Comparative Vector Competence of North American *Culex pipiens* and *Culex quinquefasciatus* for African and European Lineage 2 West Nile Viruses

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Abstract. West Nile virus (WNV) is a mosquito-borne flavivirus that is phylogenetically separated into distinct lineages. Lineage 1 (L1) and lineage 2 (L2) encompass all WNV isolates associated with human and veterinary disease cases. Although L1 WNV is globally distributed, including North America, L2 WNV only recently emerged out of sub-Saharan Africa into Europe and Russia. The spread of L2 WNV throughout and beyond Europe depends, in part, on availability of competent vectors. The vector competence of mosquitoes within the *Culex* genus for WNV is well established for L1 WNV but less extensively studied for L2 WNV. Assessing the vector competence of North American *Culex* mosquitoes for L2 WNV will be critical for predicting the potential for L2 WNV emergence in North America. We address the vector competence of North American *Culex pipiens* and *Culex quinquefasciatus* for L2 WNV. Both mosquito species were highly competent for each of the L2 WNV strains assessed, but variation in infection, dissemination, and transmission was observed. An L2 WNV strain (NS10) isolated during the Greek outbreak in 2010 exhibited a reduced capacity to infect *Cx. pipiens* compared with other L2 WNV strains. In addition, a South African L2 WNV strain (SA89) displayed a significantly shorter extrinsic incubation period in *Cx. quinquefasciatus* compared with other L2 WNV strains. These results demonstrate that North American *Culex* mosquito species are competent vectors of African and European L2 WNV and that emergence of L2 WNV is unlikely to be hindered by poor competence of North American vectors.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne flavivirus (*Flaviviridae* family) that is maintained in nature through an enzootic mosquito–bird transmission cycle.¹ West Nile viruses responsible for all human and veterinary disease cases have been phylogenetically separated into two main lineages.² Lineage 1 (L1) WNV strains are found on all continents except Antarctica and are most commonly associated with human disease outbreaks. In contrast, circulation of lineage 2 (L2) WNV strains has been historically limited to the sub-Saharan regions of Africa and Madagascar and only sporadically associated with WNV outbreaks in those regions.^{3–5} However, beginning in 2004, L2 WNV strains were first identified to have emerged into parts of central and southern Europe, Russia, and Israel, likely being maintained enzootically and causing recurrent human disease outbreaks.^{6–10}

The initial emergence of L2 WNV in Europe was recognized by the isolation of an L2 virus from a moribund goshawk in Hungary.⁹ This L2 isolate demonstrated close genetic identity to other L2 isolates from southern and central Africa, suggesting that the strain had likely been introduced through avian flyways from Africa.⁹ Subsequent bird L2 WNV isolations have been made in Hungary (2005) and Austria (2008), with a high degree of sequence conservation with the original Hungarian isolate, indicating that the introduced L2 viruses likely established autochthonous enzootic transmission cycles in central Europe.^{9,11} The first individual cases of L2 WNV infection in humans or small L2 WNV-mediated outbreaks outside of sub-Saharan Africa were observed in Russia in 2004 and 2007 and in Israel in 2009.^{6,7,12} The first large European L2 WNV outbreak occurred in 2010 in northern

Greece.¹³ Sequence analysis of an L2 WNV isolate made from a pool of *Culex (Cx.) pipiens* (L.) mosquitoes collected during the 2010 outbreak in Nea Santa, Greece (Nea Santa-Greece-2010 strain), demonstrated close sequence identity to that of the Hungarian isolate made 6 years earlier.¹⁴ Subsequent L2 WNV outbreaks were observed in Greece between 2011 and 2014, Serbia (2012), and Italy.^{15–19} The isolation of L2 WNV strains from six other countries and their corresponding genetic relatedness indicate that L2 WNV strains are being spread in central and southern Europe through autochthonous transmission and suggest the potential for further geographic dispersal.

