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Adaptation of Rabensburg virus (RBGV) to vertebrate hosts by experimental evolution

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ABSTRACT

Rabensburg virus (RBGV; *Flaviviridae, Flavivirus*) has been classified as both a novel flavivirus and a unique lineage of West Nile virus (WNV). RBGV and WNV share approximately 76% sequence homology, yet RBGV does not replicate to high viral titers within vertebrate cell lines at physiological temperatures and has not been naturally isolated from a vertebrate host. These unique genetic and biological characteristics make RBGV a viable tool to identify the genetic determinants of flavivirus infectivity and fitness in vertebrate hosts. Using experimental evolution, we characterized mutated variants of RBGV that have altered capacity for infection and replication in various cell lines. Shared genetic differences within these variants were identified throughout the genome, with a large majority found in the NS3 and NS5 genes. Our results support a role for the replication complex in host utilization and suggest that epistatic interactions likely contribute to host-specific fitness and emergence.

1. Introduction

The *Flavivirus* genus, of the family *Flaviviridae*, contains many viruses that are serious global health concerns including dengue virus (DENV), Zika virus (ZIKV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV) (Kuno et al., 1998). WNV has a worldwide distribution and is the most prevalent arbovirus in the United States. Since its emergence in 1999, there have been approximately 48,000 WNV human cases diagnosed in the U.S., resulting in over 2000 fatalities (ArboNET, 2016).

There are two primary lineages of WNV: Lineage 1 is present in Europe, Africa, the Middle East, America, Australia, and India and is further divided among three different clades (Bakonyi et al., 2006). Clade 1A contains isolates from Europe, Africa, the United States, and Israel. Clade 1B is comprised of strains from Australia (Kunjin virus) and Clade 1C from India (Bondre et al., 2007). Lineage 2 strains have been isolated from sub-Saharan Africa, Madagascar and Europe (Bakonyi et al., 2006). Up to seven other additional lineages of WNV have been proposed (Bondre et al., 2007; Hubalek et al., 1998; Bakonyi et al., 2005).

Rabensburg virus (RBGV), originally isolated from a pool of *Culex pipiens* mosquitoes in the South Moravia region, Czech Republic in 1997 (Hubalek et al., 1998), was classified as lineage 3 WNV (Bakonyi et al., 2005). In 1999, RBGV was isolated again from a pool of *Cx. pipiens* in the South Moravia region (Hubalek et al., 2000) and in 2006 from a pool of *Aedes rossicus* mosquitoes (Hubalek et al., 2010). Biological characterization and phylogenetic studies of RBGV have since proposed that it is a novel flavivirus and not a third lineage of WNV (Bondre et al., 2007; Bakonyi et al., 2005; Aliota et al., 2012; Aliota and Kramer, 2012).

Despite 75–77% nucleotide sequence similarity and 89–90% amino acid sequence similarity with WNV lineages 1 and 2, no clinical manifestations have been linked with a RBGV infection in humans and no RBGV isolate has been obtained from a vertebrate host in nature (Bakonyi et al., 2005). WNV lineages 1 and 2 are capable of producing symptoms of mild febrile illness, headache, myalgias, nausea, vomiting, and chills in humans (Rossi et al., 2010; Hernandez-Triana et al., 2014). Both lineages are responsible for WNV infections that become neuroinvasive and cause meningitis, encephalitis, and poliomyelitis-like disease (Rossi et al., 2010; Campbell et al., 2002). WNV incubation periods range from 2 to 15 days, with detectable viremia lasting until symptom onset (Rossi et al., 2010). WNV is a generalist, detected in over 75 mosquito species, 300 bird species, various reptiles, and some mammal species (Higgs et al., 2004; Marra et al., 2003; Klenk et al., 2004; Padget et al., 2007).

Characterization studies have revealed RBGV to be a biologically

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and evolutionarily unique flavivirus (Aliota et al., 2012; Aliota and Kramer, 2012). RBGV 97–103 readily infects and replicates within mosquito cells producing cytopathic effect (CPE) and viral growth kinetics similar to WNV, yet at physiological temperatures, it is unable to infect and replicate efficiently in monkey, hamster, human, and avian (Aliota et al., 2012). RBGV replication in other vertebrate cell lines including reptiles and amphibians should be tested. However, growth of RBGV 97–103 on Xenpus laevis (XTC-2) cell line at 28 °C has been documented, showing substantial replication (Hubalek et al., 2000). No viremia or antibody was detected following subcutaneous inoculations of chickens and house sparrows with RBGV (Aliota et al., 2012). Although mortality has been noted with RBGV in suckling mice inoculated intracranially and intraperitoneally, (Hubalek et al., 2010) virulence has not been observed in adult mice, regardless of route of infection (Hubalek et al., 2010). Human embryonic kidney cells (HEK-293) produced infectious virus following electroporation with RBGV RNA, suggesting that although RBGV is unable infect HEK-293 cells at physiological temperatures, it has some capacity to replicate once RNA gains access to the cytoplasm (Aliota et al., 2012). This vertebrate infection barrier can be overcome at temperatures below 35 °C, yet peak titer and replication rate of RBGV are significantly lower than WNV, suggesting it is a combination of temperature and host cell type that prevent RBGV from efficiently utilizing vertebrate hosts (Aliota and Kramer, 2012).

