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Characterization of a novel insect-specific flavivirus from Brazil: potential for inhibition of infection of arthropod cells with medically important flaviviruses

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Abstract

In the past decade there has been an upsurge in the number of newly described insect-specific flaviviruses isolated pan-globally. We recently described the isolation of a novel flavivirus (tentatively designated “Nhumirim virus”; NHUV) (Pauvolid-Correa *et al.*, in review) that represents an example of a unique subset of apparently insect-specific viruses that phylogenetically affiliate with dual-host mosquito-borne flaviviruses despite appearing to be limited to replication in mosquito cells. We characterized the *in vitro* growth potential, 3' untranslated region (UTR) sequence homology with alternative flaviviruses, and evaluated the virus's capacity to suppress replication of representative *Culex* spp. vectored pathogenic flaviviruses in mosquito cells. Only mosquito cell lines were found to support NHUV replication, further reinforcing the insect-specific phenotype of this virus. Analysis of the sequence and predicted RNA secondary structures of the 3' UTR indicate NHUV to be most similar to viruses within the yellow fever serogroup, Japanese encephalitis serogroup, and viruses in the tick-borne flavivirus clade. NHUV was found to share the fewest conserved sequence elements when compared to traditional insect-specific flaviviruses. This suggests that, despite being apparently insect-specific, this virus likely diverged from an ancestral mosquito-borne flavivirus. Co-infection experiments indicated that prior or concurrent infection of mosquito cells with NHUV resulted in significant reduction in viral production of West Nile virus (WNV), St. Louis

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encephalitis virus (SLEV) and Japanese encephalitis virus. The inhibitory effect was most effective against WNV and SLEV with over a million-fold and 10,000-fold reduction in peak titers, respectively.

INTRODUCTION

New sequencing technologies have drastically improved the capabilities for rapid genetic characterization of novel viruses and microorganisms, for both emerging pathogens of animals as well as non-pathogenic microflora and microfauna that could modulate the receptivity of hosts to infection with pathogens of medical and veterinary importance. This has been exemplified recently by the identification of numerous novel flaviviruses (Aliota *et al.*, 2012; Cook *et al.*, 2009; Crabtree *et al.*, 2009; Evangelista *et al.*, 2013; Hobson-Peters *et al.*, 2013; Hoshino *et al.*, 2009; Huhtamo *et al.*, 2009; Junglen *et al.*, 2009; Kolodziejek *et al.*, 2013; Lee *et al.*, 2013b; Parreira *et al.*, 2012; Roiz *et al.*, 2009; Sang *et al.*, 2003; Vazquez *et al.*, 2012) with an arthropod-restricted host range that, although not known to directly illicit disease in vertebrates, could alter the capacity of arthropods to transmit vector-borne pathogens. Studies demonstrating the role of mosquito microbiome in the modulation of vector competence for arboviruses capable of eliciting disease in humans underscores the potential that infection with insect-specific flaviviruses could similarly modulate transmission of human arboviral pathogens. (Bolling *et al.*, 2012; Cirimotich *et al.*, 2011; Gubler, 2002; Hobson-Peters *et al.*, 2013; Kent *et al.*, 2010a; Weiss & Aksoy, 2011).

Flaviviruses are enveloped viruses comprised of a single-stranded, positive-sense RNA genome of approximately 11 kb consisting of a 5' and 3' untranslated regions and a methylated cap that allows for direct translation of a single open reading frame (ORF) resulting in a of a single polyprotein (Markoff, 2003; Wengler *et al.*, 1978). The ORF encodes three structural proteins including the capsid (C), premembrane/membrane (prM), and envelope (E), and seven nonstructural proteins including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Castle *et al.*, 1986; Rice *et al.*, 1985) that are cleaved co- and post-translationally by host and viral proteases. Analyses of flavivirus genomes have demonstrated them to cluster phylogenetically by host preference range: insect specific flaviviruses (ISFs), dual-host tick-borne flaviviruses (TBFVs), viruses with no known vector (NKV), or mosquito-borne flaviviruses (MBFVs) (Gould *et al.*, 2003; Kuno *et al.*, 1998).

ISFs constitute a relatively novel group of flaviviruses characterized as single-host viruses that replicate in insects and have demonstrated replication incompetence in vertebrate cells. To date, the only arthropod source of insect specific flaviviruses (ISFs) has included members of the order *Diptera*, predominantly mosquitoes and sandflies. It remains to be determined if ISFs exist in alternative arthropod taxa such as ticks. TBFVs are categorized into two groups: the seabird tick-borne group and the mammalian tick-borne group. The NKV group can be sub-grouped into viruses associated with bats or rodents. MBFVs with dual hosts such as Japanese encephalitis virus (JEV) and yellow fever virus (YFV) form distinct phylogenetic clades that correlate with the mosquito genus primarily associated with viral transmission. However, some viruses, such as Entebbe bat virus (EBV) and Yokose virus (YOKV), with no apparent mosquito vector also fall into the MBFV phylogenetic

grouping. It is unclear as to whether the vector for these viruses has yet to be detected or if they have lost the ability for mosquito-borne transmission (Kuno *et al.*, 1998).

Like dual-host MBFVs, classically recognized ISFs form two phylogenetic subgroups based on whether they are vectored by *Aedes* or *Culex* mosquitoes. *Aedes* associated viruses such as cell fusing agent virus (CFAV), *Aedes* flavivirus (AeFV), and Kamiti River (KRV) virus have been isolated respectively from Puerto Rico (Cook *et al.*, 2006), Japan (Hoshino *et al.*, 2009), and Kenya (Crabtree *et al.*, 2003). However, homologous viral sequences have also been identified in Spain (Aranda *et al.*, 2009; Sanchez-Seco *et al.*, 2010), Italy (Calzolari *et al.*, 2010; Roiz *et al.*, 2009), and Canada (Pabbaraju *et al.*, 2009) indicating a widespread geographic distribution. ISFs identified to infect *Culex* mosquitoes include Quang Binh virus (QBV) isolated from Vietnam (Crabtree *et al.*, 2009), Calbertado virus (CLBOV) isolated from North America (Bolling *et al.*, 2011), and *Culex* flavivirus (CxFV), which has been isolated from Trinidad-Tobago (Kim *et al.*, 2009), Guatemala (Morales-Betoulle *et al.*, 2008), Mexico (Farfan-Ale *et al.*, 2009; Farfan-Ale *et al.*, 2010), Uganda (Cook *et al.*, 2009), USA (Blitvich *et al.*, 2009; Crockett *et al.*, 2012; Kim *et al.*, 2009), and Japan (Hoshino *et al.*, 2009). Nakiwogo virus (NAKV), a monophyletic *Culex* specific ISF, was isolated from *Mansonia africana nigerrima* (Cook *et al.*, 2009). A number of studies have highlighted the potential for ISFs to have an inhibitory effect on co-infecting flaviviruses of medical importance. For example, CxFV has been shown to suppress the capacity for *Culex* spp. to become infected and transmit WNV (Bolling *et al.*, 2012; Kent *et al.*, 2010a). Similarly, a potential role of superinfection exclusion was indicated by reduced replication of Kunjin and Murray Valley fever virus in the presence of the ISF Palm Creek virus in C6/36 cells (Hobson-Peters *et al.*, 2013).

