0. Trimming and quality filtering of reads was performed using Atropos (<https://omictools.com/atropos-tool>), removing Illumina

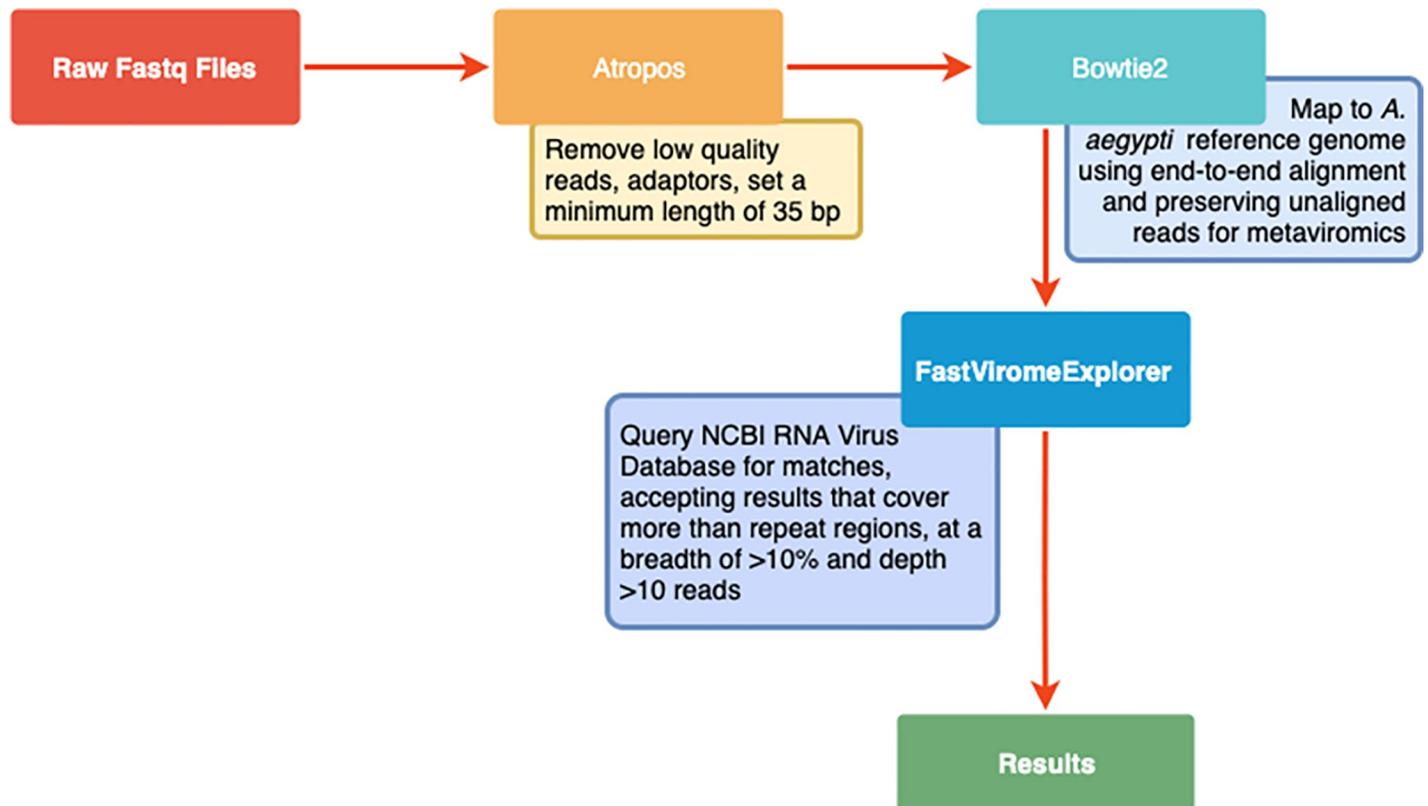


Fig 2. Metagenomic data analysis flow chart.

<https://doi.org/10.1371/journal.pone.0227998.g002>

universal adaptors, reads with base calls below Q20, and a minimum length of 35 bp. Additional host read removal was performed bioinformatically using Bowtie2 (v. 2.3.4.3). Reads were mapped to the *A. aegypti* reference genome assembly AaegL5, available at <https://www.vectorbase.org/organisms/aedes-aegypti>, using end-to-end read alignment. Non-mosquito reads were analyzed using FastViromeExplorer [50] and the NCBI RNA Virus database as a reference, which contains a total of 23,085 contigs and is available at <https://bench.cs.vt.edu/FastViromeExplorer/>. FastViromeExplorer was run using the default parameters, which accounts for potential false positives by removing results that mapped to repeat regions of the genome, cover less than 10% of the genome, or had fewer than ten reads total. Estimated abundance is expressed as total read counts adjusted for segment size.

RT-PCR verification of the metavirome results

About 200 ng of total RNA per pool (ten pools) or from individual mosquitoes was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). RT-PCR was conducted on the resultant cDNA using previously published specific oligonucleotide primers (S1 Table). PCR amplicons of expected size were extracted from gels using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Amplicons were sent to the Molecular Cloning Lab, San Francisco, California (<https://www.mclab.com/>) for Sanger sequencing. Raw sequence data were manually edited using Chromas 2.6.5 software and then compared with the sequence database using the NIH's Basic Local Alignment Search Tool (BLAST). Sequences of the Phasi Charoen-like virus (PCLV) were aligned with Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) to obtain a consensus

sequence. The S-and M-segment consensus sequence were made with seven different sequences, and the L-segment with five different sequences obtained from different mosquito pools using the primers in S1 Table [51–55].

Vertical transmission

Male *A. aegypti* mosquitoes, larva, and hatching eggs collected with oviposition traps as described in Jakob and Pratt [56] were pooled and used to determine potential vertical transmission. Larvae were confirmed as *A. aegypti* using pictorial keys for the identification of mosquitoes by Rueda [46]. RNA extraction, cDNA production and RT-PCR using PCLV primers were conducted as described above.

