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Generation of a Lineage II Powassan Virus (Deer Tick Virus) cDNA Clone: Assessment of Flaviviral Genetic Determinants of Tick and Mosquito Vector Competence

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Abstract

The *Flavivirus* genus comprises a diverse group of viruses that utilize a wide range of vertebrate hosts and arthropod vectors. The genus includes viruses that are transmitted solely by mosquitoes or vertebrate hosts as well as viruses that alternate transmission between mosquitoes or ticks and vertebrates. Nevertheless, the viral genetic determinants that dictate these unique flaviviral host and vector specificities have been poorly characterized. In this report, a cDNA clone of a flavivirus that is transmitted between ticks and vertebrates (Powassan lineage II, deer tick virus [DTV]) was generated and chimeric viruses between the mosquito/vertebrate flavivirus, West Nile virus (WNV), were constructed. These chimeric viruses expressed the prM and E genes of either WNV or DTV in the heterologous nonstructural (NS) backbone. Recombinant chimeric viruses rescued from cDNAs were characterized for their capacity to grow in vertebrate and arthropod (mosquito and tick) cells as well as for *in vivo* vector competence in mosquitoes and ticks. Results demonstrated that the NS elements were insufficient to impart the complete mosquito or tick growth phenotypes of parental viruses; however, these NS genetic elements did contribute to a 100- and 100,000-fold increase in viral growth *in vitro* in tick and mosquito cells, respectively. Mosquito competence was observed only with parental WNV, while infection and transmission potential by ticks were observed with both DTV and WNV-prME/DTV chimeric viruses. These data indicate that NS genetic elements play a significant, but not exclusive, role for vector usage of mosquito- and tick-borne flaviviruses.

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Keywords

tick-borne flavivirus; mosquito-borne flavivirus; deer tick virus; Powassan virus; West Nile virus; vector competence

Introduction

The family *Flaviviridae*, genus *Flavivirus*, comprises enveloped viruses with positive-sense, single-stranded RNA genomes of ~11 kb. Three structural proteins, envelope (E), membrane (M), and capsid (C), and seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3 (protease and helicase), NS4A, NS4B, and NS5 (methyltransferase and RNA-dependent RNA polymerase), are encoded from a single polyprotein that is co- and post-translationally cleaved by host and viral proteases into the respective viral proteins. The envelope protein acts as the primary surface antigen and mediates fusion between the virion and cellular receptors. The membrane protein is the product of proteolytic processing of the prM fragment and is essential for virus particle maturation (Lindenbach and Rice 2001). The capsid protein forms the nucleocapsid, which comprises viral genomic RNA coupled with capsid protein fragments. The entire viral genome is translated as a single polyprotein (the N terminal encodes three structural genes, and the C terminal encodes seven NS proteins) (Lindenbach et al. 2006). The open reading frame is flanked by conserved 5' and 3' untranslated regions (UTRs) that serve as cis-acting elements critical for viral replication (Lindenbach and Rice 2001).

Flaviviruses are responsible for an estimated 100 million human infections per year (Petersen and Marfin 2005). While the majority of flaviviruses are maintained in arthropod vector–vertebrate host transmission cycles, some flaviviruses have adapted to a single-host life cycle in either an arthropod or a vertebrate host. To date, flaviviruses have been separated into five groups based on host/vector-specific associations, including insect-specific flaviviruses (ISFVs), tick-borne flaviviruses (TBFVs), no known vector viruses (NKVs), mosquito-borne flaviviruses (MBFVs), and a recently described unidentified vertebrate host (UVH) group of flaviviruses (Kuno et al. 1998, Gould et al. 2003). The UVH group is unique as it phylogenetically falls within the MBFV clade, yet viruses in this clade have not demonstrated a capacity for replication in vertebrate cells (Huhtamo et al. 2009, Junglen et al. 2009, Evangelista et al. 2013, Kolodziejek et al. 2013, Lee et al. 2013, Kenney et al. 2014).

Multiple studies have been performed to characterize chimeric flaviviruses, some of which have identified specific viral genetic determinants dictating differential vector competence between viruses. For instance, chimerization between an NKV strain, Modoc virus (MODV), prME regions, and a yellow fever virus (YFV) genetic backbone has indicated that the inability of NKV viruses to infect and replicate in mosquito cells *in vitro* is not exclusively controlled by prME genetic elements (Charlier et al. 2010). Studies with other chimeric viruses consisting of donor prME regions from TBFVs and backbones from MBFVs support this assertion as all recombinant viruses demonstrated the ability to infect and replicate in mosquito cells, although (in some cases) in a reduced capacity (Pletnev et al.

1992, Engel et al. 2011, Wang et al. 2014). Utilizing a tick-borne encephalitis virus (TBEV) replicon and a Japanese encephalitis virus (JEV) prME helper system, Yoshii et al. (2008) were able to identify that JEV prME proteins allowed for internalization of virus-like particles in C6/36 mosquito cells, but that no replication was observed (Yoshii et al. 2008).

However, majority of studies lacked the direct comparison between reciprocal chimeric viruses to fully assess the heterotypic infection of structural versus NS components. Reciprocal chimeric viruses generated between more closely related viruses, West Nile virus (WNV) and St. Louis encephalitis viruses (SLEV), demonstrated an increased oral infectivity of *Culex quinquefasciatus* mosquitoes for the chimeric virus with NS genetic elements of SLEV (Maharaj et al. 2012). However, other *in vivo* studies examining chimeric viruses between distantly related flaviviruses with more divergent vector usage patterns have indicated that NS regions alone are not sufficient to allow for infection and/or replication in mosquitoes (Pletnev et al. 1992, Engel et al. 2011). This suggests that either additional structural determinants are involved in mosquito vector competence or that chimerization could have an inherent effect on vector infectivity.

