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Short Report: Comparison of Oral Infectious Dose of West Nile Virus Isolates Representing Three Distinct Genotypes in *Culex quinquefasciatus*

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Abstract

Phylogenetic analysis of West Nile virus in North America has identified replacement of the originally introduced clade, Eastern United States (NY99), by the North American clade. In addition, the transient emergence of other clades and genetic variants has also been observed. In this study, we investigated the potential role of the mosquito in the selection of these clades and genetic variants. We determined the relative susceptibility of *Culex quinquefasciatus* to infection with isolates from the Eastern U.S. clade, the North American clade, and the Southeast coastal Texas clade. Although significant differences were observed in 50% oral infectious dose values between the Eastern U.S. and two attenuated North American genetic variants compared with the North American and Southeast coastal Texas clade viruses, these differences did not correlate with persistence of the genotype in nature. These results indicate that selection of these viral genotypes was independent of viral oral infectivity in the mosquito.

The spread of West Nile virus (WNV) throughout North America has allowed detailed studies of how the virus has evolved after its introduction in 1999 and how it becomes established in new areas. This virus is typically transmitted in an avian and ornithophilic mosquito cycle, with the principle vectors being mosquitoes of the genus *Culex*.[1] Three *Culex* species have been identified as the primary vectors of WNV in the United States: *Cx. pipiens pipiens*, *Cx. tarsalis*, and *Cx. quinquefasciatus*.[2,3]

Using phylogenetic tools, Davis and others demonstrated that three distinct clades have evolved in the United States since 1999.[4] The New York 1999 (NY99), or the Eastern U.S. clade was originally introduced into New York. This virus was determined to be most closely related to a WNV isolate obtained from a dead goose in Israel in 1998.[5] As the virus moved westward, this clade was replaced by the North American clade in 2001–2002.[4] By 2004, the NY99 genotype was no longer observed in nature.[6] A third clade, Southeast coastal Texas, was identified in 2002. However, this clade has not been detected in subsequent years, which suggested that the clade has been displaced or has become extinct.[4] To test the hypothesis that the selection of viral genotypes is determined by infection of mosquito vectors, we determined the oral 50% infectious dose (ID_{50}) required to infect *Cx. quinquefasciatus* with five different

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isolates of WNV representing the three clades and two isolates that are naturally attenuated in their mouse neuroinvasive phenotype.[7]

The American prototype strain NY 382-99 belongs to the Eastern U.S. clade and was isolated from a flamingo in 1999 (GenBank accession no. AF196835).[5] The WNV 114 isolate belongs to the North American clade and was isolated from a bluejay in June 2002 from Harris County, Texas (GenBank accession no. AY185907).[8] The WNV 476 isolate, from the Bolivar peninsula located north of Galveston, Texas, was isolated in August 2002 from a bluejay and is representative of the Southeast coastal Texas clade (GenBank accession no. AY185914).[8] These three isolates have a large plaque *in vitro* phenotype and are non-attenuated for mouse neuroinvasiveness.[7] Two additional viruses, WNV v4369 and WNV 1153, which are genetic variants within the North American clade, were also examined. These isolates have a predominantly small plaque phenotype *in vitro* and are attenuated for mouse neuroinvasiveness. The WNV v4369 isolate was obtained from a *Cx. quinquefasciatus* pool (GenBank accession no. AY712948),[7] and the WNV 1153 isolate was obtained from a mourning dove (GenBank accession no. AY712945) in Harris County, Texas in 2003.[7,9]

The well-characterized colony of *Cx. quinquefasciatus* (Sebring strain) mosquitoes were used for this study. These mosquitoes were collected in Sebring County Florida in 1988 and have oral infectivity to WNV similar to that of field-collected *Cx. quinquefasciatus*.[10] This mosquito colony was maintained as previously described.[11] Groups of 125 four-day-old adult female *Cx. quinquefasciatus* mosquitoes were fed a blood meal containing dilutions of one of the five isolates of WNV to be analyzed. Vero cell culture was used to grow fresh virus from stock and the virus was harvested when 75% of the cells showed a cytopathic effect.

Serial 10-fold dilutions of viral supernatant and an equal volume of defibrinated sheep blood were mixed, heated to 37°C in a Hemotek feeding apparatus (Discovery Workshops, Accrington, United Kingdom) and presented to mosquitoes in an isolation glove box in an Arthropod Containment Level 3 insectary.[12] Fully engorged females were placed into new cartons and maintained until 14 days post-infection (dpi) when 6–30 mosquitoes were removed and held at −80°C for later titration. Mosquitoes were analyzed for percent infection on the basis of body titration and dissemination on the basis of titration of heads at 14 dpi. Briefly, 10-fold serial dilutions of triturated tissues were incubated with Vero cells on 96-well plates.[13,14] The oral ID_{50} was calculated on the basis of the percentage of mosquitoes infected at 14 dpi after exposure to four or five dilutions of blood meals that represented virus from the three clades and three dilutions of blood meals of the two genetic variants. The titers of these blood meals ranged from 0.22 to 8.22 $\log_{10}$ 50% tissue culture infectious doses ($TCID_{50}$)/5 μL. Regression lines and 95% confidence levels (CIs) were determined using PriProbit version 1.63 (Kyoto University, Kyoto, Japan).

