A Newly Emergent Genotype of West Nile Virus Is Transmitted Earlier and More Efficiently by Culex Mosquitoes

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INTRODUCTION

West Nile virus (WNV) is a member of the Japanese encephalitis group of the Flavivirus genus (family Flaviviridae).1,2 Similar to other members of this group, WNV is maintained in nature through an enzootic transmission cycle between Culex mosquitoes and birds. Although periodic epidemics occur in humans and epizootics in horses, infection of mammals does not seem to contribute to the maintenance of WNV.3

Before 1999, the geographic range of WNV was limited to Africa, Australia, and Asia, with occasional epidemics and epizootics in Europe.4 However, in 1999, WNV was first detected in New York City and has subsequently expanded its range to include most of North and Central America, the Caribbean, and recently, parts of South America.4–12 Of the two main lineages of WNV, Lineage 1 has the larger geographic range and includes the viruses currently circulating in the Americas.1 Viruses in this lineage are more pathogenic and can potentially lead to severe central nervous system infection and death. Lineage 2 viruses are found only in sub-Saharan Africa; infection with WNV belonging to this lineage rarely progresses to severe disease in humans.1

Sequence data indicate that the first WNV isolates from New York City in 1999 were 99.8% identical to an Israeli isolate from 1998.10 This introduced genotype of WNV has been termed the “Eastern” or NY99 genotype. During the subsequent spread of WNV across North America, a new genotype (“North American dominant” or WN02) emerged and rapidly became dominant among circulating WNV strains.7,13 The WN02 genotype consensus sequence contains three nucleotide sequence changes from the NY99 genotype: a U to C change at position 1442 in the E gene, a C to U change at position 2466 in the E gene, and a C to U change at position 9352 in the NS5 gene.7,13 Only the U1442C change is non-synonymous, resulting in a valine to alanine change at amino acid position 159 in the E protein. This conserved amino acid change is not located within the predicted receptor binding domain or any region predicted to be critical for efficient fusion of the viral envelope with the host cell membrane; it is therefore difficult to predict how it could be functionally important.

The WN02 genotype completely displaced the introduced NY99 genotype in the United States between 2001, when it was first detected, and 2004, when NY99 was no longer found.14 This genotype displacement could have occurred through genetic drift fixing random mutations in the viral population or by selection of advantageous variants. At least two lines of evidence, however, suggest that selective pressure, and not genetic drift, produced the observed displacement. First, the very small population sizes needed for drift to become important seem not to have existed since WNV significantly expanded its range in the United States in 2000.14 Second, and more importantly, previous experimental results have shown phenotypic differences between the genotypes, as NY99 genotype viruses have extrinsic incubation periods (EIP: the time between a mosquito imbibing an infectious bloodmeal and transmission of the virus by that mosquito) in Culex pipiens, the main WNV vector in the northeastern and northcentral United States, that are 2–4 days longer than the EIPs of WN02 genotype viruses.13 However, the previous study did not examine the mechanism leading to this reduction in EIP. In this study, we first examined whether our previous results were unique to Cx. pipiens, using an additional species of WNV vector mosquito. We examined whether NY99 and WN02 genotype viruses exhibit replicative differences in vitro and in vivo, as differences in viral replication efficiency could be involved in the reduced EIP previously observed. In addition, we determined the point in the mosquito infection pathway where the WN02 genotype gains an advantage. These studies are the first steps in understanding the mechanisms by which WNV has adapted to North American mosquito vectors and avian species.

MATERIALS AND METHODS

Viruses, cells, and mosquitoes. Aedes albopictus (C6/36) cells were used for viral growth, replication analyses, and fitness assays. Chicken embryo fibroblast (DF-1) cells were used for replication analyses. Viruses were titrated on African green monkey kidney (Vero) cells. Two viruses from each previously defined WNV genotype present in North America

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All viruses were originally derived as a standard. No significant differences were observed between the MARM strain derived from a NY99 genotype virus and exhibits neutral fitness compared with its parental virus.15

*Culex pipiens* and *Cx. tarsalis* mosquitoes were derived from laboratory colonies. The *Cx. pipiens* colony originated in 2002 from egg rafts collected in Pennsylvania, and *Cx. tarsalis* were derived from a colony maintained by WK Reisen from mosquitoes collected in California in 1953. Colonized mosquitoes were maintained on goose blood (for egg laying) and given 10% sucrose *ad libitum*. Larvae were reared and adults maintained under controlled conditions of temperature (27°C), humidity (70% RH), and light (16:8 L:D diurnal cycle), in 30.5 × 30.5 × 30.5-cm cages. All experiments involving infectious WNV were carried out in the Arbovirus Laboratories' BSL3 laboratories or insectaries.