In contrast to experiments designed to identify flaviviral mosquito competence determinants, very few experiments have been reported assessing the genetic basis for competence of flaviviruses in ticks, either *in vitro* or *in vivo*. Infection of *Ixodes scapularis*, ISE6, cells with a TBEV prME/DENV4 chimeric virus indicated that TBEV prME regions alone were not sufficient for *in vitro* infection. Similarly, *I. scapularis* larvae immersed in suspensions of chimeric TBEV prME/DENV4 viruses failed to become infected, suggesting that prME regions alone are similarly not sufficient for *in vivo* tick infection (Engel et al. 2011). This is the only study that has examined full-length chimeric flaviviral infection of tick cells with TBEV prME genes, and to our knowledge, there are no published accounts examining whether a TBEV backbone can sufficiently confer *in vitro* or *in vivo* growth in tick cells. As such, the studies described herein were undertaken to elucidate the *in vitro* and *in vivo* viral genetic determinants for either mosquito or tick competence of TBEVs (lineage II Powassan virus) and MBFVs (WNV).

Phylogenetic examination of flaviviruses proposes that both TBEVs and MBFVs originated in the old world (OW) and likely diverged ~40,000 years ago. Similarly, while WNV was introduced into the western hemisphere in 1999, Powassan virus (POWV) has been estimated as having crossed the Bering land bridge into North America between 15,000 and 11,000 years ago (Pettersson and Fiz-Palacios 2014). Powassan virus was initially isolated from a human brain in Powassan, Canada, in 1958 (Mc and Donohue 1959). A phylogenetically and ecologically distinct, yet serologically indistinguishable, strain, deer tick virus (DTV), was isolated from *I. scapularis*, a recognized vector of a multitude of pathogens and frequent feeding on humans. Recently, strains of POWV have caused an unprecedented number of cases and mortality in New Hampshire, Massachusetts, Maine, and Connecticut and are considered an emerging public health threat (Piantadosi et al. 2016, Cavanaugh et al. 2017, Hermance and Thangamani 2017, Tutolo et al. 2017).

While WNV has been isolated repeatedly from a multitude of both argasid and ixodid ticks (Lawrie et al. 2004, Mumcuoglu et al. 2005, Formosinho and Santos-Silva 2006, Moskvitina

et al. 2008, Lwande et al. 2013, 2014), laboratory studies have yet to demonstrate ixodid ticks as competent vectors (Anderson et al. 2003, Lawrie et al. 2004, Reisen et al. 2007). However, transmission by soft ticks, which are commonly associated with avian hosts, has been demonstrated (Abbassy et al. 1993, Lawrie et al. 2004, Kokonova et al. 2013). Alternatively, DTV has never been isolated from mosquitoes and only serologically detected in avian species (Dupuis et al. 2013).

This is likely explained by pronounced differences in ecology as WNV typically cycles between *Culex* mosquitoes and avians, while DTV persists in a cycle between *I. scapularis* and rodents. However, various types of ticks are found to be associated with birds and would likely be exposed to high WNV viremia of any infected bird ($>6.0 \log_{10}$ plaque-forming units [PFU]/mL), thus highlighting the importance of understanding determinants of tick vector competence for WNV (Anderson and Magnarelli 1984). Similarly, while the primary vectors of WNV have a predilection for avian bloodmeals, it is possible that mosquito species with a different host preference could encounter a DTV viremic rodent or small mammal host and acquire the virus.

Herein, methods for generation of a novel infectious clone for the DTV Spooner strain necessary to conduct vector specificity studies are described. Reciprocal chimeric viruses were generated and assessed for relative *in vitro* growth capacity and *in vivo* infection of mosquito and tick models to test the hypothesis that NS genetic elements are the critical determinants defining flaviviral vector associations.

Materials and Methods

Cell culture

Vero (African green monkey kidney fibroblast) cells and BHK-21 (baby hamster kidney fibroblast) cells were cultured as described previously (Goodman et al. 2014). Briefly, cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) with 8% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO), 1 mM sodium pyruvate (Life Technologies), 27 mM sodium bicarbonate (Life Technologies), 0.1 mM gentamicin (Lonza, Walkersville, MD), and 1 μ M amphotericin B (Sigma-Aldrich, St. Louis, MO). *Aedes albopictus* mosquito C6/36 cells were maintained at 28°C in DMEM (Life Technologies) with 10% FBS (Atlas Biologicals), 0.1 mM nonessential amino acids (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 9 mM sodium bicarbonate (Life Technologies), and 0.1 mM gentamicin (Lonza). ISE6 tick cells, derived from *I. scapularis* embryonated eggs, were obtained from Ulrike Munderloh (University of Minnesota) and maintained at 32°C, as previously described (Munderloh et al. 1994).

Construction of DTV Spooner strain cDNA clone

Before generating the DTV cDNA infectious clone, the initial 26 nt of the 5' UTR and terminal 26 nt of the 3' UTR of the Spooner DTV strain were determined by direct sequencing using 5' - and 3' -RACE Systems (Life Technologies), respectively. The viral genome was divided into four overlapping fragments spanning appropriate unique restriction

sites (Fig. 1). Viral cDNAs were synthesized from RNA by using reverse transcriptase (SuperScript II; Life Technologies). RT-PCR fragments were amplified by using high-fidelity *Pfu* Ultra Hotstart polymerase (Agilent, La Jolla, CA). The following conditions were used for PCR: 30 cycles of denaturation at 95°C for 30 s, primer annealing for 30 s at temperatures from 58°C to 63°C, followed by extension at 72°C for 1 min per 1-kb amplification. RT-PCR fragments of predicted size were cloned into the pCR-XL-TOPO vector (Life Technologies) according to the manufacturer's recommendations. To clone viral cDNA into the final construction, a customized, low-copy number kanamycin-resistant vector with convenient unique restriction sites was generated. The first fragment (T7 promoter–3612 nt–*Bst*Z17I) was cloned into this vector and denoted as DTVp1. For the second plasmid, naturally occurring restriction sites *Bst*Z17I, *Sbf*I, and *Kas*I and an introduced restriction site following the final nucleotide of the viral 3' UTR, *Not*I, were utilized to generate the 7228-bp amplicon for remainder of the genome. The three overlapping amplicons were sequentially cloned into the pCR-XL-TOPO vector (Life Technologies). The second cDNA, denoted as DTVp2, was sequenced in its entirety and used as an *in vitro* ligation template in conjunction with DTVp1 following restriction endonuclease digestion, as described below (Fig. 1).