West Nile virus is transmitted by various mosquito species within the genus *Culex*, and the significance of each species to the WNV transmission cycle can vary geographically.^{20,21} In Europe, *Cx. pipiens* and *Culex (Bar.) modestus* Ficalbi mosquitoes have been identified as the principal WNV vectors with the ability to transmit both L1 and L2 WNV strains.^{22–26} In the United States where only L1 WNV strains have been found to circulate, the most significant WNV vector in the midwest and northeast is *Cx. pipiens*, whereas *Culex quinquefasciatus* Say and *Culex tarsalis* Coquillett predominate as potential vectors in the south and northwest, respectively.²¹ *Culex univittatus* Theobald mosquitoes are the principal enzootic transmission vectors in eastern and southern Africa for L2 WNV strains.²² However, ornithophilic *Cx. quinquefasciatus* and *Cx. pipiens* have also been identified as important transmission vectors for WNV in Nigeria, Uganda, Kenya, and South Africa.^{5,27–29}

The potential for emergence of L2 WNV beyond Europe and Russia likely depends on the availability of competent transmission vectors; yet, limited information regarding the vector competence of *Culex* species outside of Europe for L2 WNV strains is available. Given the capacity of L2 WNV to emerge and establish autochthonous transmission in new environmental niches, the emergence of L2 WNV strains in North America is a potentiality. As such, the competency of North American mosquitoes to become infected with, disseminate, and transmit genetically distinctive L2 WNV strains was assessed.

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MATERIAL AND METHODS

Virus stocks. Four L2 WNV strains were used in this study (Table 1). The South African isolate (SPU116-89; SA89) used was originally isolated from a human hepatitis case during a WNV epidemic in South Africa in 1989. The patient died of necrotic hepatitis within 4 days of presenting with illness and represents the only human fatality associated with an L2 WNV in South Africa.²⁷ The Ugandan enzootic isolate, UG09, was obtained from a pool of *Culex neavei* Theobald from the Queen Elizabeth National Park in June 2009.³⁰ The Hungary isolate was obtained from a horse in 2004 (HU04; provided by Richard Bowen). The Greek 2010 isolate, NS10, was obtained from the blood of an asymptomatic blood donor who presented with myalgia, arthralgia, and severe orbital pain 2 days after the blood donation in 2010 in northern Greece.^{31,32} Compared with the SA89 L2 strain, the percent nucleotide identity for the HU04, UG09, and NS10 viruses was 97.8, 98.7, and 97.7%, respectively. Final virus stocks were prepared by inoculating C6/36 mosquito cells (*Aedes [Stg.] albopictus* Skuse) at a multiplicity of infection of 0.1. Supernatants of infected cells were harvested 4 days postinfection (dpi), centrifuged to remove cellular debris and frozen in aliquots at -80°C . Viral titers of stocks were determined by plaque assay on African green monkey kidney cells (Vero cells; ATCC no. CCL-81, Manassas, VA) as previously described.³³

Mosquito colonies. Colonized *Culex* mosquitoes have been extensively used as predictors of vector competence for L1 WNV.^{34–37} Similarly, we used well-characterized laboratory-adapted *Cx. pipiens* and *Cx. quinquefasciatus* mosquito colonies for the vector competence studies described herein. The *Cx. pipiens* colony originated in 2010 from egg rafts collected in Chicago, Illinois, that were subsequently determined to be positive for field-acquired *Culex* flavivirus (CxFV) infection. Although some reports have reported a positive association between CxFV and WNV, empirical laboratory competence assessments of CxFV in *Culex* mosquitoes have not indicated significant effects on WNV transmission.^{38–40} The *Cx. quinquefasciatus* colony was established in 1988 from Sebring County, Florida.⁴¹ The colonized mosquitoes were maintained on goose blood (*Cx. pipiens*) or calf blood (*Cx. quinquefasciatus*) for egg laying and provided 10% sucrose ad libitum. Larvae were reared and the adults were maintained at 70–80% humidity at 28°C with a 16:8 (light:dark [L:D] diurnal cycle) photoperiod.

