Genetic Determinants of Differential Oral Infection Phenotypes of West Nile and St. Louis Encephalitis Viruses in *Culex* spp. Mosquitoes

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Abstract. St. Louis encephalitis virus (SLEV) has shown greater susceptibility to oral infectivity than West Nile virus (WNV) in *Culex* mosquitoes. To identify the viral genetic elements that modulate these disparate phenotypes, structural chimeras (WNV–pre-membrane [prM] and envelope [E] proteins [prME]/SLEV.IC (infectious clone) and SLEV–prME/WNV.IC) were constructed in which two of the structural proteins, the prM and E, were interchanged between viruses. Oral dose–response assessment with the chimeric/parental WNV and SLEV was performed to characterize the infection phenotypes in *Culex* mosquitoes by artificial blood meals. The median infectious dose required to infect 50% of *Cx. quinquefasciatus* with WNV was indistinguishable from that of the SLEV–prME/WNV.IC chimeric virus. Similarly, SLEV and WNV–prME/SLEV.IC virus exhibited an indistinguishable oral dose–response relationship in *Cx. quinquefasciatus*. Infection rates for WNV.IC and SLEV–prME/WNV.IC were significantly lower than SLEV.IC and WNV–prME/SLEV.IC infection rates. These results indicated that WNV and SLEV oral infectivities are not mediated by genetic differences within the prM and E proteins.

INTRODUCTION

Culex mosquitoes have been recognized as the primary vectors for transmission of West Nile (WNV) and St. Louis encephalitis viruses (SLEV).¹ Ornithophilic species, such as *Culex pipiens* and *Culex restuans*, are the major vectors of WNV in the northeastern United States, *Culex tarsalis* is the major vector in the west, and *Culex quinquefasciatus* and *Culex nigripalpus* (mainly in Florida) are the major vectors in the south.^{2–6} SLEV has four distinct transmission cycles in North America that parallel those of WNV.^{7,8} *Cx. pipiens* transmits SLEV in the northeast, *Cx. quinquefasciatus* transmits SLEV in the south and southwest, *Cx. nigripalpus* transmits SLEV in Florida, and *Cx. tarsalis* serves as the principal vector in the west.⁸ Despite use of identical vectors for viral transmission, differential mosquito susceptibility to infection with WNV and SLEV has been documented, and oral infection thresholds for SLEV in *Culex* vectors have been shown to be much lower than for WNV.^{9–11}

To date, specific flaviviral genetic elements have not been associated with the differential infection phenotypes exhibited between WNV and SLEV in North American *Culex* mosquitoes. The flavivirus genome is composed of a single-stranded encapsulated RNA of positive polarity of approximately 11 kb in length.¹² The genome is cotranslated and post-translated as one open reading frame producing structural proteins capsid (C), pre-membrane (prM), and envelope (E) and non-structural (NS) components NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5¹²; 5' and 3' untranslated sequences are present flanking the intervening coding sequence. Previous studies have showed that specific flavivirus genetic elements can mediate mosquito infection phenotypes. For instance, glycosylation of the WNV E protein has been directly correlated with enhanced mosquito infectivity.^{13,14} Mutations to the dengue virus 2 FG loop of domain III, the fusion peptide involved in virus attachment and entry, resulted in significantly decreased infection rates in *Aedes aegypti* RexD mosquitoes.¹⁵ Additionally, studies with

yellow fever-17D vaccine virus and related chimeras revealed the importance of specific structural elements for flavivirus dissemination,¹⁶ further supporting the role of flavivirus structural proteins in mosquito infectivity.

