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The War on Arboviruses: Leveraging Co-Infection Inhibition and Endogenous Viral Elements Towards Inhibiting Arbovirus Horizontal Transmission

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SUMMARY Clinically significant arboviruses like dengue virus, zika virus, and chikungunya virus currently impose a major global health crisis due to their high frequency of transmission and from the lack of approved antivirals and vaccines against them. As current countermeasure strategies have proven insufficient in counteracting the horizontal transmission of arboviruses, there is a high demand for novel and effective strategies to reduce the incidence rate of these arboviruses. One such mechanism that could be explored is co-infection inhibition: the impairment to arbovirus fitness from co-infecting arbovirus species. Studies in humans and *Aedes aegypti* mosquitoes have revealed a trend in impairment to the viral load and infection rate of select arboviruses. While there is potential in exploiting this mechanism to inhibit arbovirus transmission, there are many limitations to using intact viruses to compete with clinically significant arboviruses, namely the requirement for a sustained infection to enable arbovirus protection. One potential solution to this would be the application of arbovirus-derived endogenous viral elements as an intrinsic measure of defense against arbovirus infection in mosquito vectors. These elements, incorporated into the genome of mosquito vectors, could encode for dysfunctional viral proteins designed to block functional viral proteins from their substrates. The efficacy of candidate endogenous viral elements could be experimentally validated in an *in vitro* C6/36 cell model and subsequently in a live *Ae. aegypti* transgenic model. Given that some arboviruses like dengue virus have been shown to negatively impact female *Ae. aegypti* survival and reproduction, it would be expected that transgenic *Ae. aegypti* would be able to outcompete wild-type strains, thus enabling the fixation of the transgene(s) into the population. Ultimately, arbovirus-derived endogenous viral elements exhibit substantial potential as an intrinsic countermeasure against arbovirus infections in mosquito vectors towards impairing the primary mode of arbovirus transmission and thus, reducing infection rates in humans.

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INTRODUCTION

Arboviruses are a category of viral species that are transmitted via mosquitoes or other bloodsucking arthropods. This mode of transmission provides direct access to the circulatory system and is utilized by numerous high-profile pathogens including dengue virus (DENV), zika virus (ZIKV), west nile virus (WNV), and chikungunya virus (CHIKV) [1].

Numerous arboviruses impose significant global health crisis due to a combination of their high annual incidences and the severity of the rarer clinical manifestations associated with many of these viruses. For example, DENV has an estimated annual incidence of 50-100 million cases spread across over 100 endemic countries, and is responsible for approximately 20,000 annual fatalities from its more severe manifestation, dengue hemorrhagic fever (DHF) [2]. Although a DENV vaccine has been approved by the FDA for commercial use, the vaccine has been shown to only be safe in patients previously infected with dengue, given that the vaccine mimics DENV primary infections and thus can put patients at risk of secondary infection [3]. For arboviruses like ZIKV and CHIKV, no antiviral treatments or vaccines have been approved for usage. Presently, the only countermeasure employed against arbovirus transmission is mosquito eradication in endemic countries – a method that has been proven to be largely ineffective [4]. As such, there is a significant demand for the development of an effective countermeasure against these high-profile arboviruses towards reducing the incidence rates.

Ideally, any effective countermeasure would inhibit the horizontal transmission of a targeted arbovirus through a mosquito vector either by inhibiting its ability to infect, be disseminated through, and/or be transmitted from the mosquito. Intriguingly, another facet of arboviruses – specifically, the interactions they can have with one another in the context of a co-infection – could potentially be exploited for that purpose. It has previously been shown that some arboviruses can have synergistic and/or inhibitory effects on the fitness of other specific co-infecting arboviruses. For example, *in vitro* C6/36 cells co-infected with Palm Creek virus (PCV) and WNV exhibited a significant reduction in WNV replication [5]. This demonstrates that some viruses, or perhaps discrete elements of these viruses, can have an inhibitory effect on viral fitness for other specific viral strains. Leveraging the inhibitory potential of viral elements on infection from related species, a potential countermeasure may lie in the application of arbovirus-derived viral elements, an endogenous defense against the infection of mosquito vectors. Thus, this paper will explore the potential application of endogenous viral elements as a broad-spectrum countermeasure against the horizontal transmission of clinically significant arboviruses through mosquito vectors.