Vector competence. Three- to five-day-old adult female *Cx. pipiens* mosquitoes or 4- to 7-day-old *Cx. quinquefasciatus* mosquitoes were deprived of sucrose sources for 24 hours and removed from water 6–8 hours before being provided an infectious blood meal. The frozen viral stocks were thawed to room temperature and added to defibrinated calf blood (Colorado Serum Company, Denver, CO) with 0.1 mM adenosine triphosphate to a final WNV titer of $7\text{--}8 \log_{10}$

plaque-forming units (PFUs)/mL blood meal. The mosquitoes were offered infectious blood meals for 1 hour by the use of a Hemotek membrane feeding apparatus with a cellulose membrane (Discovery Workshops, Accrington, United Kingdom) warmed to 37°C . An aliquot of the infectious blood meal was reserved and stored at -80°C for subsequent back-titration by plaque assay.³³ Fully engorged females were sorted under cold anesthesia, placed in 1-pint cartons, provided with 10% sucrose ad libitum, and held at 27°C at a 16:8 hour L:D photoperiod with 70–80% humidity for up to 14 days post-exposure (dpe). Mosquitoes from each group were assessed at extrinsic incubation periods (EIPs) of 5, 7, 9, and 14 dpe to evaluate potential differences in the efficiency of dissemination and transmission. At the aforementioned EIP time points, the mosquitoes were anesthetized by exposure to triethylamine (Flynap; Carolina Biological Supply Company, Burlington, NC) and their legs were removed and placed into tubes containing 500 μL mosquito diluent (Dulbecco's minimal essential media containing 10% fetal bovine sera (FBS), 5% penicillin/streptomycin [10 $\mu\text{g}/\text{mL}$], and 1% Fungizone [2.5 $\mu\text{g}/\text{mL}$]). Saliva samples were collected by placing each mosquito proboscis into a capillary tube charged with type B immersion oil (Cargille Laboratories, Inc., Cedar Grove, NJ). After a 30-minute period of expectoration, the contents of the capillary tubes were collected and expelled into 200 μL mosquito diluent by centrifugation at $5,000 \times g$ for 10 minutes and stored at -80°C until assessed for the presence of virus by plaque assay. After salivation, mosquito bodies were added to tubes containing 1 mL mosquito diluent (1 $\times$ phosphate buffered saline supplemented with 20% FBS [heat-inactivated], 50 $\mu\text{g}/\text{mL}$ penicillin/streptomycin, 50 $\mu\text{g}/\text{mL}$ gentamycin, and 2.5 $\mu\text{g}/\text{mL}$ Fungizone) and stored at -80°C . The mosquito bodies and legs were homogenized for 4 minutes at a frequency of 26 cycles per second using a Mixer Mill 300 (Retsch, Newton, PA) with the addition of two copper-coated steel shot ball barrings (Qiagen, Valencia, CA). Each body or leg sample was triturated for 2 minutes at a frequency of 26 cycles per second as described previously and clarified by centrifugation at $500 \times g$ for 10 minutes. The mosquito bodies, legs, and saliva samples were assayed for the infectious virus by plaque assay as previously described.⁴² Percentages of infection (percentage of virus-positive bodies from exposed mosquitoes), dissemination (percentages of virus-positive legs from exposed mosquitoes), and transmission (percentage of positive saliva samples from exposed mosquitoes) between virus groups were calculated.

Statistical analysis. For *Cx. pipiens* mosquitoes, three generalized linear models were fit to assess differences in percentages of WNV-positive bodies (infection), legs (dissemination), or saliva samples (transmission) between SA89, UG09, HU04, and NS10 at the four EIPs (5, 7, 9, and 14 dpe). Because of the small variation among observed responses in *Cx. quinquefasciatus* mosquitoes, two generalized linear models were fit to these data (comparing dissemination and transmission). All five models assessed time and virus strain as main effects either singly or interactively. The models were used to estimate odds ratios (ORs) for positivity and 95% confidence intervals (CIs) that were adjusted for multiple comparisons. Because there were few WNV positives for *Cx. quinquefasciatus* exposed to $7 \log_{10}$ (PFU/mL), differences in percentages between viruses and corresponding 95% simultaneous CI (adjusted for multiple comparisons) were computed for each infection percentage at each time point.

TABLE 1
Virus strains used in this study

Virus designation	Strain	Passage	Place, year of isolation	Source species
SA89	SPU116	sm(2), V(1), C6(1)	South Africa, 1989	Human
UG09	UG2274	V(1), C6(1)	Uganda, 2009	<i>Cx. neavei</i>
HU04	HU04	V(1), C6(1)	Hungary, 2004	Horse
NS10	Nea Santa	V(1), C6(1)	Greece, 2010	Human

C6 = C6/36 cells; sm = suckling mouse; V = Vero cell.

