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Brief Communication

A positively selected mutation in the WNV 2K peptide confers resistance to superinfection exclusion *in vivo*

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ABSTRACT

Molecular epidemiologic studies of North American (NA) West Nile virus (WNV; Flaviviridae, Flavivirus) have documented the displacement of the introduced NY99 genotype with WN02. In addition, these studies have shown that particular substitutions are under positive selection. One occurs in the C-terminus of the NS4A coding sequence and results in a valine to methionine substitution at position nine of the 2K peptide. 2K-V9M confers the ability to overcome superinfection exclusion *in vitro*. We hypothesized that WNV strains bearing 2K-V9M have higher fitness than wildtype in *Culex quinquefasciatus* mosquitoes. Although infection rates and viral titers were not significantly different, virus dissemination rates were significantly higher with WNV 2K-V9M. As a super-infecting virus, WNV 2K-V9M was more successful than wildtype, however, in a mixed infection, 2K-V9M was not. These data support observations that 2K-V9M confers a context-specific selective advantage in mosquitoes and provides an *in vivo* mechanism for its positive selection.

Introduction

WNV has caused the largest neuroinvasive arbovirus disease outbreaks recorded in North America (NA, reviewed in Petersen et al., 2013). Viral transmission is maintained mainly by passerine bird-*Culex* mosquito amplification (Jerzak et al., 2005; Kilpatrick et al., 2006; Komar et al., 2003; Nash et al., 2001). The WNV strain first established in NA, NY99, has been supplanted by a second strain, WN02. However rare genome variants continue to circulate regionally (Armstrong et al., 2011; Davis et al., 2005; Ebel et al., 2004; Herring et al., 2007). Ongoing molecular epidemiologic studies documenting the continuing emergence of local variants in nature have provided the opportunity to study positively selected virus variants that may alter transmission dynamics.

A number of naturally occurring positively selected WNV genome variants have been identified. For example, a positively selected mutation in the WNV NS3 protein at T249P was able to significantly increase pathogenicity in American crows (Brault et al., 2007). Another positively selected mutation in the 2K peptide confers a change from valine to methionine at amino acid position 9; it has been identified in multiple natural isolates from mosquitoes and American crows (Armstrong et al., 2011; Pesko et al., 2012). Interestingly, independent studies demonstrated that 2K-V9M allows WNV to replicate in the presence of flavivirus inhibitors in mammalian cell culture (Mertens et al., 2010; Zou et al., 2009a). In an *in vitro* replicon system it also allowed WNV to overcome superinfection exclusion (Zou et al., 2009b). An additional mutation, identified by serial passage of WNV *in vitro,* bearing a single nucleotide substitution in NS4A, K124R, also allowed the virus to overcome superinfection exclusion (Zou et al., 2009b). Superinfection exclusion occurs when a cell infected with one virus strain prevents infection with a second virus strain. This phenomenon is well described in mosquito cells (Condreay and Brown, 1986; Karpf et al., 1997), and is thought to, in part, shape transmission dynamics in regions where infection rates are extremely high (Pesko and Mores, 2009). With WNV, superinfection exclusion is most dramatic when the secondary infection is a closely related flavivirus, less clear with more distantly related flaviviruses, such as dengue, and undetectable with a nonflavivirus (Zou et al., 2009b). Therefore we hypothesized that the mechanism that underpins the observed positive selection for 2K-V9M may be an enhanced ability to overcome superinfection exclusion

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in relevant vector species. We used WNV infectious clones bearing single nucleotide mutations at 2K-V9M or NS4A K124R, as well as the double mutant 2K-V9M, NS4A K124R to characterize the impact of these mutants on the vector competence of a major WNV vector, *Cx quinquefasciatus*. Genotypes with altered vector competence compared to wildtype infectious clones were then assessed for superinfection exclusion *in vivo*. 2K-V9M conferred a slight transmission advantage in mosquitoes in isolation, and more substantially as a superinfecting virus. These results provide an *in vivo* mechanistic link between molecular evolutionary and tissue-culture based studies that have identified this mutation as positively selected.

