

# Journal Pre-proof

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PII: S2214-5745(20)30027-4

DOI: <https://doi.org/10.1016/j.cois.2020.02.005>

Reference: COIS 672

To appear in: *Current Opinion in Insect Science*

Please cite this article as: Patterson EI, Villinger J, Muthoni JN, Dobel-Ober L, Hughes GL, Exploiting insect-specific viruses as a novel strategy to control vector-borne disease, *Current Opinion in Insect Science* (2020), doi: <https://doi.org/10.1016/j.cois.2020.02.005>

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Exploiting insect-specific viruses as a novel strategy to control vector-borne disease.

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## Abstract

Novel insect-specific viruses (ISVs) are being discovered in many important vectors due to advances in sequencing technology and a growing awareness of the virome. Several *in vitro* and *in vivo* studies indicate that ISVs are capable of modulating pathogenic arboviruses. In addition, there is growing evidence that both vertical and horizontal transmission strategies maintain ISVs in vector populations. As such there is potential to exploit ISVs for stand-alone vector control strategies and deploying them in synergy with other symbiont control approaches such as *Wolbachia*-mediated control. However, before the applied potential can be realized, a greater understanding of their basic biology is required, including their species range, ability to be maintained and transmitted in native and non-native vector hosts, and the effect of infection on a range of pathogens.

## Highlights

- ISVs have been found to modulate replication and transmission of arboviruses.
- Vertical and horizontal transmission could be exploited for applied strategies.
- Similar biology to *Wolbachia* could be deployed in synergistic control strategies.

## Introduction

Arthropods possess a diverse range of microbes that play a central role in the basic biology of the host. While most research has focused on bacterial symbionts, little is known about viral symbionts. The non-pathogenic, insect-specific viruses (ISVs) of the microbiome vary in phylogeny and are now found in abundance in hematophagous arthropods. Some ISVs are closely related to human and animal pathogens, which has highlighted the rationale for further investigations into ISVs blocking pathogens through prior infections in the vector or as chimeric vaccines in mammals. The pursuit of these goals has neglected to define the most basic biological questions of ISV infection and transmission, as ISVs must employ alternative methods of transmission to arboviruses, because of their restriction in arthropods (Figure 1). An improved understanding of ISV transmission and their interactions with their arthropod hosts, as well as the pathogenic viruses that infect and are vectored by these hosts, may unlock their potential to modulate transmission of arboviruses.

## Insect-specific virus interactions with pathogens

As their name implies, ISVs are only known to replicate in insect (or arthropod) cells but not mammalian cells. A large proportion of ISVs have been discovered through surveillance for pathogenic and emerging viruses, particularly in mosquitoes [1\*]. Recent studies have sought to investigate if ISVs affect the transmission of pathogens in mosquitoes through mechanisms like superinfection exclusion, the theory that cells infected with one virus are not susceptible to subsequent infections with similar viruses. Relying on superinfection exclusion necessitates that ISVs maintain a cell tropism to directly block arboviruses at their site of attachment or replication. Experiments have demonstrated that some ISVs can modulate the replication of certain arboviruses in cells and mosquitoes [2–16]. This relationship is poorly understood and phylogeny does not appear to explain the phenotype, as diverse ISVs exert these effects. In addition to directly influencing pathogens, ISVs may indirectly upregulate the immune system either as virions or by

integration into the host genome. Endogenous flaviviral elements (EFVEs), many of which appear to be derived from insect-specific flaviviruses (ISFs) and have been hypothesized to play critical functions in arthropod antiviral immunity [17], have introgressed into the genomes of many *Aedes* and *Anopheles* mosquitoes [18,19].