Rescue of viruses from WNV and DTV cDNA clones

The two-plasmid NY99 WNV infectious clone (WNp1 and WNp2) was derived from a strain isolated from a flamingo in 1999, digested, *in vitro* ligated, and subjected to *in vitro* transcription before electroporation, as previously described (Beasley et al. 2005, Kinney et al. 2006, Maharaj et al. 2012). Briefly, 10 μ g of WNp1 was digested with *Asc*I, PCR purified, and eluted before secondary digestion with *Not*I. A total of 20 μ g of WNp2 was digested with *Mlu*I, purified, and eluted before complete digestion with *Not*I. The digested product from WNp1 and WNp2 was PCR purified and eluted. Purified WNp1 and WNp2 were *in vitro* ligated at a ratio of 1:3 and subsequently linearized with *Xba*I. For the two-plasmid DTV clone, 10 μ g of the 5' plasmid (DTVp1) and 20 μ g of the 3' plasmid (DTVp2) were each initially digested with *Bst*Z17I. Following PCR purification, DTVp1 and DTVp2 were *in vitro* ligated at a 1:3 ratio and subsequently linearized by *Not*I.

For generation of chimeras, enzymes utilized to generate fragments for *in vitro* ligation and subsequent linearization were determined by the included backbone. For NY99 WNV, DTV, and DTV-prME/WNV infectious clones, the linearized product was then utilized as a template for *in vitro* transcription of positive-sense viral RNA from a T7 RNA polymerase promoter with an analog A cap m⁷G(5')ppp(5')A (New England Biolabs) as described for WNV cDNA. Preparation and transcription of the DTV-prME/WNV construct were performed in the same manner as the parental WN99 infectious clone, as previously described (Nofchissey et al. 2013). Briefly, prME regions of DTV were cloned into the WNp1 plasmid generating the DTVprME/WNVp1 clone, which was used in conjunction with the WNp2 clone to generate the full-length virus.

For all viruses, transcribed RNA derived from each construct was electroporated into $\sim 10^7$ baby hamster kidney cells (BHK) using two pulses at 800 V, $R = 25$, $C = 25$ μ F on a BTX ECM 830 (Harvard Apparatus). Supernatants were harvested when 60–70% of cells showed

cytopathic effect (CPE), clarified by centrifugation at $6000 \times g$, aliquoted, and stored at -80°C in media with 20% FBS. At the time of harvest, $140 \mu\text{L}$ of each virus was subjected to RNA extraction and subsequent genome sequencing. RNA was extracted using QIAamp Viral RNA Mini kits (Qiagen, Valencia, CA) and amplified using the Titan One-Tube RT-PCR kit (Roche, Germany). RT-PCR products were visualized on a 1% agarose gel and bands were gel extracted. Sequencing was performed using an ABI 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA) and alignments and analysis were performed in Geneious 8 (www.geneious.com) (Kearse et al. 2012). In addition to confirming the coding sequence identity between the DTV Spooner strain and our rescued clone, we compared the phenotypes of these two viruses in C6/36 and ISE6 cells and found them to behave similarly (data not shown).

Generation of WNV-prME/DTV chimeric virus

Due to bacterial toxicity, generation of a stable 5' plasmid of the WN-prME fragment in the DTV backbone was not achieved despite efforts to introduce alternative *in vitro* ligation points to stabilize the plasmid. Alternatively, a high-fidelity Phusion enzyme (New England Biolabs, Ipswich, MA) was utilized to amplify the fragment from previous cloning intermediary fragments comprising three overlapping amplicon fragments (5' end of DTV clone to prME, complete WNV prME, and the remainder of the DTV-1 5' plasmid). These three amplicons, encompassing the entire 5' plasmid region, were generated and combined into a single fragment utilizing fusion PCR, as described previously (Charlier et al. 2003). The fusion fragment was then digested and used directly as an *in vitro* ligation product with the digested DTV-2 plasmid. RNA was subsequently transcribed and electroporated into BHK cells, as described above. The supernatant from the original electroporation was passaged an additional time to acquire a high titer stock, and the resulting virus was sequenced.

Virus infection in cell culture

Growth kinetics of each of the parental strains and the two chimeric viruses were compared on Vero, BHK, C6/36 mosquito, and ISE6 tick cells. Monolayers of each cell type were inoculated in triplicate at multiplicities of infection (MOIs) of 0.1 and 5 and allowed to adsorb for 1 h. Following incubation, cells were washed three times with phosphate-buffered saline (PBS) and overlaid with fresh cell line-appropriate media as described above. The total volume of supernatant (1 mL) was collected from each well and replaced with the same volume of fresh media at predetermined time points. Samples were frozen at -80°C until viral titers were measured by plaque assay on Vero cells. Two-way ANOVA tests with a *post hoc* multiple comparisons test with Bonferroni corrections were performed using Prism software to identify differences in mean daily growth, version 6. *p Values* ≤ 0.05 were considered significant.