Results

Viral infection phenotypes

Vector competence phenotypes were tested in adult *Cx quinquefasciatus* orally infected with one of three WNV point mutants (2K-V9M, NS4a K124R or the double mutant) compared to wildtype (NY99ic). At 7 and 14 days post-infection (dpi), mosquito carcasses, legs/wings, and salivary expectorants were collected for estimation of infection, dissemination and transmission rates, respectively, by plaque titration. There were no significant differences in infection phenotypes at 7dpi (Fig. 1A, Table 1). By 14 dpi, the 2K-V9M mutant showed a significantly higher dissemination rate than the NS4A K124R mutant or the double mutant (NS4A K124R, 2K-V9M), compared to NY99ic (Chi-Square = 11.66, $p = 0.0087$) (Fig. 1B, Table 1). However, viral titers for bodies remained higher for NY99ic infections than for all mutants (Kruskal–Wallis test, $p = 0.0002$).

Superinfection

To investigate the impact of 2K-V9M on the ability to overcome superinfection exclusion, viral fitness was assessed following a dual infection scheme. Mosquitoes were first given a primary peroral infection with a marked WNV reference strain and 5 days later, a secondary infection was established orally with either NY99ic wildtype or the 2K-V9M (Fitzpatrick et al., 2010). Midguts were collected at 7, 14, 21, and 28 days following the second feeding. At 14 dpi, via polySNP analysis (Fitzpatrick et al., 2010; Hall and Little, 2007), mosquito midguts secondarily infected with 2K-V9M had a significantly higher proportion of the superinfecting genotype than did those superinfected by wildtype (Fig. 2) (Mann–Whitney, p value = 0.0194). To assess the fitness of 2K-V9M compared to wildtype in a dual infection, but without the requirement for superinfection, mosquitoes were fed on a bloodmeal containing a 56.6%:43.3% mixture of 2K and NY99ref, respectively. The 2K-V9M genotype did not predominate in any mosquito tissue collected at 14 dpi (Table 2).