RESULTS

Vector competence of *Cx. pipiens*. The vector competence of *Cx. pipiens* (Chicago) mosquitoes for two African L2 WNV strains (SA89 and UG09) and two European L2 WNV strains (HU04 and NS10) was assessed at 5, 7, 9, and 14 dpe after an infectious blood meal ($8 \log_{10}$ PFU/mL). *Culex pipiens* mosquitoes became infected with each of the four L2 WNV strains at all time points. However, a significantly lower percentage of *Cx. pipiens* mosquitoes were infected with the NS10 strain compared with mosquitoes that were exposed to SA89, HU04, and UG09 (Figure 1A). The SA89 strain infected 70–90% of *Cx. pipiens*, the UG09 strain exhibited infection percentages ranging from 70–87%, whereas the HU04 strain demonstrated 65–92% infection through 5, 7, 9, and 14 dpe with higher dissemination observed at later time points. Comparatively, *Cx. pipiens* exposed to the NS10 strain were observed to have infection percentages of only 24–42%. The SA89, UG09, and HU04 strains had a 6- to 23-fold higher rate of infection (Table 2) in *Cx. pipiens* mosquitoes after oral exposure than the NS10 strain. Specifically, the SA89 strain had 6.3 (5 dpe)-, 9.3 (7 dpe)-, or a 7.4 (9 dpe)-fold higher infection proportion compared with the NS10 strain at all time points. The UG09 strain had a 9–13 times higher odds of infection than the NS10 strain at all time points. The second European strain used in the study, HU04, had a 23.8 (5 dpe)-, 22.7 (9 dpe)-, or 9.5 (14 dpe)-fold higher likelihood of infection than NS10 in *Cx. pipiens* mosquitoes. In contrast to the different infection percentages observed, no significant differences in dissemination between the L2 WNV strains were detected. Dissemination levels ranging from 6 to 46% were observed as early as day 5 for all L2 WNV strains and the percentage of mosquitoes with disseminated infections were observed to increase proportionally for all of the strains over time (Figure 1B). *Culex pipiens* mosquitoes were also able to transmit all viruses as early as 5 dpe and subsequent time points demonstrated a similar increased transmission rate for all strains through 14 dpe (Figure 1C), with transmission ranging from 2–25% for earlier time points (5 and 7 dpe) and 8–42% at later time points (9 and 14 dpe). No significant differences in transmission percentages between the L2 WNV strains were observed.

Vector competence of *Cx. quinquefasciatus*. To evaluate the vector competence of an alternate *Culex* mosquito species important for L1 WNV transmission in North America, the capacity of colonized *Cx. quinquefasciatus* from Florida (Sebring) to be infected with, disseminate, and transmit the same L2 WNV strains was assessed. Unlike the *Cx. pipiens* in which 23–92% of mosquitoes became infected, the infection percentage for all L2 WNV strains approximated 100% for *Cx. quinquefasciatus* orally exposed to the four L2 WNV isolates (Figure 2A). The SA89 strain was the only L2 WNV that demonstrated evidence of dissemination (25%) at 5 dpe (Figure 2B). Although dissemination was evident for all L2 WNV strains by 7 dpe, the SA89 strain continued to exhibit a significantly higher dissemination rate than the NS10 and the UG09 L2 WNV strains. Specifically, the SA89 strain had a 12.3 (7 dpe), 9.4 (9 dpe), and 17.0 (14 dpe) times higher odds of disseminating to the legs of *Cx. quinquefasciatus* mosquitoes than those of UG09. At 7 dpe, the SA89 strain had a 5.2 times higher odds of dissemination than those of NS10 (Table 2). Transmission at 5 dpe was also only observed for the SA89 strain, with 20% of mosquitoes capable of transmitting virus

FIGURE 1. Vector competence of *Cx. pipiens* mosquitoes for L2 West Nile virus (WNV). Mosquitoes were per orally exposed to $8 \log_{10}$ plaque-forming unit/mL of SA89, UG09, HU04, or NS10. (A) Infection was determined as the number of WNV-positive bodies (n_i) as a function of exposed mosquitoes (n_e). (B) Dissemination was calculated as the number of WNV-positive legs (n_d) as a function of exposed mosquitoes (n_e). (C) Transmission was calculated as the percentage of WNV-exposed mosquitoes (n_e) that were also positive for virus in saliva (n_t). Bars labeled a and b are significantly different.

