In Vitro and *In Vivo* Effects of Espirito Santo Virus on Dengue Virus Replication

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Arboviruses have been one of the leading causes of morbidity and mortality worldwide for centuries (Morens et al., 2004). Dengue virus (DENV), Zika (ZIKV), Chikungunya (CHIKV) and West Nile virus (WNV) are some of the medically important arboviruses transmitted by mosquitoes. Dengue is one of the most common arthropod diseases with over 400 million people infected yearly. Dengue is now endemic in the WHO regions of Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific. Current data estimates 3.9 billion people in 128 countries are now at risk of DENV infection. Dengue, known as break-bone fever, may be caused by one of four serotypes: DENV:1-4. Dengue is primarily transmitted by *Aedes aegypti* with *Aedes albopictus* as a secondary vector. Infection with DENV may cause a high fever, swollen glands, muscle and joint pain and nausea. Subsequent infection of DENV with a second serotype may lead to the more serious dengue hemorrhagic fever, which may

result in plasma leakage, severe bleeding, fluid accumulation, and/or organ failure. Espirito Santo virus, ESV, is an insect-infecting virus recently discovered in a patient sample in Espirito Santo, Brazil. The virus replicates in mosquito cells but is not known to replicate in vertebrate cells (tested in Vero cells, thus far). Here, we sought to study the effects of ESV on DENV-2. We hypothesized that ESV interferes with replication of DENV-2 *in vitro* and *in vivo*.

Our findings show that ESV can replicate in absence of DENV-2 and no cytopathic effects were visually observed here in mammalian (Vero) cells 6-days post infection. Immunofluorescence assay results show that during co-infection of C6/36 cells with ESV and DENV-2, ESV did not prevent DENV-2 from entering cells or expressing proteins (we did not see a difference in staining). However, plaque assays showed a decrease in infectious DENV-2 particles in co-infected cells evidenced by fewer plaques observed in DENV wells also containing ESV. *In vivo* experiments were performed with three different populations of *Aedes aegypti* mosquitoes for an incubation period of seven days. While infection rates were not statistically different in a wild-type mosquito population (Costa Rica), ESV superinfected mosquitoes had significantly lower DENV-2 body titers ($p<0.01$) and leg titers ($p<0.01$) than mosquitoes exposed to only DENV (measured via qRTPCR). In the high dissemination colony of mosquitoes, there was no significant difference in body and leg DENV-2 titers between non-ESV infected and ESV infected mosquitoes. In the low dissemination colony, DENV-2 infected mosquitoes had significantly higher body and leg titers than mosquitoes infected with DENV and ESV ($p<0.01$). These results support our initial hypothesis that mosquitoes previously infected with ESV show lower levels of DENV-2

and we expect that these findings will spur additional research to elucidate the mechanisms involved.

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CHAPTER I – INTRODUCTION AND PURPOSE OF THE STUDY

Mosquito-borne diseases are responsible for several million deaths and hundreds of millions of cases of human illness yearly (World Health Organization [WHO] 1996). Mosquitoes can be vectors of many different pathogens that may cause a variety of human and veterinary illnesses. Zika virus (ZIKV), West Nile virus (WNV), malaria, chikungunya virus (CHIKV), La Crosse virus (LACV) and dengue virus (DENV) are just a few of the pathogens spread by competent mosquito vectors. Mosquito-borne illnesses can be caused by different types of pathogens. Anthroponotic diseases, such as malaria, are caused by *Plasmodium* parasites that are transmitted via mosquito saliva during blood feeding. Similarly, chikungunya, dengue, and Zika are primarily caused by viruses transmitted from human to human by mosquitoes. Mosquito-borne diseases may have a tremendous impact on a person's health, ranging from being an asymptomatic carrier of a virus to death. Consequently, this can lead to catastrophic effects on global health and economics (Ghosh et al., 2015).

Many arboviruses are found co-circulating in the same geographic regions. Prior to 2013, chikungunya outbreaks were experienced in Africa, Asia, Europe and the Pacific Ocean regions (Furuya-Kanamori et al., 2016). Since late 2013, CHIKV has been identified in the Americas and Caribbean countries and territories with local transmission (Centers for Disease Control and Prevention [CDC] 2016). Dengue virus circulates in Africa, Asia, the Americas, and the Pacific and is primarily found in the tropics and the sub-tropics (WHO 2017). These are some of the same areas in which CHIKV is found. In addition to cases being reported via local transmission in the same

areas, the two pathogens are also transmitted by the same species of mosquitoes. Both DENV and CHIKV are transmitted mainly by *Aedes aegypti* L. and secondarily by *Ae. albopictus* Skuse. This overlap in regions and vectors could possibly lead to co-infection in a human subject. In endemic regions, concurrent infections of DENV and CHIKV have been detected in travelers (Le Coupanec et al., 2017). There have been other documented cases of concurrent infections of mosquito borne illnesses. Zika virus is an emerging pathogen in many places where DENV is endemic and is vectored by the same species involved in DENV transmission. In January 2016, a 26-year-old student became ill after returning from Port-au-Prince, Haiti (Iovine et al., 2017). The student's urine and saliva were tested and found to be positive for both DENV serotype 2 (DENV-2) and ZIKV vRNAs by RT-PCR (Iovine et al., 2017).

In Espirito Santo, Brazil, a biological sample obtained from a patient infected with DENV-2 showed infection from another virus (Huang et al., 2013). The sample was analyzed, and a new virus was discovered (i.e., Espirito Santo virus [ESV]). The virus was amplified (*in vitro*) in mosquito cells and initially found to be dependent upon co-infection with a virulent strain of DENV-2 virus (Vancini et al., 2012). Laboratory testing showed the virus replicates in C6/36 (*Ae. albopictus*) insect cells to high titers when co-infected with DENV-2 (44/2) but not Vero (African green monkey kidney; mammalian) cells (Vancini et al., 2012).

The existence of a new virus is an area meriting additional research. It is important to acquire information on how co-infection of viruses may affect one or both viruses. It is possible that one virus may be co-dependent on the other (Goertz et al.,

2017). This dependency could alter the virulence, transmission and/or replication of the virus in a host. This can potentially have tremendous public health impacts. Understanding the extent to which coinfection of ESV and DENV alters replication in one or both viruses could provide information to others interested in vaccine development or other applications. Elucidating the potential effects could also lead researchers and medical personnel to determine the extent to which virulence of a virus depends on the relationship with another virus or other types of infections.

Consequently, we will evaluate the relationship between ESV and DENV coinfection in C6/36 cells and in *Ae. aegypti* mosquitoes.

Study Objectives

1. Evaluate the ability of ESV to block replication of DENV *in vitro* when C6/36 *Aedes albopictus* cells are co-infected with DENV-2 and ESV
2. Evaluate the extent to which ESV suppresses DENV in *Ae. aegypti* mosquitoes previously infected with ESV.

CHAPTER II – LITERATURE REVIEW

More than one-third of the world's population lives in an area at risk for DENV infection (WHO 2017b). This is one of the most common human viral diseases transmitted by arthropod vectors (Hemingford et al., 2007). Dengue virus is the leading cause of human morbidity and mortality in the tropics, with as many as 400 million people infected yearly (CDC 2016). The abundance of dengue cases has grown over the years, experiencing a 30-fold increase over the past 50 years (Achee et al., 2015). This rise in cases of dengue and severe dengue (i.e., dengue hemorrhagic fever [DHF], dengue shock syndrome [DSS]) may lead to an increase in the global burden of this disease. Prior to 1970, only nine countries had experienced severe dengue epidemics. Dengue is now endemic in the WHO regions of Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific. Current data estimates 3.9 billion people in 128 countries are now at risk of DENV infection (WHO 2017b).

Dengue fever is also known as "break-bone" fever. Dengue virus causes a high fever (40°C/104°F) and is accompanied by at least two of the following symptoms: severe headache, pain behind the eyes, muscle and joint pain, nausea, vomiting, swollen glands or rash (CDC 2012). When a victim is infected with more than one serotype of DENV, the infection may be more severe and could lead to DHF. Dengue hemorrhagic fever may result in plasma leakage, severe bleeding, fluid accumulation, and/or respiratory distress and organ malfunction (WHO 2017b). Dengue hemorrhagic fever is a potentially fatal complication of dengue. Dengue virus is transmitted between humans by female *Ae. aegypti* and *Ae. albopictus* (Achee et al., 2015). After a bite from an infected mosquito, symptoms in humans usually begin in 4 – 7 days and last 3 – 10

days (Tissera et al., 2017). A mosquito must blood feed on an infected person within an approximate five-day period while the person is in the viremic phase of infection (Gubler 1997). It is during this phase where viral load is potentially the highest in the person's blood; however, the infected individual may not be showing symptoms or may be experiencing mild flu-like symptoms (Whitehead et al., 2007). The virus requires an 8-12-day extrinsic incubation period (EIP) in mosquitoes before it may be transmitted to another host. Once infected, the mosquito remains a potential vector for the rest of its life (CDC 2014).

The most important arbovirus in global public health is dengue. Dengue virus is a *Flavivirus*, which is a member of the *Flaviviridae* family (Anayansi et al., 2017). It is the most genetically diverse member of the Flaviviridae family. Dengue fever is caused by one of four anti-genetically distinct serotypes of dengue virus: DENV 1 – 4 (Cops et al., 2014). While infection with one serotype of DENV may provide lifelong immunity from the infecting serotype, it may only provide partial and temporary cross immunity from the other serotypes. Studies have shown subsequent infections of different serotypes increase the risk of developing life-threatening DHF and DSS (Muturi et al., 2017).

Dengue is a positive sense, single-stranded, enveloped RNA virus (CDC 2013). There are two structural proteins, the envelope (E) and membrane proteins (M) Alcaraz-Estrada et al., 2010). The virus has the ability to replicate in many different types of mammalian cells, such as monocytes and macrophages, dendritic cells, B and T lymphocytes, endothelial cells, bone marrow-, hepatoma, neuroblastoma- and kidney-derived cells (Alhoot et al., 2011). Dengue virus is suggested to enter target cells after

protein E binds to uncharacterized cell surface receptors (Clyde et al., 2011). The virion is internalized by receptor-mediated endocytosis, leading to subsequent fusion of the virus with the endosomal membrane, releasing the viral genome in the cell cytoplasm (Acosta et al., 2008). Because DENV viral RNA can act as mRNA, the genome is associated with rough ER where it is translated (Alcaraz-Estrada et al., 2010).

Since DENV was discovered in 1943, there has been an increase in the reported infections in the tropical and sub-tropical areas of the world (Suwanmanee et al., 2017). International travel, transport, and globalization have helped DENV expand into new geographic areas. These factors have also helped DENV re-emerge in places that had been free of the virus for years (Cops et al., 2014). For example, Peru had reported cases of DENV in the 1950's but no reported cases in the subsequent three decades. Peru was one of the countries, along with Brazil, Bolivia, Paraguay and Ecuador, that had been free of DENV for decades, yet experienced explosive epidemics of DENV-1 during the 1980's. During this epidemic, DENV-4 was also isolated in Peru (Pinheiro et al., 1997).

Dengue was first described in the continental United States (US) in Philadelphia in 1780 when a dengue-like outbreak occurred (Bouri et al., 2012). The southern US began having incidences of dengue occurring around 1827, 47 years after the dengue-like outbreak in Philadelphia (Beaumier et al., 2014). The following areas have a history of DENV outbreaks: Pensacola, Florida; Charleston, South Carolina; Savannah, Georgia; and New Orleans, Louisiana (Bouri et al., 2012). Dengue outbreaks continued to occur regularly in the US until the 1940's (Thomas et al., 2016). In 2009, after 75

years without a reported case of dengue, Florida identified a dengue case in a New York patient who had traveled to Key West (Añez at al., 2013). Dengue also reemerged in Texas in 2013. While conducting surveillance during a 2013 epidemic in northern Mexico, 53 laboratory DENV-positive human cases were found in southern Texas and 49% of patients had not traveled (Thomas et al., 2016).

There have also been a number of cases imported into the United States. Florida has recorded 392 cases from travelers from countries in Central and South America, the Caribbean, Africa, the Middle East, South and Southeast Asia since 2009 (Florida State Department of Health 2010). These cases show the importance of airports such as Miami International Airport as a gateway to the US of pathogens such as DENV and others (Añez et al., 2013). In 2001-2002, Hawaii recorded its first autochthonous cases of dengue since 1944 (Bouri et al., 2012).

Other countries, such as Costa Rica and Panama that have experienced DENV free periods have also seen a re-emergence of the virus. In 1993, both countries reported cases of indigenous transmission of DENV-1 (Pinheiro et al., 1994). In 1981, Cuba reported an epidemic of DENV-2 with 344,203 cases, including 10,312 cases of dengue haemorrhagic fever (DHF), first main DHF reported in the region (Dick et al., 2012). The same countries were not just seeing re-emergence with one serotype, but they were isolating multiple serotypes of DENV (Pinheiro et al., 1994). Dengue serotype – IV was introduced into the eastern Caribbean Islands and then expanded to the rest of the Caribbean, Mexico, Central and South America, causing epidemics in places that had experienced DENV-1 outbreaks (Dick et al., 2012). Dengue virus-3 was attributed

to a countrywide epidemic of DHF/dengue in Nicaragua in 1994 (Pinheiro et al, 1994). During this time, DENV-1 was also present (Pinheiro et al., 1994).

Vector Competence

Mosquitoes obtain arboviruses in nature via an infectious blood meal (Khoo et al., 2013). If virus particles surpass the midgut barrier, viral dissemination may occur through hemolymph to secondary organs, then finally reaching the mosquitoes salivary glands where transmission may occur (Hegde et al., 2015). Prior to virus invasion of midgut epithelial cells for replication, the virus may encounter the midgut infection barrier (MIB), which may restrict its ability to invade epithelial cells (Bennett et al., 2002). The midgut escape barrier (MEB) is another barrier in the midgut that can restrict the virus' ability to disseminate following replication in epithelial cells (Bennett et al., 2005).

The measure of a vector-borne disease's transmission potential among humans is called vectorial capacity (Ye et al., 2016). The vectorial capacity is determined by parameters such as contact rate between vector and human, vector biting rate, daily survival of vector, vector competence and the EIP (Kramer et al., 2003). Mosquitoes may maintain vector competence due to a series of both physiological and biochemical adaptations which allow viral replication to occur in the midgut epithelial cells and transfer to salivary glands (Valderrama et al., 2017).

Midgut microbiota can affect vector competence as gut bacteria can impact pathogen development (David et al., 2016). The midgut is the site of interactions that include the arthropod vector (host), vertebrate blood factors, the pathogen (virus or

parasite) and other symbiotic microbes (Ramirez et al., 2012). Mosquito gut microbiota influence DENV infection of the mosquito eliciting innate immune responses against the virus which activates the host antibacterial responses (Ramirez et al., 2012). Activation of the mosquitoes' immune system by dengue virus infection may potentiate the mosquitoes' immune homeostasis and suppress the microbiota of its midgut (Ramirez et al., 2012).