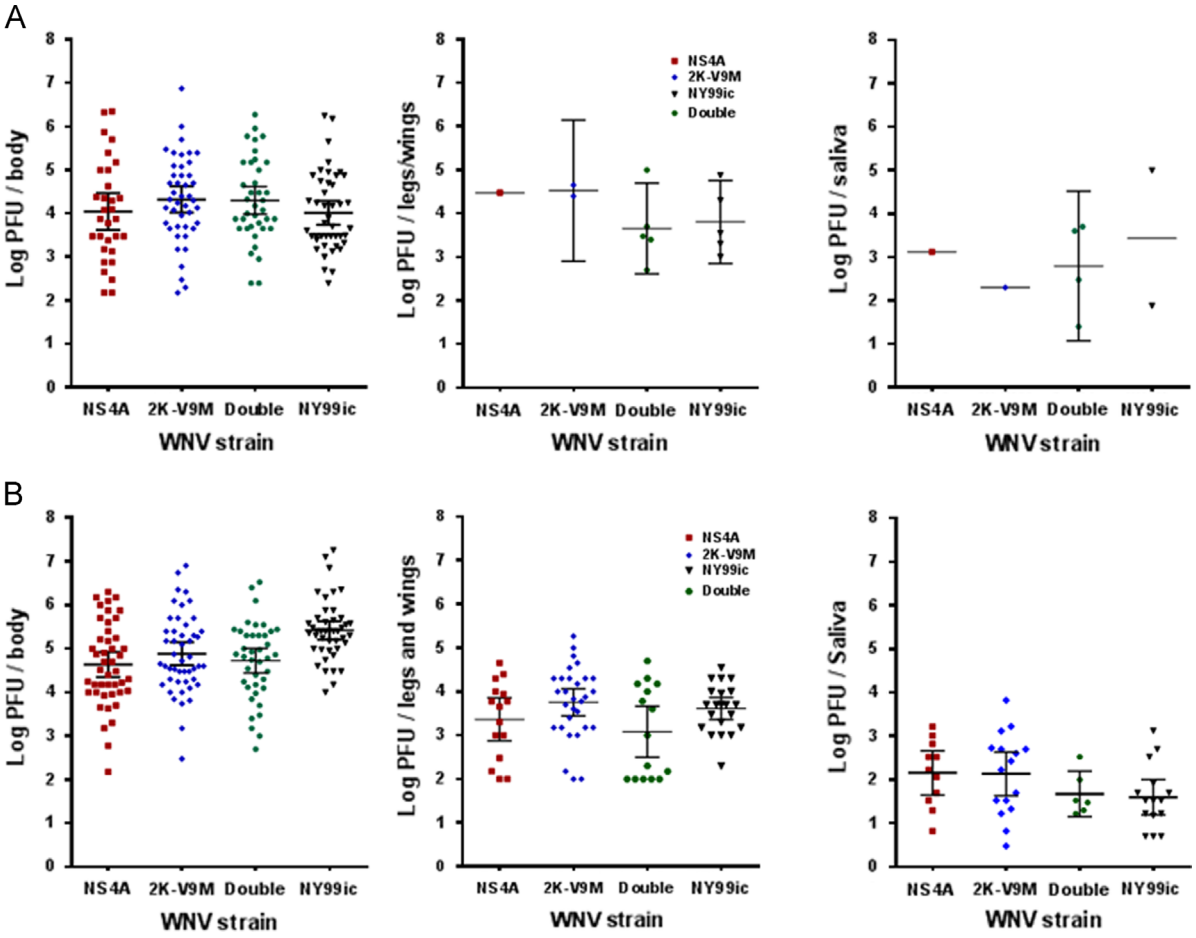

Fig. 1. At 14 dpi, dissemination rates of 2K V9M WNV were significantly higher than that of WNV NY99_ic. A. 7dpi. B. 14 dpi. Viral titers for positive samples are shown. In (A) and (B), Left panel: Infection rate is represented by carcass infections; middle panel: dissemination rate represents infection of leg/wings; right panel: transmission rates were determined from salivary expectorants. NY99ic viral titers are significantly higher than all mutants (Dunn's multiple comparisons test, alpha = 0.05; Kruskal–Wallis test, $p = 0.0002$); NS4A(K124R), 2K-V9M, 2K mutant, NY99ic, wildtype, double mutant (NS4A K124R, 2K-V9M). Bars indicate geometric mean and 95% confidence interval. These data are the compilation of two biological replicates using a total of 50 mosquitoes for each timepoint. Infection rates are shown in Table 1. Plotting and statistical analyses were performed in GraphPad Prism.

Table 1
Mosquito tissue infection rates.

Infection rate					Dissemination rate				Transmission rate			
Carcasses					Legs/wings				Saliva			
Strain	NS4A	2K	Double	WT	NS4A	2K	Double	WT	NS4A	2K	Double	WT
7 DPI %	62	88	76	88	2	4	10	10	2	2	8	4
14 DPI %	90	96	80	88	30	60[a]	32	42	26[b]	38	26	32

[a] 2K-V9M dissemination rate is significantly higher than NY99ic, (Chi-Square = 11.66, p value = 0.0087).
[b] Some saliva samples were positive by cytopathic effects assay but could not be quantitated by plaque assay. WT, wildtype. These data reflect the combined results of a total of 50 mosquitoes for each timepoint over two biological replicates.