Phylogenetic analysis of PCLV

Three phylogenetic trees were developed with the consensus sequences obtained by RT-PCR for the S, M, and L segments of PCLV. The multiple sequence alignments were produced with MAFFT version 7.0 [57] using the FFT-NS-2 progressive method. The trees were developed using neighbor-joining phylogeny based on the conserved regions and confidence testing by 1000 bootstrap replicates and visualized using Archaeopteryx.js. The phylogenetic analysis included several genera of the *Phenuiviridae* family (Order: *Bunyavirales*). A phylogenetic tree for the L segment of PCLV was also developed using the Bayesian Markov chain Monte Carlo method using MrBayes 3.2.6 (http://www.phylogeny.fr/one_task.cgi?task_type=mrbayes) (S1 Fig).

Results and discussion

RNA-seq results reveal a high prevalence of PCLV in *Aedes aegypti* in Grenada that is confirmed by RT-PCR

Assessment of Fastq files using FastQC showed an average read length of 125 bp. Total reads were 74,536,838 of which 74,273,783 remained after trimming. After depleting host reads using Bowtie2 (v.2.3.4.3), non-mosquito reads totaled 14,428,848 of which 171,792 could be classified as RNA virus. The predominant ISV in the metavirome was Phasi Charoen-like virus (PCLV) with 156,968 reads using the *A. aegypti* reference genome.

One of the limitations to metagenomic studies based on database analysis is the fact that sequence information for many viral families or genera is still limited and phylogenies based on single or short genome fragments can be misleading. This study used very stringent restrictions to establish high accuracy on the report of the virome using the *A. aegypti* assembly. PCLV was the most abundant virus and the only one that passed the restrictions (Table 1).

Table 1. *Aedes aegypti* metavirome in Grenada. Estimated abundances are presented as obtained using the reference genome for *A. aegypti*.

Virus identifier	Name	Family/Genus	Estimated Abundance
KU936056	Phasi Charoen-like virus strain Aag2-Bristol glycoprotein	<i>Phenuiviridae</i> / Phasivirus	54,980.0
KM001085	Phasi Charoen-like virus RNA-dependent RNA polymerase		31,230.2
KR003786	Phasi Charoen-like virus isolate Rio segment L RNA-dependent RNA polymerase		24,713.8
KR003784	Phasi Charoen-like virus isolate Rio segment M glycoprotein		18,332.0
KM001086	Phasi Charoen-like virus glycoprotein precursor		16,483.0
KU936055	Phasi Charoen-like virus strain Aag2-Bristol nucleocapsid		5,020.5
KR003785	Phasi Charoen-like virus isolate Rio segment S nucleocapsid		3,790.9
KM001087	Phasi Charoen-like virus nucleocapsid		2,417.6

<https://doi.org/10.1371/journal.pone.0227998.t001>

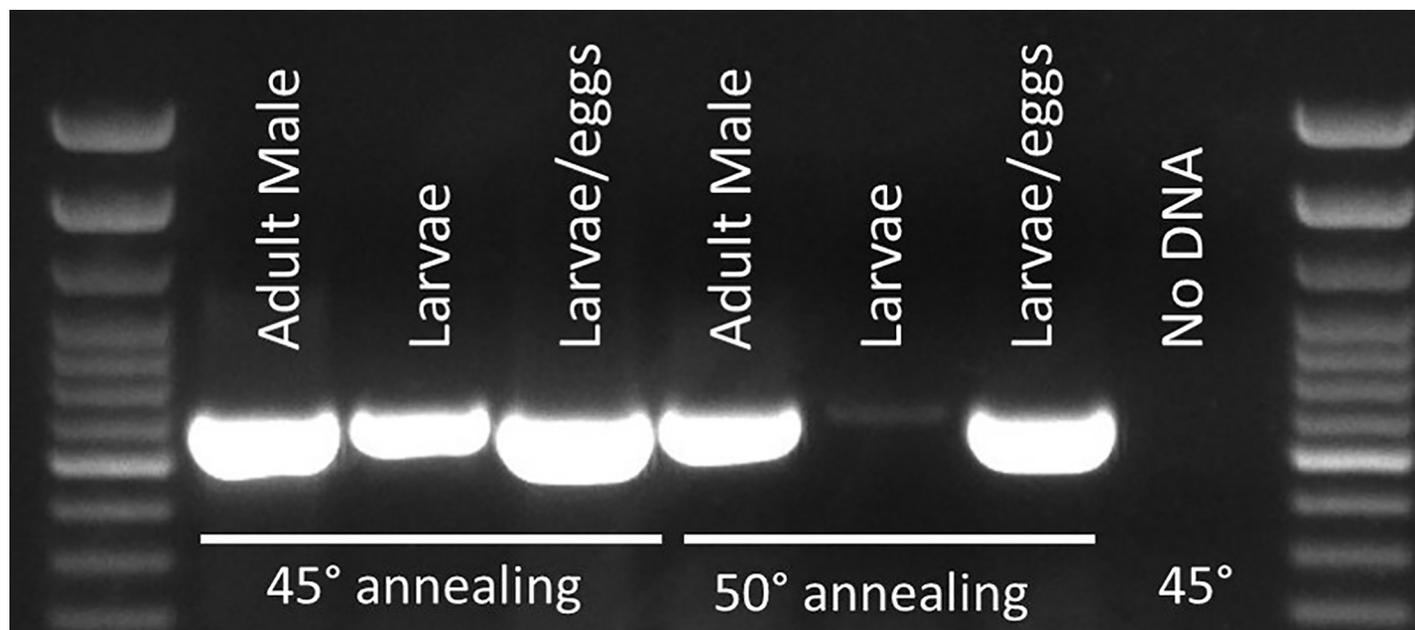


Fig 3. Detection of PCLV-Grenada in mosquito pools by RT-PCR in *Aedes aegypti* males, larvae, and hatching eggs (larvae/eggs).