(Figure 2C). Transmission of UG09, HU04, and NS10 strains was initially observed at 7 dpe and continued to be observed through 9 dpe and 14 dpe. Transmission at 7, 9, and 14 dpe for the four L2 WNV strains ranged between 5% and 95%. The odds of transmission were 10 and 17 times greater for SA89 compared with UG09 at 7 and 14 dpe, respectively.

TABLE 2
Significant odds ratios (ORs) and 95% confidence intervals (CIs) for *Cx. pipiens* and *Cx. quinquefasciatus* mosquitoes

Species	Group comparison	EIP, site	OR	95% CI
<i>Cx. pipiens</i>	HU04–NS10	5 dpe, infection	23.84	(3.04, 187.12)
	SA89–NS10	5 dpe, infection	6.33	(1.53, 26.23)
	UG09–NS10	5 dpe, infection	9.11	(1.86, 44.62)
	SA89–NS10	7 dpe, infection	9.32	(1.63, 53.31)
	UG09–NS10	7 dpe, infection	9.88	(1.74, 56.28)
	HU04–NS10	9 dpe, infection	22.75	(3.41, 151.92)
	SA89–NS10	9 dpe, infection	7.48	(1.39, 40.11)
	UG09–NS10	9 dpe, infection	13.0	(2.34, 72.22)
	HU04–NS10	14 dpe, infection	9.58	(1.72, 53.21)
	UG09–NS10	14 dpe, infection	5.47	(1.21, 24.73)
<i>Cx. quinquefasciatus</i>	SA89–UG09	7 dpe, dissemination	12.33	(1.74, 87.28)
	SA89–UG09	7 dpe, transmission	10.42	(1.04, 104.05)
	SA89–NS10	7 dpe, dissemination	5.29	(1.21, 23.18)
	SA89–UG09	9 dpe, dissemination	9.43	(2.12, 41.92)
	SA89–UG09	14 dpe, transmission	17.0	(1.68, 171.83)
	UG09–NS10*	5 dpe, infection	0.27	(0.031, 0.521)
	HU04–SA89*	9 dpe, infection	0.33	(0.040, 0.625)

EIP = extrinsic incubation period.

* For *Cx. quinquefasciatus* per orally exposed at 7 log₁₀ (plaque-forming unit/mL), with 99% CI.

In this study, *Cx. pipiens* but not *Cx. quinquefasciatus* mosquitoes demonstrated a marked decrease in infection for the NS10 strain at multiple time points. However, 100% or near 100% infection percentages were observed for all L2 WNV strains in *Cx. quinquefasciatus* mosquitoes, demonstrating that the *Cx. quinquefasciatus* mosquitoes used in this study were highly susceptible to oral infection at the 8 log₁₀ PFU/mL blood meal dose. To assess if differences in infection could be observed for *Cx. quinquefasciatus* mosquitoes exposed to a lower viral dose in an infectious blood meal, *Cx. quinquefasciatus* mosquitoes were orally exposed to 7 log₁₀ PFU/mL L2 WNV strains and infections similarly assessed (Figure 3). Although a decrease in the percentage of infected mosquitoes was observed for all of the L2 WNV strains compared with *Cx. quinquefasciatus* mosquitoes that received a 10-fold higher infectious blood meal, only small, but significant, differences in the infection percentages between the L2 WNV strains were detected (Table 2): UG09 compared with NS10 (5 dpe, 0.27 OR) and HU04 compared with SA89 (9 dpe, 0.33 OR).

DISCUSSION

The spread of a previously underrepresented WNV lineage, L2, from sub-Saharan Africa into Europe and the capacity to cause recurrent WNV outbreaks raise concerns as to whether the geographic range of L2 WNV might also expand to include North America, similar to what has occurred for L1 WNV. In this study, we demonstrated the capacity for North American *Culex* mosquito species to transmit four geographically and genetically distinct L2 WNV strains. We found that both *Cx. pipiens* and *Cx. quinquefasciatus* mosquitoes were susceptible to infection and were able to efficiently disseminate and transmit both African and European L2 WNV strains. The European L2 WNV strain, NS10, was observed to have a reduced infection capacity in *Cx. pipiens*, but not *Cx. quinquefasciatus* mosquitoes. In addition, the vector competence of *Cx. quinquefasciatus* mosquitoes was greatest for the African L2 WNV strain, SA89, because of rapid dissemination from the midgut. A previous study with an L1 WNV demonstrated similar competence to that observed here with L2 WNVs by mosquitoes from the same *Cx. quinquefasciatus* colony.³⁷ The

results herein indicate that L2 WNV strains can be efficiently transmitted by key North American WNV vectors and underscore the need for surveillance for potential L2 WNV emergence in the United States.