RESEARCH QUESTIONS

Developing a novel, effective countermeasure against arbovirus horizontal transmission is critical towards addressing the global health crisis imposed by arboviruses like DENV, ZIKV, and CHIKV. Co-infection inhibition is one potential mechanism that could be exploited in a novel countermeasure against the infection of mosquito vectors. As such, the interactions between clinically significant arboviruses – namely DENV, ZIKV, and CHIKV – during co-infections in both human and mosquito hosts will be explored. Following that, the viral exclusion potential that mosquito-based endogenous viral elements can have with related infecting viruses will be explored as a potential basis for developing a sustained intrinsic countermeasure. Lastly, it will be determined whether the incorporation of arbovirus-derived endogenous viral elements (adEVE) into mosquito vectors could serve as a practical and effective countermeasure against the horizontal transmission of targeted arboviruses.

PROJECT NARRATIVE

Co-infecting arbovirus interactions in human and *Aedes aegpyti* hosts

Presently, the viral interactions that can arise from a co-infection with high-profile arboviruses (ie. DENV, ZIKV, and CHIKV) in a human host are poorly understood. Clinical manifestations of co-infected patients (DENV/ZIKV, DENV/CHIKV, and CHIKV/ZIKV) commonly exhibit general feverlike symptoms shared between each arbovirus, with rare

occurrences of more representative symptoms like Guillain-Barré syndrome (GBS), which has been associated with ZIKV infection [6]-[11].

The occasional dominance of characteristic symptoms like GBS in some co-infection cases suggests that competition between co-infecting arbovirus species may be occurring in human hosts. This is supported by findings reported in another case study, which found that two CHIKV/ZIKV co-infected patients exhibited either CHIKV-like symptoms (prominent arthralgias) or ZIKV-like symptoms (conjunctivitis) in accordance with the dominant virus (higher viral load) [7]. In DENV-2/CHIKV co-infections, two separate case studies observed significantly higher CHIKV serum titers relative to DENV-2 [9],[10]. Few cases of DENV/ZIKV co-infection have been reported, although one study found that two co-infected patients (DENV-1/ZIKV and DENV-3/ZIKV) exhibited substantially lower ZIKV viral loads relative to DENV [11]. In all cases, the viral loads of all co-infected species were significantly lower than reported mean viral loads observed during acute single infections from ZIKV, DENV, and CHIKV [12]-[14]. However, it is unclear whether the observed differences in viral titers between co-infecting species are the result of a delay in the time of initial exposure to each virus or co-infection inhibition, warranting further investigation in a mammalian model. Nevertheless, these findings suggest that perhaps the viral load of each species is reduced as an outcome of co-infection.

In *Aedes aegpti* mosquitoes, co-exposure to both CHIKV and ZIKV resulted in a reduction in the probability of ZIKV infection in the mosquito, but not the inverse. As well, co-exposure to DENV-2 and CHIKV resulted in a reduction in the probability of DENV-2 dissemination throughout the host and CHIKV transmission from the host [15]. These findings suggest that specific elements of DENV-2 and CHIKV can impair the fitness of other clinically significant arboviruses in mosquito vectors. Collectively, these findings demonstrate the potential of co-infection inhibition in reducing viral load in humans and infection rates in mosquitoes.

Viral exclusion potential of endogenous viral elements

Rather than utilizing intact viruses to impair the transmission of co-infecting arboviruses – which would require a sustained infection in the mosquito – an alternative strategy would be to incorporate a fraction of the virus in the form of a mosquito endogenous viral element (EVE) into the mosquito genome. EVEs are fractions of viral genomes that have been incorporated into the host genome and can be derived from both retroviruses and non-retroviruses alike [16]. In the case of non-retroviral EVE incorporation, this requires three critical events to occur: a reverse transcription event, incorporation of the virus-derived DNA fragment into the genome of host germinal cells, and the fixation of the EVE into the broader host population. Given the rarity of this combination of events, this generally requires a longstanding evolutionary relationship between a viral species and the host [16].

