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## **The virome of vector mosquitoes**

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## **Abstract**

Mosquitoes are the major vectors for arthropod-borne viruses (arboviruses) of medical importance. *Aedes aegypti* and *A. albopictus* are the most prolific and widespread mosquito vectors being responsible for global transmission of dengue, Zika and Chikungunya viruses. Characterizing the collection of viruses circulating in mosquitoes, e.g. the virome, has long been of special interest. In addition to arboviruses, mosquitoes carry insect-specific viruses (ISVs) that do not directly infect vertebrates. Mounting evidence indicates that ISVs interact with arboviruses and may affect mosquito vector competence. Here, we review our current knowledge about the virome of vector mosquitoes and discuss the challenges for the field that may lead to novel strategies to prevent outbreaks of arboviruses.

## Introduction

Arthropod-borne viruses (arboviruses) are a major threat to humans worldwide. About 4 billion people live in areas at risk of arbovirus transmission. Dengue virus (DENV), the most widespread arbovirus, is estimated to cause 390 million new infections every year [1]. In the past decades, we have seen an unprecedented increase in the scale of transmission of DENV and the global emergence of other arboviruses [2]. Zika (ZIKV) and Chikungunya (CHIKV) viruses started spreading globally in the early 2000s and have since caused major outbreaks in Asia and the Americas [2]. Both ZIKV and CHIKV were known to circulate in enzootic cycles in Africa with limited spillovers for many decades but factors that determine their recent emergence are still unclear [2]. *Aedes* mosquitoes, especially *A. aegypti* and *A. albopictus*, are the major vectors for arboviruses being responsible for global transmission of DENV, ZIKV and CHIKV. Climate change, urbanization, globalized trade and human movement have allowed these anthropophilic mosquitoes to expand their global distribution, which facilitated the spread of arboviruses [3]. Virological surveillance of mosquitoes to identify viruses with zoonotic potential is an important tool to help raise preparedness and prevent outbreaks. Here we will review current knowledge about the mosquito virome, highlight the importance of studying viruses circulating in vector insects and discuss challenges for the field to control the growing threat of arboviruses.

### A historical perspective on the discovery of mosquito viruses

There are currently more than 500 known arboviruses, with about 100 that may infect humans [4,5]. Mosquitoes are the major arthropod vectors being responsible for the transmission of over a third of all arboviruses according to the comprehensive reference from the American Centers for Disease Control and Prevention (CDC arbovirus catalog) (**Supplementary Table 1**). Despite their importance for human public health, the rate of arboviruses discovery has greatly diminished and numbers have stagnated in the early 2010s [4]. Paradoxically, the number of viruses identified in mosquitoes has increased dramatically in the past decade (**Figure 1A**). An unbiased search for mosquito viruses in the NT database of Genbank retrieves approximately 300 unique entries of which 75% were deposited from 2011 to 2020 (**Supplementary Table 2**). This boom in mosquito viruses was driven by the popularization of metagenomic studies to analyze mosquitoes that jumped from one report before 2010 [6] to around 130 by the end of 2020 (**Figure 1B**). The number of articles mentioning the mosquito virome has soared from 2 [6,7] prior to 2014 to over 50 by the end of 2020 (**Figure 1B**). Of note, the 2 publications prior to 2014 were biased towards sequencing of DNA viruses that mostly included phages infecting bacteria within the mosquito [6,7]. By 2015, many groups

started applying metagenomic strategies that could also identify RNA viruses infecting mosquitoes [8–14]. These large-scale metagenomic studies helped provide a broader overview of viruses circulating in mosquitoes beyond the focus on arboviruses [8,14,15]. Among mosquito viruses obtained from Genbank, only 71 are clearly recognized as arboviruses (**Supplementary Table 2**). Although not all arboviruses transmitted by mosquitoes listed in the CDC catalog were identified in our search, the majority of them remains to be characterized and has not actually been isolated or sequenced from their presumed vectors. Even if the list from Genbank represents just a portion of the total, it is clear that the majority of viruses identified in mosquitoes over the past decade are not arboviruses (**Figure 1A**). After the boom of metagenomics, most viruses described in mosquitoes belong to a group referred to as insect specific viruses (ISVs) (**Supplementary Table 2**). In contrast, virological surveillance of vector mosquitoes and wild animals relied mostly on viral isolation methods in cell culture prior to the metagenomics era, which were methods biased towards detection arboviruses with dual host capacity [4,16].