In this study, we found that the African L2 strain, SA89, disseminated more rapidly and efficiently in *Cx. quinquefasciatus* mosquitoes compared with other L2 strains. The SA89 strain was isolated during an outbreak in South Africa in 1989.⁴³ *Culex quinquefasciatus* mosquitoes are abundant in South Africa and have been shown to contribute to the WNV transmission cycle.^{27,28,43} The ability of the SA89 strain to be more efficiently transmitted could be reflective of the relative adaption of the SA89 strain to be more efficiently transmitted by a mosquito species, *Cx. quinquefasciatus*, found in South Africa, with a more widespread geographic distribution. The colonized *Cx. quinquefasciatus* mosquitoes assayed herein were collected in southern United States (Florida), where *Cx. quinquefasciatus* serve as the main transmission vector for WNV. The adaptation of the SA89 strain to be more efficiently transmitted could also be mirrored across *Cx. quinquefasciatus* mosquitoes regardless of the geographic origin of the mosquito colony, reflecting the consequence of a potential coevolutionary history between that L2 strain and *Cx. quinquefasciatus* mosquitoes. The inability of all the HU04, UG09, and NS10 L2 strains to disseminate or to transmit at 5 dpi suggests that differences in escape rates for dissemination or transmission exist despite nearly absolute infection for all strains at that time point. The same dissemination or transmission escape rate difference was not observed in *Cx. pipiens* mosquitoes, where dissemination and transmission were observed at the same time point for the same strains (6–20% or 2–20%, respectively). The variability of these strains in eliciting differing dissemination and transmission phenotypes at early time points should be further examined. The studies performed herein were conducted using colonized *Culex* species. Additional assessments using populations of field-caught or low generation–colonized *Cx. pipiens* and *Cx. quinquefasciatus* mosquitoes should be performed to confirm these results.

The NS10 strain demonstrated lower infection rates in *Cx. pipiens* mosquitoes compared with the other three L2

FIGURE 2. Vector competence of *Cx. quinquefasciatus* mosquitoes for L2 West Nile virus (WNV). Mosquitoes were per orally exposed to $8 \log_{10}$ plaque-forming unit/mL of SA89, UG09, HU04, or NS10. (A) Infection was determined as the number of WNV-positive bodies (n_i) as a function of exposed mosquitoes (n_e). (B) Dissemination was calculated as the number of WNV-positive legs (n_d) as a function of exposed mosquitoes (n_e). (C) Transmission was calculated as the percentage of WNV-exposed mosquitoes (n_e) that were also positive for virus in saliva (n_i). Bars labeled a and b are significantly different.

WNV strains assessed. Previously published assessments with the same NS10 strain were performed with colonized *Cx. pipiens* mosquitoes originating from either the Netherlands or North America (Pennsylvania).^{26,36} Lower infection percentages after per oral exposure were demonstrated in the North American colony compared with the colony originating from

FIGURE 3. Infection rates of *Cx. quinquefasciatus* mosquitoes exposed to a 10-fold lower infectious dose ($7 \log_{10}$ WNV [PFU/mL]). Mosquitoes were per orally exposed to $7 \log_{10}$ PFU/mL of SA89, UG09, HU04, or NS10. Infection is the number of WNV-positive bodies (n_i) over WNV-exposed mosquitoes (n_e). Bars labeled a and b are significantly different. Data are not available (NA) for mosquitoes exposed to UG09 at 14 dpi. PFU = plaque-forming unit; WNV = West Nile virus.