**Viral replication in vitro.** NY99 and WN02 genotype viruses were inoculated onto confluent cultures of either DF-1 or C6/36 cells at an MOI of 0.01. The MARM strain was included as a standard against which to compare the replicative abilities of the individual viruses. Virus production was examined over 120 hours (DF-1) and 264 hours (C6/36) after infection. After 1 hour of adsorption, the inoculum was removed, and the cultures were washed three times. Fresh media were added, and the cultures were incubated at either 39°C (DF-1) or 28°C (C6/36) for up to 264 hours, with aliquots removed at indicated intervals. Virus titers at each time-point were determined by plaque titration on Vero cells.

**Fitness assays.** Relative fitness was determined by direct competition between the MARM virus derived from a NY99 genotype WNV, and each WNV isolate, essentially as previously described.16 Briefly, confluent monolayers of C6/36 cells were inoculated in triplicate with a 1:1 mixture of MARM:WNV at an MOI of 0.01 and incubated at 28°C for 96 hours. Supernatants were harvested, diluted to an MOI of 0.01, and inoculated onto fresh monolayers of C6/36 cells. Four rounds of competition were performed. Original MARM:WNV ratios and virus yields after each passage were determined by plaque titration in the presence of MAb 5H10 (Bioreliance) to determine the concentration of MARM and in the absence of MAb to determine the concentration of wildtype virus. The ratios of MARM:WNV after each competition passage were normalized to the original ratio, log-transformed, and plotted against the competition round. Linear regression was used to obtain fitness vectors, and the relative fitness values were obtained by determining the slope of the regression.

**Vector competence of *Cx. tarsalis*.** Five- to 7-day-old adult female mosquitoes were deprived of sucrose for 48 hours before feeding. Virus stocks were diluted in 5 mL defibrinated goose blood with 2.5% sucrose to a final titer of 10^7 pfu/mL. Mosquitoes were fed for 1–2 hours using a Hemotek membrane feeding apparatus (Discovery Workshops, Accrington, UK), as directed by the manufacturer. After feeding, fully engorged mosquitoes were separated under CO₂ anesthesia to 0.5-L cartons, supplied with 10% sucrose *ad libitum*, and held at 27°C, 16:8 L:D photoperiod for up to 14 days after feeding.

Transmission was evaluated *in vitro* at 5, 7, 9, and 14 days after feeding, essentially as previously described.17 Briefly, mosquitoes were anesthetized with triethylamine (Sigma, St. Louis, MO), and their legs removed into 1 mL mosquito diluent (MD: 20% heat-inactivated fetal bovine serum [FBS] in Dulbecco phosphate-buffered saline plus 50 μg/mL penicillin/streptomycin, 50 μg/mL gentamicin, and 2.5 μg/mL fungicid) zone). Mosquito mouthparts were placed into a capillary tube containing ~10 μL of a 1:1 mixture of FBS and 50% sucrose for ~30 minutes, after which time the contents of the capillary tube were removed to 0.3 mL MD. Mosquito bodies were placed into 1 mL MD, and all samples were stored at −80°C. Mosquito bodies and legs were homogenized in a mixer mill (Qiagen) and clarified by centrifugation. The proportion of mosquitoes with infected bodies, legs, and salivary secretions was determined by plaque assay on Vero cells. Infection, dissemination, and transmission were defined as the proportion of mosquitoes with infected bodies, legs, and salivary secretions, respectively. Proportions were compared using χ² analysis.

**Inoculation of *Cx. pipiens*.** Viruses were diluted in MD to a titer of 10^7 pfu/mL. Mosquitoes were inoculated intrathoracically with 10 pfu of either NY99 or WN02 under CO₂ anesthesia and held at 27°C, 16:8 light:dark photoperiod, for up to 14 days after inoculation. Transmission was evaluated at days 1, 2, 3, 4, 5, 7, 9, and 14 after inoculation as described above. The proportions of mosquitoes with infected bodies, legs, and salivary secretions were determined by plaque assay and compared using χ² analysis. The viral loads of infected mosquitoes were determined by plaque titration and compared using ANOVA.