Environmental temperature has an effect on vector competence. Experiments between DENV and its primary vector, *Ae. aegypti*, have been studied under constant temperatures in the laboratory (Carrington et al., 2013). Temperature can influence how efficiently the vector becomes infected with a parasite and how readily the parasite is transmitted by the vector (Ye et al., 2016). Temperature changes can affect the EIP, or the time lag between when a mosquito consumes an infectious blood meal to when it is capable of transmitting the virus in its saliva (Black et al., 2002). Carrington et al. (2013) conducted experiments on *Ae. aegypti*, at 26°C. During this experiment, female *Ae. aegypti* were fed blood meals using defibrinated sheep blood infected with DENV-1 with virus titers of $4.16x10^5$ pfu/mL with results showing an expected EIP for DENV as 11-12 days (Carrington et al., 2013). Another study showed DENV transmission when conducting multiple experiments at a range of constant temperatures from 13°C to 35°C (Watts et al., 1987). Extrinsic incubation periods for *Aedes aegypti* in this experiment ranged from 12 days at 30°C to 7 days at 35°C (Watts et al., 1987). Other studies have shown that higher temperatures reduced the EIP, increased mosquito mortality and resulted in higher proportions of infected *Aedes aegypti* mosquitoes (Rohani et al., 2009).

The diurnal temperature range (DTR) significantly influenced the outcome of infection and survival of mosquitoes, but not the EIP of DENV in *Ae. aegypti* (Lambrechts et al., 2011). Large diurnal temperature ranges have been found to lead to reduced immature survival and lead to extended development time in *Aedes aegypti* mosquitoes (Carrington et al., 2013). Using an experiment based off average temperatures in Mae Sot, Thailand, Carington et al. (2013) conducted an experiment with small DTR fluctuations (mean temperature 26.71°C and a 7.6°C fluctuation) and large DTR (mean 26.34°C with 18.6°C fluctuations) with relative humidity maintained at 70-80% for both groups (Carrington et al., 2013). Female mosquitoes incubated in conditions under the large DTR had the lowest survival rate versus females under constant temperatures (Carrington et al., 2013).

Genotype interactions may also play a role in vector competence. Virus genotypes may adapt to the local mosquito population genotype, although evidence of this is not consistent (Lambrechts et al., 2009). Studies have shown CHIKV transmission is affected by a three-way interaction between viral strain, environmental temperature and *Ae. albopictus* population (Zouache et al., 2014). However, it is unknown whether or not these interactions exist for DENV and *Aedes aegypti* (Soria et al., 2017). It is also possible the extent of these interactions may change for different populations and species of mosquitoes.

Another parameter that may have an effect on vector competence is insecticides. Different insecticides may exert different selective pressures on mosquito vectors due to residual spraying (WHO 2011). This has led to insecticide resistance

mutations occurring in some mosquito species including resistant alleles resulting from to frequent widespread applications of organochlorines (e.g. DDT), organophosphates (OP), carbamates (CX) and pyrethroids (PYR) (Alout et al., 2013). Two of the main mechanisms responsible for resistance are: 1) increased metabolism of detoxification, involving enhanced degradation or sequestration of insecticide molecules, and 2) modification of the insecticide at the target site, in which the site becomes less sensitive to insecticide (Labbe et al., 2011). These resistance mechanisms are conserved mutations across many insect vectors (Labbe et al., 2011). While fitness costs associated with resistant alleles may be hard to predict, they may alter the physiology of mosquito vectors, hence altering pathogen transmission (Rivero et al., 2010). Another study showed that insecticide resistant *Anopheles gambiae* Giles are more susceptible to infection with entomopathogenic fungi *Metharhizium anisopilae* and *Beauvercin basin* than insecticide susceptible mosquitoes (Howard et al., 2010). In a study comparing vector competence between susceptible and resistant strains of *An. gambiae s.s.*, results showed insecticide resistant strains were more susceptible to infection than insecticide susceptible strains (Alout et al., 2013).

The age of a mosquito may also play a role in immune response to pathogens. Another study showed larvae and newly-emerged adults of *An. gambiae* kill bacteria in their hemocoel more efficiently than older adults (League et al., 2017). These findings indicate that the immune response in larvae may be stronger than in adults, with immunity weakening after metamorphosis (i.e., five-day old adults mounted weaker immune responses than one-day old adults) (League et al., 2017). However, adult mosquitoes may boost their immune responses by ingesting blood. This may lead to

blood-meal induced hemocyte activation, which may lead to upregulation of vital immune factors (Bryant et al., 2014). In *Ae. aegypti*, blood feeding improves their ability to combat low dose *E. coli* infection but may reduce tolerance to mosquitoes experiencing a high dose *E. coli* infection as determined in a study by Castillo et al. (Castillo et al., 2011).

Simultaneous exposure of mosquitoes to different pathogens is a new avenue of study on how coinfections may affect the vector competence of mosquitoes (Ruckert et al., 2017). Molecular mechanisms such as RNA interference (RNAi) could be activated or suppressed by co-infecting viruses and thereby indirectly affect replication of another virus (Jupatanakul et al., 2017). RNA interference is a cellular mechanism that regulates gene expression in eukaryotes (van Cleef et al., 2014). Sub-genomic flavivirus RNA and RNAi suppressor function of NS4B is a mechanism for RNAi suppression in flaviviruses (Moon et al., 2015). Flavivirus NS1 is important in *Aedes aegypti* midgut infection as it suppresses immune related gene expression (Lui et al., 2016). This may lead to NS1 not only enhancing flavivirus infection but also enhancing midgut infection of heterologous viruses such as CHIKV, a virus that replicates and disseminates faster than the two flaviviruses, DENV and ZIKV (Ruckert et al., 2017). This may cause CHIKV to outcompete DENV and ZIKV *in vivo* in some cases (Dubula et al., 2009). The exposure of two closely related flaviviruses, DENV and ZIKV, could impact virus infection, dissemination and transmission in mosquitoes through superinfection exclusion (Karpf et al., 1997).

Co-circulation of viruses

Co-circulation of viruses or serotypes of viruses, such as DENV, have been noticed over the years and increase the risk of humans experiencing co-infections (Magalhaes et al., 2014). Co-infections in humans with multiple serotypes of DENV were rare prior to the 1950's (Muturi et al., 2017). In 1982, the first known naturally occurring case of co-infection in humans was reported (Cops et al., 2014). In the same study, the patient in Puerto Rico was found infected with DENV-2 and DENV-3. There have been other reported cases including six patients infected with both DENV-1 and DENV-3 in New Caledonia and an autochthonous case with DENV-1 and DENV-2 in São Paulo, Brazil (Cops et al., 2014). Increases in co-circulation of viruses and in subsequent co-infections may lead to an increase in virulence of one or both DENV serotypes (Vinodkumar et al., 2013). In the case of DENV, co-infection of multiple serotypes may lead to the development of the more severe DHF or DSS.

Aedes aegypti and *Ae. albopictus* are vectors of DENV and other arboviruses (Hiba 2011). Many arboviruses are present in the same geographic areas and this may further increase the risk of co-infection among viruses in humans. Zika virus, a re-emerging arbovirus, is also transmitted by *Ae. aegypti* and *Ae. albopictus*. Zika virus (ZIKV; genus *Flavivirus*, family *Flaviviridae*) was first isolated in Uganda in 1947. Until the 1980's, ZIKV infections in humans were found mainly in Africa and Asia (WHO 2016). Past ZIKV outbreaks were sporadic and self-limiting until an outbreak in the Yap Islands in 2007 and Zika fever in 2013 (Al-Qahtahni et al., 2016). The virus spread across continents and, in May 2015, Brazil became the epicenter of the first reported cases in the Americas (Al-Qahtahni et al., 2016). Since then, ZIKV has spread to

several countries in the Americas including Mexico, US, Panama and Puerto Rico (WHO 2016). Zika virus spread to many areas where DENV was already endemic. Zika virus circulating in the same areas as DENV, combined with ZIKV being spread by the same vectors may lead to the possibility of co-infection (Ruckert et al., 2017).

In January 2014, New Caledonia experienced their first autochthonous cases of ZIKV and multiple DENV serotypes were co-circulating in the area (Rouser et al., 2015). Later in 2014, two patients tested positive for co-infections of ZIKV/DENV-3 and ZIKA/DENV-1 (Rouser et al., 2015). In the same study, one patient had recently returned from travel to French Polynesia and the other had not traveled and the patients were infected with different DENV serotypes (Rouser et al., 2015). Genetic sequencing of ZIKV determined the strain of ZIKV from both patients belonged to the Asian lineage and had a 99% identity with sequences isolated from French Polynesia (Rouzevrol et al., 2015).

Chikungunya virus is an arbovirus (family *Togaviridae*) and is transmitted almost exclusively by *Ae. aegypti* (Chahar et al., 2009) and *Ae. albopictus*. There are three distinct evolutionary clades of CHIKV: West African, Central/East African and Asian (Power et al., 2000). Symptoms of chikungunya include muscle pain, headache, nausea, fatigue and rash (Deepa et al., 2016). Chikungunya virus has been identified in over 60 countries in Asia, Africa, Europe and the Americas (WHO 2017a). Chikungunya is a disease that can spread with ease and cause a high percentage of clinical cases with a very high attack rate, however chikungunya is overshadowed in many outbreak situations (Barde et al., 2014). Many of the areas where CHIKV is endemic are also

areas that are home to DENV. In Calcutta, India, where CHIKV and DENV overlap, cases have been reported with CHIKV and DENV co-infections (Chahar et al., 2009). The same study showed that two viruses from different families may coexist in the same host.

Not much is known about the effects of co-infections for many viruses. Reports indicate that 80% of ZIKV infections are asymptomatic (Iovine et al., 2016). Clinical manifestations of ZIKV and DENV may be similar, although ZIKV patients may have milder symptoms (Iovine et al., 2016). This similarity may make it difficult to tell the difference between infections of the two viruses where molecular testing is not carried out to identify the pathogen. The similarity may also hinder the testing for multiple viruses in patients, as only one virus' symptoms may be recognized (Chong et al., 2017). A patient in Haiti that tested positive for both ZIKV and DENV infection had severe symptoms from ZIKV (Iovine et al., 2016). Although this could be due, in part, to variations in immune responses between patients, it is recognized that DENV antibodies may enhance ZIKV infection *in vitro* (Iovine et al., 2016). The severity of co-infections has not been studied to a large extent in human patients, thus, impacts of severity have not been concluded. Impacts of co-infections on patients is a topic that is in need of further study.

A study was performed to look at the potential effects of co-infection of CHIKV and DENV in patients (Silva et al., 2017). Prior to the experiment, DENV/CHIKV co-infections reported in people from India were examined. The majority of patients experiencing co-infections showed similar symptoms (i.e., as in CHIKV or DENV mono-

infections), although there were a few patients that experienced more severe symptoms (Silva et al., 2017). In another investigation, patients infected with both DENV and CHIKV present with a clinically serious disease with a higher mortality rate when compared to mono-infections of these viruses (Deepa et al., 2016). Human peripheral blood mononuclear cells (PBMCs), from human donors, were infected with one or both viruses at various multiplicities of infection (Silva et al., 2017). This mode of infection (MOI) was done to try and mimic a “real-world” scenario. Mosquitoes experience different virus titers and can be co-infected with viruses, so the possibility of multiplicity of infections is a viable scenario. Results from the aforementioned study showed higher levels of DENV in supernatants from co-infected PBMCs than mono-infected cells. In contrast, the study also showed a decrease of CHIKV titers in co-infected cells when compared to mono-infected cells (Silva et al., 2017). The antagonistic effect produced by co-infection of CHIKV/DENV was not dependent on the replication of DENV cells in the co-infected PBMCs (Silva et al., 2017). The inhibition of CHIKV by DENV (but not the inhibition of DENV by CHIKV) was stronger and more consistent between donors and different MOI conditions (Silva et al., 2017). This study suggests that DENV suppresses CHIKV. This would have to be studied *in vivo*, to account for different mechanisms that may also affect the growth of either CHIKV or DENV in patients.

Human co-infection with viruses may occur due to multiple blood feedings by different mosquitoes on a host (Muturi et al., 2017). Furthermore, the same species of mosquito may be a vector of multiple arboviruses; hence, it is possible that co-infection may occur from the bite of one vector that is harboring both viruses (Muturi et al., 2017). An infection with DENV in a mosquito leads to the mosquito being a life-long carrier of

the virus. There are limited studies on the effects of co-infections on the mosquito. Virus-virus interactions may be 1) neutral: having no effect, 2) synergistic: at least one virus facilitates replication or transmission of the other virus, or 3) antagonistic: one virus benefits at the expense of the other (Muturi et al., 2017). Studying the types of virus-virus interactions in mosquitoes and in cell culture may help us gain knowledge and insight to begin to study these interactions in humans. Superinfection exclusion, an antagonistic reaction, where the initial virus takes hold in cells and blocks a second virus from replicating, may occur. A study investigating superinfection between DENV-2 and DENV-4 in *Ae. aegypti* found *Ae. aegypti* infected with DENV-4 were significantly less susceptible to DENV-2 (Muturi et al., 2017). Superinfection among DENV 1-4 has been tested in mosquito (C6/36 – *Ae. albopictus*) cell cultures (Muturi et al., 2017). The same study showed that DENV-4 outcompeted DENV-2. While more studies need to be done on superinfections, studies of this type may suggest superinfection could make it harder for co-infection in mosquitoes for some viruses. Viruses may be able to outcompete other viruses by having shorter EIPs (e.g., faster replication rate) in the vector. This may allow one virus to overtake cell machinery before the second virus, thus potentially limiting the effects of a superinfecting virus.

Co-circulation of viruses, among other factors, makes virus-virus interactions an important area of study. Our understanding of coinfections is limited and, as more viruses interact, it is crucial to understand how they interact in the vertebrate host and the vector (Ruckert et al., 2017). Different viruses may affect the pathogenicity and virulence of other viruses (Baba et al., 2013). Studying established viruses may give us insight to the workings of new viruses as they are discovered.

In addition to being vectors to arthropod-borne viruses that can cause disease, mosquitoes are also hosts to viruses that do not cause human disease (van Cleef et al., 2014). Espirito-Santo virus is a recently discovered virus that replicates in mosquito cells and is in the *Birnaviridae* family (Huang et al. 2013). The virus is in the monospecific genus *Entomobirnavirus* (Marklewitz et al. 2012). The virus was elucidated in mosquito cells inoculated with a virulent strain of DENV-2 obtained from a patient in Espirito Santo, Brazil (Vancini et al., 2012). Amino acid residue from ESV compared with those of other birnaviruses confirmed the uniqueness of ESV and its assignment to the genus Entomobirnavirus (Huang et al., 2013). However, the mechanism for cell infection by ESV is currently unknown. It is also unknown if the mosquito that infected the patient was previously infected with ESV prior to infection with DENV-2 (Vancini et al., 2012). ESV replication in previous studies (Vancini et al. 2012) showed the virus was co-dependent on virulent strains of DENV-2, i.e. ESV was not shown to grow on its own in mosquito cell culture. In the experiment, replication of ESV corresponded with the virulence of DENV-2 (Vancini et al., 2017). Studies are needed to show what type of viral interaction this newly discovered virus had on the DENV harvested from the patient. It is also unknown if different DENV serotypes are affected differently by ESV.