Based on these studies, it was hypothesized that differences between SLEV and WNV structural genetic elements likely dictate the *Culex* oral infection susceptibility phenotypic differences observed between WNV and SLEV. To address that hypothesis, chimeric WNV/SLEV viruses were constructed in which the structural genetic elements (prME genes) of WNV and SLEV were interchanged to create SLEV–prME/WNV.IC (infectious clone) and WNV–prME/SLEV.IC chimeric viruses.¹⁷ These chimeric viruses and viruses rescued from the parental SLEV and WNV infectious complementary DNAs (cDNAs) previously were characterized in *Aedes albopictus* (C6/36) and *Cx. tarsalis* (CT) mosquito cell lines.¹⁷ Interestingly, WNV grew to 100-fold higher titers than SLEV in both mosquito cell lines. In C6/36 cells, the growth profile of the WNV–prME/SLEV.IC chimeric virus exhibited an intermediate growth phenotype between that of WNV and SLEV, whereas the SLEV–prME/WNV.IC chimeric virus grew to almost identical titers as the WNV.IC parental virus. The growth of these chimeric viruses was slightly different in *Cx. tarsalis* cells, where WNV–prME/SLEV.IC and WNV titers were comparable with but distinctive from SLEV and SLEV–prME/WNV.IC. The variable *in vitro* growth phenotypes of these chimeric viruses in two mosquito cell lines and the failure of the mosquito *in vitro* systems to recapitulate *in vivo* infection phenotypes dictated the necessity for conducting oral infection studies in *Culex* mosquitoes. Oral infection studies were performed with *Cx. quinquefasciatus* and *Cx. tarsalis* to provide a biologically relevant characterization of midgut infection determinants of these chimeric viruses, thereby providing insights into the specific viral genetic elements involved in this observed vector infectivity of these genetically related flaviviruses.

METHODS AND MATERIALS

Cells and viruses. Vero (African green monkey kidney) cells were maintained at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA).

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Baby hamster kidney-21 (BHK-21) cells were maintained at 37°C with 5% CO₂ in Modified Eagle Medium (MEM; Gibco). Both media were supplemented with 10% fetal bovine serum (FBS) and 5% penicillin/streptomycin (100 units/mL and 100 µg/mL, respectively). Assembly and rescue of the infectious cDNA clone-derived viruses of WNV.IC (East Coast genotype),^{18,19} SLEV-prME/WNV.IC, SLEV.IC, and WNV-prME/SLEV.IC¹⁷ used in this study have been described previously.¹⁷ All viruses were passaged one time in BHK-21 cells and harvested on days 3 (WNV.IC and SLEV-prME/WNV.IC) and 7 (SLEV.IC and WNV-prME/SLEV.IC) post-infection (dpi). The complete genomes of all rescued viruses were sequenced as previously described.¹⁷

Mosquitoes. Mosquitoes from well-characterized laboratory colonies were used for all vector competence studies. The *Cx. quinquefasciatus* Sebring strain assessed for vector competence to WNV and SLEVs was originally established in 1988 from Sebring County, Florida and used at F₃₀ or later generations.²⁰ The *Cx. tarsalis* Bakersfield Field Station (BFS) strain was established from Bakersfield, Kern County, California and has been in colonization since 1952.²¹ Mosquitoes were maintained at 28°C at a 16:8 hour (light:dark) photoperiod with 10% sucrose solution provided *ad libitum*; 3- to 5-day-old females were used for oral infections and intrathoracic inoculations.

Oral dose-response study with frozen virus stocks. Frozen stocks of WNV.IC, SLEV.IC, WNV-prME/SLEV.IC, and SLEV-prME/WNV.IC viruses were thawed at room temperature and serially diluted 10-fold to 4, 5, 6, 7, and 8 log₁₀ plaque forming units (PFU)/mL. Viruses then were mixed at a ratio of 1:1 with defibrinated chicken blood (Colorado Serum Company, Denver, CO) to serve as artificial infectious blood meals. Each cohort of 100 *Cx. quinquefasciatus* mosquitoes per virus dose was sugar-starved for 18 hours and offered 3 mL blood: virus mixture using a Hemotek feeding unit (Discovery Workshops, Accrington, United Kingdom) for 1 hour in the dark. A 100-µL aliquot of the blood meal was retained and stored at -80°C for back-titration of virus doses. Fully engorged females were removed under cold anesthesia and placed in new 1-pint cartons with 5% sucrose solution provided *ad libitum*. Cartons were incubated at 28°C with a photoperiod of 16:8 hour (light:dark) through 14 dpi.

After 14 dpi, 25 mosquitoes from each virus group were anesthetized by exposure to triethylamine (Flynap; Carolina Biological Supply Company, Burlington, NC), their legs were removed, and saliva was collected by inserting each proboscis in a capillary tube containing 2.5% FBS and 2.5% sucrose solution. Mosquitoes were allowed to salivate for at least 15 minutes, after which time each capillary tube was placed in a 2-mL microcentrifuge tube containing 0.5 mL DMEM with 10% heat-inactivated FBS and 5% penicillin/streptomycin (100 units/mL and 100 µg/mL respectively). One hind leg from each mosquito was placed in a separate 2-mL microcentrifuge tube containing the same media with a sterile copper-coated steel bead (Crossman Corporation, NY). Mosquito bodies were collected after salivation. Mosquito bodies, legs, and saliva were stored at -80°C until they were triturated by mixer mill (Qiagen; Valencia, CA) and assayed for infectious virus by plaque assay on Vero cells.