### **The diversity of the mosquito virome: arboviruses and ISVs**

Studies over the past decade have established that mosquitoes harbor a rich and diverse virome composed of arboviruses and ISVs (**Figure 1A**). Arboviruses and ISVs in mosquitoes belong to diverse virus families largely composed of RNA viruses (**Figure 1C**). Even considering that current strategies might be biased towards the identification of RNA viruses, it seems unlikely that DNA viruses represent a large component of the mosquito virome. Overall, ISVs seem to be more abundant and diverse than arboviruses when directly comparing the representation of viral families (**Figure 1C**) [8–11,13,14,17–19]. The diversity of ISVs is based on metagenomic studies that tend to favor identification of viruses that are more abundant and prevalent in mosquito populations compared to arboviruses that are present in 1-2% of individuals. However, there are notes of caution when analyzing results from metagenomics. Some potential viruses are associated with a single study and often do not have a complete genome. It is also important to note that results from metagenomics may be conflated by the identification of endogenous viral elements (EVEs) that are not real viruses (**Box 1**). Here, the use of small RNA based metagenomic approaches is an important alternative that can distinguish between actively replicating viruses and EVEs in mosquitoes (**Box 2**) [14,20]. Studies based on different metagenomic strategies have consistently shown that ISVs represent a large fraction of the collection of viruses circulating in mosquitoes. If we consider the virome of *A. aegypti* mosquitoes, arguably the major insect vector for arbovirus, studies have reported a high diversity and abundance of ISVs [8,10,11,14,18,21]. Cross-referencing studies with *A. aegypti* mosquitoes from different parts of the world show

that a few ISVs are widespread such as Phasi-Charoen like virus, Humaita Tubiacanga virus, Aedes anphevirus and cell fusing agent virus (CFAV). This suggests a highly conserved core virome of *A. aegypti*, although we lack broader studies that directly compare mosquitoes from different locations. We also lack a systematic comparison between species but some studies have analyzed the virome of different mosquitoes such as *Culex* sp, *Aedes* sp and *Anopheles* sp [22–25]. These studies show variations in virus diversity and abundance between species but did not observe consistent patterns that are associated with specific mosquitoes. Rather, all mosquitoes seem to harbor a diverse and abundant virome, which contrasts with the fact that only a few species are competent vectors for arboviruses. *Anopheles* mosquitoes, for example, which possess a very rich virome, are only known to transmit a single arbovirus, o'nyong-nyong virus [19,26,27].

### **Interactions between ISVs and arboviruses in mosquitoes**

The expanding knowledge about the mosquito virome is a resource for the field that can be explored in many ways. Metagenomics has provided great insights into the diversity of viruses in mosquitoes but we are just starting to uncover the biology of ISVs and their interactions with arboviruses in the mosquito (**Figure 2**).

Phylogenetic analysis with ancestral state reconstructions have indicated that viruses circulating in insects and arthropods are basal to many viral groups, including some vertebrate specific families [28,29]. The *Bunyaviridae* family, for example, seems to have originated from viruses primarily circulating in arthropods [29]. This supports the idea that insect viruses are central to virus evolution having given rise to several virus groups that infect vertebrates and plants [28]. Insects may be a continuous source of novel viruses and by studying the biology of ISVs, we may a little better understand how host shifts happen [17,28]. More specifically, the origin of arboviruses that are able to infect phylogenetically distant hosts remains enigmatic. Yet, the ability to cycle between invertebrates and vertebrates has been selected independently within several viral families [5,16]. Similarities between ISVs and arboviruses could also be explored. Studies about the diversity and abundance of ISVs could provide information about the permissiveness of mosquitoes to viruses in general. If the detection of ISVs can be used to assess arbovirus permissiveness, their presence in mosquito vectors could be the basis for a warning system assessing the risk of arbovirus outbreaks. Furthermore, studies analyzing the diversity and abundance of ISVs in different mosquito species may help understand what is unique about some of the most efficient vectors, such as *A. aegypti* and *A. albopictus*.