Other *Entomobirnaviruses* have since been elucidated in mosquitoes. In Bad Segeberg, Germany, hibernating *Culex pipiens* complex mosquitoes were collected from a cage, analyzed and inoculated into C6/36 and Vero E6/7 (African green monkey kidney) cells (Marklewitz et al., 2012). The Vero cells showed no cytopathic effects (CPE). Due to CPE being observed in C6/36 cells, the cells were passaged and

analyzed by electron microscopy and the genome was determined by adapter based random amplification and rapid amplification (Zirkel et al., 2011; Marlewitz et al., 2012). The sequence revealed that a birnavirus, tentatively named *Culex* Y virus (CYV), had a mutation in the ORF5 (as does ESV) and showed 99% similarity to ESV (Vancini et al., 2012; Marklewitz et al., 2012). *Culex* Y viruses were tested to see if their growth depended on co-infection with DENV. The virus was co-infected with DENV-2 strain 16681 with a m.o.i of 0.001 for both viruses. No differences in CYV was observed in single and co-infected C6/36 cells (Marklwitz et al., 2012).

Virus-virus interactions may differ, depending on the similarity in family characteristics of the viruses (Ruiz-Silva et al., 2017). It is possible that different virus families may suppress or facilitate virus replication during co-infections. Further studies are needed to understand the effects of ESV on DENV and its effects on viral replication in mosquito cells. A previous study with ESV showed that this virus does not replicate in Vero cells (Vancini et al., 2012). The inability of ESV to replicate in Vero cells could have tremendous impact when studying virus-virus interactions in human and other mammalian cells. The activation and suppression of antiviral pathways in mosquitoes that are vectors for human arboviruses, may affect the ability of a vector mosquito to transmit co-infecting arboviruses (van Cleef et al., 2014). Superinfection and co-infection studies are currently being performed to establish preventative intervention strategies for blocking the transmission agents of human diseases and help gain understanding of factors that could affect vector competence (Gonzaga et al., 2015).

Wolbachia pipientis, is an endosymbiotic bacterium that affects 40-70% of all arthropod species. However, it is not naturally hosted by *Ae. aegypti*, a principal vector of dengue (Joubert et al., 2016). *Aedes aegypti* mosquitoes have been intentionally infected with *w*Mel strain of *Wolbachia* to test its ability to reduce the susceptibility of *Ae. aegypti* to DENV (Ye et al., 2016). Tests showed that *Wolbachia*-infected *Ae. aegypti* had reduced pathogen replication in both natural and transinfected insects (Joubert et al. 2016). Trials such as these show the possibility of using virus superinfection to combat viruses such as DENV. However, the potential for viruses to mutate to overcome superinfections are possible, and thus the need for greater understanding of these phenomena are needed (Joubert et al., 2016).

Chapter III – ESV Inhibits Replication of Dengue in C6/36 *Aedes albopictus* Mosquito Cells

**Note: This chapter is formatted as a complete manuscript and will be submitted to a peer reviewed journal in the future. Hence, there may be some repetition in Chapter 2 (Literature Review) and Introduction for Chapter 3*

Abstract

Dengue virus (DENV; Family Flaviviridae: Genus Flavivirus) is a single stranded RNA-positive arbovirus transmitted by mosquitoes. Globally, millions of people are sickened each year by dengue fever that is caused by infection with DENV. Approximately 3.2 million cases were reported globally in 2015. While there is currently one live recombinant tetravalent DENV vaccine on the market (CYD-TDV: Dengvaxia®), there are significant safety concerns regarding its use. The Strategic Advisory Group of Experts on Immunization currently recommends that only seropositive persons ≥ 9 years old should be vaccinated. It is common for some arboviruses to co-circulate in the same geographic regions. Espirito-Santo virus (ESV) is an Entomobirnavirus discovered in a biological sample also containing DENV serotype 2 obtained from a patient in Espirito Santo, Brazil. Here, we evaluated the interactions of ESV and DENV-2 coinfection in C6/36 (*Aedes albopictus* Skuse) cells. We show that ESV can replicate in C6/36 cells in the absence of DENV-2 and causes no cytopathic effects in mammalian (Vero) cells 6-days post infection. Immunofluorescence results of co-infected cells, showed that ESV did not affect the expression or localization of DENV proteins under the conditions of our test. However, plaque assay results from co-infection of cells with ESV and DENV-2 resulted in a significant reduction in plaques compared to cells infected with DENV-2 alone. These results suggest that ESV may inhibit DENV-2 replication in C6/36 cells.

Introduction

Mosquito-borne diseases are responsible for millions of human cases and deaths each year[1]. Greater than one-third of the global human population inhabits areas at risk for dengue virus (DENV; Family Flaviviridae: Genus Flavivirus) infection[2,3]. Dengue virus is the leading cause of human morbidity and mortality in the tropics, with as many as 400 million people infected annually[2]. Dengue is one of the most infectious human viral diseases transmitted by arthropod vectors, and the incidence of dengue has risen 30-fold over the last 30 years[4]. This increase in case incidence of dengue fever and severe dengue (i.e., dengue hemorrhagic fever [DHF] or dengue shock syndrome [DSS]) has contributed to an increase in the global burden of this disease. Facilitation of the spread of arboviruses such as DENV may be attributed to three global megatrends, i.e., urbanization, climate change, and increased intercontinental travel[5].

Dengue virus is a genetically diverse member of the Flaviviridae family (genus Flavivirus).[6] This spherically shaped virus is composed of the viral genome and capsid proteins surrounded by an envelope and a shell of proteins.[7,8] It is approximately 50 nm in diameter with a nucleocapsid of 30 nm. Two of the structural proteins (membrane protein (M) and envelope protein (E)) are inserted into the envelope. [9,10] Infection with DENV may cause fever and a variety of symptoms after an intrinsic incubation period of 3-14 d.[4] Dengue fever is caused by one of four antigenically distinct serotypes of DENV: DENV 1 – 4.[11] While infection with one serotype of DENV may provide lifelong immunity from the infecting serotype, it may only provide partial and temporary cross protective

immunity from the other serotypes. Studies have shown subsequent infections of different serotypes increase the risk of developing life-threatening DHF and DSS.[12]

The rise in the disease burden caused by DENV over the past several decades has sparked a renewed interest in developing a DENV vaccine; however, producing a successful vaccine has been challenging. For a DENV vaccine to be successful, it must induce a protective and durable immune response to all four serotypes. The vaccine must also avoid eliciting or enhancing a pathogenic immune response.[13,14] A live recombinant tetravalent vaccine (Dengvaxia®) has been developed for DENV in recent years, however the Philippine Health Ministry suspended the vaccine program in November 2017 after multiple DENV deaths in vaccinated children.[15] As of Fall 2018, the Strategic Advisory Group of Experts on Immunization recommends that only DENV seropositive persons ≥ 9 years old should be vaccinated with Dengvaxia®.

The past several years have led to the identification of a range of RNA viruses associated with hematophagous insects.[16] These discoveries, attributed to advances in sequencing technology and phylogenetics, have helped characterize RNA viruses found within mosquitoes. Espirito-Santo virus (ESV, Family Birnaviridae) is a recently discovered virus that replicates in C6/36 (*Ae. albopictus*) cells and is in the monospecific genus *Entomobirnavirus.*[17,18] The virus was first characterized in C6/36 cells from a biological patient sample containing DENV-2.[19] Amino acid residues from ESV compared with those of other birnaviruses confirmed the uniqueness of ESV and its assignment to *Entomobirnavirus.*[18] However, the mechanism for cell infection by ESV is currently undiscovered. It is possible that other mosquito genera and species may be infected with *Entomobirnaviruses*. *Culex* Y virus, another Entomobirnavirus, was

isolated from hibernating *Culex pipiens* (Linnaeus) in Germany.[17] In Australia, Palm Creek virus was isolated from *Coquillettidia xanthogaster* (Dyar) [20]. The extent to which these viruses may impact mosquito biology is currently being investigated.

Virus-virus interactions may differ, depending, in part, on virus family characteristics.[21] It is possible that viruses may suppress or facilitate replication of other viruses during co-infections. Viral interference is a well-known phenomenon where one virus blocks replication of another by several different mechanisms. This has been documented in orthomyxoviruses (e.g., influenza), retroviruses, flaviviruses, and picornaviruses, among others.[22,23] In the current study, ESV replication and the effects of viral co-infection between DENV-2 and ESV are being investigated.

Results

ESV Protein Expression

The first report of ESV indicated that ESV replication was dependent on coinfection with DENV-2 and that it corresponded with the level of DENV virulence.[19] To test viral infection, we infected C6/36 cells with ESV, multiple strains of DENV-2 isolates (strain 44/2 and 16803), or both ESV + DENV-2 (44/2 or 16803). ESV proteins were detected in cells infected with ESV alone and in cells infected with ESV+DENV-2 (44/2 or 16803) using anti-ESV antibodies made in rabbits. Western blot results showed the presence of ESV proteins in C6/36 mosquito cells even in the absence of DENV-2, indicating that ESV can at least express some viral proteins in the absence of DENV (figure 1a). Analysis also showed ESV bands of similar sizes in cells co-infected with ESV and DENV-2 (both 44/2 and 16803). Our results also demonstrated the specificity of the ESV antibody as the antibody did not bind to proteins from DENV-2 infected or

uninfected cells. To our knowledge, this is the first time anyone has detected viral proteins of ESV by Western blot (Figure 1a). Immunofluorescence assays showed ESV proteins localized in bright cytoplasmic areas, presumably viral factories in cells (Figure 1b). Western Blot analysis showed cells co-infected with ESV/DENV-2 (44/2) appeared to have a thicker DENV protein band than cells infected with ESV alone, however this apparent increase may be due to more proteins loaded into wells (Figure 1c). Figure 1d shows confocal imaging of ESV proteins in C6/36 cells infected with ESV.

ESV Replication Detected with qRTPCR.

We wanted to determine whether ESV could replicate independently in insect cells, so we infected C6/36 cells with ESV or ESV with DENV. We infected at a multiplicity of infection (m.o.i) of 1.0 for both viruses and incubated for 6 days in C6/36 cells. Since ESV does not form plaques, we measured the increase in viral genomes by qRT-PCR as a measure of replication. ESV was able to replicate independently (0.35 ± 0.3 $\log_{10}$ Focus Forming Units [FFUeq] ESV/mL), and ESV genomes were increased (0.48 ± 0.075 $\log_{10}$ FFUeq ESV/mL) when co-cultivated with DENV-2 (44/2), but the difference was not significant (p=0.679). Initial stock titer for ESV was 0.26 $\log_{10}$ FFUeq ESV/mL.

Immunofluorescence Staining

We further sought to evaluate DENV-2 when co-infected with ESV by assessing DENV-2 protein expression using an immunofluorescence assay (IFA). Using C6/36 cells in 24-well plates, we individually infected wells with either no virus (mock), DENV-2

alone (multiplicity of infection [m.o.i.] 1.0), or ESV alone (m.o.i. 1.0), or co-infected wells with both DENV-2 and ESV. After 48 h, the cells were fixed with 3% paraformaldehyde and permeabilized with 0.05 % saponin. After washing, cells were incubated with mouse monoclonal anti-DENV serotype 1+2+3+4 antibody and followed by incubation with anti-mouse IgG fluorescein isothiocyanate (FITC)-conjugated secondary antibody and examined for fluorescence. We did not detect any difference in DENV protein quantity or localization between cells infected with DENV-2 alone or co-infected with ESV (Figure 2a). When we analyzed ESV proteins (using rabbit anti-ESV antibody) in cells singly infected or co-infected with DENV-2, we observed no change in ESV proteins in cells (Figure 2b). These results suggest that viral co-infection of ESV and DENV-2 in C6/36 cells does not inhibit either virus from entering cells and making proteins.

Viral replication plaque assay.

We wanted to test whether ESV affected replication of DENV. We first infected C6/36 cells with ESV alone, DENV-2 44/2 alone, or ESV+DENV-2 together, m.o.i. of 1.0, using serial 10-fold dilutions in triplicate wells. Supernatants and cells scraped from flasks were harvested after six days. This mixture was then titered on Vero cells to enumerate plaques to measure DENV viral replication. As expected, ESV infected wells showed similar results as uninfected wells (no plaques observed) (Figure 3a). These results suggest ESV does not produce plaques in Vero cells. Next, we analyzed cells and supernatants from DENV-2 only infected wells and observed significant cytopathic effects (CPE) as shown by plaques. Analysis of serial dilutions of DENV-2 alone and ESV/DENV-2 co-infected cells showed a decrease in the number of DENV-2 plaques in

the wells co-infected with ESV. Dengue-2 virus (10^{-1} dilution) showed numerous plaques (too many to count *ca*. >200), while co-infection of ESV (stock)/DENV-2 (10^{-1} dilution) resulted in an average of 33 DENV-2 plaques/well (n=3 replicate wells). This is a significant decrease ($p<0.01$) in the number of DENV-2 plaques from wells infected with only DENV-2 compared to wells co-infected with ESV. Comparison of additional DENV-2 dilutions and ESV/DENV-2 co-infections showed similar results. For example, DENV-2 (10^{-2} dilution) resulted in *ca*. 103 plaques, while wells co-infected with ESV/DENV (10^{-2} dilution) resulted in 2 DENV-2 plaques/well, a significant reduction ($p<0.01$) (Figure 3b, Figure 3C).

Plaque assay data indicated that ESV may decrease the production of infectious DENV-2 particles. To determine whether the block occurred before or after DENV genome replication, we used qRT-PCR to detect DENV-2 genomes and tested DENV-2 infection with and without co-infection with ESV in C6/36 cells. We observed a reduction in DENV-2 genomes in the presence of ESV. At six days post co-infection with ESV, DENV-2 titers were 1.9 $\log_{10}$ pfu DENV eq/mL, an approximate 40-fold reduction in DENV-2 compared to DENV-2 titers in cells not co-infected with ESV (7.1 $\log_{10}$ pfu DENV eq/mL). These data indicate that ESV may block DENV replication prior to genome replication.

Discussion

Viral interference is a well-documented phenomenon described in many viral species.[22,23,24] The interference can occur at various stages in the virus life cycle, which progresses through sequential steps. The virus binds its cell surface receptor, internalizes, begins viral gene expression (mRNA), followed by viral protein synthesis,

and then, in the case of RNA viruses such as ESV and DENV, full length RNA genomes are produced. This is followed by stages of viral particle formation. Finally, the particles themselves may be either defective or replication competent. Viral infections that are not productive (i.e., not successful at producing infectious progeny virions) may be blocked at any of these stages.[25] Analysis of our *in vitro* assessments show co-infection of mosquito cells with the insect-infecting virus, ESV, may down-regulate the replication of DENV-2. We did not see a difference in staining during the IFA for C6/36 cells co-infected with ESV/DENV-2, inferring that ESV does not interfere with protein production of DENV-2 upon entry into cells under the conditions of this study. Further studies are needed to evaluate this. However, plaque assays show that co-infection of ESV with DENV-2 leads to a reduction in infectious DENV particles as evidenced by a reduction of plaques in ESV/DENV co-infected wells. Hence, ESV may block DENV-2 replication prior to genome replication, however this block is not complete. Further studies are needed to evaluate whether or not ESV inhibits entry of DENV-2 into the cell when different doses are used. Not much is currently known about *Entomobirnavirus* replication. It is thought that Entomobirnaviruses follow the replication cycle of Birnaviridae, which expresses peptides that destabilize the membrane during entry [27]. It is possible that this destabilization has a negative impact on receptors needed for DENV-2 entry. It may be that competition for cellular factors by ESV during replication inhibits DENV-2 replication. Mosquito infection experiments of *Culex* species with *Culex* Flavivirus have shown a decrease in susceptibility to a secondary infection with West Nile virus compared to uninfected mosquitoes.[28] Another study conducted with the insect-infecting virus, Palm Creek virus, showed a reduction in replication of Murray

Encephalitis Virus in C6/36 cells[20]. The results of the current study and those of previous studies show a necessity in determining the effects of insect-infecting viruses and the vector competence of mosquitoes. Further studies should determine at what stage ESV blocks DENV replication, the degree to which replication may be blocked, and other factors involved. It is possible that ESV inhibits DENV in the short term and DENV may be able to overcome this inhibition and have an increase in replication at later time points. Future studies should determine the mechanism by which ESV affects DENV and methods for inhibiting DENV infection.