Cohorts of 3- to 5-day-old *Cx. tarsalis* were allowed to feed on blood meals containing 4 and 7 log₁₀ PFU/mL WNV.IC, SLEV.IC, WNV-prME/SLEV.IC, and SLEV-prME/WNV.IC in the same manner described above. However, because of

high mortality, the extrinsic incubation period was shortened, and bodies, legs, and saliva were harvested as before at 4 and 8 dpi. An aliquot of each blood meal was retained at feeding to quantify virus dose. Mosquito bodies, legs, and saliva were stored at -80°C until assayed for infectious virus by plaque assay as described below.

Oral dose-response study with freshly grown virus. Because studies have shown that use of freshly grown flaviviruses that have not undergone a freeze-thaw can improve infection rates in artificial oral infectious feeds,^{11,22} a second set of oral susceptibility feeds was conducted with freshly propagated viruses to provide greater resolution of infection rates at lower viral doses. Vero cells were grown to 90% confluence, after which time they were inoculated with WNV.IC, SLEV.IC, WNV-prME/SLEV.IC, or SLEV-prME/WNV.IC at a multiplicity of infection of 0.1. Briefly, cells were washed with Dulbecco's phosphate buffered saline (DPBS; Gibco), inoculated with 200 µL virus suspension, and incubated at 37°C for 1 hour. After the incubation period, the virus inoculum was removed from the cells, the monolayer was washed two times with DPBS, and fresh medium was added. Growth curves generated in Vero cells using these four viruses indicated that, at 4 dpi, all viruses reached an approximate titer of 8.2 ± 0.5 log₁₀ PFU/mL. At 4 dpi, all four viruses were harvested by collecting cells and supernatant, and the mixture was centrifuged at approximately $1,000 \times g$ for 5 minutes at 4°C. Each virus was serially diluted 100-fold two times. Viruses then were mixed at a 1:1 ratio with defibrinated goose blood (Colorado Serum Company) to make 3 mL virus: blood mix. *Cx. quinquefasciatus* was offered the virus: blood mixture and blood-fed females were sorted according to the protocols described above. An aliquot of the blood meal was retained for confirmation of virus doses to which the mosquitoes were exposed. After 14 dpi at 28°C, mosquito bodies, legs, and expectorants were collected from 25 mosquitoes per virus per dose as described above. Blood meals and mosquito samples were stored at -80°C until assayed for infectious virus.

Intrathoracic inoculations. Sixty 5-day-old *Cx. tarsalis* were cold-anesthetized and intrathoracically inoculated with approximately 0.1 µL inoculum of the following viruses: WNV.IC, SLEV.IC, WNV-prME/SLEV.IC, and SLEV-prME/WNV.IC at 7 log₁₀ PFU/mL. Inoculated mosquitoes were placed in 1-pint cartons in a 28°C incubator with a 16:8 hour (light:dark) photoperiod and 10% sucrose supplied *ad libitum*. At 7 dpi, surviving mosquitoes were removed from each virus group and anesthetized with triethylamine, expectorant was collected, and bodies were harvested as described above. These samples were stored at -80°C until further processing.

Assessment of infection, dissemination, and transmission rates. To determine infection and dissemination rates, bodies and legs, respectively, were homogenized for 24 cycles/second for 2 minutes using a mixer mill, after which time the samples were centrifuged ($5,000 \times g$ for 5 minutes). The clarified homogenate was assayed for the presence of virus using a standard plaque assay on Vero cells.²³ To assay for transmission, expectorant from virus-positive mosquitoes was centrifuged at approximately $5,000 \times g$ for 5 minutes and then tested for virus by plaque assay on Vero cells as above.

Statistical analysis. Probit analyses were used to calculate the median infectious dose (ID₅₀; amount of virus required to infect 50% of fed females) and standard errors (SEs) from the oral dose-body infection response data. ID₅₀ values with

overlapping SEs were considered statistically indistinguishable. A 2×4 contingency χ^2 test ($P < 0.05$) was used to analyze body infection within oral doses used in the freeze–thaw virus experiment. A χ^2 test was used to analyze infection rates of *Cx. tarsalis*. A one-way analysis of variance (ANOVA) was used to test the effects of time and dose on body infection rates.