<https://doi.org/10.1371/journal.pone.0227998.g003>

PCLV presence was confirmed with the same RNA used for library construction previous to pooling. A total of ten sub-pools, obtained from 30 mosquitoes each, were tested for PCLV by RT-PCR using the S-segment primers previously designed [51,52]. All PCLV amplicons were confirmed by Sanger sequencing and BLAST analysis, with PCLV as the best match with > 96% identity. Ten individual mosquitoes were also tested for PCLV using the S-segment and L-segment primers as well as an endogenous mosquito gene (see S1 Table). The presence of PCLV in all ten sub-pools (S2 Fig) and 70% of individual mosquitoes (S3 Fig) confirms the high prevalence of PCLV in *A. aegypti* mosquitoes from Grenada. The results also suggest that some *A. aegypti* are not infected with PCLV. We cannot rule out viral RNA degradation or poor primer sensitivity as explanations for why some mosquitoes were PCLV-negative by PCR. Regardless, future research should examine whether the PCLV infection status has an effect on the vector competence for arboviruses.

PCLV was present not only in female mosquitoes, but also tested samples of males, hatching larvae (larva/eggs), and larvae of *A. aegypti* (Fig 3, S4 Fig), which suggests vertical transmission of the virus. Insect specific RNA viruses are often considered vertically transmitted due to their inability to replicate in mammalian cells and their presence in larvae and male adult forms, which do not feed on blood [42,58–60]. This points to the possibility that these viruses may have coexisted with their insect host for a long period of time [61–63] and have evolved with them [64].

Similar to our results, PCLV has been found to be the predominant ISV in *A. aegypti* in Australia, [25], Guadeloupe [23], South China [51], and Thailand [24]. *Aedes aegypti* originated in Africa [65]. There are two recognized subspecies of *A. aegypti* s.l.: 1) *A. aegypti formosus* restricted to Africa and 2) *A. aegypti aegypti*, found globally in tropical and subtropical regions typically in association with humans. Phylogenetic analyses of all mtDNA ND4 haplotypes reported to date for *A. aegypti* all support the hypothesis that *A. aegypti* populations from around the world consist of mosquitoes that arise from one of two matrilineages of African origin (reviewed in [66]). Moore et al. [66] further demonstrated this claim by sequencing

ND4 haplotypes in 426 *A. aegypti* s.l. from Senegal (West Africa) and Kenya (East Africa) and finding all haplotypes found outside Africa matches two ancestral clades in Africa. When humans started living in groups and storing water, the larval breeding sites allowed for the *A. aegypti* mosquito to exploit the new niche [67] and for the females to adapt to the most available bloodmeal: human. This domesticated form of *A. aegypti* was likely introduced by the slave trade [65] from Africa into the Americas, from where it then spread to all other inhabited continents [68]. PCLV is highly prevalent in *A. aegypti* in all metavirome studies done so far (Australia, Asia, and America), and hence may have originated from the ancestral *Aedes* found in Africa and has evolved with the vector in different parts of the planet. ISVs and arboviruses show co-evolutionary relationship suggesting arboviruses could have been ISVs that through evolution acquired the ability to expand their host-range to vertebrates [42]. For example, in the order Bunyvirales, arthropod hosts have been constructed for all nodes of the bunyavirus tree [69], suggesting that arboviruses from this order may have evolved from ISVs. Similar examples can be found in other orders [42,70–73]. Because ISVs are RNA viruses with implied high mutation rates, together with the strong evolutionary pressure exerted on *A. aegypti* to live in areas densely populated with humans and other peridomestic animals, the opportunity for host expansion is a possibility and has been previously suggested for genera like the hantaviruses [74].

The impact of PCLV on the *A. aegypti* lifecycle and vector competency requires further study. It has been reported that the presence of PCLV in the mosquito may inhibit the replication of some arboviruses. For example, dual infection with both PCLV and Cell-fusing agent virus in *A. albopictus* cell line As23 results in replication inhibition of two flaviviruses (ZIKV and DENV) and the bunyavirus La Crosse virus [75]. In contrast, a study using a pre-existing persistent PCLV infection had no major impact on the replication on DENV, ZIKV, the alphavirus Sindbis virus, or the rhabdovirus vesicular stomatitis virus [76]. The discrepancy found in studies using cell lines may be explained by the previous infection status of the cells used in the different studies, which points to the need to do studies in live mosquitoes. There have been two studies looking at the effects of ISVs on vector competence for arboviruses using live mosquitoes, both associated with WNV in *C. quinquefasciatus* [34,77] but none on PCLV.

RNA-seq and RT-PCR show absence of alphaviruses and flaviviruses in the sample pools

The mosquito metagenomic analysis did not show the presence of any of the most common endemic or recently introduced arboviruses in Grenada. To confirm the absence of alphaviruses (Family: *Togaviridae*) including chikungunya virus and flaviviruses like ZIKV and DENV in the sample pools, nested RT-PCR was run using genus-level primers as indicated in S1 Table. For the alphaviruses, previously used primers targeting the highly conserved nsP4 coding region of alphavirus RNA was chosen as described previously [54]. No amplicons for alphaviruses were detected for any of the mosquito pools. PCR amplicons of the expected size obtained with the pan-flavivirus primers were sequenced showing the bands belong to ISV-flaviviruses. The detection of low abundance viruses is likely limited in metagenomic analysis by the stringent methods described above.