As ESV and other *Entomobirnaviruses* (e.g., *Culex* Y virus, Palm Creek virus) are discovered, it is possible that additional mosquito genera, and potentially other arboviruses, could be impacted. This could lead to the development of new approaches using insect-infecting viruses to block DENV replication in mosquitoes. Transfection of the *w*Mel strain of *Wolbachia* into *Ae. aegypti* mosquitoes, blocked laboratory transmission capacity for DENV and chikungunya virus (CHIKV).[30,31] The direct mechanism by which *Wolbachia* may inhibit DENV replication is unknown. Modification of cellular membranes by DENV to form sites of viral replication complexes, leads to the virus making significant alterations to the lipid repertoire of cells.[30,31] It is possible *Wolbachia*'s need for fatty acids causes fatty acid depletion in cells, causing a cellular perturbation that disrupts viral manipulation of these pathways.[30,31] In addition to *Entomobirnaviruses*, there are several flaviviruses that only infect insects (not mammals). One such virus, Eilat virus, was isolated during an arbovirus study in the Negev desert from a pool of *Anopheles coustani* (Meigen) mosquitoes.[32,33] Erasmus et al., 2015, used the Eliat virus and created a chimeric Eilat/CHIKV virus to be used as an

antigen in diagnostic testing.[33] Continued studies on this insect-infecting virus and its uses may lead to it being used as a platform to deliver a chikungunya vaccine.[34] These types of studies could help determine new roles of insect specific viruses in reducing disease burden. A 2015 assessment of vector control for dengue prevention suggests that, along with rapidly deployable strategies, greater emphasis should be placed on proactive strategies that aim to prevent, diminish, or eliminate transmission.[29] Using insect-infecting viruses is a novel approach may help with arbovirus control in potential vectors, provide a mechanism to aid in diagnosis of arboviruses in patients, (a potential use for CHIKV that has been studied with the Eilat virus) and reducing the global public health burden of dengue[33].

Materials and Methods

Virus propagation

An ESV isolate (Brown Lab, North Carolina State University) and a Southeast Asian DENV-2 (isolate 44/2) were used for mosquito infection. Viruses were propagated in the laboratory using established methods (Richards et al., 2007). Samples not used immediately were stored at -80˚C.

Immunofluorescence assay

C6/36 (*Ae. albopictus*) cells were seeded on glass coverslips, 12 mm in diameter, in a 24 well plate, with each plate containing *ca*. 1 x 10^6 cells. The seeded plate was incubated for 48 h at 28°C and 5% CO_2. The cells were infected with DENV (isolate 44/2), and ESV at a m.o.i. of 1.0. Cell inoculations were performed using ice to slow down reaction to allow both viruses to enter the cell simultaneously. The plates

were incubated at 28°C and 5% CO_2. After 2 h, M199 medium (containing 2% fetal bovine serum [FBS] and penicillin/streptomycin) was added to each well and plates were incubated at 28°C and 5% CO_2 for 2 d. The cells were washed with phosphate buffered saline (PBS) and then fixed using 3% paraformaldehyde. The cells were then washed with PBS. Cells were subsequently permeabilized with 0.05% saponin. The cells were blocked for 30 min in a 2% FBS/PBS solution. ESV antibodies (anti-ESV (1:1000) rabbit serum were diluted in a 2% FBS/PBS (1:500 ratio). DENV antibodies were diluted in a PBS/FBS (1:1000 ratio). The cells were incubated in their respective primary antibody dilutions at room temperature for 1.5 h. Cells were then washed three times in 1 mL of PBS. Respective secondary antibody (anti-rabbit [Sigma Aldrich lot # 105K6269] and anti-mouse-antibody [Southern Biotech lot#K2915-WD97]) were prepared at a 1:1000 dilution in a PBS/FBS solution. Cells were stained with the secondary antibody, covered to prevent light exposure, and allowed to incubate at room temperature for 1 h. Cells were washed three times each in 1 mL of PBS. Cells were then mounted on glass slides, covered with coverslip and fixed with Prolong Gold and allowed to dry overnight. A Zeiss fluorescent microscope imaging system was used to visualize cells.

Western blot

C6/36 cells were seeded in a 24-well plate, 12 mm in diameter, with each plate containing 1 x 10^6 cells. The seeded plate was incubated for 48 h at 28°C and 5% CO_2. Cell inoculations were performed using ice to slow down reaction to allow both viruses could enter the cell simultaneously. Cells were infected with DENV (44/2) and ESV at an m.o.i. of 0.3. The plates were incubated at 28°C and 5% CO_2. After 2 h, 2% FBS

containing M199E with penicillin/streptomycin was added to each well and plates were culture at 28°C and 5% CO_2 for 2 d. After 2 d, media were removed into 2 mL Eppendorf tubes, centrifuged at 4000 rpm for 4 min to collect cells. The remaining cells in the 24 well plate were then lysed with 80 uL of cold disruption buffer (8% SDS, 8% β-mercaptoethanol, 1.2g/ml Tris Base, 40% Glycerol, 0.1% Bromophenol Blue) and the lysed cell solution was placed into 2 mL Eppendorf tubes with pellets retrieved from the above. The lysates were heated at 95˚ C for 5 min, and DNA sheared with 22 gauge needle before loaded to a gel. 40 µl/lane of either ESV, DENV-2, or ESV/DENV-2 lysate was loaded in 4-20% mini-protein Tgx, stain free precast gel (BIO-RAD, cat# 456-8094) and analyzed by SDS-PAGE. The proteins were transferred onto nitrocellulose membranes overnight at 4°C. The membranes were blocked with a blocking buffer (5% non-fat milk, 0.1% Tween 20 in PBS solution) at room temperature for 1 h and incubated with the following antibodies: anti-ESV (1:200) rabbit serum and anti-DENV serotype 1+2+3+4 antibody [D1-11(3)] (1:4000) (Abcam, Cambrige, MA, USA cat # ab9202). After washing with TBS-T solution once for 5 min at room temperature, the membranes were incubated at room temperature for 1 hr with secondary antibodies: anti rabbit IgG (Fc) (1:5000) (Promega, Madison, WI AP# S3738) and anti-mouse IgG (H+L) (1:5000) (Promega, AP# S3728), respectively. Membranes were washed twice with TBS-T solution at room temperature for 5 min of each washing and then Western Blue Stabilized Substance for Alkaline Phosphatase (Promega, Madison, WI USA) was added to the cover membrane. ESV bands were allowed to develop at room temperature for 1-2 h covered with aluminum foil to avoid light. Dengue bands were covered with aluminum foil and allowed to develop overnight at 4°C.

Plaque assay

A two-step process was carried out for the plaque assay. Individual T-75 cm^2 flasks, containing monolayers of C3/36 cells were inoculated with ESV (m.o.i 2.0), DENV-2 (44/2) (m.o.i 1.0), or co-infected with both viruses. Media containing M199E with 10% FBS and penicillin/streptomycin was transferred to each flask. The flasks were placed in an incubator at 28˚C and 5% CO_2 for 6 d. After 6 d, ESV, DENV, ESV/DENV infected C6/36 supernatant and cells scraped from flasks were harvested and tested. Ten-fold serial dilutions using of each infected sample were made using complete M199E (10% FBS, penicillin/streptomycin) inoculated in 6-well plates. Media was aspirated out of each Vero cell well and 0.2 mL of each infected sample (serial dilutions) were added to each well in triplicate. Plates were gently rocked back and forth to ensure Vero cell monolayers were covered with media. Plates were placed into the incubator at 35˚C for 1 hour, rocking every 15 min. The first overlay was prepared by combining 1.8 g of SeaPlaque® low melting agarose with ddH_2O, heat-inactivated FBS, non-essential amino acid solution, penicillin/streptomycin solution, L-glutamine, and fungizone. After 1 h, cells were removed from the incubator and the 1st overlay (3 mL/well of agarose solution) was carried out. Once dried, plates were incubated at 35˚C for 4 d when the 2nd overlay took place. The 2nd overlay (3 mL/well) contained SeaPlaque® low melting agarose, sodium chloride, ddH_2O, and neutral red solution. Plates were incubated for 24 h, removed from incubator, placed face down on lightbox, and plaques were counted.

qRT-PCR

Nucleic acids were extracted using a QIAmp viral RNA kit (Qiagen, Valencia, CA). Viral RNA (for both DENV and ESV) was quantified in each sample using quantitative real-time Taqman reverse transcriptase polymerase chain reaction (qRT-PCR) with the LightCycler® 480 instrument (Roche, Mannheim, Germany) and Superscript III One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). The instrument detection was programmed (for ESV and DENV-2) as follows: 48°C for 30 min, 95°C for 2 min, and 45 cycles at 95°C for 15 s, 60°C for 30 s, and finally, 40°C for 30 s. Primers and probes for DENV detection consisted of the following sequences: DENV-2 Rev 305-284: 5'- CCC CAT CTY TTC AGT ATC CCT G -3', DENV-2 FWD 237-251: 5'- CAT GGC CCT KGT GGC G -3', DENV-2 Probe254-274: 5'- /56-FAM/TCC TTC GTT / ZEN/TCC TAA CAA TCC/3IABkFQ/ -3' (IDTDNA, Coralville, IA). Primers and probes used for ESV detection consisted of the following sequences: ESV SEG A FWD: 5'-CCG CGC GGA GAC AAT CAC CT-3'; ESV SEG A REV: 5'-TTG GTC GAA CGC CCA CAC CG-3'; ESV SEG A PROBE ZENFAM: 5'-/F6-FAM/TGC TGG GTT /ZEN/CCA TTA CAG GTG GGA TGA /31ABkFQ/ -3' (IDTDNA, Coralville, IA). Standard curves for DENV were based on plaque assays used to determine titer. Standard curves for ESV were based on focus forming assays used to determine titer.

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Author Contributions

AW conducted virus and antibody experiments, analyzed results, and wrote the first draft of the manuscript. SR conducted and supervised virus experiments, analyzed results, and revised the manuscript. RR conducted and supervised antibody experiments, analyzed results, and revised the manuscript.

Competing Interests Statement

The authors declare that they have no competing interests.

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33. Erasmus JH, Needham J, Raychaudhuri S, Diamond MS, Beasley DW, Morkowski S, Salje H, Sala IF, Kim DY, Frolov I, Nasar F, Weaver S. Utilization of an Eilat virus based chimera for serological detection of chikungunya infection.

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Chapter IV - Vector Competence of *Aedes aegypti* for Dengue Virus in Mosquitoes Superinfected with Espirito Santo Virus

**Note: This chapter is formatted as a complete manuscript and will be submitted to a peer reviewed journal in the future. Hence, there may be some repetition in Chapter 2 (Literature Review) and Introduction for Chapter 4*

Abstract

Dengue virus (DENV), a mosquito-borne virus, consists of four antigenically distinct serotypes that may cause disease such as dengue fever and the more serious dengue hemorrhagic fever. Mosquitoes are known to transmit a variety of arthropod-borne pathogens. In addition to arboviruses, mosquitoes may also carry non-medically important viruses. Over the last few years, the number of recently discovered insect-infecting viruses has risen. Recent studies have shown these viruses may have an effect on the vector competence mosquitoes. Espirito Santo virus (ESV), is an insect-infecting virus that was recently discovered in Brazil, a region in which DENV is endemic. *Aedes aegypti* is the main DENV vector in Brazil. We assessed the vector competence of three different populations of *Aedes aegypti* mosquitoes (Costa Rica Wild-Type, a high dissemination colony and a low dissemination colony [dissemination classification in reference to dengue virus]) exposed to ESV and subsequently challenged with DENV. Our findings show infection rates in our wild-type population 100% and 88%, dissemination rates of 74% and 87% and transmission rates of 21% and 0% for non-ESV infected mosquitoes and ESV mosquitoes respectively. In our high dissemination colony, we observed infection rates of 100%, dissemination rates of 91% and 29% and transmission rates of 10% and 0% for non-ESV infected groups and ESV exposed groups, respectively. In our low dissemination colony, we observed infection

rates of 100% and 91%, dissemination rates of 79% and 90% for non-ESV infected mosquitoes and ESV mosquitoes. No transmission was observed in this group. Our results indicated ESV superinfection effects varied among different mosquito populations. The effects produced various differences in infection, dissemination and transmission rate and body titers.

Introduction

Since the 1950's, dengue has emerged as a world-wide problem, with over 400 million people infected yearly (Centers for Disease Control [CDC] 2016). It is endemic in 112 countries across the world (Gurgama et al., 2010). The number of dengue cases has increased five-fold between 2003 and 2013 in the Americas (World Health Organization [WHO] 2014). Dengue infection is caused by four different dengue virus (DENV) serotypes: DENV 1-4 (dos Santos et al., 2017, CDC 2016). Infection from any serotype may cause dengue fever, a flu-like illness, to the more severe dengue hemorrhagic fever, which may be fatal if its attendant plasma leakage is not given early attention (Hemungkorn et al., 2007, Vaughn et al., 2000). It is possible to become infected with multiple DENV serotypes with subsequent infections leading to antibody-mediated disease enhancement (Sanchez-Vargas et al., 2004). Lifelong homotypic immunity may be gained by infection from primary infection with a particular serotype, with heterotypic immunity to other serotypes lasting a few months (Hemungkorn et al., 2007).

The primary vector of DENV in almost all countries is the *Aedes aegypti* (Linnaeus) mosquito. In 1931, *Aedes albopictus* (Skuse) became known as a secondary vector after the virus was isolated from an *Ae. albopictus* caught in the wild (Higa 2011,

de Santos, CDC). Dengue infections experience extrinsic and intrinsic incubation periods. The intrinsic incubation period consists of the time between when an infected mosquito bites a human to the onset of symptoms from the infection, while the extrinsic incubation period (EIP) consists of when the time the mosquito takes a viremic blood meal and becomes infectious. Hence, both periods are important in the dynamics of dengue virus transmission (Chan et al., 2012). The extrinsic incubation period is an important component of vector competence of a mosquito (Anderson et al., 2007). The extrinsic incubation period (EIP) of DENV in *Ae. aegypti*, ranges from 8-12 days at a temperature of 25-28°C (Tjaden et al., 2013, Fontaine et al., 2018). Vector competence is the ability of a vector to become infected with and subsequently transmit a pathogen (Beerntsen et al., 2000). There are many factors that may affect the vector competence of a mosquito, which may also affect the EIP. Environmental temperature is one component that may influence the vector competence of *Ae. aegypti*. Studies show mosquitoes reared at low temperatures are predisposed to viral infection (Gloria-Soria et al., 2017, Turrell 1993). Genetic variation within *Ae. aegypti* may affect its susceptibility to DENV. It is not fully understood how genes mediate pathogen-vector interactions. However, some genes have been implicated in *Ae. aegypti* vector control (Caicedo et al., Osta et al., 2018). Studies performed by Chauhuan et al., 2012 suggested that a core suite of genes may play a significant role in vector competence of *Ae. aegypti* to DENV. Results from these studies showed in susceptible strains an upregulation of genes in involved in protein processing in the endoplasmic reticulum, mRNA surveillance and the proteasome. However, in refractory strains activity was shown in several metabolic processes including glycolysis, glycan biosynthesis and Wnt

pathway (Chauhan et al., 2012). The genetic variation in DENV isolates may also play a role in vector competence (Fontaine et al., 2018). Gloria-Soria et al., 2017 showed infection rate differences in *Ae. aegypti* mosquitoes were affected by the strain of DENV-2. It is possible that genotype x genotype interactions between mosquito species and virus strain may influence vector competence.