RESULTS

Oral dose–response (freeze–thaw virus). To identify viral genetic elements that may be responsible for the lower oral infection threshold for SLEV in *Culex* mosquitoes, parental WNV/SLEV and reciprocal chimeric viruses were used to orally infect colonized *Cx. tarsalis* (BFS) and *Cx. quinquefasciatus* (Sebring). *Cx. tarsalis* were orally exposed to 4 or 7 log₁₀ PFU/mL of two parental and two chimeric viruses. At both 4 and 8 dpi, body infection rates for WNV.IC, SLEV.IC, WNV-prME/SLEV.IC, and SLEV-prME/WNV.IC were significantly different at the 4-log₁₀ PFU/mL dose ($\chi^2 = 10.49$, $\chi^2 = 9.32$, $P < 0.05$) (Table 1) but not at the 7-log₁₀ PFU/mL dose ($P > 0.05$) in *Cx. tarsalis* mosquitoes. Additionally, there was a significant effect of dose and time on SLEV.IC infection rates ($P < 0.05$).

Cx. quinquefasciatus were also orally exposed to serially diluted doses (4, 5, 6, 7, and 8 log₁₀ PFU/mL) of WNV.IC, SLEV.IC, WNV-prME/SLEV.IC, and SLEV-prME/WNV.IC. Interestingly, infection rates were lower than expected for the parental clone-derived viruses and mosquito body infections were undetectable at 4 and 5 log₁₀ PFU/mL for all four viruses (Table 2). Infection was first detected at 6 log₁₀ PFU/mL with SLEV.IC (8%) and the chimeric WNV-prME/SLEV.IC (20%). Infection rates of 52% and 68% were observed at 7 and 8 log₁₀ PFU/mL, respectively, in SLEV.IC-infected mosquitoes. At the same doses, mosquitoes exposed to the WNV-prME/SLEV.IC chimeric virus showed infection rates of 52% and 84%, respectively. In contrast, infection rates at 7 log₁₀ PFU/mL for WNV.IC (20%) and SLEV-prME/WNV.IC (16%) were significantly lower than those observed for SLEV.IC and WNV-prME/SLEV.IC. Notably, infection rates for SLEV.IC and WNV-prME/SLEV.IC were not statistically significantly different at any dose. Only SLEV-prME/WNV.IC showed a significantly lower infection rate compared with the other three viruses at the 8-log₁₀ PFU/mL exposure dose. At the highest dose, no significant differences in dissemination or transmission (range = 86–100%) were observed among viruses (Table 2).

Oral dose–response (freshly propagated virus). To obtain a higher resolution of infection at lower viral doses, oral infec-

TABLE 1

Oral dose–response effect on infection of *Cx. tarsalis* mosquitoes with WNV/SLEV parental and chimeric viruses

Virus	Dose			
	4 log ₁₀ PFU/mL		7 log ₁₀ PFU/mL	
	4 dpi	8 dpi	4 dpi	8 dpi
WNV.IC	5/20 (25)	0/20 (0)	4/20 (20)	6/18 (33)
WNV-prME/SLEV.IC	2/20 (10)	1/20 (5)	4/20 (20)	5/20 (25)
SLEV.IC	0/20 (0)	3/14 (21)	5/20 (25)	8/18 (44)
SLEV-prME/WNV.IC	0/20 (0)	0/20 (0)	4/20 (20)	7/20 (35)

Mosquito bodies were collected at 14 dpi and homogenized. Clarified homogenate was plated on Vero cells to determine viral titers. Total numbers of virus-positive *Cx. tarsalis* (BFS) mosquitoes are shown.

TABLE 2

Virus-positive bodies, legs, and saliva of *Cx. quinquefasciatus* (Sebring) orally exposed to blood meals containing freeze–thaw WNV.IC, SLEV-prME/WNV.IC, SLEV.IC, and WNV-prME/SLEV.IC