Phylogenetic tree shows the relationship between PCLV-Grenada, reported PCLVs, and other *Phenuiviridae* viruses

Phylogenetic trees of all three segments (S, M, and L) of PCLV were constructed using published data of taxa from the *Phenuiviridae* Family in GenBank against the consensus of six independent sequences of PCLV found in Grenada. PCLV in this study shows a closer

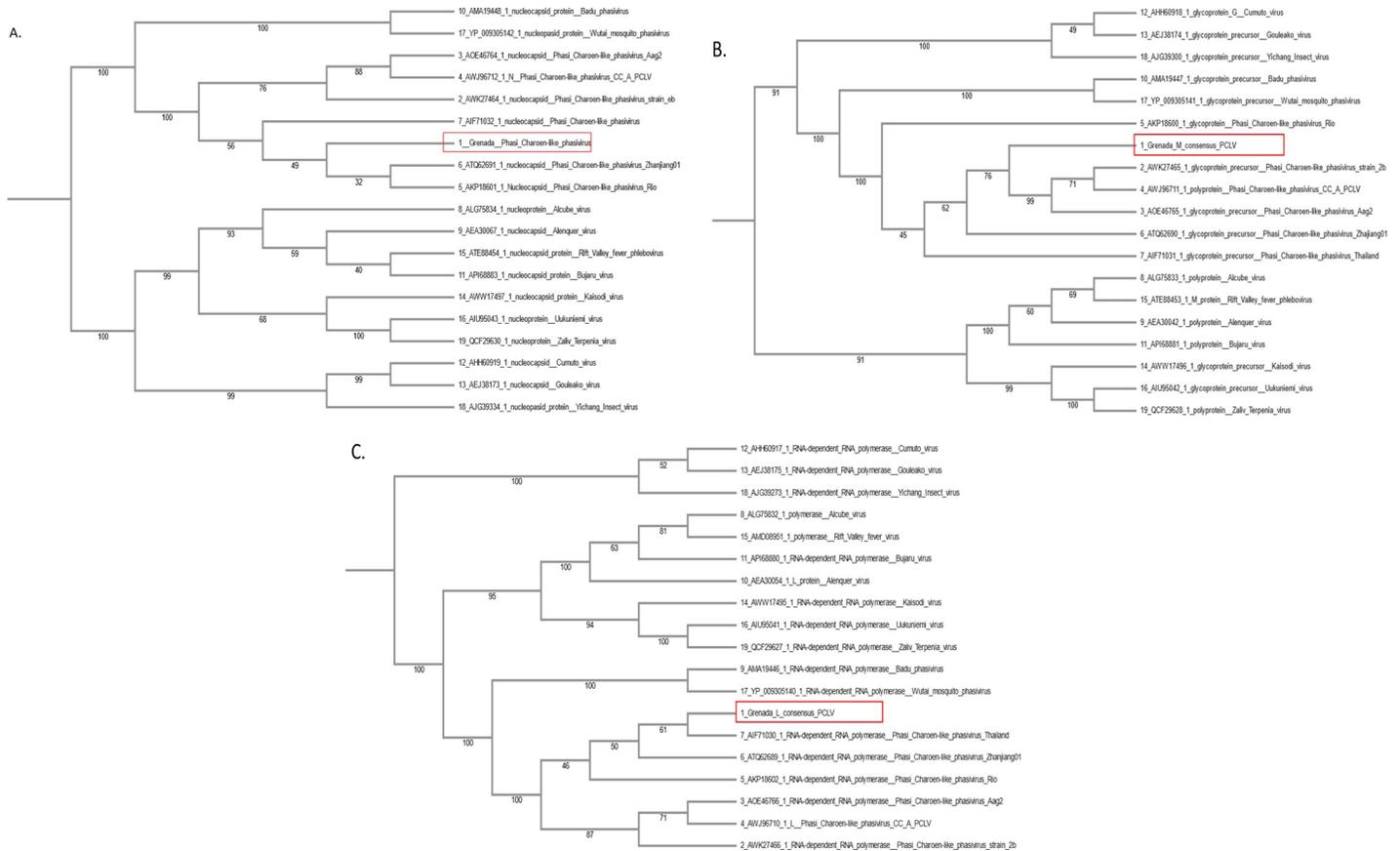


Fig 4. Phylogenetic trees showing the relationship between PCLV-Grenada and other *Phenuviridae*. Trees were developed using MAFFT version 7.0's FFT-NS-2 progressive method as the strategy. The trees were developed using NJ phylogeny based on the conserved regions and confidence testing by 1000 bootstrap replicates. A. S segment, B. M segment, and C. L segment.

<https://doi.org/10.1371/journal.pone.0227998.g004>

association to the *Phasivirus* genus, in all phylogenetic trees, than to the *Goukovirus* or *Phlebovirus* genera in this family (Fig 4 and S4 Fig) according to the latest report of ICTV released in 2018 (<https://talk.ictvonline.org/>). These findings are in disagreement with previous reports [78] where PCLV was grouped with the newly proposed genus *Goukovirus* because it clustered together with Badu virus and Gouleaku virus, but in agreement with results from similar studies in South China [51]. The consensus sequence of The PCLV obtained in Grenada had a 96–97% similarity to all other published PCLV sequences in GenBank as determined by BLAST. The sequences for the PCLV Grenada isolate have been deposited in GenBank under the accession numbers MN109951 (for the S segment/nucleocapsid coding region), MN109952 (for the M segment/glycoprotein coding region), and MN109953 (for the L segment/RNA dependent RNA polymerase coding region).

Conclusions

In summary, we have shown here that in the Caribbean, *Aedes aegypti* most abundant virus is PCLV, as has been reported previously in Guadeloupe and also in other continents. The similarities found in *A. aegypti* virome worldwide, and particularly the presence of PCLV has many implications, including the likelihood of a common ancestry. Future studies in Africa will be needed to link PCLV to ancestral origins. Due to the widespread presence of this ISVs

in *A. aegypti*, the impact of PCLV in this mosquito's biology and vector competency needs to be addressed in wild populations. This study used pooling of samples and stringent restrictions while analyzing the virome of *Aedes aegypti* which may have affected the report on low abundance viruses.

Supporting information

S1 Fig. Phylogenetic trees for the L segment of PCLV using the Bayesian Markov chain Monte Carlo method.

(PDF)

S2 Fig. Detection of PCLV-Grenada in 10 mosquito sub-pools by RT-PCR. Controls include: a mosquito pool previously determined to be PCLV-positive, extraction control (negative), and no DNA control (negative).