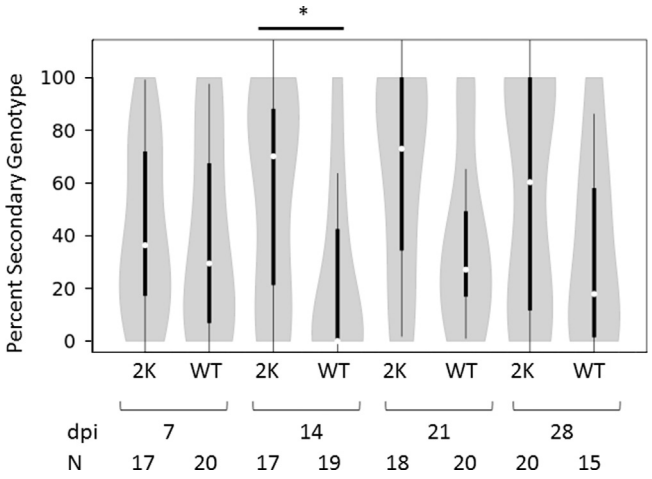

Fig. 2. Following superinfection of midguts, 2K-V9M is present at higher proportion than wildtype. Data are a compilation of 2 biological replicates. Median values, white circles; shape limits are at the 25th and 75th percentiles; black bars (Tukey whiskers) extend 1.5 times the interquartile range from 25th to 75th percentiles; shapes are representative of data density estimates. Asterisk indicates statistically significant difference (Mann–Whitney, p value = 0.0194). Plot created with http://boxplot.tyerslab.com/ (Spitzer et al., 2014). 'dpi' indicates day post-infection; *N* indicates number of midguts in each group. This data reflects two biological replicates.

Table 2
Mixed infection.

	Mixed infection[a]			2K V9M 100%			REF-100%		
Tissue	**Median % 2K-V9M genotype**	***N***	**Std dev**	**Median % 2K-V9M genotype**	***N***	**Std dev**	**Median % Reference genotype**	***N***	**Std dev**
Midguts	34.3	25	32.9	96.0	7	7.9	97.2	7	3.3
Legs/wings	7.0	20	33.0	97.8	6	26.4	97.5	8	1.5
Saliva	44.9	12	35.3	86.3	2	3.4	98.24	2	na

2K V9M genotype does not have a competitive fitness advantage over NY99ref in a mixed infection. Mosquitoes were fed a mixed virus of 56.6%: 43.3% (2K V9M: NY99ref). Percent viral genotypes were determined by polySNP.

[a] Mixed virus was 57%:43% (Mutant: Reference). *N* indicates the number of mosquitoes per group. (Std dev), standard deviation.

Discussion

Molecular epidemiologic studies have identified 2K-V9M as being a positively selected mutation, and tissue culture studies have suggested that this mutation confers resistance to superinfection exclusion. Accordingly, we evaluated whether this mutation would increase transmission fitness in relevant mosquito vectors. While the 2K-V9M genotype showed increased dissemination compared to wildtype at 14 dpi, wildtype maintained higher body viral titers. Moreover, transmission, indicated by the presence of virus in salivary expectorants, was not increased in the presence of the 2K-V9M mutant. Thus, although 2K-V9M increases spread within the mosquito host, the lack of increased transmission diminishes the impact of this gain of function mutation.

We next evaluated whether 2K-V9M would impact the ability of WNV to overcome superinfection exclusion. We found that at 14 dpi, more secondary infection genotypes representing 2K-V9M were present than those infected with wildtype. We therefore conclude that 2K-V9M confers resistance to superinfection exclusion *in vivo* in mosquitoes. In addition, these results would suggest that 2K-V9M may allow WNV to replicate more efficiently in mosquitoes infected by mosquito-only flaviviruses (Calzolari et al., 2012; Tyler et al., 2011), although this issue has not been tested directly.

Finally, in a mixed infection competition experiment where mosquitoes were exposed to wildtype and 2K-V9M simultaneously, the 2K-V9M mutant did not have a competitive advantage over NY99ic in any tissue tested. Therefore, 2K-V9M does not possess a selective advantage over wildtype when superinfection of previously infected mosquitoes is not required. Collectively, these results point to a highly context-specific fitness advantage for 2K-V9M, which supports the observations that it is positively selected in nature and its lack of fixation in the population.