Here, we exploited these unique phenotypes to gain insight into the genetic factors that limit and/or facilitate host expansion of flaviviruses. Specifically, we characterized RBGV before and after experimental passage in cell culture and utilized sequencing and reverse genetics to identify mutations associated with host and temperature-specific fitness. Our results contribute to the understanding of both the genetic and phenotypic plasticity of these viruses.

2. Results

2.1. In vitro growth kinetics and passaging

Table 1. lists the primers and probes used to quantify the growth of RBGV and WNV02–1986 isolates. Growth kinetics of unpassed RBGV 06–222, RBGV 97–103 and WNV02–1986 were compared on Vero and C6/36 cells at 28 °C (Fig. 1). WNV02–1986 replicated to significantly higher titers than both RBGV isolates at all time points on Vero cells (one-way ANOVA, $p < 0.05$ by Tukey post hoc test), with differences at peak titer of ~6 $\log_{10}$ PFU/mL. In C6/36 cells, a significant difference in viral titer was observed only at 24 h post infection (hpi; *t*-test, $p < 0.05$) and overall growth kinetics were similar (Fig. 1).

RBGV 97–103 and RBGV 06–222 were passaged multiple times at increasing temperatures in HEK 293 cells (n = 4 passages, HTP4) to select for variants with an increased capacity to infect and replicate in vertebrate cells at higher temperatures. Passages maintained at 28 °C (n = 5 passages, CTP5) were also performed as a control for determining cell-specific adaptation versus temperature-specific adaptation.

Table 1
Nucleotide sequences of primers and probes for West Nile virus (WNV) and Rabensburg virus (RBGV).

	Sequence	Genomic position
WNV		
Forward	5′-TCAGCGATCTCTCCACCAAAG-3′	1160
Probe	5′-FAM-TGCCCGACCATGGGAGAAGCTC-TAMRA-3′	1186
Reverse	5′-GGGTCAGCACGTTTGTCATTG-3′	1229
RBGV		
Forward	5′-GAGCAGATCCAGCGTACTTC-3′	1220
Probe	5′-/56-FAM/AAGGTGTTG/ZEN/ TGGACAGAGGATGGG/3IABkFQ/™-3′	1247
Reverse	5′-CGTGTCTATGCTCCCTTTACC-3′	1291

The four passaged strains of RBGV were tested in comparison to the unpassed isolates in HEK-293, PDE, and C6/36 cell lines at various temperatures to observe changes in fitness resulting from viral passaging (Figs. 2–4). There was an increase in viral replication with RBGV06-HTP4 compared to the unpassed RBGV 06–222 at all temperatures tested in HEK-293 cells (Fig. 2A-C; one-way ANOVA, $p < 0.05$ by Tukey post hoc test). In contrast, unpassed RBGV97–103 replicated to higher titers in HEK-293 cells relative to RBGV97-HTP4 (one-way ANOVA, $p < 0.05$ by Tukey post hoc test), except at 37 °C, where no growth was measured (Fig. 2D-F). Interestingly, both CTP5 strains of RBGV showed higher replicative fitness than unpassed or high temperature passed strains at all temperatures in HEK-293 cells (Fig. 2A-F). In PDE cells, RBGV 06-HTP4 grew to higher titers than the unpassed RBGV06–222 (Fig. 3A). The opposite was observed with RAB97-HTP4, which grew to significantly lower titers than the unpassed RBGV97–103 (one-way ANOVA, $p < 0.05$ by Tukey post hoc test; Fig. 3B). Both control strains (RBGV06-CTP5 and RBGV97-CTP5) had increased replicative fitness relative to high temperature passed and unpassed strains at 35 °C (one-way ANOVA, $p < 0.05$ by Tukey post hoc test; Fig. 3A-B). In C6/36 cells, an initial increased rate of replication for RBGV06-HTP4 and RBGV06-CTP5 relative to RBGV 06–222 was measured at 35 °C, with higher viral titers measured at 48–96 hpi (*t*-test, $p < 0.05$; Fig. 4A). At 28 °C in C6/36 cells, similar growth kinetics were observed for RBGV 06–222 and RBGV06-HTP4 with higher initial rates of replication observed between 24 and 96 hpi for RBGV 06-CTP5 (*t*-test, $p < 0.05$; Fig. 4B). Interestingly, decreased replication was measured for RBGV97-HTP4 at 35 °C on C6/36 relative to unpassed and control passed strains, but at 28 °C, both passed strains replicated to higher titers at early time points relative to unpassed virus (*t*-test, $p < 0.05$; Fig. 4C-D).