Tissue barriers in the mosquito play an important role in arbovirus infection and thus may have an effect on vector competence. Once an arbovirus has been ingested via blood meal, it encounters and must overcome the midgut infection and escape barriers, disseminate into secondary tissues such as nerve tissue, hemocytes and fat bodies, and surpass the salivary gland infection barrier and salivary gland escape barrier (Bosio et al., 2000, Salazar et al., 2007, Franz et al., 2015).

Co-circulation of viruses may pose an impact to vector competence in the form of virus-virus interactions. Superinfection (e.g., the acquisition of two viruses in two separate blood meals) may impact the virus-virus interaction. These virus-virus interactions may lead to different outcomes: 1) neutral, viruses have no effect on each other; 2) synergistic, at least one virus facilitates transmission or replication of the other virus; or 3) antagonistic, one virus benefits at the detriment of another virus (Muturi et al., 2017). The most common outcome between virus-virus interactions is superinfection interference, where an established primary viral infection prevents infected cells from becoming infected by a secondary virus (Nowak et al., 1994). In a superinfection experiment performed by Muturi et al. 2017, results showed a significant reduction in DENV-2 infection and dissemination rates in *Ae. aegypti* mosquitoes previously exposed to DENV-4 compared to DENV-2 infected mosquitoes not exposed to DENV-4.

However, the reverse treatment showed DENV-2 exposed mosquitoes secondarily infected with DENV-4 showed no significant effect on DENV-4 infection compared with previously unexposed mosquitoes infected with DENV-4. A DENV-2 and chikungunya co-infection study conducted by Le Coupanec et al. 2017, showed DENV-2 replication was higher in salivary glands of co-infected than single infected *Ae. aegypti* mosquitoes at 5, 10, 11, 12 days post virus exposure.

Dual infection, whether superinfection or co-infection, of viruses in mosquitoes may not be limited to the infection of multiple arboviruses. Mosquitoes may also be infected with insect-infecting viruses. Insect-specific viruses are defined as being specific to mosquitoes and unable to infect mammalian cells (Scultz et al., 2018.) This inability infect mammalian cells may be due to the lack of appropriate receptors for cell entry or possibly replication and assembly factors needed are either lacking or too structurally divergent from their invertebrate orthologs to interact with viral proteins (Halbach et al., 2017). Isolated from a cell line derived from *Ae. aegypti* embryos, cell-fusing agent virus was one of the first insect-specific viruses identified in 1974 (Stollar and Thomas 1975, Calzolari et al., 2015). Several insect-infecting flaviviruses have been isolated from natural mosquito populations over the years. Kimiti River virus, an insect infecting flavivirus, was isolated from *Aedes macintoshi* (Huang) mosquitoes in 1999 in Kenya (Lutomiah et al., 2007). *Aedes* flavivirus was isolated and detected in asymptomatic adult male and female *Ae. albopictus* and *Aedes flavopictus* (Yamada) mosquitoes in Japan, suggesting vertical transmission of insect-infecting viruses (Hoshino et al., 2009). The presence of insect-infecting viruses in areas where arboviruses exist lead to the possibility of co-infection of mosquitoes with arboviruses.

Bolling et al., 2012, isolated *Culex* flavivirus (CxFV) from *Culex* mosquitoes collected in northern Colorado from 2006 to 2007. Using these mosquitoes, they sought to determine whether persistent infection with CxFV altered vector competence in *Culex pipiens* (Linnaeus) for West Nile virus (WNV). Bolling et a., 2012 compared vector competence for WNV using laboratory *Cx*. *pipiens* originating from Colorado and an uninfected *Cx. pipiens* colony from Iowa. Results showed a significant difference in WNV dissemination rates at 7 d post infection (dpi) with the uninfected colony from Iowa having higher dissemination rates; however, these differences were no longer apparent at 14 dpi (Bolling et al. 2012). Due to the colonies' differences in geographic location, it is possible genetic variations affected vector competence (Bolling et al., 2012). Another study showed that C6/36 cells previously infected with Palm Creek virus suppressed subsequent replication of WNV (Hobson-Peters et al., 2016). Another study showed significant reduction of WNV, St. Louis encephalitis virus and Japanese encephalitis virus when these viruses were inoculated into C3/36 mosquito cells containing previous infection with Nhumirim virus, an insect-infecting flavivirus isolated from *Culex chidesteri* (Dyar) mosquitoes in Brazil, in 2010 (Kenney et al., 2014, Pauvolid-Correa et al., 2015).

A new insect-infecting virus was discovered in C6/36 mosquito cells co-infected with DENV-2. The virus, Espirito Santo virus (ESV) was characterized as an *Entomobirnavirus*, originated from a dengue fever patient from Espirito Santo, Brazil (Vacini et al., 2012). Initial studies by Vancini et al., 2012, determined ESV grew to high virus titers only during co-infection with DENV-2 44/2 in C3/36 cells. However, their studies also showed no ESV could be identified by electron microscopy when the insect-cell-infecting virus was used to inoculate Vero cells. Researchers detected the

Mosquito X virus (MXV), which was isolated from *Anopheles sinensis* (Wiedemann) in South China. Mosquito X virus is an *entomobirnavirus,* similar to ESV. Sequencing of *An. sinensis* showed no DENV in the sample, indicating the ability of the *entomobirnavirus* to replicate in absence of DENV (Huang et al., 2013).

There is evidence that insect-infecting viruses have an influence on vector competence (Vasilakis et al., 2015). The mechanism by which insect-infecting viruses may influence arboviruses is unknown. Here, we assessed the extent to which ESV affects vector competence of DENV-2 44/2 in *Ae. aegypti* mosquitoes. We hypothesized that superinfection of *Ae. aegypti* mosquitoes with ESV would decrease vector competence for DENV-2 44/2 compared to mosquitoes not exposed to ESV.

Materials and Methods

Virus propagation

A South American ESV isolate and a Southeast Asian DENV-2 (isolate 44/2) were used for mosquito infection. Viruses were propagated in the laboratory using established methods (Richards et al., 2007). ESV was inoculated into C6/36 cells and DENV was inoculated into Vero cells at an m.o.i of 1.0, incubated with M199E supplemented with 2% FBS, penicillin and streptomycin and allowed to propagate for 6 days. Samples not used immediately were stored at -80˚C.

Mosquitoes and ESV inoculation

Three different *Ae. aegypti* colonies were used in this study: 1) known for high dissemination of DENV, developed by crossing a Puerto Rico strain to Ibo strain *Ae. aegypti formosus*, generation F_{19} (BEI Resources; NR 45838), 2) known for low

dissemination of DENV, developed by outcrossing D2S3 strain to Houston strain of *Ae. aegypti*, generation F_{18} (BEI Resources; NR 45837), 3) wild-type, generation F_{48} colony from Costa Rica (BEI Resources; MRA-726). Propagation of mosquitoes consisted of placing 2.54 x 6.35 cm ovistrips containing approximately 50 eggs in clear plastic bowls approximately 6.35 cm deep and 11.43 cm diameter filled with either tap water (non-ESV infected control) or with a tap water/ESV mixture. Non-ESV infected mosquito ovistrips were placed into 300 mL of tap water. To expose mosquitoes to ESV, we used freshly propagated supernatant from flasks, inoculated with ESV and harvested 6 d post inoculation resulting in a titer volume of 1.44 $\log_{10}$ Focus Forming Unit (FFU) eq ESV/ mL. We combined 2 mL of ESV supernatant with 300 mL of tap water. Ovistrips were placed into the ESV-water solution (titer 1.10 $\log_{10}$ FFUeq ESV/ mL) for ESV exposure. The bowls were placed into incubators with a 14:10 light:dark cycle at 28°C and 85% humidity (Richards et al., 2017). Larvae were allowed to incubate in their respective ESV/water solution or tap water only and fed a mixture of yeast and liver powder (2:1 ratio) *ad libitum* until mosquitoes reached the pupal stage. Pupae were removed from bowls and placed into small clear plastic cups with tap water and cups were transferred to 30.5 x 30.5 x 30.5 cm metal cages (Bioquip, Rancho Domingo, CA) where adults starting emerging 1-2 d later. The resulting adult mosquitoes were fed a 20% sucrose solution *ad libitum*. Three to four-day old female mosquitoes were aspirated from metal cages with approximately 100 mosquitoes being transferred to 1 L cardboard cages separated by treatment. After being placed into cardboard cages, mosquitoes exposed to ESV as larvae were fed 20% sucrose solution mixed with ESV (2.27 $\log_{10}$ FFUeq ESV/ mL) via cotton pledgets for 24 h to increase the chances of ESV infection. Non-

ESV infected Control (no ESV exposure as larvae) adult mosquitoes were also fed via cotton pledgets filled with 20% sucrose solution *ad libitum*. After 24 h, all pledgets were replaced with a fresh 20% sucrose solution containing no ESV. All adult mosquitoes were deprived of sucrose solution and fed only water 24 h prior to vector competence experiments to improve blood feeding rate (Richards et al., 2017).

Vector Competence

Freshly propagated DENV-2, 6 d post inoculation in Vero cells, was mixed with defibrinated bovine blood (Hemostat, Dixon, CA) in a 1:1 ratio. Adult female mosquitoes 7-8 days old were fed warmed (35°C) DENV-2 blood meal via cotton pledgets containing 6.2 $\log_{10}$ PFUeq/mL for 1 h. After blood feeding, mosquitoes were anesthetized with cold and fully engorged females were transferred to new 1 L cardboard cages according to treatment group. All mosquitoes were incubated at 28°C with 85% humidity in a 14:10 light:dark cycle and fed a 20% sucrose solution *ad libitum* for the duration of the experiment [7 days post infection (dpi)]. At 7 dpi, live mosquitoes from each group were aspirated, anesthetize with cold, and legs and wings were removed. Mosquitoes were allowed to salivate into hematocrit tubes for *ca.* 35-45 min to test for transmission (Anderson et al., 2010). Leg, body and saliva samples for each mosquito were placed in separate 2 mL Eppendorf tubes containing 500 µL of RNALater and four 4 mm glass beads. All samples were stored at -80°C until further processing.

qRTPCR Analysis

Mosquito samples were homogenized and centrifuged using established methods (Richards et al., 2007). Nucleic acids were extracted using a QIAmp viral RNA kit (Qiagen, Valencia, CA). Viral RNA was quantified in each sample using quantitative real-time Taqman reverse transcriptase polymerase chain reaction (qRT-PCR) with the LightCycler® 480 instrument (Roche, Mannheim, Germany) and Superscript III One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). The instrument detection was programmed as follows: 48°C for 30 min, 95°C for 2 min, and 45 cycles at 95°C for 15 s, 60°C for 30 s, and finally, 40°C for 30 s. Primers and probes for DENV detection consisted of the following sequences: DENV-2 Rev 305-284: 5'- CCC CAT CTY TTC AGT ATC CCT G -3', DENV-2 FWD 237-251: 5'- CAT GGC CCT KGT GGC G -3', DENV-2 Probe254-274: 5'- /56-FAM/TCC TTC GTT / ZEN/TCC TAA CAA TCC/3IABkFQ/ -3' (IDTDNA, Coralville, IA). Primers and probes used for ESV detection consisted of the following sequences: ESV SEG A FWD: 5'-CCG CGC GGA GAC AAT CAC CT-3'; ESV SEG A REV: 5'-TTG GTC GAA CGC CCA CAC CG-3'; ESV SEG A PROBE ZENFAM: 5'-/F6-FAM/TGC TGG GTT /ZEN/CCA TTA CAG GTG GGA TGA /31ABkFQ/ -3' (IDTDNA, Coralville, IA). Standard curves for DENV were based on plaque assays used to determine titer. Standard curves for ESV were based on focus forming assays used to determine titer. Infection rate of mosquitoes is based the percentage of all blood fed mosquitoes having infected bodies. The dissemination rate is the percentage of mosquitoes that had infected bodies also having infected legs. Potential transmission rate was calculated as the number of mosquitoes that had infected bodies also having infected saliva (Anderson et al., 2010).

Statistical analysis

Chi-square statistical analysis were performed to detect differences in infection, dissemination, and potential transmission rates (p<0.05 as significance level) between groups (SPSS Institute, Chicago, Il.). Analysis of variance (ANOVA) was used to evaluate differences in body, leg, and saliva titers between groups. Body, leg, and saliva titers were log transformed to normalize data prior to performing data analysis. If significant differences were observed, a Duncan test was used to determine differences in means.

Results

Vector competence was measured in three different populations of *Ae. aegypti* mosquitoes in this study: 1) known for high dissemination of DENV, developed by crossing a Puerto Rico strain to Ibo strain *Ae. aegypti formosus*, generation F_{19} (BEI Resources; NR 45838), 2) known for low dissemination of DENV, developed by outcrossing D2S3 strain to Houston strain of *Ae. aegypti aegypti*, generation F_{18} (BEI Resources; NR 45837), 3) wild-type*,* generation F_{48} colony from Costa Rica (BEI Resources; MRA-726). Each population of mosquitoes was separated into two groups: a non-ESV exposed control group and an ESV-exposed group.

DENV-2 infection rates and body titers of *Aedes aegypti*

In the wild-type population (MRA-726), more (100%) non-ESV exposed control mosquitoes became infected with DENV-2 than treatment mosquitoes (88%) of ESV exposed mosquitoes became infected with DENV-2 (Table 1, Figure 4); however, infection rates were not significantly different between groups for this population (p=0.297, F= 5.104, df = 1,34). Body titers were significantly (p<0.01*,* F= 3.21, df= 1,32)

higher in non-ESV exposed control (1.7 ± 0.4 PFUeq DENV-2/ mL) compared to ESV-exposed mosquitoes (0.8 ± 0.2 log_{10} PFUeq DENV-2/ mL). In the high dissemination mosquito colony (NR-45838), infection rates between the ESV-exposed and non-ESV exposed control mosquitoes were 100% for both sub-groups (Table 1, Figure 4) and body titers were not statistically significant between non-ESV infected control (1.1 ± 0.3 log_{10} PFUeq DENV-2/ mL) and treatment (1.4 ± 0.4 log_{10} PFUeq DENV-2/ mL) groups (p= 0.902, F= 0.622, df= 1,23). In the low dissemination rate mosquito population (NR-45837), infection rates for the non-ESV exposed control and ESV-exposed groups were 100% and 91%, respectively (Table 1, Figure 4) and were not significantly different (p= 0.268, F= 6.360, df= 1,23); however, body titers were significantly (p<0.01, F= 1.054, df= 1,22) higher in non-ESV exposed (control) (1.2 ± 0.3 log_{10} PFUeq DENV-2/ mL) compared to ESV-exposed mosquitoes (0.6 ± 0.2 log_{10} PFUeq DENV-2/ mL).