A large proportion of ISVs are ISFs, some of which have been shown to impact titers of medically important flaviviruses in mosquitoes or cells [2–12]. These ISFs are either dual-host affiliated (dISFs) that are more closely related to pathogenic arboviruses (dual-host flaviviruses), or from a paraphyletic clade of classical ISFs (cISFs) that group among other viruses restricted to insect hosts [20] (Figure 2). Nhumirim virus (NHUV), a dISF, and Palm Creek virus (PCV), a cISF, have both been shown to decrease titers or transmission of multiple pathogenic flaviviruses in cells or mosquitoes, respectively [2–6]. These interactions appear to be virus family and vector-species dependent, as these ISFs do not reduce titers of alphaviruses in cells [4\*\*,6]. Similarly, a cell fusing agent virus (CFAV) strain isolated from *Aedes aegypti* mosquitoes reduced dissemination of both dengue virus (DENV) and Zika virus (ZIKV) in *Aedes albopictus* (C6/36) cell lines and in *Ae. aegypti* mosquitoes [7\*\*]. In contrast, a CFAV strain derived from an *Ae. aegypti* cell line (Aag2) [21] has been shown to enhance DENV replication in another *Ae. aegypti* cell line (Aa20) [8]. Other studies suggest that *Culex* flavivirus (CxFV) has little effect [9,10,22] or even enhances [11,12] West Nile virus (WNV), DENV, Japanese encephalitis virus, and Rift Valley fever virus transmission. Many of these experiments ignore the specific virus-host pairings [23] and rely on results from cell lines with a defective RNAi response [24]. The diverse outcomes from ISV-arbovirus interactions suggest that more rigorous investigations *in vivo* of interactions with pathogenic viruses in specific hosts are likely to elucidate both their natural biology and their potential to be exploited in arbovirus transmission-blocking strategies.

Limited studies have been performed to evaluate pathogen modulation of ISVs from other virus taxa. Eilat virus (EILV), an insect-specific alphavirus, was only able to delay dissemination of chikungunya virus (CHIKV) from the midgut of *Ae. aegypti* for 3 days [13]. Phasi Charoen-like virus (PCLV), an insect-specific bunyavirus, may be capable of blocking infections of bunya- and flaviviruses, but to see differences between PCLV-infected and uninfected mosquitoes, PCLV was infected at high titers, suggesting the effect may be subtle and not biologically relevant [14,15]. Menghai rhabdovirus and Shinobi tetravirus ISVs, which were isolated from mosquito cells, were also able to inhibit replication of multiple flaviviruses [16]. Despite the evidence mentioned above regarding ISV-pathogen interactions, the majority of ISVs remain unevaluated, including those discovered in ticks, sand flies and midges [25–29].

While there is some experimental evidence indicating that ISVs influence viral pathogens, we have a limited understanding regarding the role of ISV infection on other arthropod-borne pathogens. A survey of the microbiome of *Ixodes scapularis* identified a positive co-occurrence and correlation between the presence of tick-specific bunyaviruses, South Bay virus and blacklegged tick phlebovirus, and *Borrelia burgdorferi*, the causative agent of Lyme disease [30\*]. These ISVs have been found in ticks across the globe, including areas endemic with Lyme disease [29,31], but experimental infections have not been undertaken to determine if the ISVs promote *B. burgdorferi*, or vice versa. There are also a range of ISVs that infect *Anopheles* mosquitoes that transmit *Plasmodium* [32–37] and phlebotomine sand flies that transmit *Leishmania* [26–28], warranting further investigations into potential interactions with malaria parasites in these systems.

## Maintenance and transmission

The variable prevalence of ISVs in mosquitoes suggests that different types of transmission modes may be maintaining these viruses in vector populations. Many reports have suggested vertical transmission as the mode for maintenance for ISVs in mosquito populations, with a small

number of studies providing direct experimental evidence. CFAV, NHUV and Kamiti River virus (KRV), all ISFs, were tested through intrathoracic injection or oral exposure of female mosquitoes for detection of virus in the offspring and to initiate transmission to naïve colonies [3,38–40]. Transmission rates varied greatly from 4% for KRV to 93% for CFAV. When naturally infected hosts were observed, transovarian transmission rates were 100% for both CFAV in *Ae. aegypti* and CxFV in *Culex pipiens* [38,39]. The attempt to initiate vertical transmission of CxFV in a naïve laboratory colony of *Cx. pipiens* was unsuccessful, showing a narrow host range may even be restricted to sub-populations of a species [39]. ISV infections transstadially transmitted from immature life stages to adults have been attributed to vertical transmission from their parents. However, it has not yet been determined whether the aquatic environment during early development also facilitates horizontal transmission of ISVs [33\*]. The difference in infection and prevalence may be due to host and viral genetics, which could dictate host range at the species and strain level. In all of these cases, vertical transmission was confirmed by RT-PCR of parents and offspring without taking into account environmental factors, so additional methods are required for the mechanism(s) to be elucidated. Investigations into aphid-borne viruses have used a variety of methods, such as immunofluorescence of dissected reproductive organs and tissues, and binding assays with recombinant viral proteins, to detail the process of transmission with more precision [41\*]. Similar studies could provide insights in mosquito-ISV systems.