2.2. Sequencing and reverse genetics

Full genome sequences were performed and novel sequences of RBGV 06–222, 97-CTP5, 97-HTP4, 06-CTP5, 06-HTP4 were compared to RBGV97–103 and WNV02–1986. A total of 9 nucleotide differences were observed between RBGV 97–103 and RBGV06–222, resulting in 3 amino acid changes (Table 2). Most of the nucleotide changes (Bakonyi et al., 2005) were in the NS5 gene, including the non-synonymous changes G7982T in the methyltransferase (Mtase) and G8842A in the RNA-dependent RNA polymerase (RdRp). Ten and 12 nucleotide substitutions accumulated throughout the genome during passage of RBGV97–103, resulting in 4 and 6 amino acid changes in control (97-CTP5) and high temperature (97-HTP4) strains, respectively (Table 2). All mutations except 2 identified in 97-HTP4 were found in 97-CTP5. Six and 8 nucleotide substitutions accumulated throughout the genome during passage of RBGV06–222, resulting in 5 and 6 amino acid changes in control (06-CTP5) and high temperature (06-HTP4) strains, respectively (Table 2). Three amino acid changes identified in 06-HTP4 was not present in 06-CTP5. Two non-synonymous changes, A5716G and A6901G, were identified in all passed strains, resulting in serine to glycine substitutions in the NS3 and 2K peptides, respectively. Interestingly, G5716 is also conserved among WNV strains.

2.3. Reverse genetics and mutant characterization

To determine if the change at position 5716 in the NS3 gene contributed to the limited host range of RBGV, we utilized a WNV infectious clone (WNV-IC) to reverse engineer a G5716A mutation in WNV, resulting in a glycine to serine substitution at amino acid 350 in the helicase domain of the NS3. Although the effect was modest, WNV G5716A showed decreases in viral titers in HEK-293 cells at 28 °C and at 37 °C compared to WNV-IC (*t*-test, $p < 0.05$; Fig. 5A-B).

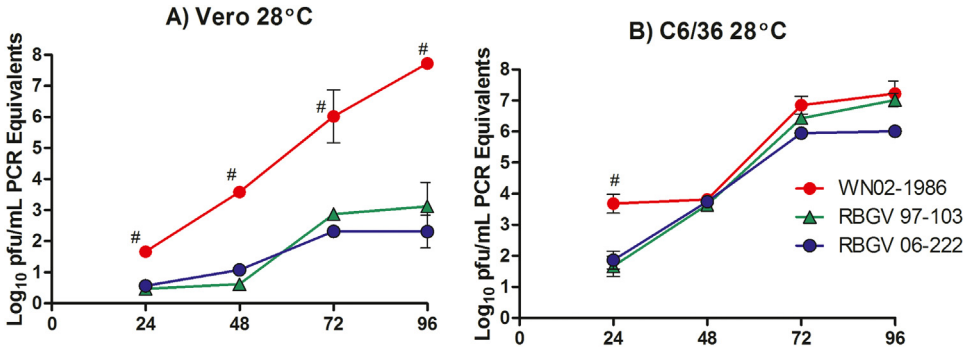

Fig. 1. Growth kinetics of West Nile virus 1986 (WNV02–1986) and Rabensburg virus (RBGV) 97–103 and RBGV 06–222 in **A)** Vero cells at 28 °C and **B)** C6/36 cells at 28 °C. Viral RNA was quantified by real-time RT-PCR using plaque forming units per milliliter (PFU/mL) standards for individual strains. Data points are a mean of triplicate experimental measurements with standard deviations. # Denotes significant difference (one-way ANOVA, $p < 0.05$ by Tukey post hoc test) between WN02–1986 and both RBGV isolates at indicated time points.