Europe.²⁶ Interestingly, the same North American (Pennsylvania) *Cx. pipiens* colony demonstrated high infection percentages for several strains of L1 WNV and no differences in infection percentages between the L1 WNV strains assessed were observed.³⁶ In our study, the L2 NS10 strain similarly demonstrated low infection with North American *Cx. pipiens* mosquitoes compared with the other three L2 WNV strains. The concordance of this finding with that previously observed for the North American *Cx. pipiens* with the NS10 strain could indicate a reduced infectivity of North American *Cx. pipiens* with this recently emergent L2 strain.²⁶ Curiously, the same midgut infection barrier observed for *Cx. pipiens* mosquitoes orally exposed to NS10 was not observed for *Cx. quinquefasciatus* mosquitoes infected with the same strain, indicating that the midgut infection barrier observed for NS10 is specific to *Cx. pipiens* mosquitoes. Further investigation with low generation *Cx. pipiens* mosquitoes or field-caught *Cx. pipiens* populations is warranted to follow up on these observations.

The NS10 was isolated during the first L2 WNV outbreak in Europe in 2010.³² Apart from a few individual cases of human L2 WNV infections in Russia (2004 and 2007) and Israel (2009), all previous outbreaks of L2 were limited to the sub-Saharan region of Africa.^{6,7,12} Notably, previous sequence analysis of the NS10 strain identified a proline substitution at the NS3-249 locus.³² L2 WNV strains previously isolated in Africa and Europe all have been found to have a histidine at the NS3-249 locus.⁴⁴ Sequence analyses of L1 WNV strains have previously indicated that the NS3-249 locus has been subjected to strong positive selection effects.⁴⁵ Phylogenetic analysis of L1, L2, L3, and L4 WNV strains has demonstrated considerable amino acid heterogeneity at the NS3-249 locus present between and within lineages.⁴⁵ For example, threonine, proline, and alanine residues are found at NS3-249 locus in L1 WNV strains. However, a threonine to proline substitution at the NS3-249 site has evolved more frequently in recently emergent L1 WNV isolates, arising on at least three independent occasions.⁴⁵ Notably, each substitution event

directly preceded an epidemic (Egypt, 1950; Romania and Russia, 1996 and 1999, respectively; Israel, 1997–1998).⁴⁴ It is intriguing that the NS3-249Pro mutation observed at this locus in an L2 WNV strain was also associated with the emergence of L2 WNV in Greece between 2010–2014.¹⁴ In the context of L1 WNV genetic backbones, the NS3-249 has been demonstrated to modulate pathogenesis in corvids and modulate WNV growth at elevated temperatures. Previous studies have established that a threonine to proline substitution at the NS3-249 locus results in significantly elevated viremia titers and mortality in American crows (*Corvus brachyrhynchos*).⁴⁵ The results presented herein demonstrated the NS10 strain to be less infectious for *Cx. pipiens* mosquitoes relative to the other L2 WNV strains assessed. Among the four L2 WNV strains examined, the NS10 strain is the only L2 WNV strain to have a proline at the NS3-249 locus, whereas the SA89, UG09, and HU04 strains have an NS3-249His at this locus. Previous work by Lim et al.⁴⁶ demonstrated that the NS10 strain containing the NS3-249Pro substitution elicited elevated viremia and mortality in European carrion crows (*Corvus corone*). The relative importance of the NS3-249Pro substitution in the NS10 strain in affecting either changes in pathogenic outcomes in avian species or in altering vector competence phenotypes of *Cx. pipiens* mosquitoes needs to be further addressed.

Current molecular methods of surveillance for L1 WNV may not capture L2 WNV emergence events or low-intensity transmission levels for L2 WNV transmission. For example, a five-nucleotide mismatch (20%) is present between the binding sequence of the probe used in the standard reverse transcription polymerase chain reaction assay designed to detect L1 WNV strains and all four L2 WNV strains tested herein.⁴⁷ WNV surveillance methods based on nucleic acid amplification and probe binding for L1 WNV, including those typically used in North America, would be unlikely to detect the evidence of L2 WNV transmission and highlight the need for a more comprehensive approach for WNV surveillance. The use of sentinel birds that could be assessed for seroconversion to both L1 and L2 WNV or more comprehensive molecular detection methods that could detect and/or distinguish multiple WNV lineages could offset these potential surveillance limitations.^{47–51} Together, these studies demonstrate that geographically and genetically distinct L2 WNV strains have the potential to be transmitted by North American *Culex* mosquitoes and emphasize the need to maintain surveillance and vector control efforts to monitor and/or control further geographic spread of L2 WNV strains.

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