Virus/oral dose (log ₁₀ PFU/mL)	Body (%)	Leg (%)	Saliva (%)
WNV.IC			
4	0/25 (0)	0/25 (0)	0/25 (0)
5	0/25 (0)	0/25 (0)	0/25 (0)
6	0/25 (0)	0/25 (0)	0/25 (0)
7	5/25 (20)	2/5 (40)	2/5 (40)
8	20/25 (80)	20/20 (100)	19/20 (95)
SLEV-prME/WNV.IC			
4	0/25 (0)	0/25 (0)	0/25 (0)
5	0/25 (0)	0/25 (0)	0/25 (0)
6	0/25 (0)	0/25 (0)	0/25 (0)
7	4/25 (16)	4/4 (100)	3/4 (75)
8	5/16 (31)	5/5 (100)	5/5 (100)
SLEV.IC			
4	0/25 (0)	0/25 (0)	0/25 (0)
5	0/25 (0)	0/25 (0)	0/25 (0)
6	2/25 (8)	2/2 (100)	2/2 (100)
7	12/23 (52)	10/12 (83)	6/12 (50)
8	15/22 (68)	15/15 (100)	15/15 (100)
WNV-prME/SLEV.IC			
4	0/25 (0)	0/25 (0)	0/25 (0)
5	0/25 (0)	0/25 (0)	0/25 (0)
6	5/25 (20)	4/5 (80)	4/5 (80)
7	13/25 (52)	11/13 (85)	10/13 (77)
8	21/25 (84)	18/21 (86)	18/21 (86)

Percentages of mosquitoes positive for infection and infected mosquitoes positive for dissemination and transmission are designated in parentheses.

tions of *Cx. quinquefasciatus* were repeated using freshly propagated viruses. Because viruses were used when freshly harvested, titers of infecting blood meals were variable among virus groups (Table 3). SLEV.IC and WNV-prME/SLEV.IC established infections at oral doses of 2.5 and 2.4 log₁₀ PFU/mL, respectively, after which infection rates increased with increasing blood meal titer (Table 3). This fresh virus propagation experiment showed a significant increase in the susceptibility of *Cx. quinquefasciatus* at low doses compared with the experiment using thawed frozen virus suspensions. In contrast, no infection was observed for females feeding at similar

TABLE 3

Oral dose–response of *Cx. quinquefasciatus* fed freshly propagated viruses: WNV.IC, SLEV.IC, WNV-prME/SLEV.IC, and SLEV-prME/WNV.IC

Virus/oral dose (log ₁₀ PFU/mL)	Body (%)	Leg (%)	Saliva (%)
WNV.IC			
1.0	0/24 (0)	0/24 (0)	0/24 (0)
3.1	0/25 (0)	0/25 (0)	0/25 (0)
6.0	3/25 (12)	3/3 (100)	2/3 (67)
8.5	17/25 (68)	17/17 (100)	8/16 (50)
SLEV.IC			
2.5	5/25 (20)	4/5 (80)	4/5 (80)
3.8	8/25 (32)	8/8 (100)	7/8 (88)
6.9	2/2 (100)	2/2 (100)	1/2 (50)
WNV-prME/SLEV.IC			
2.4	1/25 (4)	0/1 (0)	0/1 (0)
4.8	4/25 (16)	4/4 (100)	3/4 (75)
6.1	16/19 (84)	15/16 (94)	14/16 (88)
SLEV-prME/WNV.IC			
2.7	0/9 (0)	0/9 (0)	0/9 (0)
5.1	1/9 (11)	1/1 (100)	0/1 (0)
7.0	7/25 (28)	6/7 (86)	6/7 (86)
7.5	11/25 (44)	5/11 (45)	5/11 (45)

Data represent numbers of virus-positive bodies, legs, and saliva. Infection, dissemination, and transmission rates are in parentheses.

TABLE 4

Median infectious doses (ID₅₀ values) for *Cx. quinquefasciatus* orally exposed to freshly propagated or freeze-thaw WNV.IC, SLEV-prME/WNV.IC, SLEV.IC, and WNV-prME/SLEV.IC suspensions

Virus	Freshly grown virus		Frozen virus	
	Median infectious dose (log ₁₀ PFU/mL)	± SE	Median infectious dose (log ₁₀ PFU/mL)	± SE
WNV.IC	8.0	0.1	7.5	0.4
SLEV.IC	5.2	2.4	7.3	0.4
WNV-prME/SLEV.IC	5.4	0.4	6.9	0.3
SLEV-prME/WNV.IC	8.1	1.3	9.1	1.8

doses on WNV.IC- and SLEV-prME/WNV.IC-spiked blood meals (Table 3).