Recent discoveries in plant arboviruses, viruses that circulate between plants and arthropod host, have demonstrated that infection of either male or female parental insects can result in vertical transmission. Interestingly, this has been attributed to interactions with both structural and non-structural viral proteins [41\*–43]. These mechanisms were discovered in plant reoviruses, and reovirus ISVs have also been found in mosquitoes [44]. This family of viruses is non-enveloped, therefore capsid-derived interactions may not be applicable for many ISVs, the majority of which are enveloped. However, ISVs that are not enveloped or have exposed capsid proteins, such as

reoviruses, birnaviruses, densoviruses, and negevirus, may exploit similar mechanisms of vertical transmission in mosquitoes compared to their plant vector relatives. If vertical transmission is not observed, then these ISVs may only require minimal engineering of their capsid proteins to instigate the known mechanism of vertical transmission. For enveloped ISVs, more experiments should be undertaken to investigate the contribution of non-structural proteins in vertical transmission. To pinpoint interactions and replication, future studies may consider using virus isolates expressing visual reporters as a means to track infection through parent and offspring.

Severe host restrictions may provide hurdles when trying to colonize diverse species or locations with ISVs but can be advantageous for specific targeting of major vectors and limiting spread to non-target insects. Nonetheless there are a couple of reports of Aedes-specific flaviviruses (AeFVs) being detected in *Culex* mosquitoes [33\*,45], indicating that cross-species infection of ISVs occurs, but mosquito species-specific ISV infections are more likely to persist, as evidenced by mosquito genus- and species-specific ISF clades (Figure 2). Nonetheless, instances of cross-species infection may assist in the transfer of ISVs between species, with subsequent co-evolution [46]. Though the mechanism for such cross-species transmission remains unknown, such findings suggests that there may be secondary modes of transmission through shared environments. Unnatural routes of ISV transmission, such as intrathoracic injections and artificial blood meals, have successfully initiated infection in new host species [10,27]. Although vertical transmission would be advantageous for long-term colonization of vector insects with ISVs that block pathogens, horizontally transmissible ISVs could be used to quickly colonize new mosquito populations.

### **ISV interactions with other microbial symbionts**

It is evident that microbial interactions are ubiquitous in arthropod vectors [32,47–50] and it will be important to determine the extent of interactions between ISVs and other symbionts that are currently being used in vector and pathogen control strategies. Multiple studies have been carried



out to define a relationship between ISVs and *Wolbachia*, an insect symbiont that has been artificially introduced into *Ae. aegypti* colonies. A survey of field-caught and laboratory-reared mosquitoes evaluated the inevitable and ongoing relationship between the introduced bacteria and the native virome [51\*\*]. The study found that field-caught mosquitoes with *Wolbachia* had higher titers and rates of ISV infections, and *Wolbachia* infected mosquitoes reared in the laboratory had higher ISV titers compared to their uninfected counterparts. These results contrast *in vitro* studies where *Wolbachia* infections reduce CFAV below detection limits and have no effect on PCLV titers [52–54]. Further investigations are required to determine the interactions of ISVs with other symbionts.

It is possible that ISVs could be used in synergy with *Wolbachia* population replacement control approaches. In theory, a vertically transmitted ISV could hitchhike with *Wolbachia* into naïve populations and enhance the anti-viral effect by a distinct mechanism. For example, while *Wolbachia* does not inhibit the bunyavirus PCLV, PCLV is the only ISV to show inhibition against a negative strand RNA virus, La Crosse virus [14,52,54]. Similarly, it would be interesting to determine if ISVs have played an incidental role in DENV transmission reduction in *Wolbachia*-colonized mosquitoes in the field.

### **Other applied uses**

Aside from using wild-type ISVs to interfere with pathogens, engineered ISVs may be employed in both insects and mammals to directly prevent or identify infection. Both a diagnostic kit and a vaccine candidate have been developed for CHIKV based on a CHIKV chimera with EILV [55,56]. Chimeric PCV expressing WNV structural proteins replicate in mammalian cells but do not produce infectious virions, offering potential for effective attenuated vaccines for flaviviruses [57]. Paratransgenic approaches using chimeric ISVs may also be useful to interfere

with arboviruses either by producing an antiviral molecule, priming the RNAi responses in the vector, producing double-stranded RNAs, or by sequestering receptors required for viral infection.