In the case of mosquitoes, multiple flavivirus-derived EVEs (fdEVE) have been identified within the genomes of various *Anopholes* species, including a *Nienokou*-derived EVE in the *An. Minimus* genome and a *Culex*-derived EVE in the *An. sinensis* genome [17]. Given the need for an EVE to be fixed into the host population, it is possible that these fdEVEs confer an evolutionary benefit to the host by providing a level of protection against infection from related viral species, although this has yet to be verified through experimentation. This phenomenon has been demonstrated in sheep, where a sheep endogenous retroviral element derived from Jaagsiekte sheep retroviruses (JSRV) was found to encode for a defective JSRV Gag polyprotein, which was shown to associate with and block the intracellular trafficking of wild-type JSRV Gag and thus, late replication steps of the virus [18]. This demonstrates one possible mechanism through which an EVE can deter infection from related viral species – that being the targeted impairment of a specific step in the viral lifecycle. Another potential mechanism that has been suggested is that some transcriptionally active EVEs may encode for miRNA, which could target viral RNA [19], although this has yet to be substantiated with data. Regardless of the mechanism, it stands to reason that an EVE derived from an arbovirus like ZIKV, DENV, or CHIKV may be capable of conferring protection against infection from the respective virus.

Incorporating engineered endogenous arbovirus elements into *Aedes aegpyti* mosquitoes

Presently, no EVEs derived from clinically significant arboviruses have been discovered in *Ae. aegytpi* mosquitoes (or other known mosquito vectors). Thus, the use of an adEVE would necessitate the artificial insertion of the element into the vector genome. This operation could be performed using CRIPSR-Cas9 gene-editing technology paired with the intrinsic homology-directed repair mechanism [20]. However, developing such a countermeasure would require the careful consideration of three key factors for each virus being targeted: the mosquito vector, the site of insertion in the vector genome, and the design of the EVE.

In designing an adEVE for distribution amongst the mosquito population, it is essential that the optimal vector mosquito species be chosen. Between DENV, ZIKV, and CHIKV, the primary vector of these arboviruses is the *Ae. aegypti* mosquito [21]-[23], making it an ideal vector to target in the first iteration of this countermeasure. Given that all three of these arboviruses utilize this vector, it also has the potential to be used to selectively impair the horizontal transmission of each of these arboviruses simultaneously through the insertion of multiple EVEs, each derived from a different arbovirus. In order to provide continuous protection against infection from target arboviruses, an EVE would need to be under the control of a constitutively active promoter in the vector genome. The *Ae. aegypti* polyubiquitin (PUb) promoter would be an ideal candidate, as the promoter has previously been used to drive the constitutive expression of transgenes in *Ae. aegypti* [24].

Along with the choice of vector and promoter, the specific design of the arbovirus-derived EVE is critical in determining the effectiveness of the EVE in conferring resistance against infection from related species. The EVE design would be dependent on both the target arbovirus and the intended mechanism of impairment. For instance, a dengue-derived EVE (ddEVE) might encode for an NS3 helicase with a mutation in the conserved P-loop motif of the RNA helicase domain to remove ATPase activity, but maintain binding properties to allow for competitive sequestering of viral dsRNA intermediate and replication impairment [25]. Alternatively, a loss of function (LOF) mutation in the C-terminal protease domain of NS3 may enable competitive sequestering of the DENV polyprotein and DENV NS2B, preventing the release of viral proteins necessary for progression of the lifecycle (depicted