DENV-2 dissemination rates and leg titers of *Aedes aegypti*

In the wild-type population (MR-726), dissemination rates for non-ESV exposed control and ESV-exposed mosquitoes were 74% and 87%, respectively, and were not significantly different (p= 0.168, F= 10.782, df = 1,31) (Table 2, Figure 5). However, for this population, leg titers were significantly higher (p=0.010, F= 3.294, df= 1,25) in non-ESV exposed control (3.0 ± 0.3 log_{10} PFUeq DENV-2/ mL) compared to ESV-exposed (0.8 ± 0.2 log_{10} PFUeq DENV-2/ mL) mosquitoes. Mosquitoes in the high dissemination colony (NR-45838) showed significantly higher (p= 0.022, F= 0.729, df= 1,24) dissemination rates for non-ESV exposed control (91%) than for ESV-exposed (29%) groups (p=0.170, F= 0.729, df= 1,24) (Table 2, Figure 5). However, leg titers for non-ESV exposed control (3.0 ± 0.5 log_{10} PFUeq DENV-2/ mL) and ESV-exposed (2.8 ± 0.2

log_{10} PFUeq DENV-2/ mL) mosquitoes were not significantly different (p= 0.878, F= 2.953, df= 1,12). In the low dissemination colony (NR-45837), no significant differences were observed in dissemination rates between non-ESV exposed control (79%) and ESV-exposed (90%) mosquitoes (p= 0.8548, F= 0.152, df= 1,23) (Table 2, Figure 5). For the same colony, leg titers were significantly higher (p=0.008, F= 0.604, df= 1,18) in non-ESV exposed control (3.9 ± 0.2 log_{10} PFUeq DENV-2/ mL) compared to ESV-exposed (2.9 ± 0.3 log_{10} PFUeq DENV-2/ mL).

DENV-2 transmission rates and saliva titers of *Aedes aegypti*

Wild type mosquitoes (MRA-726), had transmission (21%) only in non-ESV exposed control mosquitoes and saliva titers were 1.1 ± 0.4 log_{10} PFUeq DENV-2/ mL (Table 3, Figure 6). Similarly, in the high dissemination colony (NR-45838), transmission (10%) was observed in only non-ESV exposed control mosquitoes with saliva titers of 0.4 ± 0.0 log_{10} PFUeq DENV-2/ mL. In the low dissemination colony (NR-45837), no transmission was observed in either non-ESV exposed control or treatment groups.

Detection of ESV in mosquitoes

Previous experiments conducted by our lab were unable to consistently detect ESV in single adult mosquitoes (when mosquito larvae were exposed to ESV). Here, we detected ESV in the wild-type mosquito population (MRA-726), with 65% infection and 91% dissemination (Table 5). ESV titers were calculated for bodies (0.1 ± 0.2 log_{10} FFUeq ESV/mL) and legs (0.2 ± 0.1 log_{10} FFUeq ESV/mL); however, no ESV was detected in saliva samples. In the high dissemination colony (NR-45838), we detected ESV in mosquitoes with a 7 % infection rate and body titers of 0.6 log_{10} FFUeq ESV/mL

(Table 5). Neither ESV dissemination nor transmission was detected in this colony. For the low dissemination colony (NR-45837), we did not detect ESV infection, dissemination, or transmission in ESV-exposed mosquitoes.

Discussion

We investigated vector competence of three different *Ae. aegypti* populations exposed to an insect-infecting virus (ESV) prior to being fed a DENV-2-infected blood meal. In this study, we show that ESV may play a role in the vector competence of DENV-2 in multiple populations of mosquitoes. Although our sample size was relatively small, we did not observe differences in infection, dissemination and transmission rates between non-ESV exposed control and ESV-exposed groups within each population. Prior exposure to ESV did not inhibit mosquitoes from becoming infected with DENV-2. However, reduced body, leg and saliva DENV-2 titers were observed in ESV-exposed (superinfected) mosquitoes compared to non-ESV exposed control mosquitoes, indicating that ESV may partially block DENV-2 replication in mosquitoes. Further studies are needed to evaluate these effects at different doses of DENV. This *in vivo* experiment follows the results of our previous *in vitro* experiments showing that ESV did not inhibit DENV-2 entry into mosquito cells under the conditions of our test. However, plaque assays showed a reduction in infectious DENV-2 particles when C6/36 cells were co-infected with DENV-2 and ESV prior to plaque assay on Vero cells. The observation in cell culture is supported by our mosquito experiments, with ESV-exposed mosquitoes having significantly lower DENV-2 titers compared to non-ESV infected control mosquitoes. Furthermore, the method for ESV infection of mosquitoes may impact measures of vector competence. In our study, we first reared mosquitoes in ESV

larval water and then exposed mosquitoes to ESV via sugar feeding. This may have increased the ESV in some mosquitoes that fully engorged on sugar containing ESV versus mosquitoes that did not receive a full sugar-ESV meal.

With the discovery of insect-infecting viruses, additional attention should be paid to determine the extent to which these viruses may impact vector competence. In a previous study (Bolling et al., 2012), mosquitoes persistently infected with *Culex* Flavivirus (CxFV) had significantly lower dissemination of West Nile virus among of CxFv infected mosquitoes than single-infected mosquitoes at 7 dpi. However, at 14 dpi, infection, transmission and dissemination rates of WNV did not differ between CxFv-exposed and control groups (Bolling at al., 2012). Hence, it is possible that some insect-infecting viruses may inhibit replication of other viruses during the early stages of infection. In a case control study using *Culex pipiens* mosquito pools collected from the southwestern Chicago suburbs, results indicated a four-fold increased probability of infection of WNV-positive pools with CxFV relative to spatiotemporally matched WNV-negative pools. This study indicated a positive ecological association between CxFV and WNV (Newman et al.2011). Genetic variations in mosquito populations and/or the viral isolate may impact the host effects of insect-infecting viruses. Co-infection experiments with simultaneous infection of Honduras *Cx. quinquefasciatus* (Say) with WNV from Guatemala and CxFv Izabal resulted in increased transmission of WNV rates of co-infected mosquitoes. A similar study performed on a different population of mosquitoes (Sebring *Cx. quinquefasciatus*) infected with WNV (Guatemala) showed no significant difference in transmission rates of single (CxFv) and co-infected (CxFv and WNV) mosquitoes (Kent et al., 2010). Consequently, insect-infecting viruses may

enhance transmissibility of certain flaviviruses (Blitvich et al., 2015). This should be tested under a variety of environmental conditions.

Superinfection exclusion, in which a cell infected with one virus cannot be secondarily infected with another virus, is of particular interest in studying effects of certain insect-infecting viruses and medically important arboviruses (Kenney et al., 2014). Superinfection studies have indicated that the effect of exclusion of the secondary virus generally does not take effect until at least one hour following infection of the initial virus (Eaton, 1979; Johnston et al., 1974). In addition, some insect-infecting viruses (such as ESV) may not replicate in some vertebrate cells. If this is consistent across all types of vertebrate cells, this could lead to novel approaches using insect-infecting viruses to combat medically important arboviruses. New approaches are being generated such as using Eilat virus (EILV), an insect-infecting alphavirus, as a platform for chikungunya virus (CHIKV) vaccine. A study showed that a chimera of EILV/CHIKV protected two mouse models from all measures of CHIKV up to 292 days post vaccine (Erasmus et al., 2018). With the growing number of insect-specific viruses being discovered it is imperative to explore ways they may be used to reduce the impact of medically important arboviruses. With the re-emergence and expansion of viruses such as DENV and ZIKV it is imperative we discover new ways to combat these public health threats.

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Chapter V – Conclusion

Arbovirus surveillance has led to the discovery of insect-infecting viruses in mosquitoes. Initially studies of these insect-infecting viruses were limited as the viruses were deemed to be of little importance. In recent years, renewed interest in these viruses have been born out of their potential effects and possible interference with viral replication of medically important arboviruses. Espirito Santo virus (ESV), an insect-infecting virus, was recently discovered in a patient sample from Brazil. The virus was discovered in a biological sample containing DENV, leading to the current study on the potential effects of ESV on DENV-2. We have shown in our *in vitro* experiments (illustrated by plaque assay) that ESV may inhibit DENV from entering cells. Our findings also show that ESV is able to replicate in the absence of DENV-2 in the mosquito under the conditions of this test. This finding suggests that ESV may be able to replicate in the mosquito in the absence of co-infection of DENV-2. Further studies are planned to investigate the possibility of transovarial transmission of ESV in mosquitoes. Simultaneous co-infection of C6/36 cells with ESV and DENV-2 (44/2), show neither virus inhibits the other from entering the cell. Observations of IFAs show the presence of DENV-2 proteins in cells co-infected with ESV and no differences in staining; however, plaque assays assessing co-infections of ESV and DENV-2 in C6/36 cells showed a reduced amount of DENV-2 plaques. These results indicate, that ESV may affect the replication process of DENV-2. Further studies are needed to elucidate these observed differences. Although C6/36 cells lack a functional antiviral RNAi response, we expected these results to be duplicated to an extent in *in vivo* studies (Brakney et al., 2010).

In vivo studies using three different populations of *Ae. aegypti* allowed us to study viral superinfection. Using a wild-type population of mosquitoes from Costa Rica, high dissemination population from Puerto Rico and a low dissemination population from the United States, we infected larval mosquitoes with ESV, and subsequently blood fed adult mosquitoes with an infectious blood meal containing DENV-2. We did not observe lower rates of DENV-2 infection, dissemination and transmission; however, we did observe lower DENV-2 titers in ESV-exposed compared to control (non-ESV exposed) mosquitoes. While, our sample size was relatively small, these results highlight the need for additional testing on the effects of insect-infecting viruses against medically important arboviruses, such as DENV. As insecticide resistance increases globally, geographic ranges of mosquitoes continue to increase, and international travel continues to increase, novel approaches are needed to combat arboviral diseases impacting public health.

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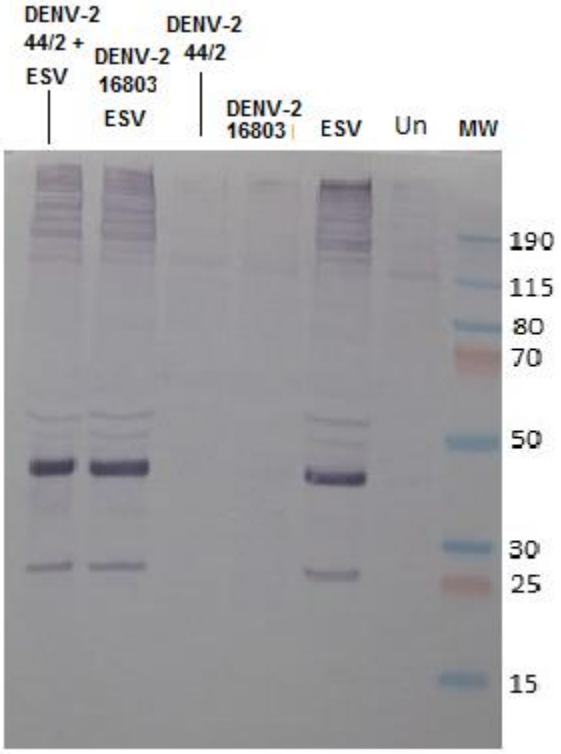
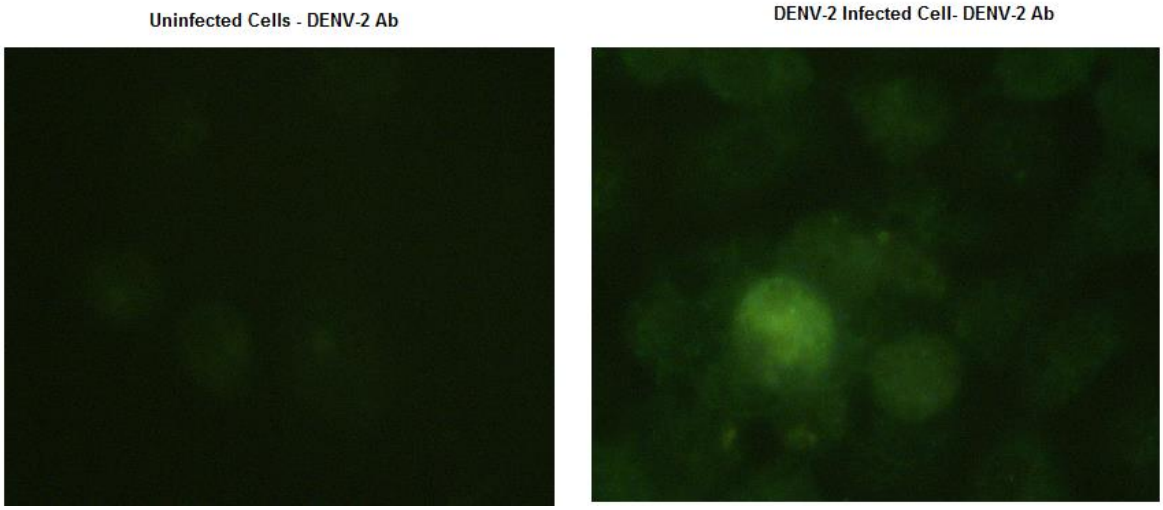
Appendix

Figure 1.*Detection of ESV Proteins*

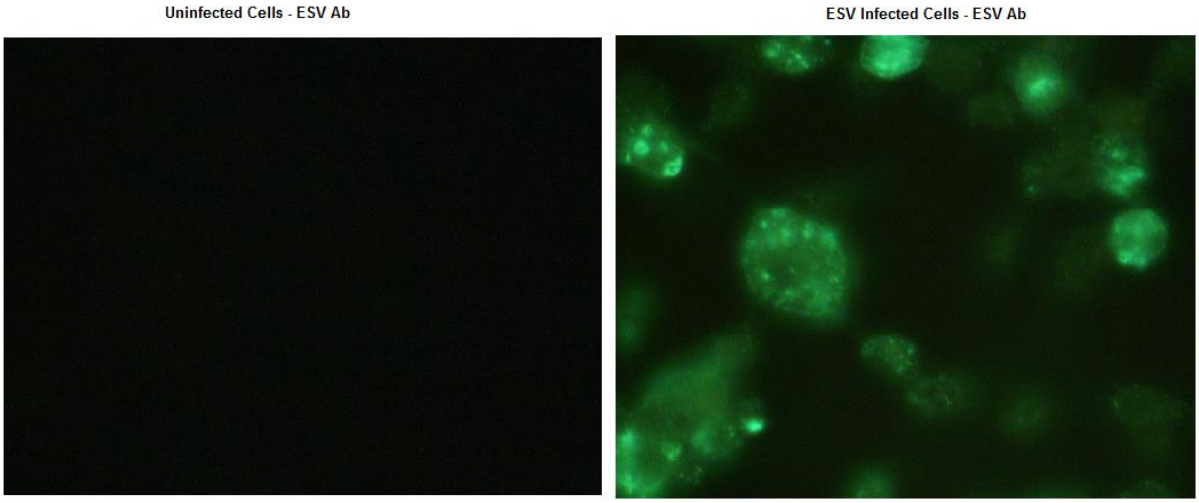
a. Western blot showing ESV proteins (anti-ESV antibody) expressed in ESV and ESV/DENV-2 infected C6/36 cells



b. Immunofluorescence assay showing DENV-2 (44/2) proteins in DENV-2 infected C6/36 cells