Interestingly, there is a growing number of ISF-like isolates that appear to be phenotypically insect-specific with no indication of replication in vertebrates, yet are phylogenetically distinct from the ISF clade, as they group with other dual-host MBFVs. These isolates, characterized for the purposes of this manuscript as unidentified vertebrate host (UVHs) viruses in the MBFV group, are limited to replication in arthropod cells include: Nounané virus (NOUV) (Junglen *et al.*, 2009), Lammi virus (LAMV) (Huhtamo *et al.*, 2009), Chaoyang virus (CHAOV) (Lee *et al.*, 2013a), Barkedji virus (BJV) (Kolodziejek *et al.*, 2013), and Nanay virus (NANV) (Evangelista *et al.*, 2013). Like viruses from the ISF phylogenetic cluster, these viruses have been isolated from a wide geographic range including Israel, Peru, Finland, Côte d'Ivoire, the Republic of Korea, and China.

Continued isolation and characterization of these unique flaviviruses will provide key insights into the evolution of vector/host adaptation and, potentially, flavivirus origins. Herein, we describe the characterization of a novel mosquito-borne flavivirus, tentatively designated Nhumirim virus (NHUV), isolated from the Pantanal region of Brazil (Pauvolid-Correa *et al.*, in review) that appears to be most closely related to other novel flaviviruses that have insect-specific host replication capabilities but differ from their projected phylogenetic relationships by grouping within dual-host MBFVs. We evaluated its phylogenetic relationship to other flaviviruses, identified permissive cell lines *in vitro*, analyzed the predicted secondary structure of the 3' UTR, and demonstrated the virus's

ability to suppress replication of representative *Culex* spp. vectored pathogenic flaviviruses *in vitro*.

RESULTS

Virus Isolation and *in vitro* characterization

A novel flavivirus tentatively designated Nhumirim virus (NHUV) was isolated from a single pool of 43 non-engorged adult female *Culex chidesteri* collected in April 2010 in Brazil (Pauvolid-Correa *et al.*, in review). Evidence of cytopathic changes were definitively identified six days following initial inoculation onto C6/36 cells, whereas no CPE was observed from initial inoculation on Vero cells. Upon secondary passage in C6/36, NHUV manifested CPE in the form of rounded cells still attached to the monolayer, observable within 3 days post-infection (dpi), and limited syncytia development by 6 dpi (Fig. 1). The C6/36 TCID₅₀ of the stock isolated from the second passage of NHUV was 9.1 log₁₀ TCID₅₀/ml and was used to inoculate additional cell lines. While NHUV was able to replicate in other mosquito cell lines including *Ae. albopictus* C7/10, C6/36, and *Cx. quinquefasciatus* cells, the virus did not replicate in alternative invertebrate cells. Inoculation of ISE6 tick cells failed to generate detectable infectious virus assayed on C6/36 cells as screened by IFA (Fig. 2). Furthermore, RNA extracted from culture supernatants of the second passage were RT-PCR negative using pan-flavivirus primers. Attempted culturing in vertebrate cell lines, including Vero, BHK21, DF-1, and *Xenopus laevis*, proved unsuccessful as confirmed by negative RT-PCR amplification of the second passage and lack of detectable antigen detection by IFA using a pan-flavivirus (4G2) monoclonal antibody developed from a Dengue 2 (New Guinea C) strain (Gentry *et al.*, 1982) (Table 1).

Sequence and phylogenetic analysis

The complete NHUV genome, including the 5' and 3' UTRs, was sequenced and identified to be 10,891 nucleotides (nt) in length. The predicted open reading frame (ORF) was 10,338 nt, while the 5' UTR was 102 nt, and the 3' UTR 451 nt. Three flavivirus-type structural proteins C, prM, E, and seven flaviviral non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 were identified (Table 2) and polyprotein cleavage sites were predicted (Table 3). The full viral sequence has been deposited in GenBank under accession number KJ210048 (Pauvolid-Correa *et al.*, in review). The coding region of NHUV had the greatest nucleotide identity to BJV (65.9%) and NOUV (56.2%), both recent isolates that have demonstrated the unique phenotypic characteristics of UVH viruses (Table 4). Maximum likelihood phylogenetic analysis of the ORF regions of 59 flavivirus sequences similarly indicated that NHUV was most closely related to a group of novel UVH viruses that cluster within the dual-host mosquito borne flaviviruses with strong bootstrap support (Fig. 3). Interestingly, viruses that have been shown to be restricted to growth in insect cells within the MBFV clade cluster in two distinct branches with robust bootstrap support: one consisting of DGV, LAMV, and CHAOV, with the other branch containing NHUV, BJV, and NOUV (Fig. 3).

3' UTR characterization

Studies of the flavivirus 3' UTR have identified a number of direct repeats that appear to have evolved from a progenitor of six long repeated sequences (LRS) that are homologous to extant TBFV 3' UTR sequences (Gritsun & Gould, 2006c; 2007b). Alignments between the flavivirus 3' UTRs have indicated the evolutionary remnants of these LRSs can be identified in MBFV, NKV, and ISFV and tend to show conservation within each group (Gritsun & Gould, 2007a; b). Comparison of the NHUV 3' UTR to representative viruses for each flavivirus group MBFV, TBFV, NKV, and ISFV were made in order to identify homologous regions of conservation (Fig. 4). NHUV was identified to have several conserved structural elements in common with viruses in the MBFV group including a 3' terminal long-stem loop (3'LSH) with conserved pentanucleotide sequence CACAG, a conserved stem loop (SL2), and a conserved dumbbell shaped element (DB1) with an internal conserved sequence element CS2 (Fig. 4). A third MBFV conserved sequence element, previously identified as CS1, was found to be incorporated with a small stem loop structure (SL3) (Markoff, 2003). The absence of a Y-shaped structure typically conserved between TBFVs, NKVs, and ISFVs was noted (Fig. 4). The only common features that NHUV had with ISFVs was the 3' LSH structure and a pentanucleotide sequence although only four out of five nucleotides were conserved. Comparisons of NHUV to other representative UVH viruses were inconclusive as many of these have incomplete 3' UTR sequences available.

Codon usage frequencies

Studies of codon usage have indicated that certain codon dinucleotide pairings are utilized preferentially and this codon bias can often be correlated to match an organism's transfer RNA pool (Akashi, 1994; Clarke, 1970; Ikemura, 1981). In particular, TA dinucleotides have been found to be proportionally underutilized for encoding amino acids in both vertebrate and invertebrate hosts in order to minimize targeting by endoribonucleases. In addition, vertebrate hosts underutilize CG dinucleotides to reduce DNA methylase targeting and subsequent increased mutation rates at these sites (Zhao & Jiang, 2007). Examination of RNA viruses have indicated that they have evolved optimized codon usage for facilitated replication in hosts utilized (Lauring *et al.*, 2012). In order to utilize these potential codon usage biases for the assessment of the potential vertebrate host infectivity by NHUV, we generated histograms of leucine (TA dinucleotide containing codons) and threonine, proline, and arginine codon usage frequencies (CG dinucleotide containing codons) for NHUV, WNV, CxFV, and MODV. As would be expected, all four viruses demonstrated an underutilization of TA (CTA and TTA codons) dinucleotides (Fig. 5a; Leu panel). MODV was found to highly underutilize CG dinucleotides with the mean codon frequency of these codons encoding Arg (CGT, CGC, CGA and CGG) to be $4.5\% \pm 0.1$. In contrast, CxFV exhibited a usage of $19.3\% \pm 2.8$ and $11.4\% \pm 6.3$ for CG and non-CG codons. NHUV was identified to mirror the codon usage frequencies of CG dinucleotide codons (threonine, leucine, and proline) of WNV. Both WNV [$11\% \pm 2.9$ (non-CG) / $28\% \pm 7.9$ (CG)] and NHUV [$13.5\% \pm 3.7$ (non-CG) / $22.3\% \pm 21.4$ (CG)] demonstrate a bias against CG dinucleotide codons that was less significant than that of MODV (Fig 5).