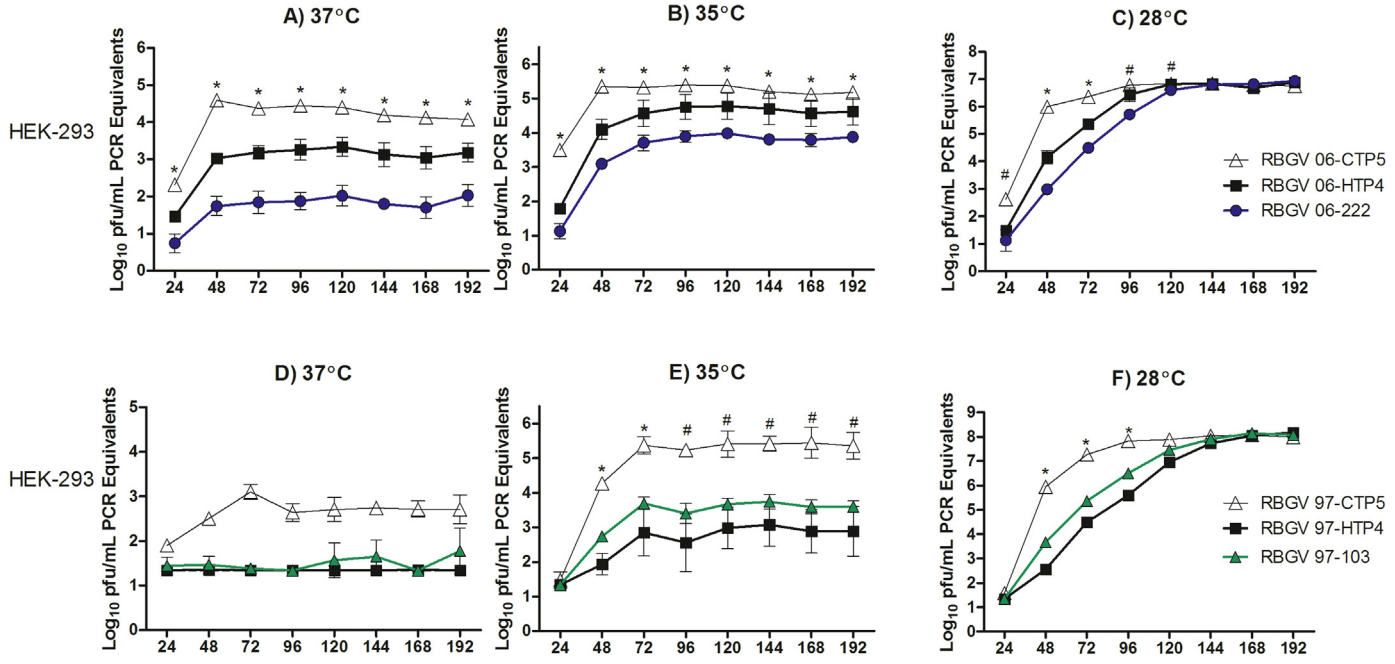

Fig. 2. Growth kinetics of unpassed Rabensburg Virus (RBGV) 06–222, RBGV 97–103, control temperature (CTP5), and high temperature (HTP4) passed RBGV strains in HEK-293 cells at three different temperatures. Viral RNA was quantified by real-time RT-PCR using plaque forming units per milliliter (PFU/mL) standards for individual strains. Data points are a mean of triplicate experimental measurements with standard deviations. * Denotes significant difference (one-way ANOVA, $p < 0.05$ by Tukey post hoc test) between all viruses at indicated time points. # Denotes significant difference (one-way ANOVA, $p < 0.05$ by Tukey post hoc test) between control temperature passed and unpassed RBGV strains at indicated time points. A) RBGV 06 strains in HEK-293 cells at 37 °C. B) RBGV 06 strains in HEK-293 cells at 35 °C. C) RBGV 06 strains in HEK-293 cells at 28 °C. D) RBGV 97 strains HEK-293 cells at 37 °C. E) RBGV 97 strains in HEK-293 cells at 35 °C. F) RBGV 97 strains in HEK-293 cells at 28 °C.

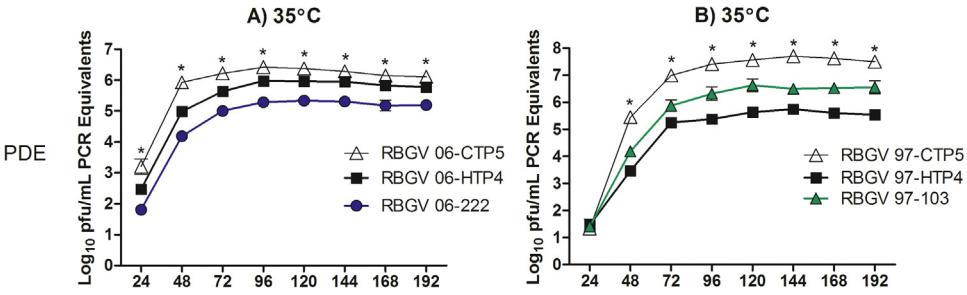

Fig. 3. Growth kinetics of unpassed Rabensburg virus (RBGV) 06–222, RBGV 97–103, control temperature (CTP5), and high temperature (HTP4) passed RBGV strains in avian cells (PDE) at 35 °C. Viral RNA was quantified by real-time RT-PCR using plaque forming units per milliliter (PFU/mL) standards for individual strains. Data points are a mean of triplicate experimental measurements with standard deviations. * Denotes significant difference (one-way ANOVA, $p < 0.05$ by Tukey post hoc test) between all viruses at indicated time points. A) RBGV 06 strains in PDE cells at 35 °C. B) RBGV 97 strains in PDE cells at 35 °C.

3. Discussion

Our results generally support the mosquito-specific nature of RBGV and demonstrate that this virus has a limited capacity to adapt to replication in vertebrate cells at higher temperatures. Our initial characterization confirmed that RBGV infection and replication in vertebrate cell culture occurs at 28 °C, yet peak viral loads remain significantly lower than WNV. Efficient replication of RBGV in tested vertebrate cells at temperatures above 28 °C has been achieved but requires electroporation or isolation following inoculation of suckling