Infection and dissemination rates were determined for each of the five viruses after oral infection of *Cx. quinquefasciatus* mosquitoes. When higher titers of the different viruses were used to orally infect mosquitoes, the infectivity rates were higher. Mosquitoes orally exposed to undiluted virus mixed with an equal volume of blood and analyzed by body titration on Vero cell culture at 14 dpi were positive for WNV at a high percentage, which ranged from 94% to 100% (Table 1). There was no significant difference between the five viruses with respect to dissemination rates at 14 dpi in mosquitoes fed blood meals with WNV titers ranging from 4.2 to 6.2 $\log_{10}TCID_{50}$/5 μL. At 14 dpi, dissemination rates were determined for WNV-infected mosquitoes. Mosquitoes were analyzed for WNV in mosquito heads titrated on Vero cell culture. The dissemination rate ranged from 86% to 100%.

Oral ID_{50} values for the five viruses were determined in *Cx. quinquefasciatus* mosquitoes; overlap of the 95% CIs was used as a conservative test of statistical significance (Figure 1).

There was no significant difference in the oral ID_{50} values for WNV NY99 clade virus and the two attenuated genetic variants WNV v4369 and WNV 1153. Similarly, there was no significant difference between the non-attenuated North American clade virus, WNV 114, and the Southeast Coastal Texas clade virus WNV 476. However, at the 95% CI, there was a statistically significantly difference identified between these two groups (Figure 1).

Plaque assays on Vero cell monolayers were used to determine if the plaque morphology phenotype had changed during replication in the mosquito. On the basis of these plaque assays of whole body homogenates at 14 dpi, the small plaque phenotype of the two attenuated viruses WNV 1153 and WNV v4369 was maintained after replication in mosquitoes.

We compared the phenotype of five isolates of WNV in *Cx. quinquefasciatus*. The predominant North American clade, represented by the WNV 114 isolate, had a significantly higher oral ID_{50} in *Cx. quinquefasciatus* mosquitoes than the NY99 isolate, which it replaced (5.56 and 3.88 $\log_{10}TCID_{50}$/5μL, respectively). This finding suggests that the lower oral dose, which enabled NY99 to infect this mosquito species, did not confer a selective advantage in nature. Previous studies have found that the oral ID_{50} of WNV 114 presented to field collected *Cx. quinquefasciatus* F_0 mosquitoes was 5.33 $\log_{10}TCID_{50}$/5 μL,[10] which is comparable to the oral ID_{50} value of 5.56 $\log_{10}TCID_{50}$/5 μL for WNV 114 virus in the *Cx. quinquefasciatus* colony used in this study.

There was no significant difference in the oral ID_{50} values of the North American clade (WNV 114) and Southeast Coastal Texas clade (WNV 476) viruses in *Cx. quinquefasciatus* mosquitoes (5.56 and 5.21 $\log_{10}TCID_{50}$/5 μL, respectively), although the Southeast Coastal Texas clade did not persist in nature beyond 2002. Interestingly, the two isolates that have naturally attenuated phenotypes in mice (WNV 1153 and WNV v4369) had oral ID_{50} values similar to that of to NY99 rather than to the North American clade virus from which they are genetically derived (Figure 1). In nature, these isolates did not persist beyond 2003, which indicated that a lower oral infectious dose for mosquitoes apparently conferred no selective advantage to these isolates.

Previous studies have attempted to identify the source of the genetic variability and the mechanism of selection of one virus genotype over another. A study examining the source of the genetic variability of WNV found that WNV sequences in mosquitoes were more variable than WNV sequences isolated from birds.[15] Jerzak and others speculated that mosquitoes in nature may be responsible for the genetic variation.[15] Studies examining a possible mechanism of selection of WNV isolates in mosquitoes suggested that the rate of transmission may be important. In one study, 2% of orally infected *Cx. tarsalis* mosquitoes transmitted a North American clade isolate (WN02) four days earlier than mosquitoes infected with NY99.[16] A similar transmission study in *Cx. pipiens* found that the WN02 genotype virus was transmitted 2–4 days earlier than the NY99 genotype virus.[17] In both of these studies, the infection and dissemination rates of WNV in *Cx. tarsalis* and *Cx. pipiens* mosquitoes were substantially lower than the infection and dissemination found in colonized and F_0 *Cx. quinquefasciatus* mosquitoes orally infected for this and a previous study.[10] These differences in infection and dissemination between the mosquito species and time points examined prohibit a direct comparison of these data.