Inhibition of WNV, JEV, and SLEV growth

C6/36 cells were inoculated at an MOI of 5 with NHUV and challenged at day 0 (co-infected), day 1, day 3, and day 5 post infection with WNV at an MOI of 0.1. Similar studies were performed with JEV and SLEV infection at 0 and 3 days post NHUV infection to determine if NHUV affects alternative representative MBFV flaviviruses isolated from *Culex spp.* mosquitoes. A two-way repeated measures ANOVA indicated a significant difference between NHUV co-infected groups and control viruses for WNV, JEV, and SLEV ($p < .0001$). A secondary Dunnett's multiple comparison test with a corrected p-value, found all control infections of WNV, JEV, or SLEV alone to have a significantly higher ($p < 0.0001$) average titer for each daily time point sample (day 2 post-infection through day 7 post-infection) as compared to groups pre- or co-infected with NHUV (Fig. 6). Comparison of SLEV and SLEV+NHUV d0pi, also from the D1 time point, was the only comparison in which a NHUV co-infected group did not show significantly reduced replication as compared to the control. The control viruses WNV, JEV, and SLEV alone achieved a $6.2 \log_{10}$ (PFU/ml), $1.2 \log_{10}$ (PFU/ml), and $4.3 \log_{10}$ (PFU/ml) higher mean peak titer than matched groups coinfecting with NHUV (Fig. 6). These differences in peak titer translate to 1.5 million-fold reduction for WNV, an 80-fold reduction for JEV, and a 15,000-fold reduction for SLEV in the presence of NHUV *in vitro*.

DISCUSSION

We describe the characterization of a novel mosquito-borne virus, Nhumirim virus (NHUV), from the Pantanal region of Brazil and establish with high degree of certainty that it segregates with MBFVs within the genus *Flavivirus*. While the source mosquito, *Culex chidesteri*, has not been shown to be a disease vector, WNV has been isolated from this species (Kent *et al.*, 2010b). Field studies have indicated this mosquito feeds on a range of hosts including humans, chickens, rabbits, and turtles (Almiron & Brewer, 1995). The NHUV isolate is part of a novel group of flaviviruses that we are tentatively designating as unidentified vertebrate host (UVH) viruses that have been isolated from a wide geographic range including China (Wang *et al.*, 2009), Republic of Korea (Lee *et al.*, 2013a), Côte d'Ivoire (Junglen *et al.*, 2009), Finland (Huhtamo *et al.*, 2009), Israel (Kolodziejek *et al.*, 2013) and Peru (Evangelista *et al.*, 2013). Upon phylogenetic characterization, we found that NHUV clustered most closely with these viruses and had the same apparent inability to replicate in mammalian vertebrate cells, despite being grouped within the dual-host mosquito vectored clade of flaviviruses. Interestingly, NHUV, NOUV, and BJV form a distinct clade from the branch encompassing CHAOV, LAMV, and DGV, which may be due to vector/host species differences. CHAOV, LAMV, and DGV viruses were all reportedly isolated from *Aedes spp.* while NANV, NHUV, and BJV were isolated from either *Culex spp.* or *Uranotaenia spp.* (Evangelista *et al.*, 2013; Junglen *et al.*, 2009; Kolodziejek *et al.*, 2013; Lee *et al.*, 2013a; Wang *et al.*, 2009). While there is a precedent for vector/host preference being correlated with phylogenetic divergence throughout the *Flavivirus* genus, research into the vector preference or transmission mechanism (i.e. transovarial or oral infectious) of these viruses has yet to be performed.

The phylogenetic branching pattern indicates that NHUV, NOUV, and BJV share a more recent common ancestor with viruses from the MBFV group than with the other members of the distinct clade UVH viruses including CHAOV, LAMV, and DGV in that phylogenetic cluster. The phenotypic and phylogenetic contrast is what makes these viruses of particular interest because historically, flaviviruses have been found to cluster by host/vector preference. There are three possible explanations for this anomaly: 1) these viruses are a distinct group of ISFs that never evolved the ability to replicate in vertebrate hosts, 2) these viruses are part of the dual-host mosquito vectored clade and have lost the ability to replicate in vertebrates, or 3) these viruses are part of the dual-host mosquito vectored clade and replicate in an as of yet to identified non-insect secondary host. 3' UTR analysis in concert with phylogenetic findings indicate that NHUV has the most conserved structures and sequences (present and absent) with viruses of the MBFV group. Specifically, the complete conservation of the 3' LSH pentanucleotide with other MBFVs, the presence of SL2 which is conserved between TBFV, MBFV, and NKV, but not ISFV (Gritsun & Gould, 2007b), and the absence of Y-1 which is conserved in NKV (Charlier *et al.*, 2002), TBFV (Gritsun & Gould, 2007b; Gritsun *et al.*, 1997; Proutski *et al.*, 1997), ISFV (Gritsun *et al.*, 2014) but not MBFV, supports the likelihood that NHUV is a member of the mosquito-borne flavivirus group and has either lost its ability to replicate in vertebrates or has an as yet unidentified vertebrate host. Similar observations were made upon informal analysis of codon usage preferences in that NHUV codon usage by amino acid more closely resembled that of WNV than the insect specific model utilized, CxFV. A study by Lobo et al. indicated that *Flaviviridae* members which persist in a single host cycle have codon usage profiles more similar to their hosts than to closely related *Flaviviridae* (Lobo *et al.*, 2009). The dissimilar codon usage profiles between the insect-specific virus CxFV and NHUV in concert with the similarities between the codon profile of NHUV and WNV supports the theory that NHUV is not a mosquito-specific virus, but either a dual-host virus with an as of yet undiscovered vertebrate host or a virus that has recently lost its ability to replicate in vertebrates.