**RESULTS**

**Replicative ability of WNV genotypes in cell culture.** We first examined whether the NY99 and WN02 genotypes differed in their replication efficiency and/or kinetics *in vitro*, using a NY99 genotype MARM as a standard. No significant differences in growth among any of the viruses in DF-1 cells were observed (Figure 1A). Interestingly, the MARM virus exhibited decreased growth compared with the other viruses in C6/36 cells between 120 and 216 hours after infection, but by 264 hours after infection had reached similar levels of virus yield (Figure 1B). However, there was no difference in replicative ability between the genotypes (Figure 1) in either cell type examined.

**Fitness of WNV genotypes in C6/36 cells.** Competitive fitness assays can be used as a more sensitive comparison of differences in replicative ability. Therefore, viruses with similar replication efficiencies as measured by growth curves may have different fitness values.18 We examined whether differences in the relative fitness of the NY99 and WN02 genotypes could be involved in strain displacement. The MARM strain was used as the NY99 reference strain for the *in vitro* competition assays, because it could be easily differentiated from the WNV isolates by plaque assay in the presence of MAb.14,15 As controls, we examined whether the NY99 isolates exhibited fitness differences compared with the MARM

(Eastern genotype: NY99; North American dominant genotype: WN02) were chosen for these studies. NY99-2094, NY99-3557, WN02-1986, and WN02-1956 (GenBank accession numbers AY369435, AY590222, AY590223, and AY590210, respectively).13 All viruses were originally derived from the kidneys of American crows collected in New York State and were isolated on Vero cells, followed by a single round of amplification on C6/36 cells. The complete genome sequences of the viruses used in these studies have been determined previously. The MARM strain was previously derived from a NY99 genotype virus and exhibits neutral fitness compared with its parental virus.15

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strain. There were no consistent fitness differences between the WN02 isolates and the MARM strain, as one of the isolates exhibited increased fitness over the MARM strain, while the second isolate was outcompeted by the MARM strain (Figure 2). In addition, one of the NY99 isolate controls exhibited a nearly identical fitness increase over the MARM strain. These data indicate that there are no detectable differences in fitness between the NY99 and WN02 genotypes in vitro.

Vector competence of WNV genotypes in Cx. tarsalis. To evaluate the impact of WNV genotype on EIP for a major western US vector of WNV, we examined the effect of viral genotype on the vector competence of Cx. tarsalis. At all time-points examined, a higher proportion of mosquitoes became infected and transmitted infectious WNV after feeding on WN02 genotype viruses compared with mosquitoes that fed on NY99 genotype viruses (Table 1). In addition, mosquitoes infected with WN02 genotype viruses transmitted infectious WNV 4 days earlier than those infected with NY99 genotype viruses, similar to previous results with Cx. pipiens. The data presented here, in combination with previous data, suggest that early transmission of WN02 genotype viruses is common among Culex species, contributing to the displacement of the NY99 genotype through increases in genotype-specific vectorial capacity.

Vector competence of Cx. pipiens after intrathoracic inoculation. Adult female mosquitoes were inoculated with NY99 or WN02 genotype virus to determine whether bypassing midgut infection reduced or eliminated genotype-specific differences in vector competence. There was no difference in infection rate between the genotypes at any time-point examined, and viruses belonging to both genotypes were transmitted as early as 2 days after inoculation (Table 2). In addition, we determined the viral load in inoculated mosquitoes. Titers in WN02-infected mosquitoes were significantly higher than those of NY99-infected mosquitoes at all times beginning at 2 days after inoculation, indicating that WN02 genotype viruses replicate more efficiently in vivo (Figure 3).

DISCUSSION

The introduction of WNV to North America at a relatively defined time and location has provided a unique opportunity to observe the evolution and adaptation of an emerging virus to a naïve environment. During its spread across North America, a variant genotype of WNV emerged (WN02) and has since become dominant. WN02 genotype viruses were first detected in 2001, and since 2003, no viruses belonging to the NY99 genotype have been detected in North America, indicating that it has been completely displaced by the WN02 genotype. We have begun studies to determine the point(s) in the enzootic transmission cycle where WN02 gains an advantage over NY99. In this study, we examined whether differences between the genotypes in viral replication or interaction with mosquito vectors could have contributed to the strain displacement observed in nature.