(TIFF)

S3 Fig. Detection of PCLV-Grenada in 10 individual mosquitoes by RT-PCR. A. PCLV S-segment. B. PCLV L-segment. C. Mosquito endogenous gene (AAEL004181). Controls include: a mosquito pool previously determined to be PCLV-positive, extraction control (negative), and no DNA control (negative).

(TIF)

S4 Fig. Uncropped Fig 3.

(TIF)

S5 Fig. Uncropped S3 Fig. A. Gel electrophoresis for PCLV S-segment primer PCR. B. Gel electrophoresis for PCLV L-segment primer PCR (top) and mosquito endogenous gene (bottom).

(TIF)

S1 Table. Primers used in this study.

(PDF)

Acknowledgments

We acknowledge Jaelene Haynes from SGU Veterinary School for her contribution to capturing mosquitoes, and to Dr. Svetlana Kotelnikova from SGU-School of Medicine for her feedback on the original study proposal.

Special thanks to all the people of Grenada that provided access to the collection sites used in this study.

Author Contributions

Conceptualization: Maria E. Ramos-Nino, Sonia Cheetham.

Data curation: Maria E. Ramos-Nino, Scott Tighe, Korin M. Eckstrom, Julie Dragon.

Formal analysis: Maria E. Ramos-Nino, Daniel M. Fitzpatrick.

Funding acquisition: Maria E. Ramos-Nino.

Investigation: Maria E. Ramos-Nino, Daniel M. Fitzpatrick, Lindsey M. Hattaway, Andy N. Hsueh, Sonia Cheetham.

Methodology: Maria E. Ramos-Nino, Daniel M. Fitzpatrick.

Project administration: Maria E. Ramos-Nino.

Visualization: Maria E. Ramos-Nino.

Writing – original draft: Maria E. Ramos-Nino.

Writing – review & editing: Maria E. Ramos-Nino, Daniel M. Fitzpatrick, Diana M. Stone, Sonia Cheetham.

References

1. Tandina F, Doumbo O, Yaro AS, Traoré SF, Parola P, Robert V. Mosquitoes (Diptera: Culicidae) and mosquito-borne diseases in Mali, West Africa. *Parasit Vectors*. 2018. <https://doi.org/10.1186/s13071-018-3045-8> PMID: 30103823
2. Richard V, Cao-Lormeau VM. Mosquito vectors of arboviruses in French Polynesia. *New Microbes New Infect*. 2019. <https://doi.org/10.1016/j.nmni.2019.100569> PMID: 31316821
3. Braack L, Gouveia De Almeida AP, Cornel AJ, Swanepoel R, De Jager C. Mosquito-borne arboviruses of African origin: Review of key viruses and vectors. *Parasit Vectors*. 2018. <https://doi.org/10.1186/s13071-017-2559-9> PMID: 29316963
4. Weaver SC, Reisen WK. Present and future arboviral threats. *Antiviral Res*. 2010. <https://doi.org/10.1016/j.antiviral.2009.10.008> PMID: 19857523
5. Weaver SC. Urbanization and geographic expansion of zoonotic arboviral diseases: Mechanisms and potential strategies for prevention. *Trends Microbiol*. 2013. <https://doi.org/10.1016/j.tim.2013.03.003> PMID: 23910545
6. Patterson J, Sammon M, Garg M. Dengue, zika and chikungunya: Emerging arboviruses in the new world. *West J Emerg Med*. 2016. <https://doi.org/10.5811/westjem.2016.9.30904> PMID: 27833670
7. Weaver SC, Charlier C, Vasilakis N, Lecuit M. Zika, chikungunya, and other emerging vector-borne viral diseases. *Annu Rev Med*. 2017; <https://doi.org/10.1146/annurev-med-050715-105122> PMID: 28846489
8. Fitzpatrick DM, Hattaway LM, Hsueh AN, Ramos-Nino ME, Cheetham SM. PCR-based bloodmeal analysis of *Aedes aegypti* and *Culex quinquefasciatus* (Diptera: Culicidae) in St. George Parish, Grenada. *J Med Entomol*. 2019; 1–6. <https://doi.org/10.1093/jme/tjy197>
9. Simmons CP, Farrar J, Nguyen van VC, Wills B. Current Concepts Dengue. *N Engl J Med*. 2012; <https://doi.org/10.1056/NEJMra1110265>
10. Jentes ES, Pomeroy G, Gershman MD, Hill DR, Lemarchand J, Lewis RF, et al. The revised global yellow fever risk map and recommendations for vaccination, 2010: Consensus of the Informal WHO Working Group on Geographic Risk for Yellow Fever. *Lancet Infect Dis*. 2011. [https://doi.org/10.1016/S1473-3099\(11\)70147-5](https://doi.org/10.1016/S1473-3099(11)70147-5)
11. Boyer S, Calvez E, Chouin-Carneiro T, Diallo D, Failloux AB. An overview of mosquito vectors of Zika virus. *Microbes Infect*. 2018; <https://doi.org/10.1016/j.micinf.2018.01.006> PMID: 29481868
12. Leparco-Goffart I, Nougairède A, Cassadou S, Prat C, de Lamballerie X. Chikungunya in the Americas. *Lancet*. 2014; [https://doi.org/10.1016/s0140-6736\(14\)60185-9](https://doi.org/10.1016/s0140-6736(14)60185-9)
13. Guégan M, Zouache K, Démichel C, Minard G, Tran Van V, Potier P, et al. The mosquito holobiont: fresh insight into mosquito-microbiota interactions. *Microbiome*. 2018. <https://doi.org/10.1186/s40168-018-0435-2> PMID: 29554951
14. Atyame CM, Alout H, Mousson L, Vazeille M, Diallo M, Weill M, et al. Insecticide resistance genes affect *Culex quinquefasciatus* vector competence for West Nile virus. *Proc R Soc B Biol Sci*. 2019; <https://doi.org/10.1098/rspb.2018.2273> PMID: 30963855
15. Scolari F, Casiraghi M, Bonizzoni M. *Aedes* spp. and their microbiota: a review. *Front Microbiol*. 2019; <https://doi.org/10.3389/fmicb.2019.02036> PMID: 31551973
16. Olmo RP, Martins NE, Aguiar ERGR, Marques JT, Imler JL. The insect reservoir of biodiversity for viruses and for antiviral mechanisms. *An Acad Bras Cienc*. 2019; <https://doi.org/10.1590/0001-3765201920190122> PMID: 31166476
17. Ramirez JL, Souza-Neto J, Cosme RT, Rovira J, Ortiz A, Pascale JM, et al. Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota, innate immune system and dengue virus influences vector competence. *PLoS Negl Trop Dis*. 2012; <https://doi.org/10.1371/journal.pntd.0001561> PMID: 22413032
18. Hill CL, Sharma A, Shouche Y, Severson DW. Dynamics of midgut microflora and dengue virus impact on life history traits in *Aedes aegypti*. *Acta Trop*. 2014; <https://doi.org/10.1016/j.actatropica.2014.07.015> PMID: 25193134