The WNV 2K peptide is a 23 amino acid transmembrane peptide at the carboxyterminus of NS4A that spans the endoplasmic reticulum (ER) between NS4A and NS4B; in addition, it serves as a signal peptide for NS4B. 2K is cleaved from NS4A by the NS2B–NS3 viral protease and from NS4B by a host signal peptidase (Nall et al., 2004; Shiryaev et al., 2007). In its mature form as a structural peptide, 2K may alter the topology of the ER during WNV infection. The 2K peptide has been described to be present *in vivo* fused to the carboxy-terminus of NS4A or the amino-terminus of NS4B (Mertens et al., 2010). Importantly, a study of WNV in human cell culture found that, at the light microscope level of resolution, localization and processing of NS4A-2K and 2K-NS4B remained unaltered with V9M (Mertens et al., 2010). To assess the possible implications of the V9M mutation for membrane topology, the secondary structure of the 2K peptide was assessed upon total cleavage and in the context of the NS4A-2K or 2K-NS4B conformations using a structural prediction algorithm (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) (Mitaku et al., 2002). No significant changes to hydrophobicity or overall secondary structure were found. However, the first transmembrane domain of the 2K-V9M-NS4B helices was altered from a primary helix motif to a secondary type helix (Suppl. Fig. 1). Primary transmembrane helices contain amphiphilic helical side chains with high hydrophobicity in central residues, and secondary helices have been proposed to play a role in protein active sites (Hirokawa et al., 1998). Another study has shown that WNV NS4B interacts with NS1, and possibly affects replication complexes (Youn et al., 2012). Therefore, one hypothesis

for the gain-of-function of 2K-V9M is that it alters function of 2K-NS4B, perhaps by changing protein–protein interactions required for the replication complex formation. In this scenario, 2K-V9M could induce more stable or efficient replication complexes than wildtype, which allow superinfection to occur. Given the data presented, these complexes, however, would not allow mutant viruses to replicate to higher titers than wildtype.

Given that 2K V9M enables WNV to replicate in the presence of the anti-viral agents lycorine and 2′, 5′ oligoadenylate synthetase 1b (Oas1b) in mammalian cells, some have proposed that structural changes to ER membranes occur in the presence of V9M that physically enhance formation of replication complexes (Mertens et al., 2010; Zou et al., 2009a). This hypothesis was based on the interpretation that, in the presence of lycorine, 2K-V9M does not affect viral translation but rather RNA synthesis. In addition, Oas1b activates RNase L, which then cleaves viral dsRNA replicative intermediates (reviewed in Silverman, 2007), suggesting that perhaps V9M prevents degradation of replicative intermediates. Therefore, an alternative hypothesis for the viral gain of function mutation imposed by 2K-V9M is that alteration of ER membranes protects virus replicative intermediates from degradation by host defense mechanisms, which could increase viral particle packaging and egress without increasing the replication rate *per se*.

These experiments describe the phenotypic changes associated with a single amino acid mutation, however, in nature, WNV exists as a mixture of quasispecies that make up a fluid fitness landscape (Jerzak et al., 2005). Individual genotypes may rise to dominance under specific selective constraints and environmental conditions. However, a genotype that is advantageous under a specific set of environmental conditions may not remain dominant when conditions change. Moreover, the combinatorial effects imposed by multiple selective pressures to produce a given virus genotype must be considered. Thus, with 2K-V9M as an individual component of the overall viral population, the data presented here are consistent with the hypothesis that 2K-V9M contributes to fitness increases compared to wildtype, depending on the coinfection status of the mosquito.

In summary, our results demonstrate that the 2K-V9M genotype was similar to wildtype in replication phenotype in mosquitoes, but disseminated more quickly and established secondary infections more readily than wildtype. Therefore, this point mutant seems to alter intra- or intercellular transport mechanisms to evade superinfection exclusion and/or enhance virus dissemination. However, the evidence that 2K-V9M was unable to outcompete wildtype in a mixed infection, restricts the conditions in which it could positively affect virus transmission dynamics in nature. Moreover it seems likely that, 2K-V9M, arising as part of the WNV mutant swarm present in a mosquito, could superinfect previously infected cells, ultimately appearing in the transmitted pool of WNV, where it can be transmitted to a new host. That this mutation has not become fixed in the WNV population supports the observation that its fitness advantage is highly context specific and it frequently disappears from the consensus WNV sequence due to stochastic or selective mechanisms.