While the relatedness of ISVs to pathogenic arboviruses may limit applications based on identical structure, such as using an insect-specific alphavirus to mimic pathogenic alphaviruses, other ISV classifications may provide ideal platforms for paratransgenic applications.

Mesoniviruses and negeviruses have wide geographic and host ranges, can be grown to high titers in cell culture, and can be genetically tractable [26,27,58–60]. Additionally, these virus groups are strictly insect-specific, negating concerns over reversion or recombination to pathogenic strains.

## Conclusions

Gene drives and the use of *Wolbachia* endosymbionts to manipulate wild populations show promise as valuable tools for reducing pathogen transmission. Adding ISVs to the list of available tools will lead to more effective and synergistic methods to limit transmission rates. Additional layers of pathogen control will also hinder the rise of resistance, a likely consequence of any singular control method.

The potential of ISVs for vector control can only be realized once there is a greater understanding of their basic biology. However, the ability of ISVs to interfere with pathogenic arboviruses and be maintained in vector populations warrants further investigations into these viruses. While genomics studies have been critical for identifying the ubiquitous nature of ISVs, future studies need to build upon these sequencing results and investigate host-microbe, tripartite interactions, and phenotypes in their native and non-native vectors. These basic studies will undoubtedly provide valuable foundation to use ISVs as a tool to combat infectious diseases and offer a platform for vaccines and diagnostics.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

EIP was supported by the Liverpool School of Tropical Medicine Director's Catalyst Fund award. JV and JNM were supported by the ANTI-VeC Pump-Priming Award (AV/PP12) sub-awarded by the University of Glasgow from UK government Global Challenges Research Fund (GCRF) Networks in Vector Borne Disease Research funds and *icipe* institutional funding from the UK's Department for International Development (DFID), the Swedish International Development Cooperation Agency (SIDA), the Swiss Agency for Development and Cooperation (SDC), and the Kenyan Government. GLH is supported by the NIH (R21AI129507 and R21AI138074), the BBSRC (BB/T001240/1), a John S. Dunn Foundation Collaborative Research Award, and a Royal Society Wolfson Fellowship (RSWF\R1\180013). We would like to thank Manuela Bernardi for preparation of the summary figure.

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### Special interest references

[1] Calisher and Higgs, 2018\*

This review provides a comprehensive list of known ISVs, including a history of their discovery.

[4] Romo et al., 2018\*\*

This study used NHUV to reduce titer and transmission of flaviviruses in *Aedes* cells and mosquitoes. These experiments demonstrated that NHUV could interfere with pathogenic virus infections in multiple hosts, building on previous results from WNV in *Culex* mosquitoes.

[7] Baidaliuk et al., 2019\*\*

This study observed reduced infection of DENV and ZIKV in *Ae. aegypti* mosquitoes attributed to CFAV infection. CFAV is the most widely documented ISF, often being found in laboratory colonies and cell culture stocks.

[30] Cross et al. 2018\*

This study identified components of the microbiome of field caught ticks. The results demonstrated a relationship with the occurrence of insect-specific bunyaviruses and *B. burgdorferi*.

[33] Ajamma et al. 2018\*

This study found diverse ISFs in *Aedes* and *Anopheles* mosquitoes that were naturally infected in native aquatic stages, then reared to adults. As *Aedes*-specific flavivirus was also isolated from *Culex* mosquitoes, ISFs may have the potential to be transmitted both vertically and horizontally, possibly through shared aquatic environments.

[41] Mao et al., 2019\*

This study determined a mechanism of reovirus vertical transmission in aphid vectors. Many experiments were used to extensively characterize the interaction between virus and host proteins during paternal transmission.

[51] Amuzu et al., 2018\*\*

This study surveyed the virome of laboratory and field-caught mosquitoes in the context of *Wolbachia* infections. Although *Wolbachia* is known to interfere with pathogenic arboviruses, mosquitoes with *Wolbachia* infection had greater rates of ISF infection as well.

## Figure legends

Figure 1. Putative overview of using Insect-specific viruses for vector control. ISVs have been discovered in arthropod vectors. There is evidence that ISVs modulate arbovirus infections. Many modes of transmission may be used in natural and laboratory settings, but aspects of the natural biology of ISVs remain unknown. Combining these attributes will inform methods used for vector control.