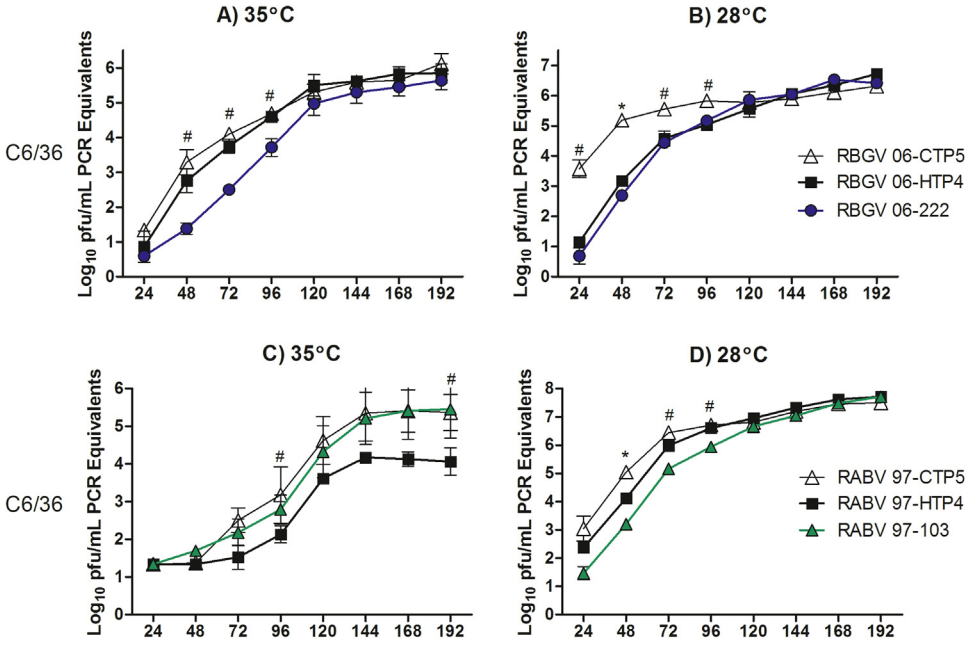

Fig. 4. Growth kinetics of unpassed Rabensburg virus (RBGV) 06–222, RBGV 97–103, control temperature (CTP5) and high temperature (HTP4) passed RBGV strains in C6/36 cells at 35 °C and 28 °C. Viral RNA was quantified by real-time RT-PCR using plaque forming units per milliliter (PFU/mL) standards for individual strains. Data points are a mean of triplicate experimental measurements with standard deviations. * Denotes significant difference (one-way ANOVA, $p < 0.05$ by Tukey post hoc test) between all viruses at indicated time points. # Denotes significant difference (one-way ANOVA, $p < 0.05$ by Tukey post hoc test) between RBGV97-CTP5 and unpassed isolate at indicated time points. **A)** RBGV 06 strains in C6/36 cells at 35 °C. **B)** RBGV 06 strains in C6/36 cells at 28 °C. **C)** RBGV 97 strains in C6/36 cells at 35 °C. **D)** RBGV 97 strains in C6/36 cells at 28 °C.

mouse brains (Aliota and Kramer, 2012). Our results further demonstrate that the barrier to vertebrate utilization is likely both temperature and host-dependent, yet there is also evidence for variability among vertebrate hosts. For instance, although RBGV is not known to infect birds in nature or experimentally (Aliota et al., 2012), it grows to significantly higher titers on avian cells as compared to mammalian cells at near physiological temperatures, suggesting adaptation to avian host may be attainable.

Results also demonstrate phenotypic variability among 1997 and 2006 RBGV isolates. Previous studies suggest increased virulence of RBGV 06–222 relative to RBGV 97–103 in sucking mice (Aliota et al., 2012). There are 9 total base differences between these strains and just 3 amino acid substitutions, one in the M gene and two in the NS5 gene. The non-synonymous mutations in the NS5 gene include individual substitutions in the MTase and RdRp. These enzymes interact with one another, are both critical for viral replication, and have been implicated in host-specific fitness differences previously (Li et al., 2014; Zhang et al., 2008; Van Slyke et al., 2012, 2015). These results together suggest that more recent RBGV strains may have modestly higher fitness potential in vertebrate hosts. Consistent with this, we found that RBGV

Table 2
Genetic differences among unpassed Rabensburg virus (RBGV 97, RBGV 06), control temperature (97-CTP5, 06-CTP5), high temperature (97-HTP4, 06-HTP4) and West Nile virus (WNV02).