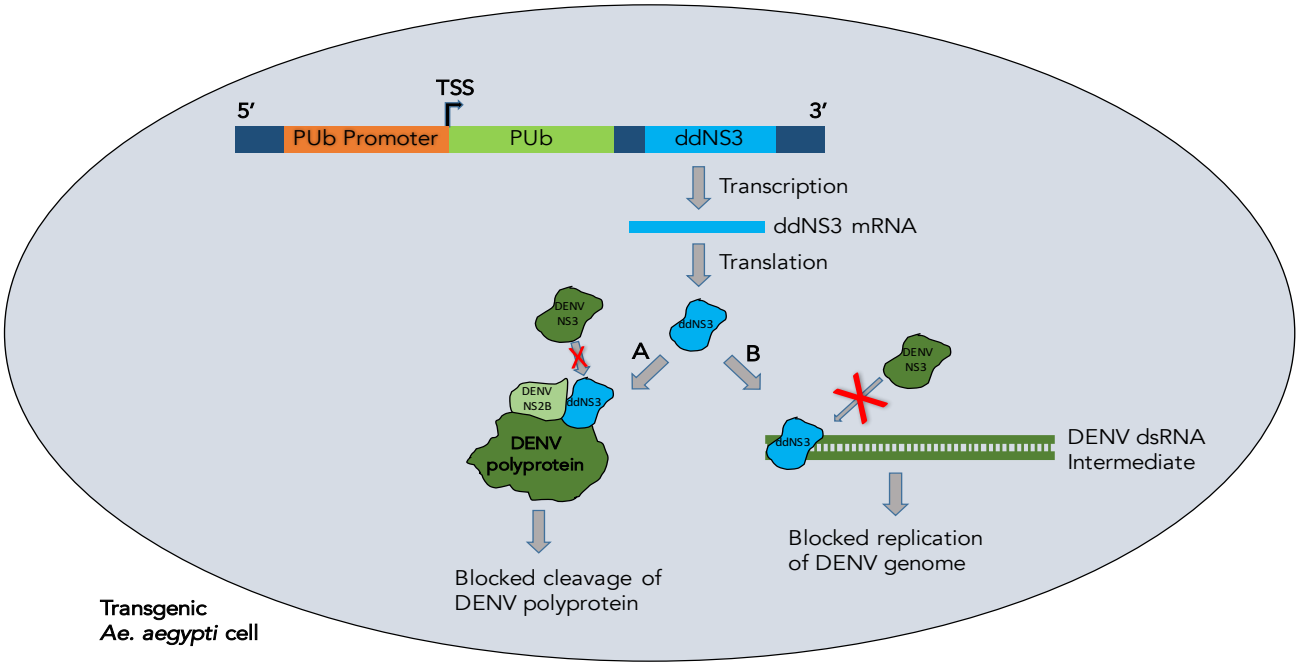

FIG. 1 Potential mechanisms of EVE-directed impairment of DENV infection in transgenic *Ae. aegypti* cells. One proposed ddEVE could encode for defective dengue-derived NS3 (ddNS3) under the control of the *Ae. aegypti* PUb promoter, with A) a LOF mutation in the C-terminal protease domain, blocking DENV NS3p from binding to NS2B and subsequent cleavage of the DENV polyprotein and B) a LOF mutation in the conserve P-loop, blocking DENV NS3h from separating DENV dsRNA intermediates.

below in Figure 1). An adEVE could also contain elements implicated in facilitating co-infection inhibition, adding an additional layer of intrinsic resistance against infection in the *Ae. aegypti* vector.

Regardless of the effectiveness of the specific adEVE in warding off infection from related arboviruses, the adEVE must ultimately be capable of being effectively disseminated throughout a population to be effective as a long-term countermeasure. As such, the EVE must both be heritable and exhibit a high likelihood of being fixed into the broader *Ae. aegypti* population. The heritability of the EVE can easily be addressed by incorporating the EVE into the genome of germinal *Ae. aegypti* cells and subsequently genotyping the F1 generation. The fixation of the EVE into the *Ae. aegypti* population, however, is contingent on the evolutionary advantage conferred by the EVE to the host. Interestingly, it has been shown that female *Ae. aegypti* mosquitoes infected with DENV-2 exhibit reduced fecundity and lifespan [26]. Thus, ddEVEs, if proven effective in deterring DENV infection in the vector, would be expected to have a positive impact on the fitness of female *Ae. aegypti* compared to wild-types, increasing the likelihood that the ddEVE will be fixed in the population. Although the impact of ZIKV and CHIKV infection on survival and reproductive capacity in *Ae. aegypti* has yet to be validated, it is possible that zika-derived EVEs (zdEVE) and chikungunya-derived EVEs (cdEVE) may confer similar benefits to the mosquito.

SUMMARY AND CONCLUSION

High-profile arboviruses like DENV, ZIKV, CHIKV impose a substantial global health threat, particularly due to the lack of approved antivirals or vaccines [1],[2]. Currently, there is a high demand for effective countermeasures against arbovirus horizontal transmission from mosquito vectors to human hosts, given that current countermeasures have proven insufficient in addressing the epidemic [4].