Immunofluorescence assay showing ESV proteins in ESV infected C6/36 cells



c. Western blot showing ESV band present (anti-ESV antibody) when ESV alone and ESV co-infected with DENV-2 (44/2) in C6/36 cells. Apparent increase may be due to more proteins loaded onto wells.

d. Confocal imaging of ESV proteins in C6/36 cells

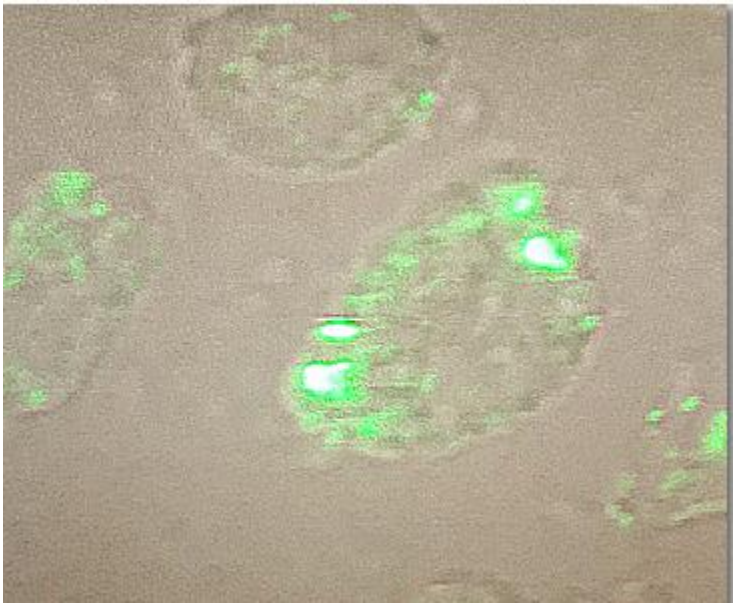
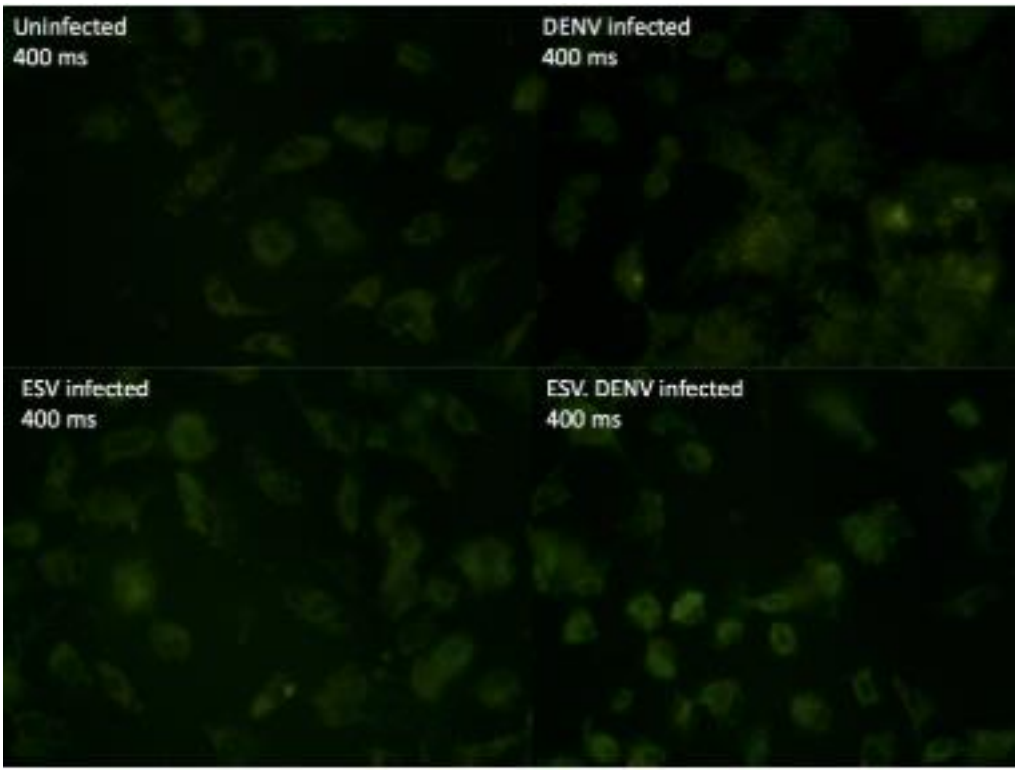


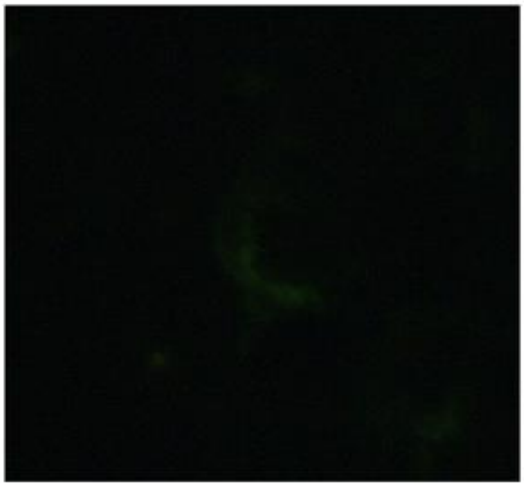
Figure 2. No difference in DENV-2 (44/2) proteins

a. Immunofluorescence showing no difference in DENV-2 (44/2) proteins (anti-DENV antibody) for DENV-2 alone compared to DENV-2 co-infected with ESV in C6/36 cells.

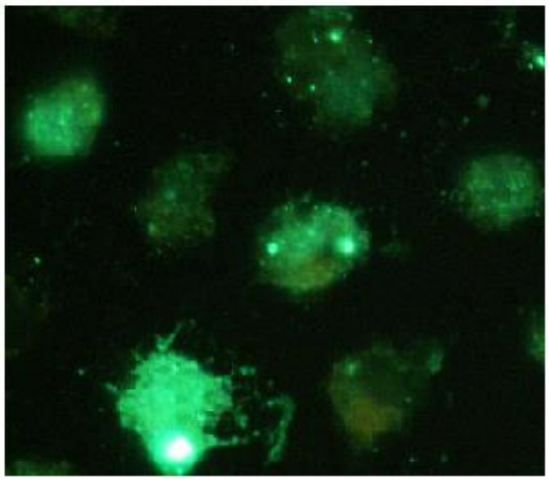


b. ESV protein expression in ESV + DENV-2 (44/2) co-infected C6/36 cells

Uninfected Cells -ESV Ab



ESV Infected Cells - ESV Ab



DENV-2 + ESV Infected Cells -ESV Ab

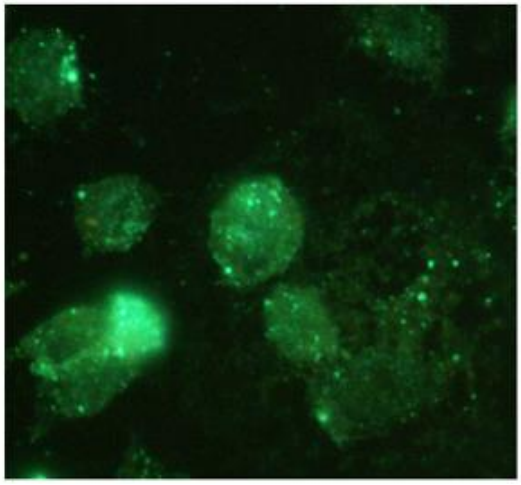
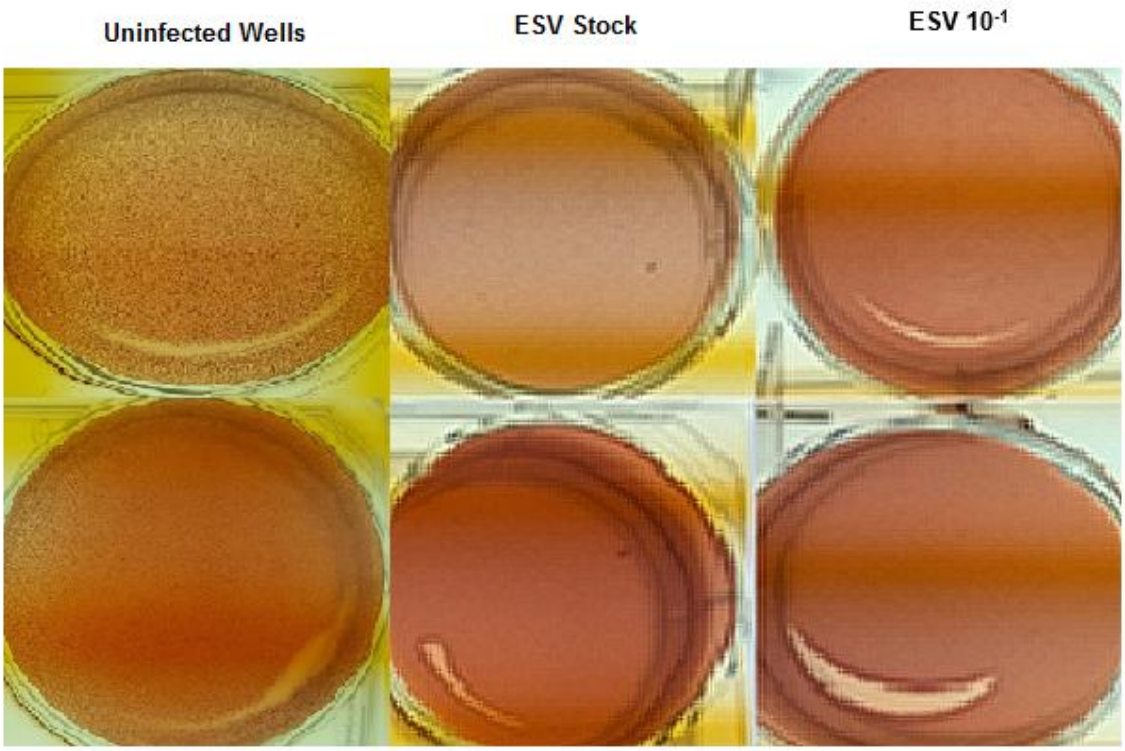
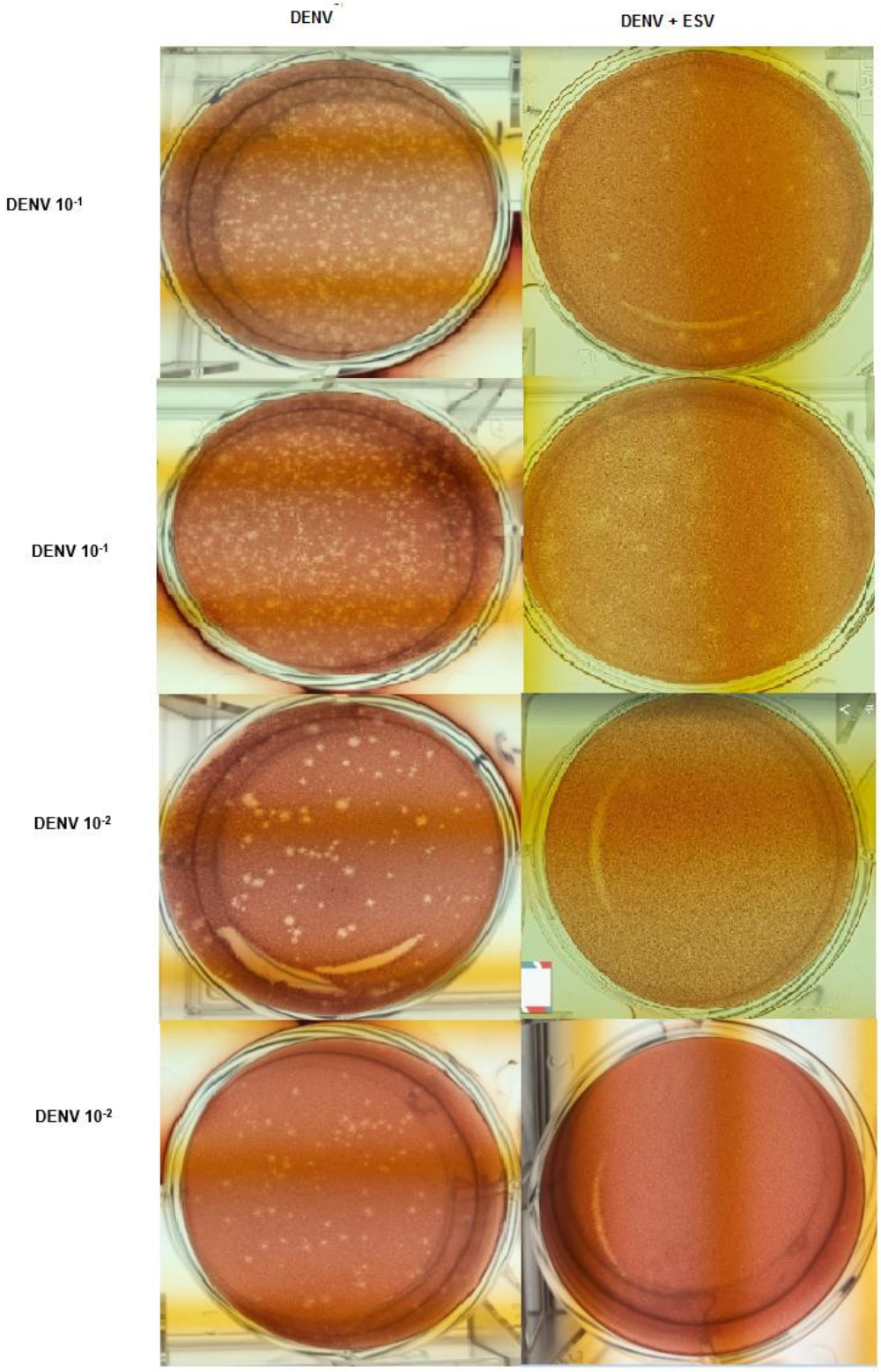


Figure 3. ESV suppresses DENV-2 replication in plaque assay

a. Comparison of ESV infected wells and non-ESV infected control wells showing no plaques, verifying that ESV does not cause plaques in Vero cells.



b. Serial dilutions of DENV-2 (44/2) alone and DENV-/ESV (stock) wells showing fewer plaques in wells containing ESV in Vero cells reduction.



c. Bar graph showing plaque assay data of counts of DENV-2(44/2) plaques in wells with DENV-2 alone, ESV alone, and wells co-infected with ESV and DENV-2