Involved Gene	Genomic Position	Nucleotide (Amino Acid)						
		RBGV 97	RBGV 06	97-CTP5	97-HTP4	06-CTP5	06-HTP4	WNV02
5' UTR	84	**C**	**T**	**T**	**T**	T	T	C
M	582*	**A (Ile)**	**G (Met)**	A (Ile)	A (Ile)	G (Met)	**A (Ile)**	G (Met)
M	720	**C** (Arg)	**A** (Arg)	**A** (Arg)	**A** (Arg)	A (Arg)	A (Arg)	C (Arg)
ENV	2234*	A (Asp)	A (Asp)	A (Asp)	**C (Ala)**	**G (Gly)**	**C (Ala)**	A (Asp)
NS1	2694	C (Leu)	C (Leu)	C (Leu)	C (Leu)	**T** (Leu)	**T** (Leu)	G (Leu)
NS3	5716*	A (Ser)	A (Ser)	**G (Gly)**	**G (Gly)**	**G (Gly)**	**G (Gly)**	G (Gly)
NS3	5909*	T (Ile)	T (Ile)	**C (Thr)**	**C (Thr)**	**C (Thr)**	T (Ile)	C (Thr)
NS4A	6590*	G (Arg)	G (Arg)	**C (Thr)**	**C (Thr)**	G (Arg)	G (Arg)	G (Arg)
2K	6901*	A (Ser)	A (Ser)	**G (Gly)**	**G (Gly)**	**G (Gly)**	**G (Gly)**	A (Ser)
NS5 (MTase)	7840*	T (Ser)	T (Ser)	T (Ser)	**C (Pro)**	**C (Pro)**	**C (Pro)**	C (Pro)
NS5 (MTase)	7982*	**G (Arg)**	**T (Ile)**	G (Arg)	G (Arg)	T (Ile)	**G (Arg)**	G (Arg)
NS5 (RdRp)	8842*	**G (Glu)**	**A (Lys)**	G (Glu)	G (Glu)	A (Lys)	A (Lys)	G (Ala)
NS5 (RdRp)	9136	**C** (Leu)	**T** (Leu)	**T** (Leu)	**T** (Leu)	T (Leu)	T (Leu)	C (Leu)
NS5 (RdRp)	9630	**C** (Thr)	**G** (Thr)	**G** (Thr)	**G** (Thr)	G (Thr)	G (Thr)	C (Thr)
NS5 (RdRp)	9843	**G** (Leu)	**C** (Leu)	**C** (Leu)	**C** (Leu)	C (Leu)	C (Leu)	G (Leu)
NS5 (RdRp)	10143	A (Val)	A (Val)	**G** (Val)	**G** (Val)	A (Val)	A (Val)	T (Val)
NS5 (RdRp)	10341	**C** (Tyr)	**T** (Tyr)	C (Tyr)	C (Tyr)	T (Tyr)	**C** (Tyr)	C (Tyr)

* Denotes amino acid (aa) change. Bold lettering denotes nucleotide and/or aa differences between unpassed RBGV strains. Red lettering denotes nucleotide and/or aa differences between unpassed and control/high temperature RBGV strains.

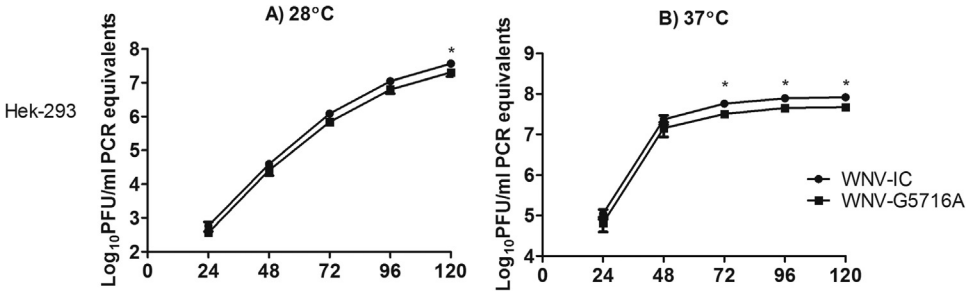

Fig. 5. Growth kinetics of West Nile virus infectious clone (WNV-IC) and WNV NS3 mutant (WNV-G5716A) in HEK-293 cells at **A)** 28 °C and **B)** 37 °C. Viral RNA was quantified by real-time RT-PCR using plaque forming units per milliliter (PFU/mL) standards for individual strains. Data points are a mean of triplicate experimental measurements with standard deviations. * Denotes significant difference between WNV-IC and WNV-G5716A at indicated time points (*t*-test, $p < 0.05$).

06–222 has an increased capacity for adaptation to vertebrate cell culture.

Attempts were made to passage RBGV isolates on mammalian (Vero and HEK-293) and avian (PDE) cell lines. Virus could only be consistently recovered at temperatures above 28 °C on HEK-293 cells, therefore; we chose these cells for subsequent passage studies.

Experimental evolution was only modestly successful. While just 4–5 passages were achieved for both RBGV isolates, attempts to passage far exceeded this as most additional passages and/or temperature increases failed to recover virus, likely due to a lack of adaptive change combined with the detrimental effects of repeated bottlenecks resulting from low titer outputs (Muller, 1964). Only one high temperature lineage was maintained through passage 4, while a duplicate passage series was attempted but was unsuccessful. Since we only analyzed RNA from cell culture supernatants, perhaps RBGV replication is impaired at higher temperatures within the cell or that viral maturation is inhibited due to high temperatures. Given that there is approximately 10% amino acid divergence between WNV and RBGV, and clearly multiple barriers to host utilization, it is perhaps not surprising that limited passaging and/or adaptation was achievable. Even though RBGV may often encounter vertebrate hosts through transmission events and, like all RNA viruses, possesses a high capacity for adaptive evolution due to the high levels of error-prone replication (Drake and Holland, 1999; Sanjuan et al., 2010; Garcia-Villada and Drake, 2012), it has never been isolated from a vertebrate host (Aliota et al., 2012). These results suggest that complex, epistatic interactions are likely responsible for host restriction of RBGV, and therefore, its capacity to utilize the WNV transmission cycle and/or spillover to cause human disease is likely limited.