The ID₅₀ for the SLEV-prME/WNV.IC (8.1 ± 1.3 log₁₀ PFU/mL) was statistically indistinguishable ($P > 0.05$) from the ID₅₀ for WNV.IC (8.0 ± 1.0 log₁₀ PFU/mL) (Table 4). Similarly, the ID₅₀ values for SLEV.IC (5.2 ± 2.4 log₁₀ PFU/mL) and WNV-prME/SLEV.IC (5.4 ± 0.4 log₁₀ PFU/mL) were indistinguishable ($P > 0.05$) but statistically lower ($P < 0.05$) than WNV.IC and SLEV-prME/WNV.IC (Table 4). Although small sample sizes of infected mosquitoes and variable input doses confounded the statistical assessment of dissemination and transmission rates, most infected mosquitoes had virus in the legs (range = 63–100%) and expectorant (range = 53–85%) (Table 4).

The ID₅₀ values for SLEV.IC and WNV-prME/SLEV.IC that were frozen and thawed were significantly greater (7.3 ± 0.4 and 6.9 ± 0.3 log₁₀ PFU/mL) than for the freshly propagated virus described above. Both sources of virus showed ID₅₀ values for these two viruses that were not statistically distinguishable ($P > 0.05$) but were significantly different from ID₅₀ values calculated for WNV.IC and SLEV-prME/WNV.IC (Table 4). The ID₅₀ values for freeze-thaw WNV.IC and SLEV-prME/WNV.IC (7.5 ± 0.4 and 9.1 ± 1.8 log₁₀ PFU/mL, respectively) were statistically indistinguishable ($P > 0.05$), and also, they were indistinguishable from ID₅₀ values calculated for SLEV.IC and WNV-prME/SLEV.IC (Table 4). In contrast, the ID₅₀ values for SLEV.IC and WNV-prME/SLEV.IC were significantly lower for mosquitoes exposed to freshly propagated virus compared with freeze-thaw suspensions.

Intrathoracic inoculations. After infection by intrathoracic inoculation of *Cx. tarsalis* to bypass midgut infection and escape barriers, there were no statistically significant differences ($P > 0.05$) in infection and transmission rates or titers observed in the triturated bodies or salivary expectorants among the four viruses assayed (Table 5). Because *Cx. quinquefasciatus* mosquitoes exposed to high oral doses of all four viruses assayed developed disseminated infections and were capable of transmission, similar intrathoracic inoculations were not performed, because a midgut escape barrier was not evident.

DISCUSSION

WNV has been shown to exhibit comparatively higher thresholds for oral infection of North American *Culex* mosquitoes than SLEV.^{9,24} Although adaptation studies in *Culex* mosquitoes and chickens (*Gallus gallus*) have shown greater genetic plasticity of SLEV compared with WNV,^{25,26} the viral genetic determinants that dictate the comparatively greater oral susceptibility for WNV are unknown. Because SLEV is an endemic North American flavivirus that generally elicits low titers in avian hosts, it is likely that this virus has adapted over time to infect North American *Culex* at low blood meal titers. West Nile virus, in contrast, has recently been introduced into the Western Hemisphere and produces elevated virus titers in many birds. The development of high viremias in avian hosts would be predicted to reduce the selective force for adaptation of WNV strains for increased oral infectivity of *Culex* mosquitoes, especially if it was negatively affecting fitness in avian hosts. Nevertheless, the shortened extrinsic incubation period of the North American WN02 genotype in *Cx. pipiens* and *Cx. tarsalis* indicates that WNV may be adapting to mosquitoes in the United States.²⁷

Consistent with previous findings, WNV showed higher ID₅₀ values than SLEV in *Cx. quinquefasciatus* in both oral dose-response experiments. In the freeze-thaw virus study, WNV was only capable of establishing infection in mosquitoes exposed to ≥ 7 log₁₀ PFU/mL virus, whereas SLEV infected mosquitoes at 6 log₁₀ PFU/mL. Similarly, infection rates with freshly propagated WNV and SLEV indicated a significantly higher infection threshold dose for infection with WNV; however, the disparity between viruses was more pronounced than that observed in the freeze-thaw virus study, with SLEV exhibiting an ID₅₀ of 5.2 ± 2.4 log₁₀ PFU/mL, whereas the ID₅₀ for WNV was determined to be 8.0 ± 1.0 log₁₀ PFU/mL. Higher oral infection rates were achieved at lower viral doses using freshly grown flaviviruses than freeze-thaw preparations. This result was similar to previous results from reports with other flaviviruses.^{11,28} Interestingly, both SLEV and WNV infection rates from our study were not as high as the rates reported by in the work by Pesko and Mores,¹¹ which reported infection rates of 100% with SLEV and WNV at initial oral doses of 3.3 ± 0.1 and 5.6 ± 0.1 log₁₀ PFU/mL, respectively.¹¹ At similar doses in this study, SLEV showed a 30% infection rate, whereas WNV infected only 10% of the mosquitoes. Intraspecific differences between colonies of *Cx. quinquefasciatus* could potentially dictate the dissimilarity in infection rates in these two studies. In agreement, markedly different vector competence rates for SLEV and WNV were documented in field-collected *Culex* from diverse regions of California over a 5-year period.²⁹ Pesko and Mores¹¹ used mosquitoes collected from Gainesville, Florida in 1996 that were in colony for 50 generations, whereas the mosquitoes used in this study were from Sebring

