VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 9, Number 3, 2009

DOI: 10.1089/vbz.2007.0281

Effect of Sequential Exposure on Infection and Dissemination Rates for West Nile and St. Louis Encephalitis Viruses in *Culex quinquefasciatus*

Kendra Pesko* and Christopher N. Mores†

Abstract

West Nile virus has spread rapidly throughout the United States since its introduction in 1999, into some areas that are also endemic for St. Louis encephalitis virus (SLEV). These viruses are in the same antigenic complex within the genus *Flavivirus*, family Flaviviridae. Further, both viruses are transmitted primarily by *Culex* spp. mosquitoes and use birds as amplifying hosts. These viruses could contemporaneously coinfect individual vectors wherein changes in mosquito immune responses might occur. To explore this possibility, we evaluated the effect of sequential infection with both West Nile virus and SLEV on the infection and dissemination rates of these viruses in the vector mosquito, *Culex quinquefasciatus*. Prior exposure to either virus lowered susceptibility to infection with the second virus, and lower dissemination rates of the second virus, compared to controls. Exposure to one virus followed by a second virus resulted in similar infection rates for the first virus to those of controls, but higher SLEV dissemination rates when exposed first to SLEV than in singly SLEV infected controls. While some mosquitoes became infected with both viruses, only one of those viruses disseminated from the midgut into the legs, indicating a midgut infection barrier to secondary infection. Lower infection rates in mosquitoes exposed to both viruses could change transmission patterns when these viruses are present at epizootic levels.

Key Words: Culex quinquefasciatus—Saint Louis encephalitis virus—West Nile virus—Coinfection.

Introduction

St. Louis encephalitis virus (SLEV) and West Nile virus (WNV) are neurotropic arboviruses (Flaviviridae: *Flavivirus*) in the Japanese encephalitis antigenic complex, which includes Japanese, Murray Valley, and Kunjin encephalitis viruses (Kimura-Kuroda and Yasui 1986). Both viruses cycle primarily between avian hosts and *Culex* spp. mosquitoes, with typically cryptic epizootics occurring before outbreaks in human populations (Gerhardt 2006). SLEV is principally transmitted by *Culex quinquefasciatus* in the central and southern United States (Savage et al. 1993). WNV was introduced into the United States in 1999 and has since spread rapidly throughout the nation, vectored by a variety of mosquito species, including those that transmit SLEV (Mackenzie et al. 2004).

Surveillance data have shown that WNV can become established in areas where SLEV is endemic (Lillibridge et al. 2004). In these regions, the shared hosts and vectors used by WNV and SLEV may result in transmission to the same individual vertebrate hosts or vectors, resulting in potential interactions between these viruses. Interactions may be indirect, such as exposure to the heterologous antibodies present in a previously infected host, or direct, as within a doubly infected host or vector. At a population level, high infection rates in hosts with one virus might also remove (by death or immunization) potential hosts for another virus (Derouich et al. 2003). The co-occurrence of these viruses in bird populations provides the opportunity for one mosquito to feed initially on a blood meal containing one virus, become infected with that virus, oviposit, and then seek out a second blood meal from a bird infected with a different virus. WNV is

Florida Medical Entomology Laboratory, University of Florida, Vero Beach, Florida.
Present address: Biomedical Sciences Graduate Student, SOM Office of Research, University of New Mexico, Albuquerque, New Mexico.
†Present address: Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana.

now present in many of the same geographic regions as SLEV (Mackenzie et al. 2004). Although these viruses have not been simultaneously isolated from a mosquito vector, and infection rates in wild-caught mosquitoes tend to be fairly low, the potential for interaction does exist and these interactions should be further characterized.