The majority of mutations accumulated during passaging were non-synonymous, consistent with selective sweeps during adaptive evolution. Despite this, RBGV 97–103 passed at increasing temperatures showed no evidence of adaptation and was in fact further attenuated in multiple host cell types and temperatures following cell culture passage. This is consistent with the fact that this strain (97-HTP4) replicated to lower titers throughout passaging and was therefore subjected to the most stringent bottlenecks, yet all consensus substitutions identified in 97-HTP4 were shared with at least one of the adapted strains. Phenotypic consequences, therefore, either resulted from interactions among this specific combination of mutations or from unidentified variability in mutant swarm breadth and/or composition. The phenotypic impact of the viral swarm is well documented and future studies should consider the minority genetic signatures that accompany passage and adaptation to new host environments (Ciota et al., 2012; Patterson et al., 2018).

Interestingly, temperature passed strains of RBGV exhibited lower titers than control strains, a result that was not anticipated. This suggests that to some extent further adaptation to the host can overcome temperature barriers. Since the control passage was completed at the optimal temperature for RBGV replication (28 °C), these strains replicated to higher titers and subsequently more opportunity for adaptation to mammalian cells. Surprisingly, although passage was completed in mammalian cell culture, replicative fitness also increased in mosquito cell culture for both strains. Since RBGV 06-HTP4 has increased replicative fitness at 35 °C but not 28 °C, this can, to some extent, be explained by temperature adaptation. Yet, this is not the case for RBGV-97–103, for which passage at both temperatures resulted in increased fitness at 35 °C. Although it is generally thought that host-specific fitness gains result in adaptive trade-offs in divergent hosts, many studies have shown that beneficial mutations generated in individual hosts can result in a range of phenotypes (detrimental, neutral, or beneficial) in alternate hosts (Ciota et al., 2014, 2015; Turner et al., 2010).

A serine to glycine change at position 5617 was observed in all passed strains. Interestingly, the glycine residue, which is in the helicase domain of the NS3 protein, is also present in WNV (Luo et al., 2008). Mutations within this domain could impact the ability of RBGV to replicate, as the helicase interacts with the protease domain of NS3 to cleave the polyprotein during replication, as well as with NS5 proteins within the replication complex. This mutation may be important for host expansion or fitness of RBGV. Brault et al. showed that a single nucleotide change found within the helicase domain of NS3 in WNV was prone to adaptive evolution and was responsible for a higher virulence and viremia is avian hosts (Brault et al., 2007). Ciota et al. reported multiple changes within the NS3 region of St. Louis encephalitis virus through cell passaging in a novel host, supporting the idea that changes in NS3 may play a role in host utilization (Ciota et al., 2014). We engineered a serine change in place of the glycine in a WNV infectious clone to determine whether this change influenced host restriction. Although the effect was modest, WNV G5716A did replicate to lower titers compared to the WNV infectious clone in HEK-293 cells at 37 °C and at 28 °C. Interactions with other mutated residues, particularly other positions in the NS3 (5909), NS5 (7840, 7982), or envelope (2234) identified in one or more passed strains likely contribute to increased host breadth in evolved strains. Epistatic interactions between mutations are known to play a role in the ability of viruses to emerge in new hosts (Ciota et al., 2012), and the role of the flavivirus replication complex in host-specific fitness has been documented (Van Slyke et al., 2012).

Questions remain regarding RBGV transmission and host range. Although previous studies suggest that the capacity for vertical transmission in mosquitoes may be enhanced (Aliota et al., 2012), it is not clear if this would be sufficient for maintenance in nature. While experimental studies suggest an avian or mammalian host is unlikely (Aliota et al., 2012), temperature constraints and efficient growth in cell culture suggest an amphibian or reptilian host is possible (Hubalek et al., 2000; Aliota and Kramer, 2012). Additional surveillance and experimental infections are needed to clarify RBGV host usage and identify additional genetic markers associated with flavivirus host range.

4. Materials and methods

4.1. Cells

All cell stocks, base cellular media, and supplements to cellular media were provided by the Wadsworth Center's Cell Culture and

Media Core Facility (Albany, NY, USA). Human embryonic kidney cells (HEK-293), African Green monkey kidney cells (Vero), and Pekin duck embryonic cells (PDE) were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 0.1 mM Non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin for complete growth medium. *Aedes albopictus* cells (C6/36) were cultured in Minimum Essential Medium (MEM) supplemented in identical fashion as EMEM for complete growth medium. Maintenance media for all cell lines were created by identical supplementation as their respective growth medium, except supplementing with 2% FBS instead of 10% FBS.