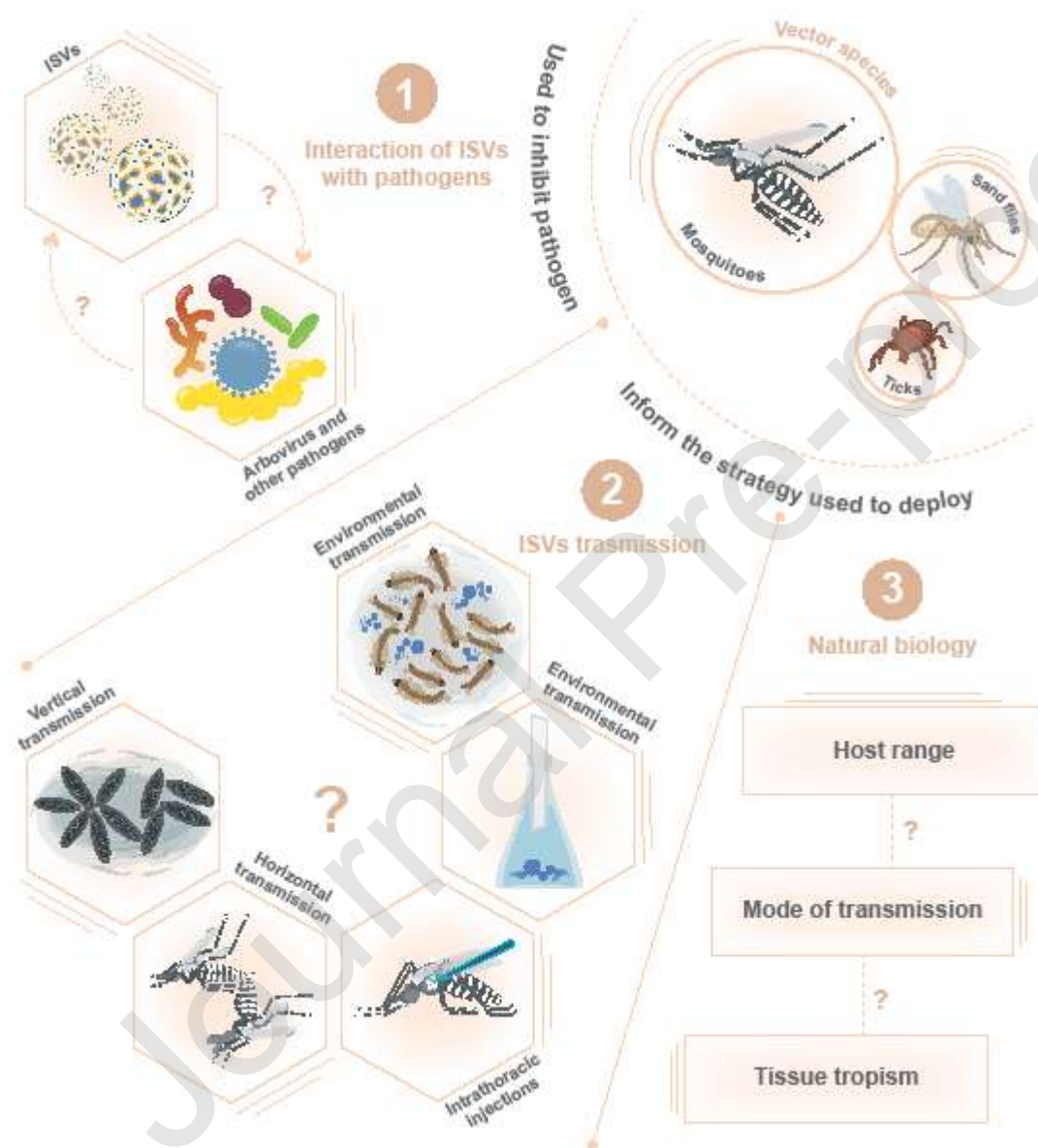


Figure 2. Midpoint-rooted phylogeny of 875-910 nt dual-host flavivirus and insect-specific flavivirus (ISF) NS5 gene sequences. Virus designations followed by GenBank accessions and mosquito species from which they were isolated are indicated. Key interactions of ISFs that inhibit or enhance arbovirus transmission are indicated in green and blue, respectively. The phylogeny was

generated using PhyML version 3.0 [61], employing the Akaike information criterion [62] for automatic selection of the general time reversible (GTR) sequence evolution model. Tree topologies were estimated using nearest neighbour interchange (NNI) improvements over 1000 bootstrap replicates. The branch length scale represents substitutions per site.