Natural coinfection has been detected in the past for other serotypes and species of arboviruses that have overlapping geographical ranges and share transmission cycles. Mourning Doves with antibodies against WNV and currently viremic for western equine encephalitis virus (WEEV, another *Culex* vectored arbovirus commonly infecting birds) have been detected in Kern County, CA (Reisen and Hahn 2007). Studies have shown that different serotypes of dengue virus (DENV), a flavivirus that circulates between *Aedes* spp. mosquitoes and human hosts, may simultaneously infect the same host, especially during epidemics (Lorono-Pino et al. 1999, Wang et al. 2003, Wenming et al. 2005). Concurrent infections with two distinct DENV serotypes have been detected recently from human patients in Sri Lanka (Wenming et al. 2005), Taiwan (Wang et al. 2003), and Brazil (Araujo et al. 2006). Chikungunya virus, an alphavirus also transmitted primarily by *Aedes* spp., has been isolated simultaneously with DENV-2 from a patient in India (Myers and Carey 1967), and SLEV has more recently been isolated along with DENV-3 from a human patient in Brazil (Mondini et al. 2007). More ecologically relevant to this study of doubly infected invertebrates has been the discovery of concurrent infections of DENV-2 and DENV-3 isolated from field-caught *Aedes albopictus* and *Aedes aegypti* in southern Thailand (Thavara et al. 2006), and an *Ae. aegypti* dually infected with chikungunya virus and DENV-1 was collected in India (Mourya et al. 2001).

Previous research describing interactions between different arboviruses has shown that inhibition of one virus by another is a possible outcome in both mosquito cell culture (Zebovitz and Brown 1968, Eaton 1979, Karpf et al. 1997) and mosquitoes (Chamberlain and Sudia 1957, Altman 1963, Lam and Marshall 1968, Rozeboom and Kassira 1969, Beaty et al. 1985, Sundin and Beaty 1988, Borucki et al. 1999). This inhibition of one virus by another is often related to the order of viral exposure. Once infection has occurred with one virus, the superinfecting or second virus is unable to infect. This inhibiting interaction has been referred to as "superinfection exclusion," which refers only to exclusion of the second virus, and "intrinsic interference," for an inhibition of either virus, or simultaneously introduced viruses (Eaton 1979, Karpf et al. 1997).

We hypothesized that sequential infections of both WNV and SLEV in the vector *Cx. quinquefasciatus* could affect the infection and dissemination rates of the superinfecting virus. Specifically, we quantified the effects of prior exposure to one virus on the subsequent infection and dissemination rates of a different virus.

Materials and Methods

Mosquitoes

Cx. quinquefasciatus collected in Gainesville, FL, in 1996 and kept in our laboratory since 2004 were used for these experiments (total generations in colony >50). Larvae were reared at 27.5°C, 14 light–10 dark cycle, and fed a 1:1 yeast:albumin mixture. Adults were provided with 10% sucrose (*ad libitum*).

Viruses

A 2003 isolate of WNV from a pool of *Culex nigripalpus* in Indian River County, FL, designated as strain WNFL03p2-3 was used. SLEV strain TBH28, isolated from a human in Florida in 1962, was used. Stock viruses were produced by inoculating virus into African green monkey kidney (Vero) cell culture, and harvesting cell culture supernatant. The SLEV strain was passed twice in Vero cells, and the WNV strain was passed five times in Vero cells and twice in baby hamster kidney cells. Both strains were stored at −80°C in 1 mL aliquots.

Infectious blood meal preparation

Freshly propagated virus was mixed with blood and fed to mosquitoes to enhance infection rates (Richards et al. 2007). Freshly propagated viruses were produced by inoculating T75 tissue culture flasks containing confluent Vero cells and 10 mL of M-199 medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and fungizone (Invitrogen™, Carlsbad, CA) with 250 μL of WNV or SLEV stock. Viral growth curves were determined before the experiment, and virus was harvested from cells at the peak production period. Respective WNV and SLEV flasks were maintained at 35°C and 5% CO_2 for 48 h (WNV) or 96 h (SLEV) postinoculation, at which point the cytopathic effects were observed and the supernatant was harvested for addition to blood meals. Infectious blood meals were prepared by mixing citrated bovine blood (Hemostat, Dixon, CA) 1:1 with fresh SLEV stock, or 1:1 with fresh WNV stock that was already diluted 1:10 in BA-1 medium, prepared as described (Lanciotti et al. 2000).