We compared the ability of NHUV to inhibit representative *Culex* spp. vectored MBFV replication upon simultaneous co-infection and delayed secondary infection. We were able to determine that NHUV had a significant inhibitory effect on the replication of WNV, SLEV, and JEV in culture by decreasing peak titers anywhere from 6.2 log₁₀ (PFU/ml) to 1.2 log₁₀ (PFU/ml). This inhibitory effect was observed as early as one-day post-infection for both WNV and JEV, and by day two post-infection for SLEV, which is not unexpected as SLEV is a slower growing virus. Inhibition of WNV growth *in vitro* following co-infection with an insect specific flavivirus, CxFV, has been demonstrated previously by Bolling et al., in which ~1.0 log₁₀ reduction of WNV in co-infected C6/36 cells (Bolling *et al.*, 2012) and Hobson-Peters et al showed inhibition of up to 1.6 log₁₀ for Palm Creek Virus (PCV) inhibition of Murray Valley encephalitis virus and a 1.0 log₁₀ reduction in WNV replication (Hobson-Peters *et al.*, 2013). Such inhibition upon dual-infection has often been described by superinfection exclusion, the phenomenon in which a cell infected with one virus cannot be secondarily infected with another, closely related virus. It has been previously demonstrated *in vitro* and *in vivo* for both alphaviruses and flaviviruses (Bolling *et al.*, 2012; Eaton, 1979; 1981; Hobson-Peters *et al.*, 2013; Karpf *et al.*, 1997; Kent *et al.*, 2010a; Pepin *et al.*, 2008; Pesko & Mores, 2009). However, while we did observe inhibition

with secondary infection with WNV, SLEV, and JEV that would be consistent with superinfection exclusion, we also saw equally marked MBFV inhibition when the viruses were infected with NHUV simultaneously. This is of particular interest as studies of superinfection have indicated the exclusion of secondary infection generally does not take effect until at least one hour following infection with the initial virus (Eaton, 1979; Johnston *et al.*, 1974). Therefore, it is likely that NHUV has a distinct mechanism that interferes with replication of these *Culex spp.* vectored MBFV as demonstrated *in vitro*. It is also of note that NHUV was found to have a high TCID₅₀ in C6/36 cells, which may also contribute to the efficiency of the inhibition effect. Because the range of species, rate, or geographic spread of NHUV infection remains unknown, we cannot draw conclusions regarding any potential impact this inhibition phenomenon may have in the field. Further studies will need to examine whether these same inhibitory effects translate into *in vivo* mosquito infection, dissemination, and transmission blockage. Previous studies investigating the inhibition of CxFV on transmission of WNV in *Culex spp.* have shown mixed results, indicating that observed replication interference *in vitro* may not necessarily be indicative of *in vivo* findings (Bolling *et al.*, 2012; Kent *et al.*, 2010a). However the increased phylogenetic relatedness between MBFV and NHUV as compared to that of MBFV and CxFV could be an important contributing variable to the degree of inhibition. The phylogenetic relatedness between NHUV and MBFV also improves prospects for vaccine development, as recombinant and chimeric viruses are more likely to be stable and viable. Ongoing studies are focusing on examining chimeras between WNV and NHUV in order to evaluate regions responsible for co-infection inhibition, as well as determine regions responsible for ablated vertebrate replication. Identification of these regions could have implications for improved attenuation strategies, which would allow for an additional safety factors as well as shed light on fundamental genetic determinants that dictate host range differences of flaviviruses.

METHODS

Virus isolation and sequencing

Adult mosquitoes were collected between 2009 and 2010 in the Nhecolândia sub-region of the Pantanal, within the State of Mato Grosso do Sul, Brazil as previously described in Pauvolid-Correa *et al.* (Pauvolid-Correa *et al.*, 2013). Pools of mosquitoes were homogenized in 300 μ l Dulbecco's modified Eagle medium (DMEM) complete with penicillin (100U/ml), streptomycin (100mg/ml), 10% fetal bovine serum (FBS), and 50 μ g/ml amphotericin B. Clarified supernatants from triturated mosquito pools were used to inoculate both C6/36 (mosquito) and Vero (mammalian) cells in 24-well plates. Inoculated cells were observed daily and harvested upon the appearance of cytopathic effect (CPE) or following ten days incubation. *Ae. albopictus* C6/36 cells were maintained at 28°C with complete DMEM supplemented with 10% FBS, and penicillin/streptomycin. Viral RNA was extracted from 140 μ l of the harvested supernatant using QIAamp RNA mini kit (Qiagen, Inc., Valencia, CA). RT-PCR was performed on the extracted RNA using flavivirus-specific primers as previously described (Pauvolid-Correa *et al.*, 2013; Pauvolid-Correa *et al.*, in review). The full coding sequence was acquired with second generation sequencing (SGS) using a Mi-Seq system (Illumina Inc., San Diego, CA, USA) and the NHUV virus genome was constructed via an automated computational pipeline as previously described (Langevin

et al., 2013; Pauvolid-Correa *et al.*, in review). The 5' and 3' untranslated regions (UTRs) were confirmed using the corresponding kit for rapid amplification of cDNA ends (RACE) (Invitrogen, Carlsbad, CA, USA).

Phylogenetic analysis/codon usage frequency calculations

The NHUV polyprotein open reading frame sequence was aligned with available flavivirus sequences in the NCBI database using MUSCLE on the Cipres Science Gateway (Edgar, 2004; Miller *et al.*, 2010). Maximum likelihood inference was performed using RAxML 7.06 on the Cipres Science Gateway (Stamatakis *et al.*, 2008). 1000 replicates of bootstrapping resampling were utilized to assess the accuracy of tree topologies. Output trees were manipulated using Fig Tree v1.4. Codon frequency calculations were performed using MacVector (v10.6) (MacVector, Inc, Cary, North NC, USA) software based on the coding regions of the following arboviral strains; WNV (382-99; AF196835), MODV (M544; AJ242984), CxFV (FJ663034) and NHUV (KJ210048) strain characterized herein.

IFA and TCID₅₀

To confirm and quantify the growth of the non-plaque forming NHUV flavivirus isolate, immunofluorescence assays (IFAs) were performed in conjunction with the Reed and Muench method for titrating endpoints (Biacchesi *et al.*, 2005; Reed & Muench, 1938). C6/36 cells were inoculated with 10-fold serial dilutions in a 96-well format and fixed with 20% acetone 24 hours post-infection. Once fixed, cells were washed with phosphate buffered saline (PBS), incubated with a pan-flavivirus monoclonal (Dengue 2, New Guinea C; 4G2) antibody, washed with PBS, and incubated with a FITC-labeled secondary antibody (goat anti-mouse IgG; Jackson ImmunoResearch Laboratories, West Grove, PA). After a final wash, cells were examined for the presence of viral antigen with an inverted fluorescent microscope.

In vitro characterization

In vitro propagation of the isolate was attempted in various cell lines including *Aedes albopictus* mosquito (C6/36 and C7/10), *Culex quinquefasciatus*, *Ixodes scapularis* tick cells (ISE6), African green monkey (Vero), and hamster (BHK21-clone 15), chicken (DF-1), and *Xenopus laevis* (South African clawed toad) cells. Each cell monolayer was inoculated at a multiplicity of infection of 10 TCID₅₀ units from supernatant isolated from the original passage of the triturated mosquito pool sample as determined by titration on C6/36 cells. Cultures were observed for CPE for 7 days prior to harvest. Virus was serially blind passaged three times each on Vero cells, ISE6 cells, and BHK21 clone15 cells as no initial CPE was identified following a single passage. To confirm the presence or absence of viral replication, RT-PCR was performed on supernatant taken from the third passage using pan-flavivirus primers, FU1 and CFD3R, designed to amplify a ~1085 nt portion of the NS5 gene region (Kuno *et al.*, 1998). Negative RT-PCR samples were confirmed by IFA.