19. Dennison NJ, Jupatanakul N, Dimopoulos G. The mosquito microbiota influences vector competence for human pathogens. *Curr Opin Insect Sci*. 2014; <https://doi.org/10.1016/j.cois.2014.07.004> PMID: 25584199
20. Johnson KN. Bacteria and antiviral immunity in insects. *Curr Opin Insect Sci*. 2015; <https://doi.org/10.1016/j.cois.2015.01.008>
21. Saraiva RG, Kang S, Simões ML, Angleró-Rodríguez YI, Dimopoulos G. Mosquito gut antiparasitic and antiviral immunity. *Dev Comp Immunol*. 2016; <https://doi.org/10.1016/j.dci.2016.01.015> PMID: 26827888
22. Kumar S, Krishnani KK, Bhushan B, Brahmane MP. Metagenomics: Retrospect and Prospects in High Throughput Age. *Biotechnol Res Int*. 2015; <https://doi.org/10.1155/2015/121735> PMID: 26664751
23. Shi C, Beller L, Deboutte W, Yinda KC, Delang L, Vega-Rúa A, et al. Stable distinct core eukaryotic viromes in different mosquito species from Guadeloupe, using single mosquito viral metagenomics. *Microbiome*. 2019; <https://doi.org/10.1186/s40168-019-0734-2> PMID: 31462331
24. Chandler JA, Thongsripong P, Green A, Kittayapong P, Wilcox BA, Schroth GP, et al. Metagenomic shotgun sequencing of a Bunyavirus in wild-caught *Aedes aegypti* from Thailand informs the evolutionary and genomic history of the Phleboviruses. *Virology*. 2014; <https://doi.org/10.1016/j.virol.2014.06.036> PMID: 25108381
25. Zakrzewski M, Rašić G, Darbro J, Krause L, Poo YS, Filipović I, et al. Mapping the virome in wild-caught *Aedes aegypti* from Cairns and Bangkok. *Sci Rep*. 2018; <https://doi.org/10.1038/s41598-018-22945-y> PMID: 29549363
26. Villegas LEM, Campolina TB, Barnabe NR, Orfano AS, Chaves BA, Norris DE, et al. Zika virus infection modulates the bacterial diversity associated with *Aedes aegypti* as revealed by metagenomic analysis. *PLoS One*. 2018; <https://doi.org/10.1371/journal.pone.0190352> PMID: 29293631
27. Nasar F, Gorchakov R V., Tesh RB, Weaver SC. Eilat virus host range restriction is present at multiple levels of the virus life cycle. *J Virol*. 2014; <https://doi.org/10.1128/jvi.01856-14> PMID: 25392227
28. Vasilakis N, Guzman H, Firth C, Forrester NL, Widen SG, Wood TG, et al. Mesoniviruses are mosquito-specific viruses with extensive geographic distribution and host range. *Viol J*. 2014; <https://doi.org/10.1186/1743-422X-11-97> PMID: 24884700
29. Sangdee K, Pattanakitsakul SN. Comparison of mosquito densovirus: two clades of viruses isolated from indigenous mosquitoes. *Southeast Asian J Trop Med Public Health*. 2013;
30. Cook S, Bennett SN, Holmes EC, De Chesse R, Moureau G, de Lamballerie X. Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. *J Gen Virol*. 2006; <https://doi.org/10.1099/vir.0.81475-0>
31. Calzolari M, Zé-Zé L, Růžek D, Vázquez A, Jeffries C, Defilippo F, et al. Detection of mosquito-only flaviviruses in Europe. *J Gen Virol*. 2012; <https://doi.org/10.1099/vir.0.040485-0>
32. Parry R, Asgari S. *Aedes anphevirus*: an insect-specific virus distributed worldwide in *Aedes aegypti* mosquitoes that has complex interplays with *Wolbachia* and dengue virus infection in cells. *J Virol*. 2018; <https://doi.org/10.1128/jvi.00224-18> PMID: 29950416
33. Romo H, Kenney JL, Blitvich BJ, Brault AC. Restriction of Zika virus infection and transmission in *Aedes aegypti* mediated by an insect-specific flavivirus. *Emerg Microbes Infect*. 2018; <https://doi.org/10.1038/s41426-018-0180-4> PMID: 30429457
34. Bolling BG, Olea-Popelka FJ, Eisen L, Moore CG, Blair CD. Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex pipiens* laboratory colony and effects of co-infection on vector competence for West Nile virus. *Virology*. 2012; <https://doi.org/10.1016/j.virol.2012.02.016> PMID: 22425062
35. Hobson-Peters J, Yam AWY, Lu JWF, Setoh YX, May FJ, Kurucz N, et al. A new insect-specific flavivirus from northern Australia suppresses replication of West Nile Virus and Murray Valley encephalitis virus in co-infected mosquito cells. *PLoS One*. 2013; <https://doi.org/10.1371/journal.pone.0056534> PMID: 23460804
36. Fujita R, Kato F, Kobayashi D, Murota K, Takasaki T, Tajima S, et al. Persistent viruses in mosquito cultured cell line suppress multiplication of flaviviruses. *Heliyon*. 2018; <https://doi.org/10.1016/j.heliyon.2018.e00736> PMID: 30167494
37. Zhang G, Asad S, Khromykh AA, Asgari S. Cell fusing agent virus and dengue virus mutually interact in *Aedes aegypti* cell lines. *Sci Rep*. 2017; <https://doi.org/10.1038/s41598-017-07279-5> PMID: 28761113
38. Kuwata R, Isawa H, Hoshino K, Sasaki T, Kobayashi M, Maeda K, et al. Analysis of mosquito-borne flavivirus superinfection in *Culex tritaeniorhynchus* (Diptera: Culicidae) cells persistently infected with *Culex flavivirus* (Flaviviridae). *J Med Entomol*. 2015; <https://doi.org/10.1093/jme/tju059> PMID: 26336307