4.1.1. Viruses

RBGV isolates 97–103 and 06–222 (GenBank accession no. AY765264, GQ421359, respectively) were isolated from *Culex pipiens* in 1997 and *Aedes rossicus* in 2006, respectively, in the Czech Republic and obtained from Zdenek Hubalek (Institute of Vertebrate Biology, Academy of Sciences, Brno, Czech Republic). Viral stocks were created by one passage through C6/36 cells at 28 °C. Titration of the stocks were done by plaque assay on Vero cells at 28 °C. Titers for RBGV 97–103 and RBGV 06–222 were $\log_{10}$ 7.34 and $\log_{10}$ 6.30 plaque forming units per milliliter (PFU/mL), respectively. WNV isolate WNV02–1986 (GenBank accession no. DQ164189) was isolated from an American Crow collected in Albany, New York in 2003 and subsequently passed in C6/36 cells. Titration of the WNV02–1986 stock was done on Vero cells at 37 °C, yielding a titer of $\log_{10}$ 9.18 PFU/mL. West Nile virus infectious clone (WNV-IC) and NS3 mutant clone (WNV G5716A) were both grown on BHK cells at 37 °C, producing titers of $\log_{10}$ 9.2, and $\log_{10}$ 8.0 PFU/mL, respectively.

4.1.2. In vitro growth kinetics and quantification of viruses

Viral growth kinetics were assessed on confluent C6/36, Vero, HEK-293, and PDE cell monolayers in 6-well plates infected with viruses in triplicate at a multiplicity of infection (MOI) of 0.01. After one hour of absorption at 28–37 °C, inoculum was removed, washed 3 times and overlaid with 3mls of appropriate maintenance media for each cell line. Cell culture supernatants were collected at various time points starting from 24 h, and RNA was extracted using MagMAX viral isolation kit (Ambion, Foster City, CA, USA) on a Tecan Evo 150 liquid handler (Tecan, Morrisvelle, NC, USA) according to the manufacturer's instructions. Real time quantitation was performed using TaqMan one-step RT-PCR master mix (Applied Biosystems, Carlsbad, CA, USA) and analyzed on ABI Prism 7500 (Applied Biosystems, Carlsbad, CA, USA). RBGV primers and probes were designed to detect both strains of RBGV using PrimerQuest Tool (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). WNV primers and probes were designed by Lanciotti et al. (2000). RNA was extracted from RBGV 06–222, RBGV 97–103, and WNV02–1986 stock isolates to generate PFU/mL genome equivalent standards tested alongside harvested samples for quantitation. Linear regression analysis was done to calculate a PCR equivalent of PFU/mL for harvested samples. For viral growth kinetics, calculated titers of triplicate biological replicates were plotted over time and compared by using one-way analysis of variance (ANOVA), Tukey post hoc test, and *t*-test (GraphPad Prism version 5.0; GraphPad software, La Jolla, CA, USA).

4.1.3. Experimental evolution

RBGV 97–103 and 06–222 isolates were inoculated on Vero cells at 28 °C (MOI of 0.1). Supernatant was harvested, quantified by real-time RT-PCR, and used as inoculum for subsequent passages in HEK-293 cells. RBGV 97–103 and RBGV 06–222 were passed four times at increasing temperatures, from 29 °C to 35 °C, on HEK-293 cells in an attempt to select for variants with an increased capacity to infect and replicate in vertebrate cells at higher temperatures. As a control, both RBGV isolates were passed five times on HEK-293 cells at 28 °C to differentiate between cell-specific adaptation and temperature-specific adaptation. Growth kinetics were performed with passed and unpassed strains on Vero, C6/36, HEK-293, and PDE at different temperatures (37 °C, 35 °C, and 28°). The temperature for viral adsorption was the same as the holding temperature. Four passaged strains were created: RBGV 97 high temperature passage 4 (RBGV 97-HTP4), RBGV 97 control temperature passage 5 (RBGV 97-CTP5), RBGV 06 high temperature passage 4 (RBGV 06-HTP4), RBGV 06 control temperature passage 5 (RBGV 06-CTP5).

4.1.4. Sequencing of viruses

Primers were designed according to GenBank accession no. AY765264 to sequence all RBGV including control and high temperature passed strains. The complete genomes were amplified using SuperScript™ III One-Step RT-PCR System (Qiagen Inc., Valencia, CA, USA) to create 4 overlapping PCR fragments. Sanger sequencing was performed at the Applied Genomics Technology Core Facility, (Wadsworth Center, Albany, NY, USA) using primers to give a minimum of two-fold redundancy. Consensus sequences were compiled and edited by using DNASTAR software package (Lasergene, Madison, WI, USA).

4.1.5. Site directed mutagenesis and viral growth kinetics

The West Nile virus infectious clone (WNV-IC) was derived from WNV strain 3356 (Shi et al., 2002). The NS3 G5716A mutation was engineered into the WNV-IC using the QuikChange XlII SDM kit (Stratagene, La Jolla, CA, USA) as per the manufacturer's protocol. E. coli cells were used to amplify the plasmids and sequencing was performed to verify the engineered changes. Plasmids were harvested by High-speed Midiprep (Qiagen, Valencia, CA, USA), linearized, and viral RNA was synthesized using in-vitro transcription with MEGAscript kit (Invitrogen, Waltham, MA, USA) as per manufacturer's protocol with the addition of 1.5ul ARCA cap analogue (Invitrogen, Waltham, MA, USA). WNV-IC and WNV-NS3 G5716A RNA were electroporated into separate BHK cells using a GenePulser (BioRAd, Hercules, CA, USA). Viruses were collected 5 days post-transfection and titers were quantified by plaque assay on Vero cells.

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Declaration of interest

None

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