Inhibition of West Nile virus growth *in vitro*

West Nile virus utilized for co-infection studies was derived from an infectious clone of the New York 1999 strain (Kinney *et al.*, 2006). Twelve well plates of C6/36 cells all originally

seeded at the same time and density, were inoculated at an MOI of 5 with NHUV. These cultures were subsequently inoculated with WNV, JEV, or SLEV at an MOI of 0.1 on day 0 (simultaneous co-infection) and day 3 following initial NHUV infection. Additional pre-inoculation of NHUV was performed at -1 and -5 dpi for WNV inhibition studies. All infections were performed in duplicate with mock WNV, JEV, and SLEV infection controls for each experimental time-point group. Additionally, a positive infection control for each virus was inoculated at 0.1 MOI on C6/36 cells that were split at the same time as the experimental dual infection replicate cultures. Supernatant samples were observed and collected daily from triplicate cultures and subsequently titered by plaque assay. A two-way ANOVA with an *a posteriori* Tukey's multiple comparison was utilized to assess statistical differences in viral titers between the control and dual-infection groups.

3' UTR characterization

It has been previously proposed that an ancestral form of the flavivirus 3' UTR has evolved in such a way that divergence of the TBFV, MBFV, NKV, and ISF groups can be distinguished by the presence and number of long repeated sequences (LRS) and shorter direct repeats (DR), as well as the characterization of secondary structure RNA elements that are found in the 3' UTR (Grard *et al.*, 2007; Gritsun & Gould, 2006a; b; c; 2007a; b; Hahn *et al.*, 1987). As such, the 3' UTR of the NHUV isolate was compared to 3' UTRs of representative members from other flaviviruses representing the distinctive phylogenetic and phenotypic grouping viruses in order to identify homologous secondary structures and repeat elements that could associate with phylogenetic or phenotypic patterns. R-Coffee (Moretti *et al.*, 2008) was utilized to generate multiple alignments between available 3' UTR regions of flaviviruses for identification of conserved repeat regions and location of homologous secondary structure RNA elements in concert with direct comparison to structural elements and sequences identified from previous studies (Gritsun & Gould, 2006a; b; c; Markoff, 2003). Mfold web server was utilized to predict secondary structure formation with the maximum distance between paired bases set to 80 as previously described by Gritsun *et al.* 2014 (Gritsun *et al.*, 2014; Zuker, 2003).

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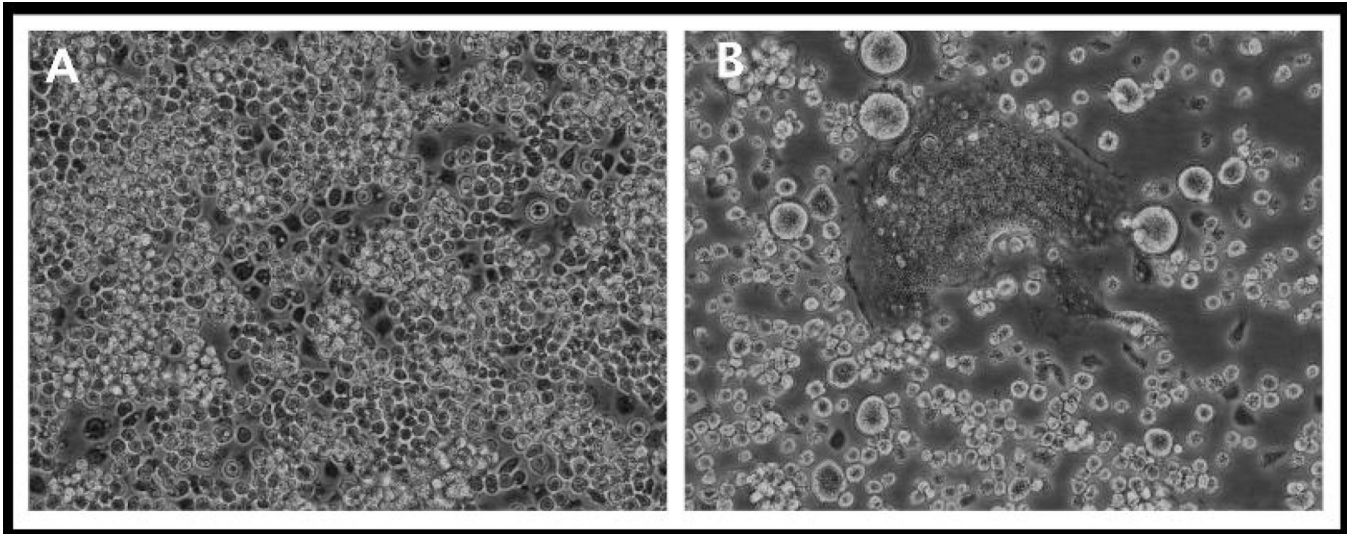


Figure 1.
Phase contrast image depicting NHUV cytopathology in C6/36 cells *in vitro*; A) negative control mock infected, B) NHUV infected cells with syncytia.