Assessment of mosquito oral infectivity

Four cohorts of colonized adult female *Culex pipiens pipiens* (Chicago) mosquitoes maintained at the Division of Vector-Borne Diseases since 2010 (Kothera et al. 2010) were transferred to a BSL-3 environmental chamber and maintained at 28°C with 75% relative humidity. At ~ 3 –5 days postemergence, each group was offered an infectious blood-meal

(DTV, WNV, DTV-prME/WNV, or WNV-prME/DTV) with a titer of 1.0×10^7 PFU/mL at a 1:1 v:v ratio with de-fibrinated sheep blood (Colorado Serum Company, Boulder, CO). Artificial bloodmeals were encased in a collagen membrane and warmed in a Hemotek feeder (Discovery Workshops, Accrington, United Kingdom) before being placed on the screened lids of 0.45-L paper cartons. After feeding, mosquitoes were cold-anesthetized, sorted, and held at 28°C at a relative humidity of 70–75% for an extrinsic incubation period of 11–14 days. After the extrinsic incubation period, legs and wings were removed from cold-anesthetized mosquitoes and placed in a centrifuge (Eppendorf, Hamburg, Germany) tube with 350 μ L of Dulbecco's modified Eagle's essential medium (DMEM) with 10% FBS and amphotericin B (50 μ g/mL). The proboscis of each immobilized mosquito was inserted into a 10- μ L capillary tube containing Type B immersion oil (Cargille Laboratories, Cedar Grove, NJ) to induce salivation for ~45 min. After salivation, mosquito bodies and legs were triturated for 4 min at a frequency of 24 cycles per second in 350 μ L of DMEM, 10% FBS, and amphotericin B using a Mixer Mill 300 (Retsch, Newton, PA). Expectorants were added to a tube containing 100 μ L of 10% FBS/DMEM and centrifuged at $5000 \times g$ for 2 min before transfer of supernatants to fresh tubes. Collected supernatants from each sample were analyzed for the presence of virus by induction of CPEs on Vero cells.

Synchronous infection of *I. scapularis* ticks

Three days before synchronous infection, uninfected *I. scapularis* adult ticks were transferred to a 26°C environmental chamber for dehydration and desiccation. Infection was performed by a modification of an immersion feeding method previously described (Policastro and Schwan 2003). After 3 days of desiccation, ticks were divided into four groups and suspended in 1 mL of each of the four virus stocks. Each vial of virus containing submerged ticks was incubated at 34°C for 45 min. The ticks were washed twice with PBS and dried of excess moisture using filter paper. The virus-exposed ticks were transferred to glass vials where they were stored inside a desiccator at 26°C to allow extrinsic incubation of respective viruses.

Tick salivary gland dissection and RNA extraction

After 4 weeks of incubation at 26°C, salivary glands from each tick were dissected and stored in TRIzol (Life Technologies, Carlsbad, CA). The midgut and Malpighian tubules were also harvested from each tick and labeled as other organs; abbreviated [OTH]. The dissected organs were homogenized in TRIzol and viral RNA extraction was performed by a combination of TRIzol reagent and QIAamp Viral RNA mini (Qiagen) protocols (Hernance and Thangamani 2015) (Heinze et al. 2012).

Viral load determination from tick samples by quantitative RT-PCR

All tick salivary glands and OTH organs were screened by real-time RT-PCR. The iTaq Universal Probes One-Step assay (Bio-Rad) was performed on an iCycler iQ5 real-time PCR instrument (Bio-Rad). The master mix and reverse transcriptase from the iTaq Universal Probes One-Step assay were mixed with template RNA and primer–probe pairs. Probes contained a 5'-reporter FAM and a 3'-quencher TAMRA. The following primer and probe set was used for detection of the NS5 region of the DTV genome: 5'-GA TCATGAGAGCGGTGAGTGACT-3' (+); 5'-GGATCTCA CCTTTGCTATGAATTCA-3'

(-); Probe 5'-TGAGCACC TTCACAGCCGAGCCAG-3'. For detection of the conserved region of the 5' UTR and part of the capsid gene of the WNV genome, the following primer and probe set was used: 5'-CCTGTGTGAGCTGACAACTTAGT-3' (+); 5'-GCGT TTTAGCATATTGACAGCC-3' (-); Probe 5'-CTGGTTT CTTAGACATCGAGATCT-3'. Reactions were performed in IQ PCR 96-well plates (Bio-Rad) that were sealed and run on an iCycler iQ5 real-time PCR instrument (Bio-Rad) with the following cycling protocol: 10 min at 50°C; 3 min at 95°C; 15 s at 95°C, followed by 30 s at 60°C for 45 cycles; and an 81-cycle (+0.5°C/cycle) 55–95°C melt curve. RNA was extracted from BHK transfection stocks of known titer (PFU/ μ g of RNA) that were used to synchronously infect the ticks. Serial dilutions were made from the resulting RNA. For each virus stock, a linear equation was generated by plotting the cycle threshold (Ct) values of the standard curve against the corresponding log titer. Viral load Ct values from the unknown salivary gland and OTH samples were determined by real-time PCR and converted to log₁₀ PFU equivalents using the linear equation determined for the standard curve. Results were analyzed utilizing Student's *t*-test examining differences in mean titers between surviving ticks.

Results

Characterization of rescued viruses

As previously described (Beasley et al. 2005, Kinney et al. 2006), clone-generated WNV showed ~70% CPE 3 days postelectroporation (dpe) in BHKs and derived a stock of 8.8 log₁₀ PFU/mL in Vero cells. Plaques were small and clear 4 days postinfection (dpi), following a 3-dpi overlay with neutral red (Fig. 2). Recombinant DTV derived from electroporated BHK cells showed initial signs of CPE at 4 dpe, was harvested 6 dpe, and found to have a titer of 7.0 log₁₀ PFU/mL. On Vero cells, DTV plaques were large and poorly defined following a 7-dpi overlay on Vero cells (Fig. 2). The DTV-prME/WNV chimeric virus exhibited a similar pattern to the parental WNV and was harvested at a titer of 7.4 log₁₀ PFU/mL 3 dpe. Plaque size and the day of overlay were the same as that for WNV (Fig. 2). Initial electroporation of WNV-prME/DTV RNA in BHK cells was blind passaged in Vero cells and CPE was observed 7 dpi and supernatant harvested 9 dpi. A titer of 7.5 log₁₀ PFU/mL was detected in Vero cells and plaques appeared only slightly larger than those of WNV or those of the DTV-prME/WNV clone, although plaque margins were less defined (Fig. 2).