Overall, our findings indicate that although there are phenotypic differences between the viruses *in vitro* and in mice, these differences are unrelated to the mosquito infectivity phenotype in terms of the oral ID_{50} values of these viruses. The selective advantage of a lower oral ID_{50} in *Cx. quinquefasciatus* mosquitoes does not appear to be sufficient to influence the selection of this particular viral phenotype. Therefore, we conclude that the replacement of the

Eastern U.S. clade with the North American clade has occurred by a mechanism not directly related to viral infectivity for *Cx. quinquefasciatus* mosquitoes.

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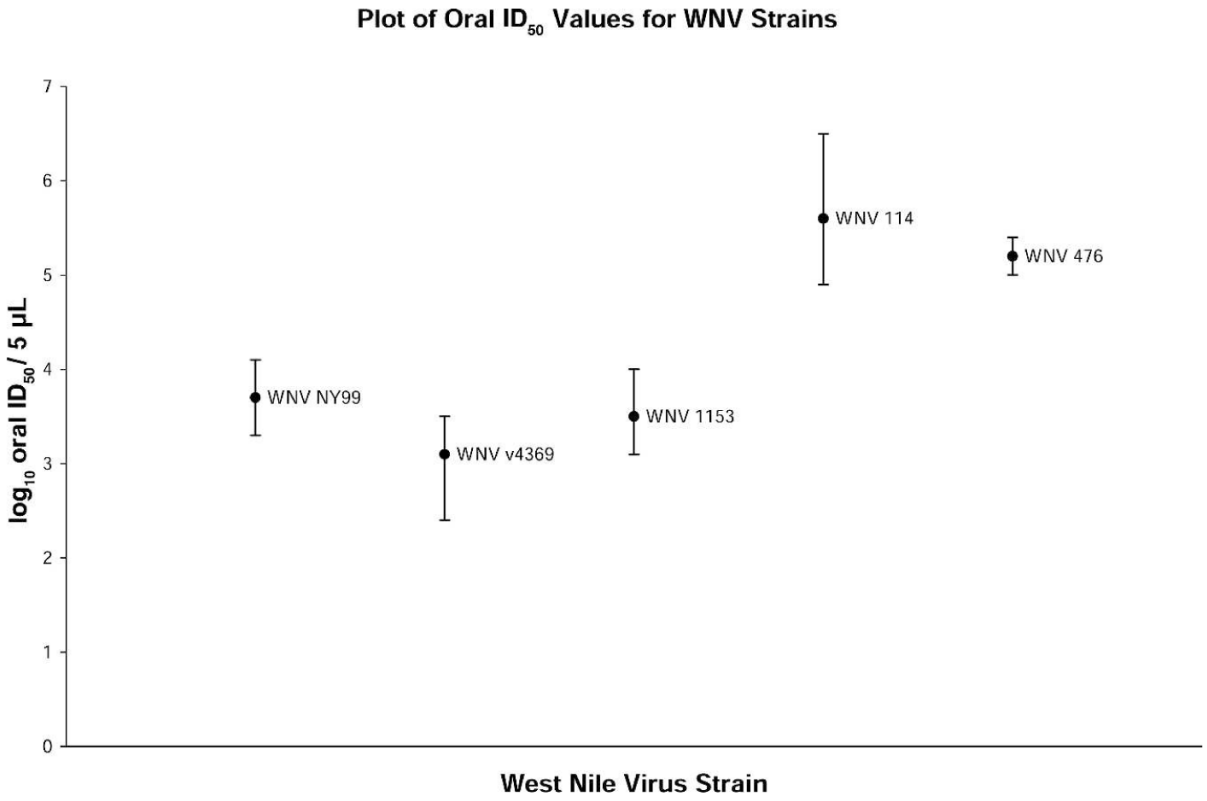

Figure 1.
Oral 50% infectious dose values of five isolates of West Nile virus in *Culex quinquefasciatus* (Sebring colony). Error bars represent 95% confidence intervals. Titers are reported as $\log_{10}$ 50% tissue culture infectious doses_0/5 µL, which is the estimated volume of ingested virus.

Table 1

Infection rates of *Culex quinquefasciatus* mosquitoes orally exposed to five West Nile virus isolates at various viral titers

Blood meal titer*	NY99 Eastern U.S. infection, no. (%)	v4369 attenuated† infection, no. (%)	1153 attenuated† infection, no. (%)	114 North American infection, no. (%)‡	476 Southeast Coastal Texas infection, no. (%)
8.22				6/6 (100)	
6.22	30/30 (100)			13/22 (59)	29/30 (97)
5.22		25/26 (96)	17/18 (94)		17/30 (57)
4.65	27/30 (90)				
4.22		15/20 (75)			0/30 (0)
3.65			12/24 (50)	5/30 (17)	
3.22	4/30 (13)				
2.65		14/35 (40)			0/30 (0)
2.22			4/26 (15)	1/24 (4)	
1.22	2/30 (7)				
0.22				0/18 (0)	

*Reported as $\log_{10}$ 50% tissue culture infectious dose/5 μL.

†Viruses are attenuated in their mouse neuroinvasive phenotype.

‡Infection rates were determined by whole body titration of the mosquito and reported as number positive/total number tested (%).