TABLE 5

Infection and transmission rates in *Cx. tarsalis* intrathoracically inoculated with WNV.IC, SLEV-prME/WNV.IC, SLEV.IC, and WNV-prME/SLEV.IC

Viruses	Body (%)	Mean body titer (log ₁₀ PFU/mL)	Saliva (%)	Mean saliva titer (log ₁₀ PFU/mL)
WNV.IC	15/15 (100)	7.4 ± 0.2	15/15 (100)	2.3 ± 0.3
SLEV-prME/WNV.IC	21/25 (84)	7.4 ± 0.2	21/21 (100)	2.3 ± 0.5
SLEV.IC	12/15 (80)	7.6 ± 0.2	12/12 (100)	2.2 ± 0.4
WNV-prME/SLEV.IC	24/24 (100)	7.2 ± 0.2	24/24 (100)	2.2 ± 0.2

County, Florida and have been in colonization since 1988.²⁰ Vector competence of WNV can also vary depending on interspecies differences and seasons, which may also explain the discrepancy between the two studies.^{5,6,30–33}

Prior *in vitro* infection studies with WNV.IC, SLEV.IC, SLEV-prME/WNV.IC, and WNV-prME/SLEV.IC in C6/36 (*Ae. albopictus*) and CT (*Cx. tarsalis*) cell lines indicated that viral structural genetic elements could potentially mediate viral growth in cultured mosquito cells.¹⁷ However, oral mosquito infections revealed that the infection threshold phenotype was modulated by viral genetic elements exclusive to the prME structural proteins. This finding further supports the lack of biological relevance of mosquito cell lines, such as the C6/36 cells, that have an impaired RNA interference (RNAi) pathway and therefore, do not model natural arboviral infections.³⁴ In the freeze–thaw virus study, the infection rate of the WNV-prME/SLEV.IC chimeric virus was statistically indistinguishable from that of SLEV.IC. Furthermore, the infection rates observed with this virus were statistically different from those of WNV.IC and SLEV-prME/WNV.IC at 6- and 7-log₁₀ PFU/mL doses. Similar phenotypes were observed in the freshly grown virus study, where the chimeric virus containing the WNV non-structural proteins, SLEV-prME/WNV.IC, was unable to establish infection at the lower oral doses. In contrast, SLEV.IC and WNV-prME/SLEV.IC exhibited lower ID₅₀ values and were able to successfully infect midguts at comparable lower doses. Lack of significant differences in ID₅₀ values at higher doses in both oral dose–response studies can be attributed to the known dose dependency of the viral barriers to infection.³⁵ Together, these data indicate that viral genetic elements exclusive of the prME genes are associated with the differential midgut infection phenotypes observed between WNV and SLEV, which is supported by previous studies that have identified specific non-structural genetic determinants of flavivirus infectivity for mosquitoes. For example, in dengue virus-4 (DENV-4), a single mutation (C to U) in the NS4B gene at amino acid position 101 resulted in a non-conservative change from a proline to leucine that restricted midgut replication in *Ae. aegypti* and consequently, suppressed dissemination compared with wild-type DENV-4 virus.³⁶ Similarly, prME chimeric viruses generated between WNV and DENV4 assessed in *Cx. tarsalis* provided additional evidence that flavivirus mosquito infection rates can be mediated by non-structural genetic elements.³⁷ McElroy and others³⁸ also attributed the lack of dissemination of the yellow fever virus (YFV-17D) from *Ae. aegypti* midguts to mutations in the NS2A and NS4B genes. Both flaviviral NS2A and NS4B proteins are required for efficient flavivirus replication and potential suppression of the interferon pathway in vertebrate hosts, but little is known about the function of these proteins in mosquitoes.^{12,39} In our study, infectivity of *Cx. quinquefasciatus* was increased by the expression of SLEV NS gene proteins; using more refined viral chimeras will provide greater insight into the molecular determinants of flaviviral infectivity of mosquito midgut epithelium.