Vertebrate cell growth (Vero and BHK cells)

Both of the parental clone-derived viruses and the two chimeric viruses were capable of growth in Vero and BHK vertebrate cell lines with at least 7 log₁₀ PFU/mL titers observed. In Vero cells, WNV demonstrated a 10-fold higher peak mean titer compared with DTV (8.5 and 7.5 log₁₀ PFU/mL, respectively). Comparison of growth profiles of the WNV-prME/DTV chimeric virus and DTV only showed growth differences in Vero cells at early time points (dpi 1 and 2 for the lower MOI and dpi 1–3 for the higher MOI); however, mean peak titers were indistinguishable (7.5 log₁₀ PFU/mL). Similarly, the DTV-prME/WNV chimeric virus and WNV demonstrated indistinguishable mean peak titers of 8.3 and 8.5 log₁₀ PFU/mL. However, multiple comparison analysis showed the DTV-prME/WNV chimeric virus to have a significantly lower growth potential than WNV at dpi 5–7 ($p < 0.05$;

$p < 0.0001$; $p < 0.001$), suggesting that inclusion of heterologous DTV structural regions could affect growth at later time points in Vero cells (Fig. 3A).

In BHK cells, no significant growth differences were observed between viruses with DTV NS genetic elements. DTV and WNV-prME/DTV reached statistically indistinguishable mean peak titers of 8.2 and 8.4 \log_{10} PFU/mL, respectively, whereas peak mean titers of WNV (9.5 \log_{10} PFU/mL) and DTV-prME/WNV (8.2 \log_{10} PFU/mL) were statistically different ($p < 0.01$). In comparison with WNV, the DTV-prME/WNV chimera reached significantly lower titers on dpi 2–7 ($p < 0.0006$; $p < 0.0011$; $p < 0.0001$; $p < 0.0001$; $p < 0.0001$) at an MOI of 0.1 and on dpi 1–7 ($p < 0.0057$; $p < 0.0001$) in the MOI 5 treatment group (Fig. 3B).

Mosquito cell growth (C6/36)

In mosquito cells, viruses with NS regions derived from DTV (DTV and WNV-prME/DTV) failed to grow at or above the minimal level of detection (1.8 \log_{10} PFU/mL), whereas constructs with WNV NS regions (WNV and DTV-prME/WNV) grew to peak mean titers of 9.6 \log_{10} PFU/mL versus 7.6 \log_{10} PFU/mL, respectively ($p < 0.0015$). Despite sharing the same NS regions, DTV-prME/WNV grew to significantly lower titers compared with WNV at all days postinfection at both MOIs ($p < 0.0001$) (Fig. 3C).

Tick cell growth (ISE6)

Growth profiles of WNV, DTV, and the two chimeric viruses exhibited considerable variability in ISE6 tick cells (Fig. 3D). At an MOI of 5, DTV reached a peak mean titer 10,000-fold higher than the WNV peak titer. There was no evidence of WNV or DTV-prME/WNV replication on ISE6 cells at either MOI. In general, DTV showed increased replication fitness compared with WNV-prME/DTV, which was more pronounced at the higher MOI. At MOI of 5, the parental DTV strain achieved a peak mean titer of 6.5 \log_{10} PFU/mL, while the WNV-prME/DTV chimeric virus reached a lower peak mean titer of 4.4 \log_{10} PFU/mL ($p < 0.01$). Titer differences were observed by dpi 2 at MOI of 5 and at dpi 4 at an MOI of 0.1.

In vivo mosquito infection

Culex pipiens females were orally exposed to 6–6.5 \log_{10} PFU/mL bloodmeal of the two parental and two prME chimeric viruses and were tested for infection (bodies), dissemination (wings), and transmission potential (saliva) by CPE assay on Vero cell monolayers and verified by quantitative RT-PCR. Of the 12 mosquitoes exposed to DTV that survived the 14-day extrinsic incubation period, none demonstrated infection or dissemination. Accordingly, transmission was not assessed for any of the saliva samples from these mosquitoes (Table 1). Similarly, none of the 24 surviving *C. pipiens* mosquitoes exposed to chimeric WNV-prME/DTV showed evidence of infection or subsequent dissemination (Table 1). As expected, the WNV parental virus was found to have infected 50% (6/12) of orally exposed females and, of those, 83% (5/6) and 67% (4/6) demonstrated successful dissemination and transmission, respectively (Table 1). In contrast, 0 of the 20 surviving DTV-prME/WNV chimeric virus-exposed mosquitoes demonstrated infection, dissemination, or transmission potential (Table 1).

***In vivo* tick infection**

Following immersion of adult *I. scapularis* with the two parental and two chimeric viruses, organs were dissected out of the ticks and viral loads were quantified by quantitative RT-PCR to estimate viral titers. Three to five females and one to four males per virus group survived submersion and incubation and were tested for viral RNA. Data by sex were combined for analysis. Both DTV and WNVprME/DTV were detected in tick salivary glands with peak titers of $4.8 \log_{10}$ PFU/ μg of RNA [$\bar{x} = 3.5 \log_{10}$ (PFU/ μg of RNA)] and $4.2 \log_{10}$ PFU/ μg of RNA [$\bar{x} = 3.0 \log_{10}$ (PFU/ μg of RNA)], respectively (Fig. 4A). Alternatively, neither WNV nor the DTVprME/WNV chimera was detected in salivary glands of exposed ticks. Interestingly, RNA from DTV, WNVprME/DTV, and WNV was detected in other organs tested, while the DTVprME/WNV chimera was not. Specifically, DTV, WNVprME/DTV, and WNV reached a peak titer of $5.8 \log_{10}$ PFU/ μg of RNA [$\bar{x} = 3.7 \log_{10}$ (PFU/ μg of RNA)], $4.7 \log_{10}$ PFU/ μg of RNA [$\bar{x} = 3.0 \log_{10}$ (PFU/ μg of RNA)], and $5.0 \log_{10}$ PFU/ μg of RNA [$\bar{x} = 2.3 \log_{10}$ (PFU/ μg of RNA)], respectively (Fig. 4B). Further analysis of infection observed in other organs of the WNV exposure group demonstrated that WNV RNA was only detected in males and not in females. This was the only difference observed between males and females during the tick challenge experiments. A Student's *t*-test examining the difference in mean titers between surviving ticks following exposure to DTV and WNV-prME/DTV showed no difference regardless of the organ tested.