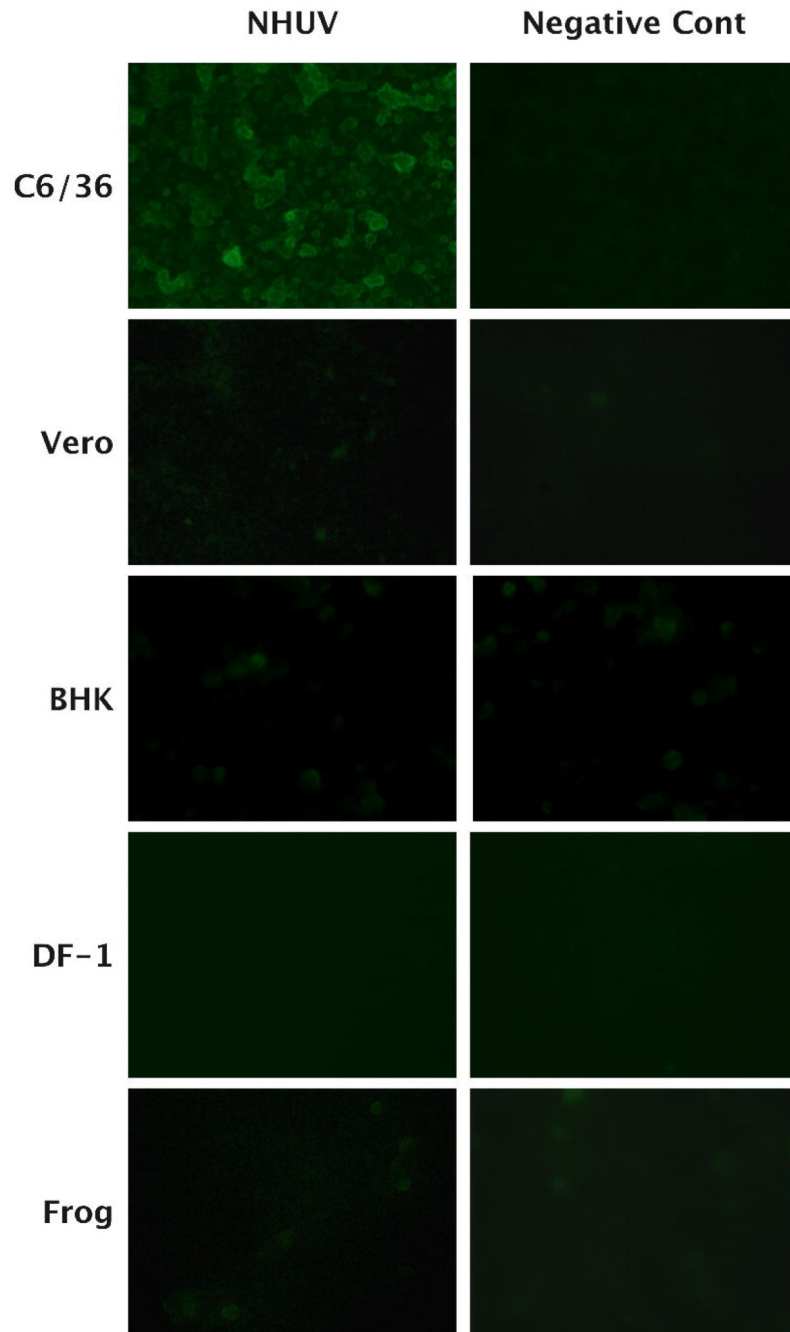


Figure 2.
Epifluorescent images of IFA tests in the various cell types examined

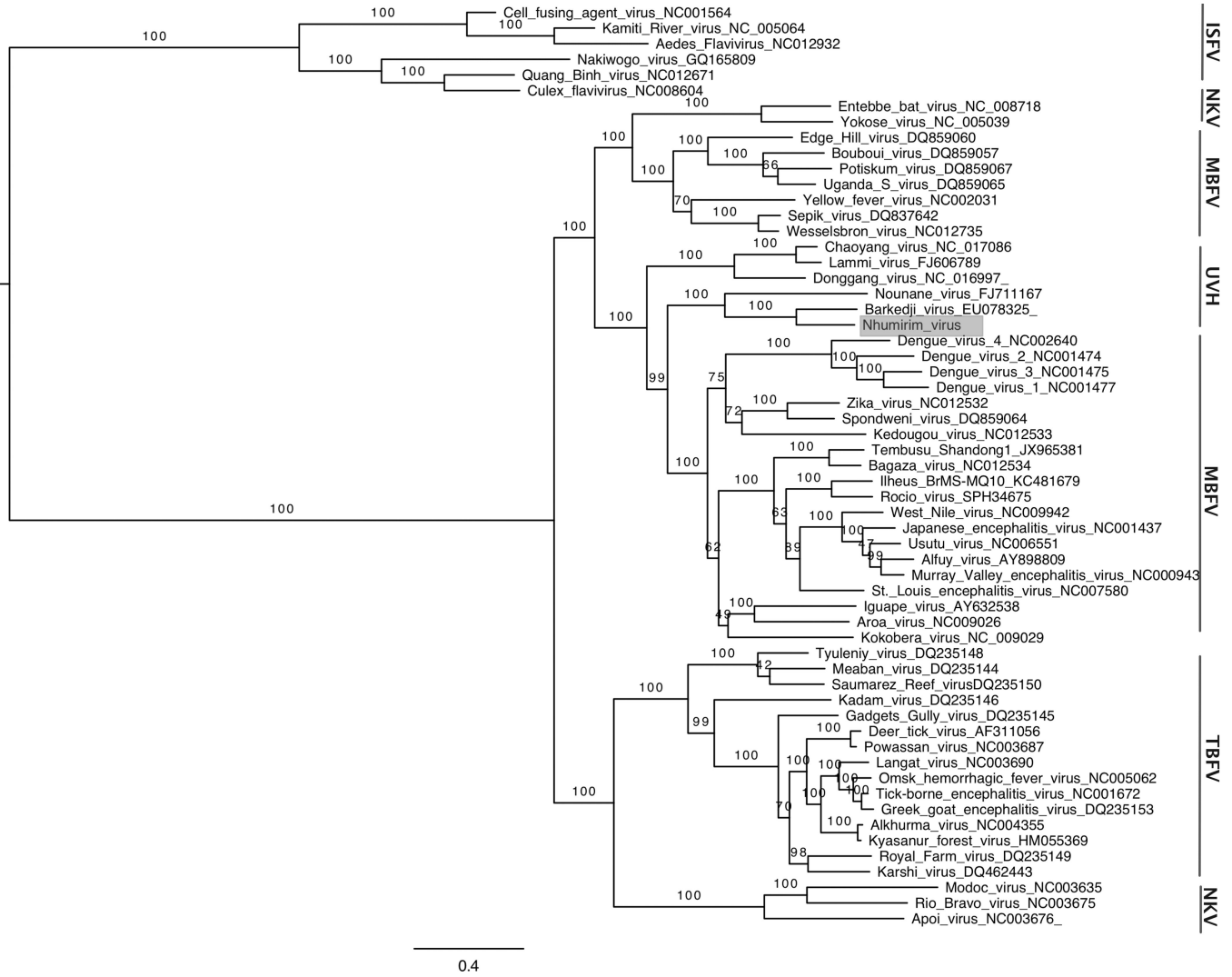
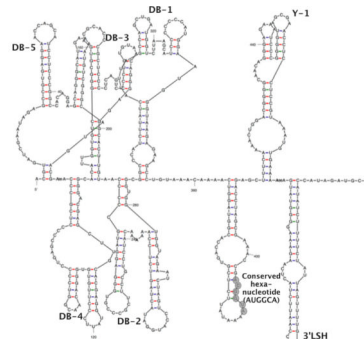
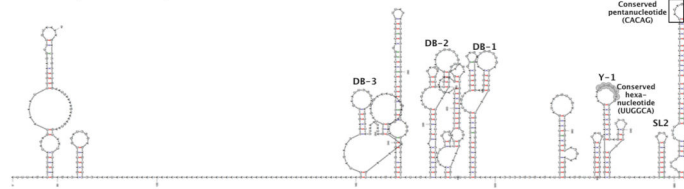


Figure 3. Phylogenetic analysis based on nucleotide sequences of complete polyprotein coding sequences. Phylogenies were constructed using the maximum likelihood method with labeled bootstrap percentages as support. Labels include taxon name and accession number. NHUV is highlighted in gray and clades are labeled by host association designations on the far right of the figure.

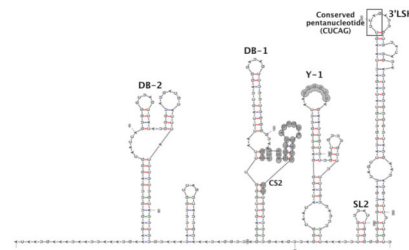
A. CFAV (ISFV)



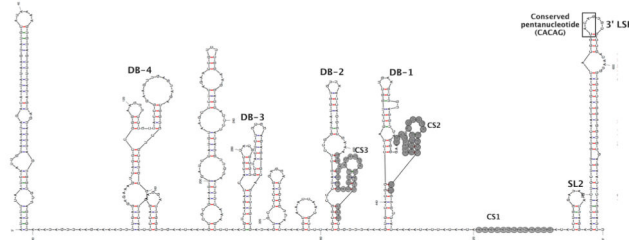
B. TBEV (TBFV)



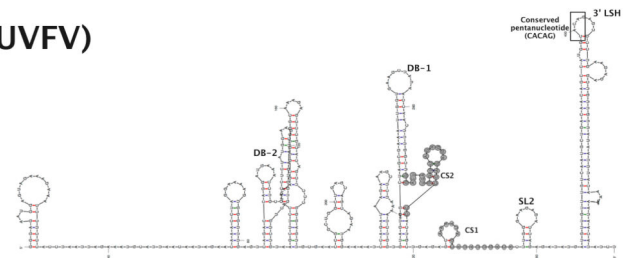
C. MODV (NKV)



D. WNV (MBFV)



E. NHUV (UVFV)

**Figure 4.**

Mfold generated prediction and labels denoting conserved secondary structure and sequence elements for CFAV (shown in alternating display for clarity), TBEV, MODV, WNV, and NHUV. Nucleotides included in conserved MBFV sequences such as the pentanucleotide, conserved sequence 1 (CS1), and CS2 are highlighted with grey circles. A) Key structures identified in CFAV include the 3' LSH with an internal conserved pentanucleotide (CACCG), a Y-shaped element, and a conserved hexanucleotide sequence element. B). TBEV had the 3'LSH, pentanucleotide (CACAG), SL2, and Y-1 with an internal

hexanucleotide sequence. C) MODV demonstrated the 3' LSH, pentanucleotide (CUCAG), and Y-1 with internal hexanucleotide sequence.multiple. D) WNV showed a 3'LSH, the conserved pentanucleotide sequence (CACAG), SL2, conserved sequences CS1, CS2, and CS3. E) NHUV was found to have a 3' LSH, a conserved pentanucleotide (CACAG), SL2, and only CS1 and CS2.