We first examined replication efficiencies of the NY99 and WN02 genotypes in vitro in avian (DF-1) and mosquito (C6/36) cells, using two isolates belonging to each genotype. Although there were minor differences in replication efficiencies and growth kinetics between individual isolates, there was no consistent difference between replication of NY99 isolates compared with WN02 isolates in either cell type (Fig-
In vitro/H11505/H11505 after peroral infection 0.015 (Table 1), indicating that, although the overall phenotype differences between the genotypes could have contributed to the strain displacement observed. When C6/36 cells were infected with a 1:1 mixture of WN02 isolate and NY99-MARM, there was no consistent fitness increase (Figure 2). These results suggest that a difference in replication efficiency between the NY99 and WN02 genotypes was unlikely to have been involved in strain displacement. However, because in vitro results are not necessarily predictive of in vivo differences, we moved our assays into an in vivo system.

We previously reported that viruses belonging to the WN02 genotype were transmitted 2–4 days earlier than NY99 genotype viruses by Cx. pipiens mosquitoes,13 the main WNV vector in the northeastern and northcentral United States. However, it was possible that this was a species-specific phenotype and would therefore not be applicable to other species of WNV vector mosquitoes. Because the displacement of the introduced genotype was evident throughout the United States, we examined whether there were phenotypic differences between NY99 and WN02 genotype viruses in Cx. tarsalis, a major WNV vector in western US agricultural areas. After peroral infection, we found that WN02 viruses infected a larger proportion of Cx. tarsalis than NY99 viruses (Table 1). In addition, NY99 viruses were transmitted later and by a smaller proportion of infected mosquitoes than WN02 viruses. These results are similar to, but more dramatic than, those of our previously reported study.13 Although the differences in infection and transmission rates between the genotypes had disappeared by 9 days after feeding in Cx. pipiens, they were still apparent at 14 days after feeding in Cx. tarsalis (Table 1), indicating that, although the overall phenotypic differences between the WN02 and NY99 genotypes were the same in different Culex species, species-specific differences in genotype–vector interactions exist.

We next examined at what stage of mosquito infection the NY99 and WN02 genotypes were interacting differently with the vector mosquitoes. All previous experiments had examined transmission after feeding, which requires that the virus enter and replicate in the midgut before dissemination to secondary tissues, including the salivary gland. To determine if infection of the midgut was the point in mosquito infection that the genotypes were interacting differently with the vector, we used intrathoracic inoculation of Cx. pipiens mosquitoes. This method of infection bypasses the requirement for initial midgut infection and leads directly to an infection of secondary tissues. If the genotypes were transmitted with similar kinetics and efficiency after inoculation, it would suggest that the WN02 genotype viruses were better able to infect, replicate in, or disseminate from the midgut. However, if the NY99 genotype viruses were transmitted less efficiently and/or later than the WN02 genotype viruses, as was previously seen after peroral infection, it would suggest that the WN02 genotype viruses were more efficient at some aspect of salivary gland infection, although it would not exclude midgut involvement. Mosquitoes infected with either genotype transmitted virus beginning at 2 days after inoculation (Table 2), in contrast to the 2- to 4-day delay in NY99 transmission after peroral infection. These data suggest that WN02 genotype viruses are more easily able to overcome the midgut barrier. However, WN02-infected mosquitoes exhibited significantly higher viral titers than NY99-infected mosquitoes beginning at 2 days after inoculation (Figure 3), and a greater proportion of WN02 mosquitoes transmitted virus on days 2 and 3, although this difference was not significant. Taken together, these data suggest that WN02 viruses are more efficient than NY99 viruses during one or more stages of viral replication in the midgut, enabling them to more quickly disseminate, infect

### Table 1

**Vector competence of Cx. tarsalis after peroral infection**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I*</td>
<td>D</td>
<td>T</td>
<td>I</td>
</tr>
<tr>
<td>NY99</td>
<td>29 (n = 181)</td>
<td>7</td>
<td>0</td>
<td>31 (n = 180)</td>
</tr>
<tr>
<td>WN02</td>
<td>41 (n = 200)</td>
<td>9</td>
<td>2</td>
<td>44 (n = 189)</td>
</tr>
<tr>
<td>P value†</td>
<td>0.022</td>
<td>0.612</td>
<td>0.057</td>
<td>0.008</td>
</tr>
</tbody>
</table>

* I, percent infected; D, percent disseminated; T, percent transmitted.