The results herein have focused mostly on the effects of viral genetic determinants that modulate *Cx. quinquefasciatus* and *Cx. tarsalis* susceptibility to infection by SLEV and WNV; however, other barriers exist that also may preclude transmission of a virus by a particular vector,⁴⁰ such as the midgut infection barrier (MIB) and the midgut escape barrier (MEB).⁴¹ Because there were no differences in infection and

transmission rates of *Cx. tarsalis* mosquitoes intrathoracically infected with WNV.IC, SLEV-prME/WNV.IC, SLEV.IC, and WNV-prME/SLEV.IC, we concluded that both WNV and SLEV were equally efficient at establishing infections in secondary tissues after bypassing the midgut. Differences in midgut infectivity could result from (1) the presence or absence of appropriate cell surface receptors on microvilli cells,⁴¹ (2) the presence of susceptible midgut epithelial cells at differing proportions of the epithelium, resulting in a variable dose dependency for infection, or (3) differential capacity for intracellular replication of viruses in exposed midgut epithelial cells. The fact that the non-structural genetic elements of SLEV were shown to dictate establishment of midgut infection indicates the probability that hypothesis 3 results in the variable infection phenotype.

Gene expression in mosquito midgut epithelial cells is up-regulated after ingestion of blood.^{42,43} Ingestion of virus along with the blood meal also is associated with altered gene expression in the midgut, which was observed in WNV-infected *Cx. quinquefasciatus*, where expression of the putative protein, CQ G12A2, increased throughout the extrinsic incubation period.⁴⁴ The CQ G12A2 protein is similar to a Toll-like receptor (TLR) involved in resistance to dengue virus infections in *Ae. aegypti*, suggesting the potential role of this protein in mosquito antiviral innate immune response.⁴⁵ Up-regulation of specific genes imparting innate mosquito immunity in midgut cells may depend on virus genotype and thereby, influence vector competence.⁴⁶ Similar experiments have not been performed with SLEV, and therefore, additional investigation is needed to assess the effect of increased expression of CQ G12A2 and/or other genes on the innate immune response in SLEV-infected mosquitoes.

The discordance between *in vitro* and *in vivo* mosquito infection phenotypes of WNV and SLEV could indicate that RNAi responses *in vivo* within the mosquito midgut epithelial cells could restrict WNV infection disproportionately compared with SLEV. The lack of an intact RNAi response in C6/36 cells³⁴ would likely negate these observed differences¹⁷ if alternative evasion of this response serves as a factor distinguishing *in vivo* mosquito replication phenotypes between the viruses. It is also possible that direct antagonism of the innate immune response or functional compartmentalization of the replication complexes of SLEV in mosquitoes could result in more efficient intracellular replication in midgut epithelial cells. Innate mosquito immunity studies have indicated that RNAi pathways influence viral infection of mosquitoes.⁴⁷ Because formation of double-stranded RNA (dsRNA) is non-structurally mediated and the RNAi response is sequence-specific, differential immune evasion responses based on non-structural viral genomic differences could be important antagonists of the RNAi response in mosquitoes and thus, influence vector infectivity phenotypes.^{46,48} *Cx. quinquefasciatus* infected with SLEV previously has been reported to produce lower levels of viral RNA compared with WNV.^{35,49} Lower initial RNA levels could delay the induction of the RNAi pathway in an SLEV-infected mosquito. In contrast, increased replicative capacity of WNV could result in the production, consequent recognition, and degradation of viral RNA, effectively dampening viral RNA production and reducing oral infection rates in mosquitoes comparatively earlier than in SLEV-infected mosquitoes. Virus-encoded protein suppressors of RNAi (VSRs) are expressed by a number

of plant and insect pathogenic viruses during replication.⁵⁰ Flaviviral small subgenomic RNAs (sfRNAs) have the capacity to impede RNAi pathways, inhibiting dsRNA degradation by dicer⁵¹ in mosquito cells. Furthermore, microRNA-like elements in the 3' untranslated region (UTR) of WNV have been associated with transcriptional regulation of genes that can facilitate replication in mosquito cells.⁵² It is possible that flaviviral VSRs related to differential effects of WNV versus SLEV sfRNAs or microRNAs present in the 3' UTR differentially antagonize the RNAi response or alter gene expression profile in *Culex* spp. mosquitoes. Additional studies on such phenomena will provide insight into the mechanisms that dictate the differential ability of arboviruses to establish infections in mosquitoes.

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