Oral infection of mosquitoes

Six different treatment groups of four cages each were fed on infectious blood meals (Table 1). Twenty-four hours before feeding, 3–6-day-old mosquitoes were placed into cylindrical cardboard cages (14 cm high and 11 cm diameter) (Dade Paper, Miami, FL), with the lid removed and replaced by bridal veil, in groups of approximately 50 female mosquitoes per cage. A 3 mL medicine cup was glued to the inside wall of each cage to provide an oviposition receptacle. Sugar was removed from cages 24 h before feeding. The cages were kept

Table 1. Infectious Blood Meal Exposure and Number Tested on Final Day

Group	*First exposure (blood meal titer, average freshly blood-fed mosquito body titer [n = 3] ± SE*[a]*)*	*Second exposure (blood meal titer, average freshly blood-fed mosquito body titer [n = 3] ± SE*[a]*)*
SLEV-WNV	SLEV (3.3, 1.5 ± 0.1)	WNV (5.4, 3.6 ± 0.3)
WNV-SLEV	WNV (5.6, 3.6 ± 0.1)	SLEV (3.0, 1.3 ± 0.2)
SLEV-X	SLEV (3.3, 1.5 ± 0.1)	None
X-SLEV	None	SLEV (3.0, 1.3 ± 0.2)
WNV-X	WNV (5.6, 3.6 ± 0.1)	None
X-WNV	None	WNV (5.4, 3.7 ± 0.3)

[a]Log_{10} plaque forming units per 0.1 mL.

in 28°C incubators (Percival, Perry, IA) with a 14 light–10 dark cycle, with dusk/dawn periods of 1 h.

Infectious blood meals were offered to 4–7-day-old mosquitoes using a silicon membrane feeder system (Butler et al. 1984). Approximately 1 mL of blood was placed into each membrane feeder. Two membrane feeders containing infectious blood were heated at 35°C for 20 min, then placed on top of each cage from which female *Cx. quinquefasciatus* were allowed to feed for 30 min. A 100 μL aliquot of each blood meal was diluted with 900 μL of BA-1 both pre- and postheating, and stored at −80°C until further processing to detect the blood meal titer.

After feeding, only fully engorged females were kept in a 28°C incubator with 10% sucrose provided (*ad lib.*). Several freshly blood-fed mosquitoes were collected from each group immediately after feeding and stored in 900 μL of BA-1 at −80°C until further processing. Forty-eight hours after the first blood meal, the oviposition cups were filled with tap water, and females were given an additional 48 h to oviposit. Approximately half of the mosquitoes in each cage laid an egg raft during this period (20–30 egg rafts/cage). Four days after the first blood feeding, treatment mosquitoes were offered a second blood meal containing the other virus (i.e., those fed WNV first were offered SLEV blood meals at this second blood feeding, and vice versa). Control groups of mosquitoes, reared simultaneously and treated in every way the same, except only exposed to a single blood meal, were fed on the first infectious blood meal or the second infectious blood meal only for each virus, and kept under the same conditions to quantify infection and dissemination rates for singly introduced virus (Table 1).

At 10 days after feeding on the second infectious blood meal (14 days after imbibing the first infectious blood meal), all surviving mosquitoes were killed in a −20°C freezer, and legs and bodies were triturated in 900 μL of BA-1 and stored at −80°C.

RNA extraction

Samples were homogenized at 25 Hz for 3 min using a Tissuelyzer® tissue homogenizer (Qiagen, Valencia, CA) and then clarified by centrifugation (3148 *g* for 4 min). Viral RNA was extracted using MagNA Pure LC Instrument (Roche Diagnostics, Chicago, IL), and the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics) following the manufacturer's instructions.