Discussion

The genetic determinants for flavivirus adaptation to replication in mosquito or tick vectors form an area requiring additional investigation. Understanding genetic determinants and any adaptive constraints these factors impart on flaviviruses will enhance our ability to design attenuated, host-restricted, yet replication-competent, live-attenuated vaccine candidates. DTV and WNV were selected because of their distinct evolutionary history and ecological phenotypes. Chimeric viruses between DTV and WNV were generated to assess genetic determinants of infection and growth within *in vitro* and *in vivo* models for each virus's respective arthropod vectors. Based on limited previous studies, we hypothesized that NS regions largely determine replicative fitness for both MBFV and TBFV in their respective vectors. In support of this hypothesis, it was observed that matching NS proteins were essential for replication in each respective natural invertebrate model as viruses with a DTV backbone did not grow within *in vitro* or *in vivo* mosquito cells and chimeric viruses with a WNV NS backbone were unable to grow in ISE6 cells or infect *I. scapularis*. However, we did observe infection in other organs of three of four male ticks exposed to WNV. This implies that NS regions of the WNV genome are driving infection of nonsalivary organs in males as the only chimera unable to infect ticks was the chimera lacking WNV NS regions. There is an established precedent for WNV infection in ticks as previous laboratory infection studies demonstrated that *I. scapularis* and *Ixodes pacificus* became infected and maintained the virus transstadially following oral exposure to viremic host models (Anderson et al. 2003, Reisen et al. 2007). Nevertheless, further examination of the potentially sex-dependent infection of tick organs by WNV is warranted.

While the NS regions were shown to be primary predictors of replication competence, attenuation was demonstrated by each chimeric virus in Vero cells, C6/36, and ISE6 cells, suggesting that inclusion of heterologous structural regions had a mitigating effect on replication. A sequence alignment between the prME regions of DTV and WNV indicates a nucleotide identity of 50% and an amino acid identity of only 37.6%, highlighting the genomic and potentially functional divergence of these proteins. Conserved elements within this region have been implicated with playing a role in prM-E interactions and prM/M cleavage (Guirakhoo et al. 1992, Heinz et al. 1994). Similarly, the lack of conservation in the α -helical domain in the M protein has also been shown to be important for proper maturation of viral particles, viral entry, and formation of E homodimers following pr-M cleavage (Wengler and Wengler 1989, Stiasny et al. 1996, Hsieh et al. 2014).

Vector group-specific characteristics of the structural E protein have also been described that could account for attenuation of chimeric viruses described herein. Initial studies of domain III of the E protein, a region largely implicated in receptor binding, have demonstrated that characteristics of this region may be important for mediating vector-specific interactions, including the proposed receptor-binding site for TBFV. It has previously been shown that the pH-dependent conformational rearrangement of flavivirus particles from E dimers to E trimers is required for subsequent membrane fusion (Stiasny et al. 1996). Examinations of the dynamic properties of TBFV and MBFV recombinant E domain III proteins have suggested that TBFV has an increased tolerance to a wider range of environmental conditions, including low pH, which may contribute to their ability to persist in tissues and fluids such as milk and still be viable for transmission (Yu et al. 2004).

While the hypothesis that NS proteins largely drive flavivirus host association was supported in both *in vivo* and *in vitro* tick cells, *in vitro* mosquito cells, and Vero cells, the determinants of mosquito competence and growth in BHK cells were not fully explained. Specifically, DTV-prME/WNV growth was largely attenuated compared with WNV in mosquitoes and BHK cells, yet the WNV-prME/DTV chimera behaved no differently from DTV. BHK cells were utilized in an attempt to model the ecology of DTV that utilizes a rodent reservoir in nature. These findings could indicate different receptor-mediated effects of structural genes between WNV and DTV in BHK cells.

Alternatively, examination of the interaction between flaviviruses and host type I IFN response has indicated likely virus–host specificity. For instance, TRIM79 α , an interferon-stimulated gene, has been previously shown to restrict TBFV through targeted lysosomal-dependent decay of NS5, a known type I IFN antagonist (Best et al. 2005). Interestingly, this targeted degradation of NS5 was specific to TBFV and did not act on WNV-derived NS5 (Taylor et al. 2011). However, a study comparing type I IFN restriction of multiple flaviviruses in primary cells from *Peromyscus leucopus*, a predicted reservoir host for DTV, and *Mus musculus* showed that while replication restriction as a result of IFN was limited to *P. leucopus* cells, both TBFV and MBFV were attenuated (Izuogu et al. 2017). This highlights the complex arms race occurring between virus replication and its reservoir host's innate immunity.