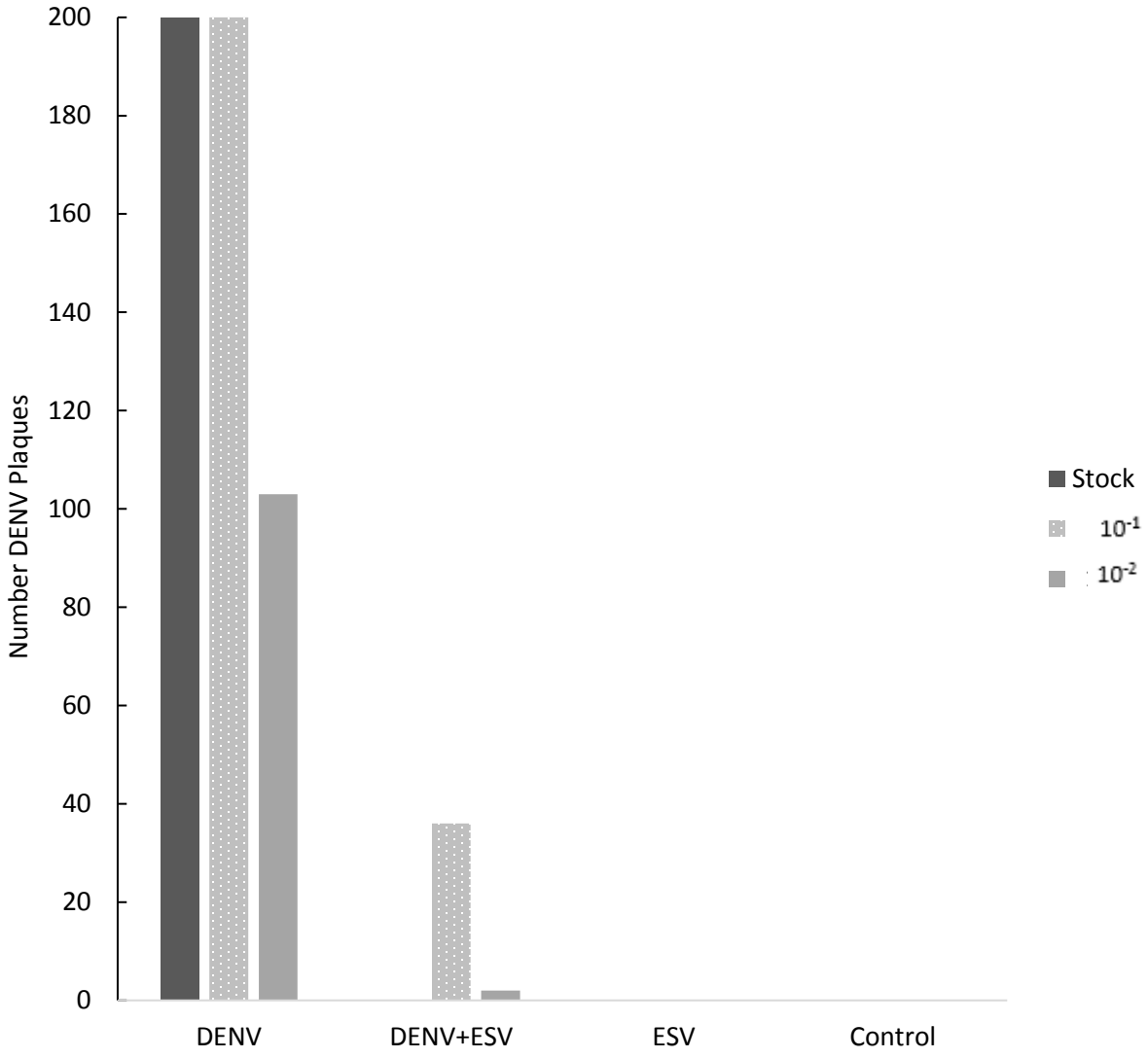


Figure 4. Rates of DENV-2 (44/2) infection, dissemination and transmission potential, 7 days post blood meal in *Aedes aegypti*.

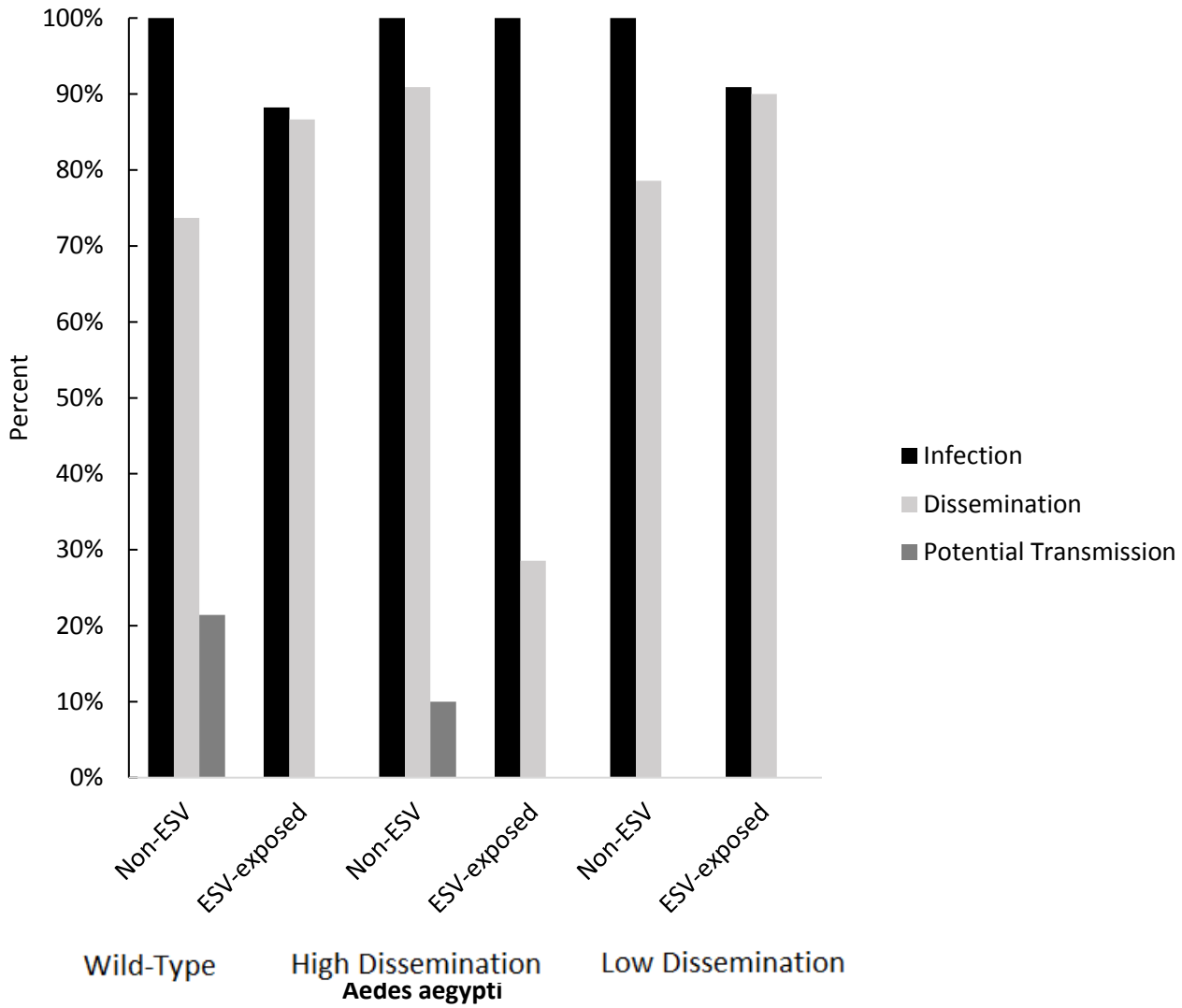


Figure 5. Rates of ESV infection, dissemination and transmission potential 7 days post blood meal in *Aedes aegypti*. *Aedes aegypti* reared in larval water inoculated with ESV and adults fed 20% sucrose solution containing ESV after emergence.

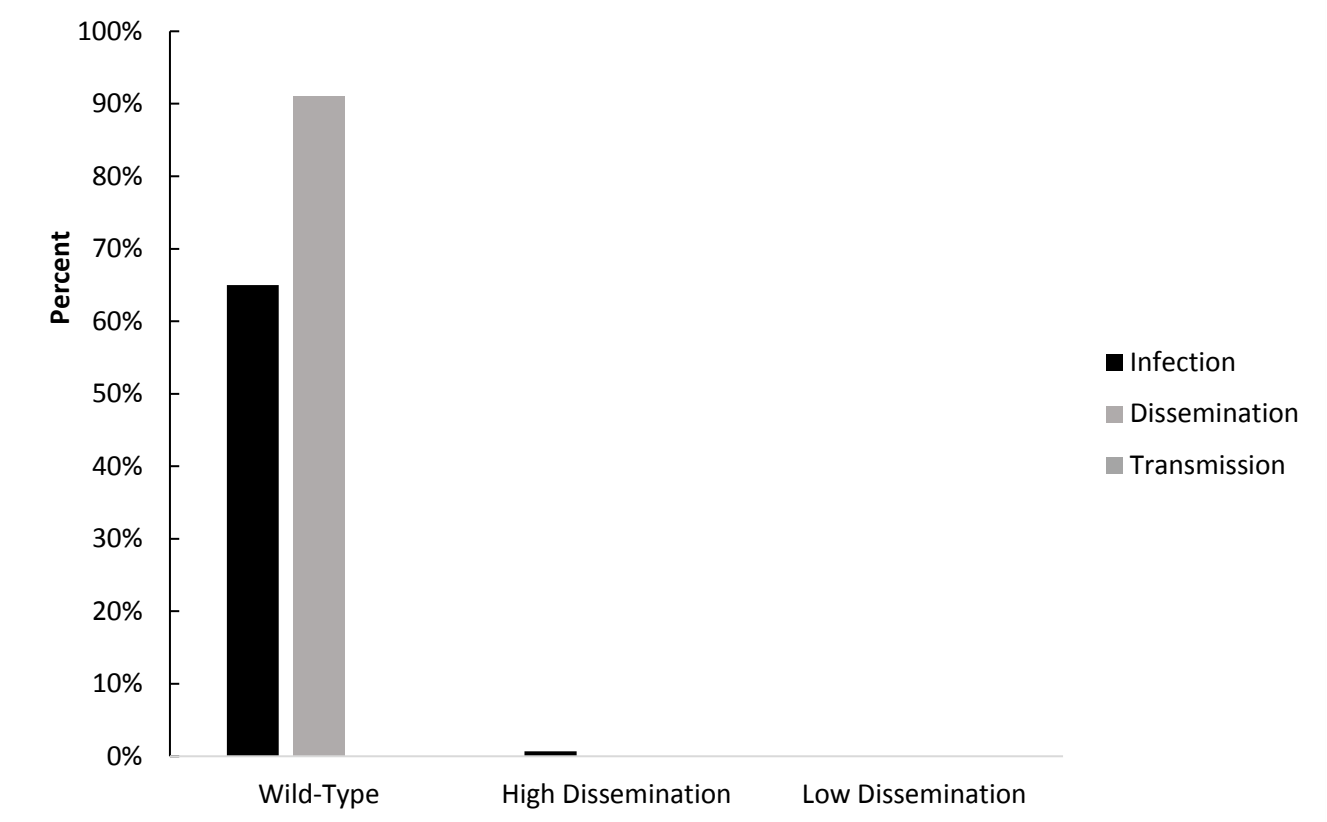


Table 1. The mean DENV-2 (44/2) qRTPCR detected plaque forming equivalents (log_{10} PFUeq DENV/ mL) ± standard error and rates of infection (% with DENV-2-positive bodies) for *Aedes aegypti* (larval rearing water inoculated with ESV for ESV-exposed mosquitoes) fed DENV-2-infected blood meals and incubated at 28°C for 7 d. Significantly higher values within mosquito populations for titer indicated with asterisk.

DENV-2 Infection Rates and Body Titers			
MRA-726 (wild-type colony)			
	No. tested	No. Body Infection (%)	Body Titer
control	19	19 (100%)	1.7 ± 0.4*
ESV	17	15 (88%)	0.8 ± 0.2
NR-45838 (high dissemination colony)			
	No. tested	No. Body Infection (%)	Body Titer
Control	11	11 (100%)	1.1 ± 0.3
ESV	14	14 (100%)	1.4 ± 0.4
NR-45837 (low dissemination colony)			
	No. tested	No. Body Infection (%)	Body Titer
Control	14	14(100%)	1.2 ± 0.3*
ESV	11	10 (91%)	0.6 ± 0.2

Table 2. The mean DENV-2 (44/2) qRTPCR detected plaque forming equivalents (log_{10} PFUeq DENV/ mL) ± standard error and rates of dissemination (% with DENV-2-positive legs) for *Aedes aegypti* (larval rearing water inoculated with ESV for ESV-exposed mosquitoes) fed DENV-2-infected blood meals and incubated at 28°C for 7 d. Significantly higher values within mosquito populations for dissemination and/or leg titer indicated with asterisk.

DENV-2 Dissemination Rates and Leg Titers			
MRA-726 (wild-type colony)			
	No. Tested	No. Dissemination (%)	Leg Titer
Control	19	14 (74%)	3.0 ± 0.3*
ESV	15	13 (87%)	0.8 ± 0.2
NR-45838 (high dissemination colony)			
	No. tested	No. Dissemination (%)	Leg Titer
Control	11	10 (91%)*	3.0 ± 0.5
ESV	14	4 (29%)	2.8 ± 0.2
NR-45837 (low dissemination colony)			
	No. tested	No. Dissemination (%)	Leg Titer
Control	14	11 (79%)	3.9 ± 0.2*
ESV	10	9 (90%)	2.9 ± 0.3

Table 3. The mean DENV-2 (44/2) qRTPCR detected plaque forming equivalents (log_{10} PFUeq DENV/ mL) ± standard error and rates of potential transmission (% with DENV-positive saliva) for *Aedes aegypti* (larval rearing water inoculated with ESV for ESV-exposed mosquitoes) fed DENV-2-infected blood meals and incubated at 28°C for 7 d.

DENV-2 Transmission Potential Rates and Saliva Titers			
MRA-726 (wild-type colony)			
	No. Tested	No. Transmission (%)	Saliva Titer
Control	14	3 (21%)	1.1 ± 0.4
ESV	13	0 (0%)	0
NR-45838 (high dissemination colony)			
	No. tested	No. Transmission (%)	Saliva Titer
Control	10	1 (10%)	0.4
ESV	4	0 (0%)	0
NR-45837 (low dissemination colony)			
	No. tested	No. Transmission (%)	Saliva Titer
Control	11	0 (0%)	0
ESV	9	0 (0%)	0

Table 4. The mean detected qRTPCR focus forming equivalents (log_{10} FFUeq ESV/ mL) ± standard error and rates of infection, dissemination and transmission (% with ESV-positive bodies, legs and saliva) for *Aedes aegypti*. Mosquitoes were reared in ESV inoculated larval water and new emerged adults were allowed to feed on a 20% sucrose solution containing ESV for 24 h. Mosquitoes were fed DENV-2 (44/2) infected blood meals and incubated at 28°C for 7 d.

Rates of ESV Infection, Dissemination, and Transmission Potential, and Body, Leg, and Saliva Titers			
	No. Tested	MRA-726	ESV Titer
Infection (bodies)	17	11 (65%)	0.1 ± 0.2
Dissemination (legs)	11	10 (91%)	0.2 ± 0.1
Transmission (saliva)	10	0	0
	No. Tested	NR-45838	ESV Titer
Infection (bodies)	14	1 (7%)	0.6
Dissemination (legs)	1	0	0
Transmission (saliva)	0	0	0
	No. Tested	NR-45837	ESV Titer
Infection (bodies)	11	0	0
Dissemination (legs)	0	0	0
Transmission (saliva)	0	0	0