39. Talavera S, Birnberg L, Nuñez AI, Muñoz-Muñoz F, Vázquez A, Busquets N. *Culex flavivirus* infection in a *Culex pipiens* mosquito colony and its effects on vector competence for Rift Valley fever phlebovirus. *Parasit Vectors*. 2018; <https://doi.org/10.1186/s13071-018-2887-4> PMID: 29792223
40. Kuno G, Chang GJJ. Biological transmission of arboviruses: Reexamination of and new insights into components, mechanisms, and unique traits as well as their evolutionary trends. *Clin Microbiol Rev*. 2005. <https://doi.org/10.1128/CMR.18.4.608-637.2005>
41. Souza-Neto JA, Powell JR, Bonizzoni M. *Aedes aegypti* vector competence studies: A review. *Infect Gen Evol*. 2019. <https://doi.org/10.1016/j.meegid.2018.11.009> PMID: 30465912
42. Öhlund P, Lundén H, Blomström AL. Insect-specific virus evolution and potential effects on vector competence. *Virus Genes*. 2019. <https://doi.org/10.1007/s11262-018-01629-9> PMID: 30632016
43. Lee Y-M, Tscherne DM, Yun S-I, Frolov I, Rice CM. Dual mechanisms of pestiviral superinfection exclusion at entry and RNA replication. *J Virol*. 2005; <https://doi.org/10.1128/jvi.79.6.3231-3242.2005>
44. Aldridge RL, Britch SC, Allan SA, Tsikolia M, Calix LC, Bernier UR, et al. Comparison of volatiles and mosquito capture efficacy for three carbohydrate sources in a yeast-fermentation CO₂ generator. *J Am Mosq Control Assoc*. 2017; <https://doi.org/10.2987/16-6609.1> PMID: 28206863
45. Darsie JR, Ward R. Identification and geographical distribution of the mosquitoes of north America, north of Mexico, University Press of Florida, 2005. <https://doi.org/10.1017/s0031182005228834>
46. Rueda LM. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with dengue virus transmission. *Zootaxa*. 2004; <https://doi.org/10.11646/zootaxa.589.1.1>
47. Gaffigan T, Wilkerson R, Pecor J, Stoffer J, Anderson T. Systematic catalog of Culicidae-Walter Reed Biosystematics Unit, 2015. Available: <http://www.mosquitocatalog.org/default.aspx> [Internet].
48. Beckmann JF, Fallon AM. Decapitation improves detection of *Wolbachia pipientis* (Rickettsiales: Anaplasmataceae) in *Culex pipiens* (Diptera: Culicidae) mosquitoes by the polymerase chain reaction. *J Med Entomol*. 2012; <https://doi.org/10.1603/me12049> PMID: 23025192
49. Sultan M, Amstislavskiy V, Risch T, Schuette M, Dökel S, Ralsler M, et al. Influence of RNA extraction methods and library selection schemes on RNA-seq data. *BMC Genomics*. 2014; <https://doi.org/10.1186/1471-2164-15-675> PMID: 25113896
50. Tithi SS, Aylward FO, Jensen R V., Zhang L. FastViromeExplorer: A pipeline for virus and phage identification and abundance profiling in metagenomics data. *PeerJ*. 2018; <https://doi.org/10.7717/peerj.4227> PMID: 29340239
51. Zhang X, Huang S, Jin T, Lin P, Huang Y, Wu C, et al. Discovery and high prevalence of Phasi Charoen-like virus in field-captured *Aedes aegypti* in South China. *Virology*. 2018; <https://doi.org/10.1016/j.virol.2018.07.021> PMID: 30077072
52. Schnettler E, Sreenu VB, Mottram T, McFarlane M. *Wolbachia* restricts insect-specific flavivirus infection in *Aedes aegypti* cells. *J Gen Virol*. 2016; <https://doi.org/10.1099/jgv.0.000617> PMID: 27692043
53. Scaramozzino N, Crance JM, Jouan A, DeBriel DA, Stoll F, Garin D. Comparison of Flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. *J Clin Microbiol*. 2001; <https://doi.org/10.1128/JCM.39.5.1922-1927.2001>
54. Grywna K, Kupfer B, Panning M, Drexler JF, Emmerich P, Drosten C, et al. Detection of all species of the genus *Alphavirus* by reverse transcription-PCR with diagnostic sensitivity. *J Clin Microbiol*. 2010; <https://doi.org/10.1128/JCM.00317-10> PMID: 20504990
55. Klasson L, Kambris Z, Cook PE, Walker T, Sinkins SP. Horizontal gene transfer between *Wolbachia* and the mosquito *Aedes aegypti*. *BMC Genomics*. 2009; <https://doi.org/10.1186/1471-2164-10-33> PMID: 19154594
56. Jakob W, Pratt H. Oviposition trap reference handbook. *Aedes aegypti* Handbook Series No 6. 1967.
57. Katoh K, Rozewicki J, Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform*. 2017; <https://doi.org/10.1093/bib/bbx108> PMID: 28968734
58. Bolling BG, Eisen L, Moore CG, Blair CD. Insect-specific flaviviruses from *Culex* mosquitoes in Colorado, with evidence of vertical transmission. *Am J Trop Med Hyg*. 2011; <https://doi.org/10.4269/ajtmh.2011.10-0474>
59. Saiyasombat R, Bolling BG, Brault AC, Bartholomay LC, Blitvich BJ. Evidence of efficient transovarial transmission of *Culex flavivirus* by *Culex pipiens* (Diptera: Culicidae). *J Med Entomol*. 2011; <https://doi.org/10.1603/me11043> PMID: 21936322
60. Haddow AD, Guzman H, Popov VL, Wood TG, Widen SG, Haddow AD, et al. First isolation of *Aedes flavivirus* in the western hemisphere and evidence of vertical transmission in the mosquito *Aedes (Stegomyia) albopictus* (Diptera: Culicidae). *Virology*. 2013; <https://doi.org/10.1016/j.virol.2012.12.008> PMID: 23582303