Co-infection inhibition, the observed inter-viral impairment in human and mosquito hosts co-infected with a DENV, ZIKV, and/or CHIKV, is one mechanism that could potentially be exploited [7],[9]-[11],[15]. However, the usage of attenuated arbovirus strains to inhibit arbovirus infections in mosquito vectors may not be effective enough to be considered, given that it requires a sustained infection with the attenuated strain to provide any protective effect. Rather, a more effective alternative could leverage discrete adEVEs incorporated into the genome of a mosquito vector (e.g. *Ae. aegypti*), providing a sustained intrinsic deterrent against infection from related viral species. These could potentially act through adEVEs comprised of elements implicated in co-infection inhibition or through intra-viral impairment, utilizing defective viral proteins to block viral protein function by sequestering substrates.

In future experiments, the effectiveness of an adEVE would need to be experimentally validated prior to usage as a countermeasure against the horizontal transmission of target arboviruses. For any given adEVE, the capacity of the adEVE to impair a target arbovirus' lifecycle could be evaluated *in vitro* through the insertion of the adEVE into C6/36 cells. Candidate adEVEs that exhibit antiviral activity could then be incorporated into the genome of *Ae. aegypti* germinal cells to establish a hereditary transgene. F1 progeny could then be screened for resistance to infection from target arboviruses and for a reduction in the transmission of the target arbovirus to mammals following exposure to the virus. Transgenic

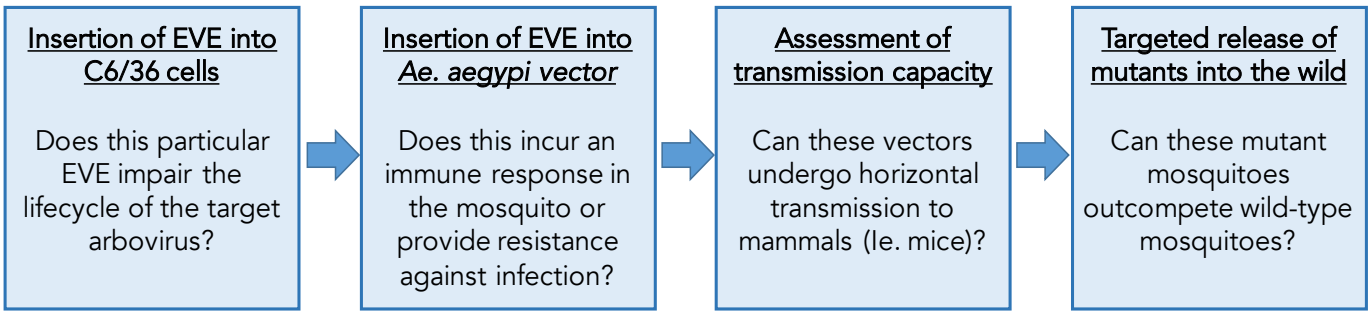

FIG. 2 Proposed experimental series to investigate the capacity of a given EVE in impairing the horizontal transmission of a target arbovirus.

Ae. aegypti that demonstrate viral resistance and reduced viral transmission could then be released into endemic regions as a means to reduce horizontal transmission of the virus through mosquito vectors in the region (see Fig. 2 for a summary of the proposed workflow).

Ultimately, this application has the potential to vastly reduce the incidence rate of infections from targeted arboviruses and directly combat the ongoing health crisis imposed by them. As well, it demonstrates a broadly applicable countermeasure technique against viral transmission through biological intermediates.

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ACRONYMS

CHIKV – Chikungunya Virus; DENV – Dengue Virus; DENV-1 – Dengue Virus (Serotype 1); DENV-2 – Dengue Virus (Serotype 2); DENV-3 – Dengue Virus (Serotype 3); DHF – Dengue Hemorrhagic Fever; EVE – Endogenous Viral Element; adEVE – Arbovirus-Derived Endogenous Viral Element; cdEVE – Chikungunya-Derived Endogenous Viral Element; ddEVE – Dengue-Derived Endogenous Viral Element; zdEVE – Zika-Derived Endogenous Viral Element; fdEVE – Flavivirus-Derived Endogenous Viral Element; GBS – Guillain-Barré Syndrome; JSRV – Jaagsiekte Sheep Retrovirus; LOF – Loss of Function; NS3h/p – Non-structural Protein 3 Helicase/Protease; ddNS3 – Dengue-Derived Non-structural Protein 3; PCV – Palm Creek Virus; PUb – Polyubiquitin; WNV – West Nile Virus; ZIKV – Zika Virus