Methods

Viruses

Mutant virus infectious clones (ic), wildtype NY99ic and the marked reference genotype have been described elsewhere (Fitzpatrick et al., 2010; Shi et al., 2002; Zou et al., 2009b). Virus infectious clones were reverse transcribed from plasmids using standard methods (Shi et al., 2002). Virus stocks were prepared by electroporation of the transcription reactions into baby hamster kidney cells (BHK-21) (ATCC) maintained in MEM supplemented with 10% fetal bovine serum at 5% CO_2. Passage 0 virus stocks were used for all experiments described herein. Virus titers were determined by plaque titration on Vero cells using standard methods (Lindsey et al., 1976).

Mosquitoes

Colony *Cx quinquefasciatus* were maintained at 26–27 °C, 60–70% humidity and a 15:9 (L:D) light cycle. Adult female *Cx quinquefasciatus* (5–7 days old) were infected by peroral administration of 8 logs plaque-forming units per ml p0 passage mutant, wildtype (NY99ic), or reference virus stocks diluted into defibrinated sheep blood (Colorado Serum Company) and provided to mosquitoes in a water-jacketed membrane feeder using hog gut. At indicated times post-infection, mosquitoes were anesthetized with cold, and carcasses, legs/wings, and salivary expectorants were collected into cell culture media or in viral RNA extraction buffer and stored at −80 °C until processing. Following collection of legs and wings, saliva expectorants were collected by inserting each mosquito proboscis into an individual capillary pipette prefilled with type B immersion oil (Electron Microscopy Sciences). After 30 min, the capillary was removed and placed directly into a collection tube containing cell culture medium or RNA extraction buffer. After saliva collection, the carcass or midgut was collected, as well. For the vector competence experiments, infection rates were determined by calculating the number of bodies with positive plaque titration results divided by the total number tested. Dissemination rates were determined by calculating the number of legs/wings samples with positive plaque titration results divided by the total number tested. Transmission rates were determined by calculating the number of bodies with positive plaque titration results divided by the total number tested.

Superinfection exclusion

Adult female mosquitoes were fed a primary bloodmeal of 8 logs PFU/ml of the marked reference genome WNV NY99ic. Engorged females were retained. Egg cups were provided at 2 dpi. Five days after the primary feed, mosquitoes were fed 8 logs PFU/ml of a secondary genotype, either wildtype NY99ic or the WNV 2K-V9M point mutant. At 7, 14, 21, and 28 days post-secondary infection, samples were collected for genotype analysis.

Virus genotyping

Total RNA was extracted from individual mosquito tissues using the manufacturer's recommendations for the Ambion Viral RNA extraction kit (Life Technologies) on the Kingfisher Flex (ThermoScientific). RT-PCR amplification of a portion of the WNV genome was used for genotype analysis using methods previously described (Fitzpatrick et al., 2010). A portion of the WNV genome was amplified by reverse transcriptase PCR (Qiagen) (5′ primer- GTGGAAGAGGCGGTTGGTGTTACT; 3′ primer- TACTTCACTCCTTCTGGCGGTTCA) and subjected to Sanger sequencing using the primer, GCTCTGCCCCTACATGCCGAAAGT. polySNP analysis, which uses PHRED analysis to read the area under a chromatogram peak, was used to differentiate virus genotypes at positions 8313–8317 (Hall and Little, 2007). The median of all five marked nucleotide positions was used to calculate the proportion of mutant genome present. Median values for each timepoint were entered into Graphpad Prism for statistical calculations.

Mixed infection

Viruses were mixed and confirmed to be 56.6%: 43.3% (2K-V9M: NY99ref) by Sanger sequencing, followed by polySNP analysis. *Cx quinquefasciatus* were fed the 2K-V9M / NY99ref mixture at 8 logs pfu/ml in sheep blood, and tissues were collected at 14 dpi for assessment of viral genotype by polySNP.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.07.009.

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