† Calculated using χ² test.

### Table 2

**Vector competence of Cx. pipiens after intrathoracic inoculation**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>14</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>I*</td>
<td>T</td>
<td>I</td>
<td>T</td>
<td>I</td>
<td>T</td>
<td>I</td>
<td>T</td>
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<td>NY99</td>
<td>76</td>
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<td>4</td>
<td>100</td>
<td>4</td>
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<td>44</td>
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<tr>
<td>WN02</td>
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<td>0</td>
<td>92</td>
<td>16</td>
<td>96</td>
<td>16</td>
<td>100</td>
<td>52</td>
</tr>
<tr>
<td>P value†</td>
<td>0.270</td>
<td>1.000</td>
<td>0.384</td>
<td>0.157</td>
<td>0.312</td>
<td>0.157</td>
<td>0.297</td>
<td>0.571</td>
</tr>
</tbody>
</table>

* I, percent infected; T, percent transmitted.

† Calculated using χ² test.
the salivary glands, and be transmitted. Future work is focusing on viral infection of the midgut in more detail to determine if the WN02 genotype viruses are more efficient at entry into midgut epithelial cells, RNA replication and packaging, or egress from the midgut. It is also possible that differences between the genotypes at each of these points in the viral life cycle contribute to the apparent increased \textit{in vivo} replication efficiencies of WN02 viruses.

WNV is maintained in nature in an enzootic cycle between \textit{Culex} mosquitoes and avian reservoir hosts. Our results suggest a possible model for displacement of the NY99 genotype by the WN02 genotype, in which the vectorial capacity of WN02 is increased. Vectorial capacity is described by the equation \( C = m^a p^b / -\ln p \), where \( m \) is the vector density, \( a \) is the feeding rate of the mosquito, \( p \) is the daily survival probability, and \( b \) is the proportion of mosquitoes that will become infectious.\(^{20}\) The decrease in the length of the EIP leads to an increase in the probability that a WN02-infected mosquito will survive the EIP to infect new hosts (\( p^b \)), increasing the vectorial capacity \( C \) of the WN02 genotype. As a result, a WN02-infected mosquito would have the potential to infect more birds than a NY99-infected mosquito, leading to higher WN02 infection rates in birds. Because WN02 viruses are more efficient than NY99 viruses at mosquito infection and transmission, \( b \) is also increased, magnifying the effect of the shorter EIP of the WN02 genotype on the circulating WNV population. Parallel comparative studies in an important avian host of WNV, the house sparrow (\textit{Passer domesticus}), suggest further enhancement of the WN02 advantage (AP Dupuis II, unpublished data). Decreased EIP in the mosquito vector plus enhanced infection in the avian host work synergistically and explain displacement of the NY99 genotype.

Our model of genotype displacement is similar to the mechanism that has been proposed for another flavivirus, Dengue virus (DENV)\(^{21}\); however, in our studies, viral transmission was directly measured, yielding unambiguous values for EIP. Displacement of DENV genotypes and serotypes has been extensively documented in nature.\(^{22-24}\) Dengue virus 2 (DENV-2) genotypes have different efficiencies of infection and transmission in \textit{Aedes aegypti}, with viruses belonging to the Southeast Asian genotype being more efficient at both infection and transmission than American genotype viruses, suggesting a possible mechanism for displacement of the American genotype by the Southeast Asian genotype.\(^{25,26}\)

In addition, Southeast Asian genotype DENV-2 can outcompete American genotype viruses during co-infection in both mosquitoes and human dendritic cells.\(^{27}\) Studies evaluating the competitive \textit{in vivo} fitness of the WN02 and NY99 genotypes are currently underway in \textit{Cx. pipiens}, and parallel studies are planned in house sparrows. Both the DENV-2 model and our WNV model describe the potential for virus strains to significantly affect vectorial capacity, thereby contributing to the displacement of one virus strain by another.

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