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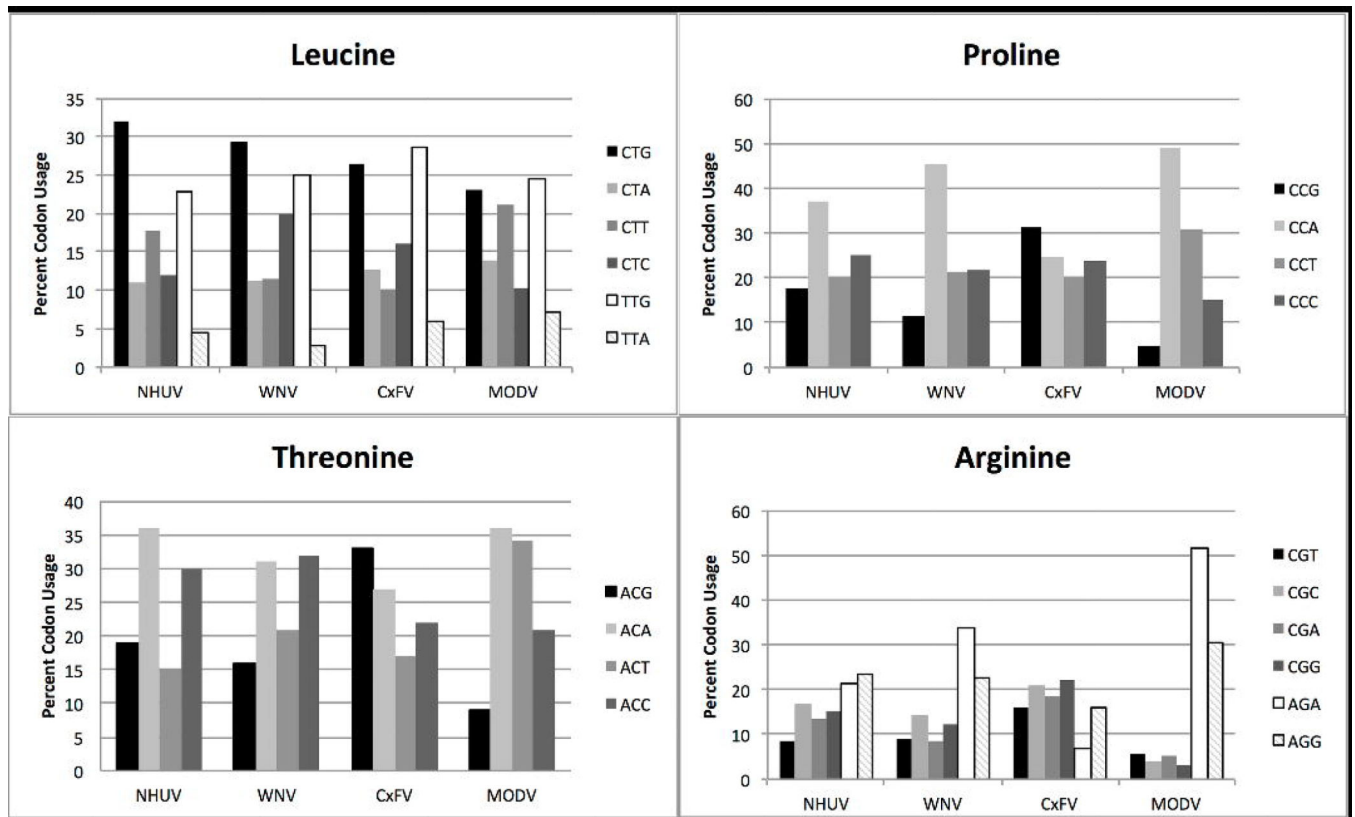


Figure 5. Histograms demonstrating threonine, arginine, leucine, and proline codon usage frequencies for NHUV, WNV, CxFV, and MODV.