The observed phenotypes of infection in mosquitoes also did not support the hypothesis that NS elements are the primary determinants of competent infection and replication as the DTV-prME/WNV chimera showed no infection in *C. pipiens*. It should be noted that this experimental finding was based on a small number of mosquitoes ($N = 12$), so while we are not confident saying that the DTV-prME/WNV chimera is unable to infect mosquitoes, we can conclude that the ability to infect is significantly diminished compared with the WNV parental. It is possible that this is related to the absence of the fusion loop region in the DTV-prME. Interestingly, the fusion loop region of 4 amino acids in domain III of the E glycoprotein is present and conserved in MBFV, but absent in TBFV. Mutagenesis studies have indicated that the absence of this fusion loop drastically reduced MBFV replication in Vero cells and the infection rate in *Aedes aegypti* mosquitoes, but not growth in C6/36 cells (Erb et al. 2010). The growth characterization results demonstrate that DTV-prME/WNV was able to grow in C6/36 cells, but was unable to infect *Culex* mosquitoes *per os*, which could be explained by the absence of this fusion loop in DTV-prME regions of the chimeric virus.

Although not assessed in this study, future studies will consider the role of noncoding determinants of *in vivo* vector specificity. A conserved feature identified in the 3' UTR of flaviviruses is elements that generate subgenomic flavivirus RNA (sfRNA) upon interaction with host 5'–3' exoribonuclease. Following oral infection of sfRNA-deficient WNV mutants, reduced rates of *C. pipiens* infection and transmission were observed, whereas uninhibited transmission and infection rates were observed following intrathoracic inoculation in *C. pipiens*. This suggests that sfRNA is likely associated with viral evasion of the midgut infection or escape barrier (Goertz et al. 2016). While, the role of TBFV sfRNA for knockdown of RNAi for *in vitro* tick cell models has also been demonstrated (Schnettler et al. 2014), it is unclear if the sfRNA of each TBFV and MBFV is uniquely adapted to their respective vectors, thus acting as a limiting factor for infection/replication in the nontraditional insect vector.

The work described herein shows that NS regions are largely responsible for determinants of replication competence for *in vitro* vector models. It also highlighted different key determinants for replication in ticks and mosquitoes. Future studies will focus on *in silico* structural modeling and mutational analysis of 3' UTR sfRNA sequences between MBFV and TBFV, targeted site-directed mutagenesis of heterologous prME elements of interest, and detailed determination of genomic sites under selective pressures by way serial passaging and temperature sensitivity studies.

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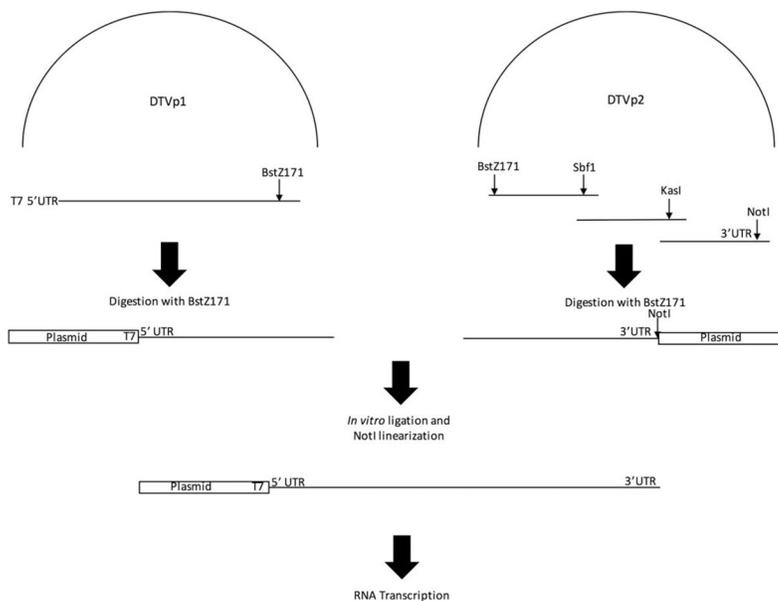


FIG. 1. Construction of the DTV Spooner strain infectious clone and viral rescue. A T7 promoter was added to a 3597-bp amplicon terminating at a *BstZ171*, which was subsequently cloned into a low-copy number plasmid with kanamycin resistance and designated DTVp1. DTVp2 was generated by cloning overlapping fragments (7228 bp) with naturally occurring restriction sites *BstZ171*, *SbfI*, and *KasI*. *NotI* was introduced as a ligation site following the 3' UTR. Both DTVp1 and DTVp2 were digested with *BstZ171* before *in vitro* ligation and linearization with *NotI*. The resulting linear DNA was transcribed to RNA for subsequent electroporation. DTV, deer tick virus.

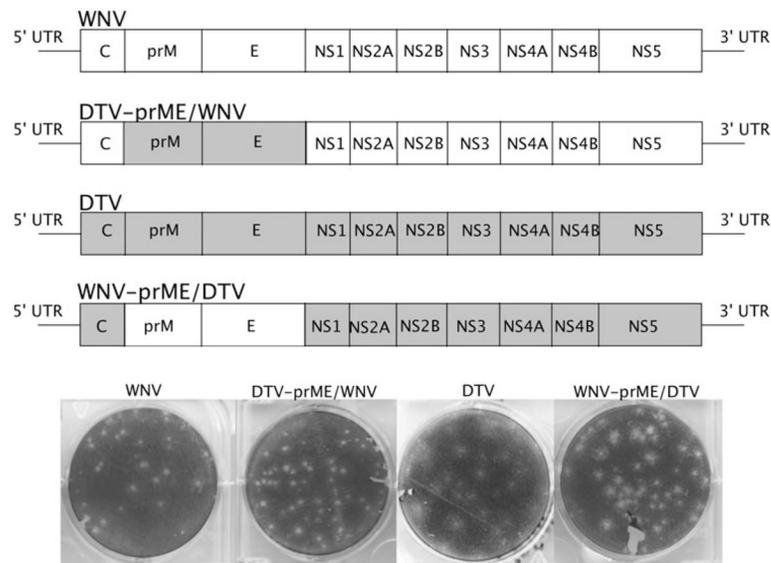


FIG. 2. Schematic illustration of the WNV, DTV-prME/WNV, DTV, and WNV-prME/DTV genomes and their corresponding plaque phenotypes. Clone-derived WNV was generated using *in vitro* ligation of WNVp1 and WNVp2 and subsequent linearization and *in vitro* transcription, whereas DTV-prME/WNV was generated from ligation, linearization, and *in vitro* transcription of DTV-prME/WNVp1 and WNVp2. DTV was generated as described with DTVp1 and DTVp2. WNV-prME/DTV was generated from ligation of a 3727-bp PCR amplicon (T7-DTVcapsid-WNVprME-DTVNS1-*Bstz17I*) with DTVp2 and subsequent *in vitro* transcription. WNV, West Nile virus.