Although ISVs were perceived to lack medical relevance, recent evidence shows they may affect vector competence of mosquitoes by direct competition with arboviruses or indirectly affecting insect physiology (**Figure 2**) [30]. A good example is provided by CFAV, the first ISV identified in mosquito cell culture [31], which was recently shown to reduce levels of both DENV and ZIKV infections in adult mosquitoes [32]. This is likely a case of superinfection exclusion whereby there is competition for common resources between similar viruses especially considering that CFAV belongs to the *Flaviviridae* family like DENV and ZIKV. Other examples of ISVs affecting arboviruses mostly come from experiments in cell lines [33] and very few have been performed under more natural conditions *in vivo* [32,34]. We therefore still lack good examples of ISVs modulating vector competence and indirectly affecting arboviruses *in vivo*. This is a challenge for the field since studies in cell lines are unlikely to shed light into molecular mechanisms involving systemic effects and tissue communication *in vivo*.

Finally, the intimate relationship between ISVs and their insect host allied to their widespread presence could be useful to help trace mosquito speciation or the timing of separation for different populations. The higher mutation rate of ISVs would provide a more sensitive molecular clock compared to their host although cross-population infections could weaken these types of analyses. Notably, a recent analysis showed that the phylogeny of CFAV in different *A. aegypti* populations did not exactly correspond to the genetics of the host [35]. This suggests we need to understand better how ISVs evolve compared to the host, especially in cases where the virus may be transmitted to other mosquito species. It is possible that only a few ISVs may provide a good alternative molecular clock.

## Conclusions

In this review, we described current knowledge about the mosquito virome and the importance of continuous virological surveillance of vector insects. The rate of arbovirus discovery has diminished over the past decade, which increases the risk that we may face unknown challenges in the future [4]. Previous knowledge of viruses that may cause massive outbreaks can help expedite solutions and the lack of information can be a major threat to public health worldwide. Hence, investigating the diversity of viruses in mosquitoes and optimizing the identification of new arboviruses is a challenge for the field. To achieve this goal, we will need to develop new metagenomic strategies that improve the detection of arboviruses. This may be achieved through the combined use of direct sequencing coupled to enrichment steps that favor dual host viruses. In addition, virological surveillance of mosquitoes will expand our understanding about ISVs thus providing insights into virus

evolution and the molecular mechanisms affecting vector competence. This knowledge could lead to potential biotechnological applications of ISVs as tools to control arbovirus transmission.



**Box 1: Endogenous viral elements in metagenomic studies**

Metagenomic studies have expanded our knowledge about the diversity of viral sequences but experimental validation is often required to confirm the presence of a real virus. Mosquitoes, like many organisms, possess EVEs that are remnants of viral integrations into their genomes, including many derived from RNA viruses (**Figure 2**) [36–38]. These EVEs are often transcribed and can be detected by metagenomic approaches based on DNA and RNA sequencing [20,39]. It is often not possible to distinguish between an active virus and an integrated element based solely on sequence analysis, although EVEs tend to represent just fragments of a viral sequence that often lost coding potential [37]. Hence, EVEs can easily contaminate the analysis and inflate estimates of the diversity and abundance of viruses identified in mosquitoes. For example, EVEs derived from Chuviruses such as Kaiowa virus and Chuvirus Mos8Chu0 that are integrated into the *A. aegypti* genome [36,37] have been considered viruses in previous metagenomic studies [8,9,12]. Correctly identifying EVEs in metagenomic studies is a challenge for the field and will also help understand possible functions for these integrated viral sequences [40]. For example, EVEs could represent a historical timeline of viruses that circulated in mosquito populations or species, giving a historical perspective of the mosquito virome.

**Box 2: The advantages of small RNA based-metagenomic strategies**

Different metagenomic strategies have been used to characterize the virome in mosquitoes. These included approaches based on DNA and RNA sequencing of whole mosquitoes or filtered extracts to enrich for viral particles [6,7,11,13,17,18,22,25]. However, these approaches can be confounded by the detection of EVEs, integrated viral sequences that do not represent real viruses (**Box 1**). In 2015, our group applied an alternative approach to analyze the virome of mosquitoes that relied on the production of virus-derived small RNAs by the host antiviral response [14]. These small RNAs can be used to re-construct full viral genome sequences without any loss in sensitivity compared to other methods. Furthermore analyzing the size distribution and molecular features of small RNAs derived from each sequence also allows the distinction between EVEs and exogenous viruses. While EVEs generate small RNAs with sizes ranging from 24-29 nt and characteristics of PIWI-interacting RNAs, actively replicating viruses infecting the mosquito produce small interfering RNAs that are 20-22 nt in size [20].