61. Ballinger MJ, Bruenn JA, Hay J, Czechowski D, Taylor DJ. Discovery and evolution of Bunyavirids in arctic phantom midges and ancient Bunyavirid-like sequences in insect genomes. *J Virol*. 2014; <https://doi.org/10.1128/jvi.00531-14> PMID: 24850747
62. Dudas G, Obbard DJ. Are arthropods at the heart of virus evolution? *Elife*. 2015; <https://doi.org/10.7554/eLife.06837> PMID: 25751043
63. Fort P, Albertini A, Van-Hua A, Berthomieu A, Roche S, Delsuc F, et al. Fossil rhabdoviral sequences integrated into arthropod genomes: Ontogeny, evolution, and potential functionality. *Mol Biol Evol*. 2012; <https://doi.org/10.1093/molbev/msr226> PMID: 21917725
64. Li C-X, Shi M, Tian J-H, Lin X-D, Kang Y-J, Chen L-J, et al. Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses. *Elife*. 2015; <https://doi.org/10.7554/eLife.05378> PMID: 25633976
65. Brown JE, Evans BR, Zheng W, Obas V, Barrera-Martinez L, Egizi A, et al. Human impacts have shaped historical and recent evolution in *Aedes aegypti*, the dengue and yellow fever mosquito. *Evolution (N Y)*. 2014; <https://doi.org/10.1111/evo.12281> PMID: 24111703
66. Moore M, Sylla M, Goss L, Burugu MW, Sang R, Kamau LW, et al. Dual african origins of global *Aedes aegypti* s.l. populations revealed by mitochondrial DNA. *PLoS Negl Trop Dis*. 2013; <https://doi.org/10.1371/journal.pntd.0002175> PMID: 23638196
67. Powell JR. Perspective piece mosquito-borne human viral diseases: Why *Aedes aegypti*? *Am J Trop Med Hyg*. 2018. <https://doi.org/10.4269/ajtmh.17-0866> PMID: 29557341
68. Kraemer MUG, Sinka ME, Duda KA, Mylne AQN, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. *Elife*. 2015; <https://doi.org/10.7554/eLife.08347> PMID: 26126267
69. Marklewitz M, Zirkel F, Kurth A, Drosten C, Junglena S. Evolutionary and phenotypic analysis of live virus isolates suggests arthropod origin of a pathogenic RNA virus family. *Proc Natl Acad Sci U S A*. 2015; <https://doi.org/10.1073/pnas.1502036112> PMID: 26038576
70. Bolling BG, Weaver SC, Tesh RB, Vasilakis N. Insect-specific virus discovery: Significance for the arbovirus community. *Viruses*. 2015. <https://doi.org/10.3390/v7092851> PMID: 26378568
71. Cook S, Moureau G, Kitchen A, Gould EA, de Lamballerie X, Holmes EC, et al. Molecular evolution of the insect-specific flaviviruses. *J Gen Virol*. 2012. <https://doi.org/10.1099/vir.0.036525-0>
72. Nasar F, Palacios G, Gorchakov R V., Guzman H, Travassos Da Rosa AP, Savji N, et al. Eilat virus, a unique alphavirus with host range restricted to insects by RNA replication. *Proc Natl Acad Sci U S A*. 2012; <https://doi.org/10.1073/pnas.1204787109> PMID: 22908261
73. Hermanns K, Zirkel F, Kopp A, Marklewitz M, Rwegu IB, Estrada A, et al. Discovery of a novel alphavirus related to Eilat virus. *J Gen Virol*. 2017; <https://doi.org/10.1099/jgv.0.000694> PMID: 28206905
74. Plyusnin A, Sironen T. Evolution of hantaviruses: Co-speciation with reservoir hosts for more than 100MYR. *Virus Res*. 2014; <https://doi.org/10.1016/j.virusres.2014.01.008> PMID: 24463501
75. Schultz MJ, Frydman HM, Connor JH. Dual Insect specific virus infection limits Arbovirus replication in *Aedes* mosquito cells. *Virology*. 2018; <https://doi.org/10.1016/j.virol.2018.03.022> PMID: 29625404
76. Fredericks AC, Russell TA, Wallace LE, Davidson AD, Fernandez-Sesma A, Maringer K. *Aedes aegypti* (Aag2)-derived clonal mosquito cell lines reveal the impact of pre-existing persistent infection with the insect-specific bunyavirus Phasi Charoen-like virus on arbovirus replication. *PLoS Negl Trop Dis*. 2019; <https://doi.org/10.1371/journal.pntd.0007346> PMID: 31693659
77. Kent RJ, Crabtree MB, Miller BR. Transmission of West Nile virus by *Culex quinquefasciatus* say infected with *Culex flavivirus izabal*. *PLoS Negl Trop Dis*. 2010; 4. <https://doi.org/10.1371/journal.pntd.0000671> PMID: 20454569
78. Hobson-Peters J, Warrilow D, McLean BJ, Watterson D, Colmant AMG, van den Hurk AF, et al. Discovery and characterisation of a new insect-specific bunyavirus from *Culex* mosquitoes captured in northern Australia. *Virology*. 2016; <https://doi.org/10.1016/j.virol.2015.11.003> PMID: 26773387