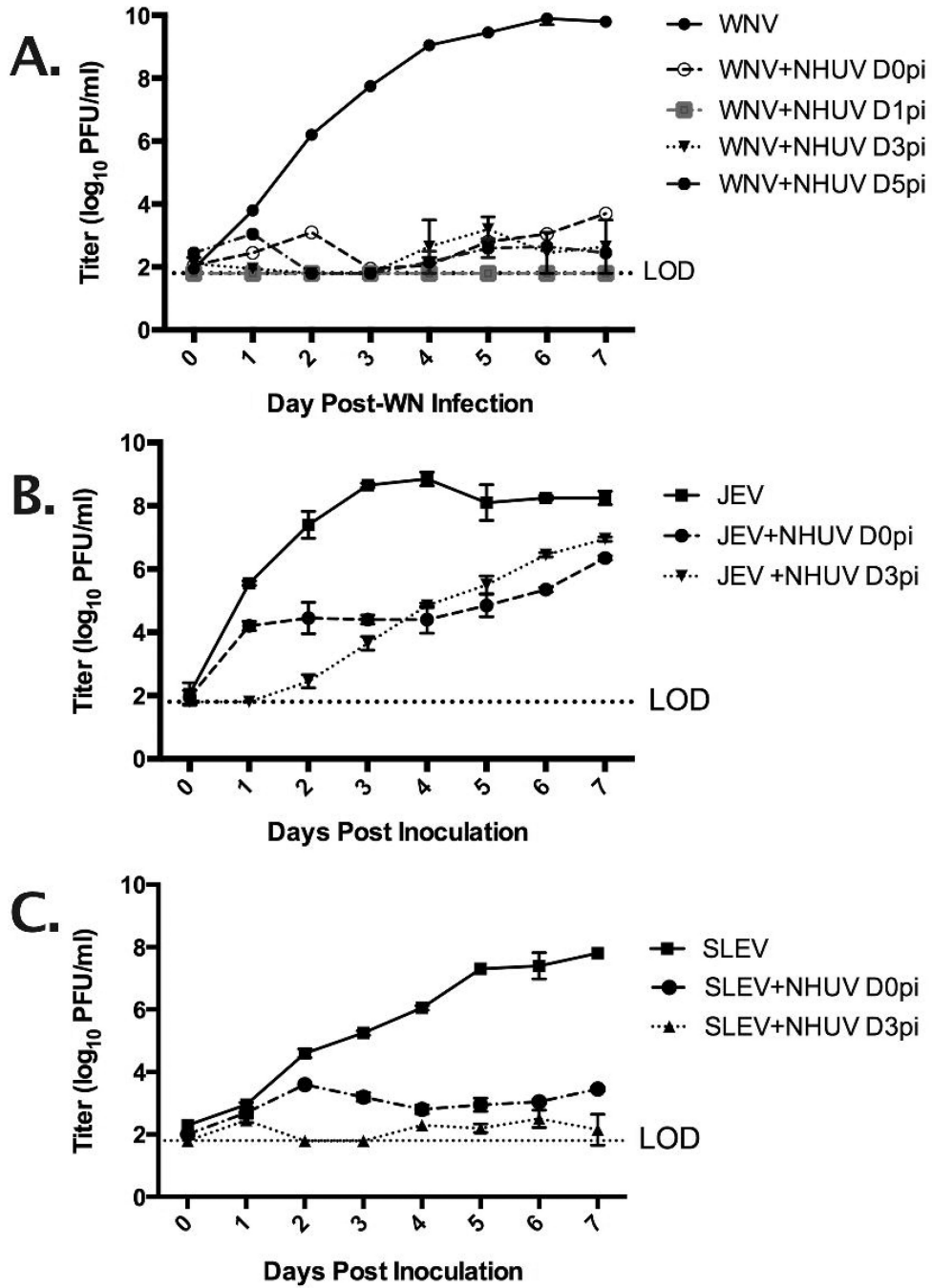


Figure 6. Inhibition of WNV, JEV, and SLEV on C6/36 cells in the presence of NHUV. A) Replication kinetics of WNV compared with WNV dual-infection with NHUV at day 0, day 1, day 3, and day 5 post-NHUV inoculations of C6/36 cells. B) JEV dual-infection with NHUV at day 0, and day 3 post-NHUV infection. C) SLEV dual infection with NHUV at day 0 and day 3 post-NHUV infection. Cells were inoculated with an MOI of 0.1 of the

representative MBFV and exposed to NHUV at an MOI of 5. Time points were collected daily for seven days following infection with each MBFV.

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Table 1Summary of NHUV *in vitro* infection of various cell types

	IFA	RT-PCR ^a	CPE
Vero	-	-	-
BHK21-15	-	-	-
DF-1	-	-	-
<i>Xenopus laevis</i>	-	-	-
C6/36	+	+	+
C710	+	+	+
<i>Cx. quinquefasciatus</i>	+	+	+
ISE6	-	-	-

^aRT-PCR on supernatant from second passage

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Table 2

Genome organization of NHUV virus

Region	Gene	Position in genome (nt)	Protein size (aa) ^a
5' UTR		1-102	
	C	103-486	128
	pr	487-768	94
Structural	M	769-993	75
	E	994-2502	503
	NS1	2503-3555	351
	NS2A	3556-4251	232
	NS2B	4252-4641	130
Non-structural	NS3	4642-6507	622
	NS4A	6508-6954	149
	NS4B	6955-7719	255
	NS5	7720-10440	907
3' UTR		10441-10891	

^a aa, amino acids

Table 3
Putative polyprotein cleavage sites of NHUV and other closely related flaviviruses

Cleavage	NHUV	NOUV	BJV	JEV	SLEV	WNV
AnchC/ virion C	NRTRR/ARRGM	VSKRR/GSASL	KTSKR/GLQQSF	RKQNKR/GGNEGS	PSKKR/GGTRS	SKQKKR/GGKTGI
C/prM	TMVAC/VTVGT	GVASA/VTFTT	WTMAAC/ATLGMF	IAYAGA/MKLSNF	GLASS/LQLST	IASVGA/VTLSN
prM	RRRRR/SVALSP	QRRRR/SYGIS	KRRSKR/SVAIA	SKRSRR/SVSVQT	RRSRR/SISVQ	SRRSRR/SLTVQT
prME	VAPAYS/THCVR	IPAYS/MKCIG	VAPAYS/LHRSV	VAPAYS/FNCLGM	APAYS/FNCLG	VAPAYS/FNCLGM
E/NS1	TSAHA/EVGCS	TSVSA/ELGCS	TTVAG/DVGCNL	TNVHA/DTGCAI	TSVQA/DSGCA	VNVHA/DTGCAI
NS1/NS2A	WVTAG/QMITGI	LGVLAM/TMMF	WTTAG/NATGID	QVDAF/NGEMV	SRVTA/GVAGG	QVNAY/NADMID
NS2A/NS2B	KSGKR/SVSMG	KTTKR/SVPQS	GSGKR/SVSMGE	PNKKR/GWPATE	PNGKR/SWPAS	PNRKR/GWPATE
NS2B/NS3	SATQR/AGAMW	ENRKR/SNDTP	EKGTQK/AGAMWD	LKTTKR/GGVFWD	KHSKR/GGALD	LQYTKR/GGVLWD
NS3/NS4A	AEGRR/GAMD	AGGKR/SAVDL	AEGRR/GASDIW	AAGKR/SAISFI	AA GKR/SALGM	ASGKR/SQIGLI
NS4A/NS4B	TLMIAA/NEKGLL	GAVAA/NEYGM	LAVTA/NEKGL	GVVAA/NEYGM	GVVAA/NEMGL	SAVAAA/NEMGW
NS4B/NS5	KSARR/GTPGG	AYKKR/GIWEV	KSARR/GTPGG	PSLKR/GRPGG	PKGKR/GGGKG	PGLKR/GGAKG

Table 4
 Percentage of nucleotide and amino acid identity between NHUV and representative flavivirus ORF sequences.

	BJV		NHUV		NOUV		JEV		SLEV		WNV		LAMV		YFV		MODV		POWV		
	% nt	% AA	% nt	% AA	% nt	% AA	% nt	% AA	% nt	% AA	% nt	% AA	% nt	% AA	% nt	% AA	% nt	% AA	% nt	% AA	
NHUV	65.9	70.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NOUV	56.1	53.0	56.2	53.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
JEV	53.4	49.2	53.9	49.2	52.8	47.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SLEV	54.0	49.4	54.1	49.8	53.5	48.0	64.2	66.9	-	-	-	-	-	-	-	-	-	-	-	-	-
WNV	53.8	49.1	54.3	49.4	52.5	46.9	69.1	76.5	65.1	67.8	-	-	-	-	-	-	-	-	-	-	-
LAMV	53.2	48.0	53.2	47.7	52.6	46.5	54.6	49.7	55.1	50.6	54.5	49.5	-	-	-	-	-	-	-	-	-
YFV	51.2	44.4	51.1	44.2	50.8	43.8	51.9	45.3	52.0	45.9	51.6	45.1	51.7	45.6	-	-	-	-	-	-	-
MODV	44.5	36.1	44.5	35.8	44.4	35.2	45.0	36.5	45.8	36.2	45.0	36.6	45.4	35.6	47.0	38.2	-	-	-	-	-
POWV	47.7	39.3	47.2	38.9	46.5	38.3	48.0	40.5	47.9	40.6	48.6	40.6	47.8	40.6	49.5	42.2	48.7	41.6	-	-	-
CFAV	36.7	24.8	36.5	24.2	36.0	24.5	36.6	24.9	36.6	24.9	36.9	24.5	35.8	24.6	36.1	24.4	35.5	24.0	36.0	24.1	-