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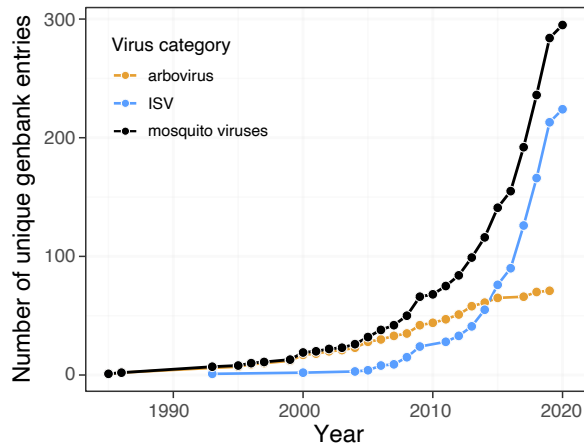
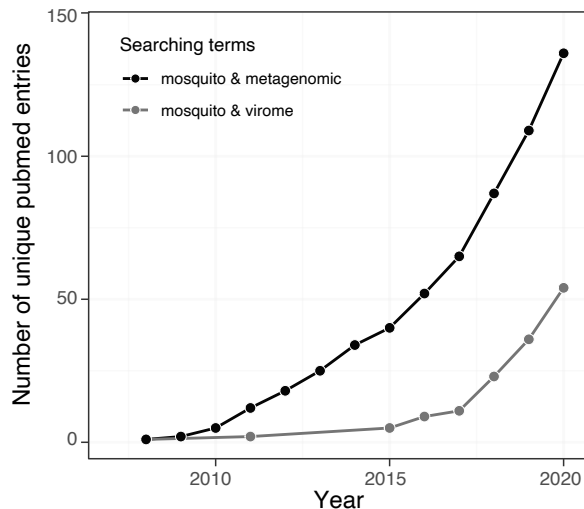
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## Figure Legends

**Figure 1 – Overview of viruses circulating in mosquitoes.** (A) Cumulative numbers of unique entries in Genbank for viruses associated with mosquitoes. Numbers for arboviruses and ISVs are also indicated separately. (B) Cumulative number of articles in Pubmed containing the terms ‘mosquito metagenomics’ or ‘mosquito virome’. (C). Diversity of viral families of arbovirus and ISVs in the list of mosquito viruses in Genbank. Unclassified viruses are shown together. Genome structure of viral families and unclassified viruses are indicated. The list of mosquito viruses was obtained from the NCBI Nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide/>, accessed in May 07, 2020) using the query: mosquito[All Fields] AND virus[All Fields] AND "Viruses"[Organism] AND viruses[filter]. Data were exported and parsed using in-house scripts. The list was curated to remove redundancy, and, in the case of multiple entries, the oldest entry date was considered. The full list of mosquito viruses is shown in **Supplementary Table 2**. In order to identify arboviruses, the list obtained from Genbank was cross-referenced against the CDC arbovirus catalog (<https://wwwn.cdc.gov/arbovat/>) summarized in **Supplementary Table 1**. Original articles where the virus was described were also consulted to identify additional arboviruses. Remaining viruses were considered ISVs and classified as ‘ISV’ or ‘Probably ISV’ in **Supplementary Table 2**, based on current information available in the literature.

**Figure 2 – Interactions between components of the mosquito virome.** Schematic view of a mosquito, showing possible positive and negative interactions between ISVs and arboviruses. ISVs are not infectious to vertebrates but may affect different steps of the arbovirus cycle. ISVs may facilitate or block arbovirus replication in the mosquito thus interfering with transmission to the vertebrate host or maintenance of infection in the vector population by vertical transmission. Mosquito viruses, both ISVs and arboviruses, also interact with integrated viral sequences, EVEs, that may affect replication of viruses with whom they show sequence similarity. More details of these interactions are described in the body of the text.



**A****B****C**