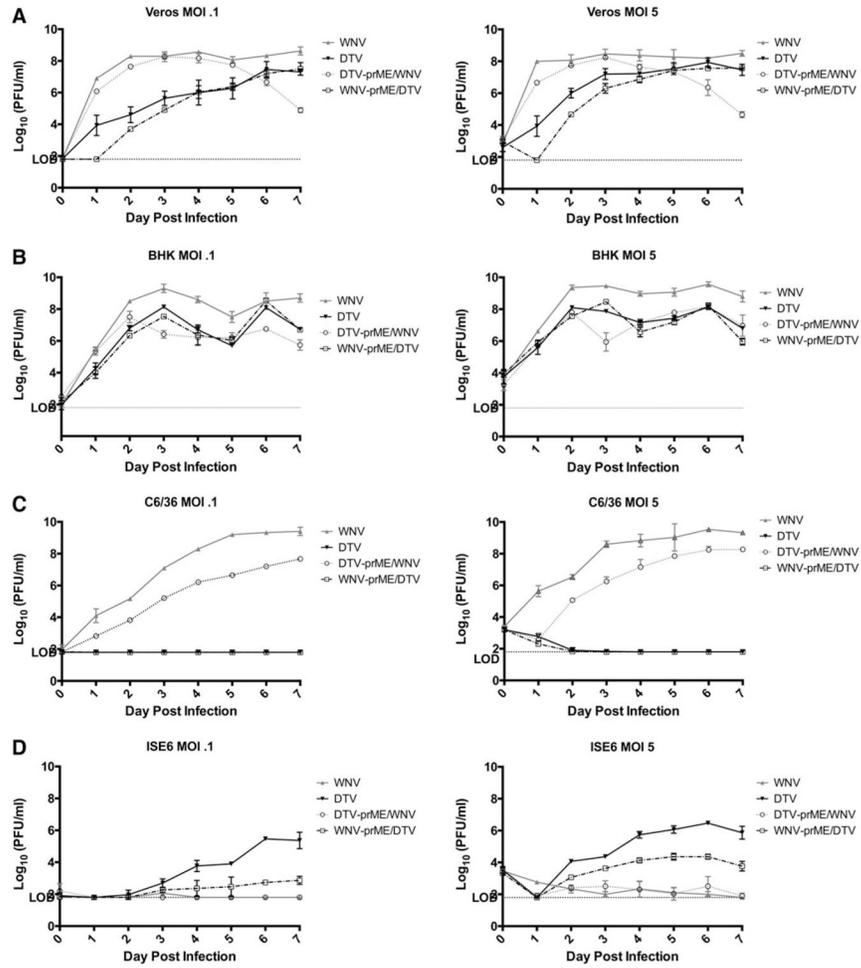


FIG. 3. Growth comparison of the DTV and WNV infectious clone-derived parental and chimeric WNV-prME/DTV and DTV-prME/WNV viruses in various vertebrate and invertebrate cell lines. Inoculations were performed at MOIs of 0.1 and 5 on (A) Vero, (B) BHK, (C) C6/36, and (D) ISE6 cells. LOD was 1.8 log₁₀ (PFU/mL). Titers were determined by plaque assay on Vero cells. BHK, baby hamster kidney cells; LOD, limit of detection; PFU, plaque-forming units; MOIs, multiplicities of infection.

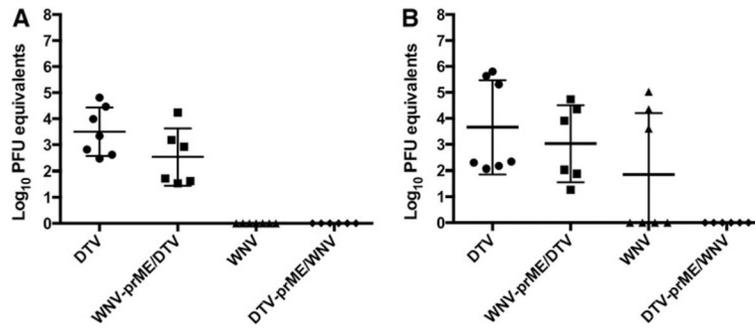


FIG. 4. Comparison of PFU equivalents determined by RNA loads derived from quantitative RT-PCR converted to titers based on a standard curve in (A) salivary glands and (B) other organs in between of *Ixodes scapularis* following immersion and synchronous infection.

Table 1

Results of Oral Exposure of *Culex pipiens* Mosquitoes

<i>Virus</i>	<i>N</i>	<i>Inoculum stock (PFU/mL) log₁₀</i>	<i>Infection (%)</i>	<i>Dissemination infection (%)^a</i>	<i>Transmission (%)^{b,c}</i>
DTV	12	6	0 (0)	0 (0)	0 (0)
WNV-prME/DTV	24	6.4	0 (0)	0 (0)	0 (0)
WNV	12	6.3	6 (50)	5 (83)	4 (67)
DTV-prME/WNV	20	6.5	0 (0)	0 (0)	0 (0)

^aNo. of disseminated/no. of infected.

^bTransmission determined by salivation.

^cNo. of positive transmissions/no. of infected.

DTV, deer tick virus; PFU, plaque-forming units; WNV, West Nile virus.