Quantitative RT-PCR

One-step quantitative RT-PCR (qRT-PCR) with dual detection TaqMan® probes was used to detect infection and dissemination of infected mosquitoes. In a final volume of 20 μL, 5 μL of extracted nucleic acid was added to 4 pmol of each TaqMan probe, 10 pmol of each primer, 40 ng of bovine serum albumin (New England Biolabs™, Ipswich, MA), and 0.4 μL of SuperScript TM III Platinum one-step (Invitrogen). Reactions were carried out in a LightCycler 480 (Roche Diagnostics) under the following conditions: 48°C for 20 min (reverse transcription), 95°C for 2 min (initial denaturation), and 40 cycles of 60°C for 15 s and 95°C for 10 s (amplification), followed by cool down at 50°C for 30 s. Previously published primers amplified a noncoding region of the SLEV genome and part of the WNV envelope protein: SLEV forward [nt 834-852]: 5′-GAA AAC TGG GTT CTG CGC A-3′; SLEV reverse [nt 905c-889c]: 5′-GTT GCT GCC TAG CAT CCA TCC-3′; SLEV probe [nt 857-880]: 5′-/Cy5/TGG ATA TGC CCT AGT TGC GCT GGC/3BHQ_2/-3′ (Lanciotti and Kerst 2001); WNENV-forward [nt 1160-1180]: 5′-TCA GCG ATC TCT CCA CCA AAG-3′; WNENV-reverse [nt 1209-1229]: 5′-GGG TCA GCA CGT TTG TCA TTG-3′; WNENV-probe [nt 1186-1207]: 5′-/56-FAM/TGC CCG ACC ATG GGA GAA GCT C/3BHQ_2/-3′ (Lanciotti et al. 2000) (IDT DNA, Coralville, IA).

The primers and probes used were specific to SLEV or WNV, and no cross hybridization was detected in preliminary trials (data not shown). The dual probe system could not detect SLEV with high titers of WNV present; therefore, for samples of treatment mosquitoes that ingested both WNV and SLEV, any WNV-positive mosquitoes were assayed separately for SLEV, using only the SLEV probes and primers. The results from qRT-PCR were standardized with a plaque assay performed on serial dilutions (Gargan et al. 1983), to estimate virus present in a given sample, measured in plaque forming units per 0.1 mL (Bustin 2000).

Statistical analysis

Standard curve and estimation of freshly blood-fed body titers by qRT-PCR were calculated with LC-480 software (Roche, Chicago, IL). Virus found in the body but not in the legs was considered a nondisseminated infection limited to the midgut, and virus found in both the body and legs indicated a disseminated infection. Infection rates were calculated by dividing the number of infected bodies by the total number of mosquitoes tested. Dissemination rates were calculated as the number of mosquitoes with positive legs divided by the total number of positive mosquito bodies. Wilcoxon ranked test was used to look for differences in freshly blood-fed mosquito body titers. Pairwise comparisons with the chi-squared statistic were used to detect differences in proportions of infection and dissemination between groups ($p > 0.05$) (SAS/STAT 2002).

Results

Blood meal titer and freshly blood-fed mosquito titer analysis

Blood meal titers and freshly blood-fed average body titers are listed in Table 1. Average body titers of freshly blood-fed mosquitoes did not differ for secondarily infected mosquitoes compared with singly infected controls (SLEV: $z = 0.87$, $p = 0.423$; WNV: $z = 1.75$, $p = 0.089$; Table 1). The freshly blood-fed mosquitoes showed average body titers about two logs below the blood meal titers to which they were exposed, indicating that they imbibed around 2–3 μL of blood, similar to previous estimates of blood meal volume (Calheiros et al. 1998).

Effects of prior WNV exposure on SLEV superinfection

At 36%, SLEV infection rates were significantly lower in mosquitoes exposed to WNV before SLEV exposure, relative to the 93% infection rates in mosquitoes only exposed to SLEV ($\chi^2 = 25.22$, df = 1, $p < 0.001$; Tables 1 and 2). No mosquitoes in the superinfection treatment group showed disseminated SLEV infections, which was significantly lower than the 21% dissemination rates of the control ($\chi^2 = 0.81$, df = 1, $p < 0.001$) (Table 2).

Table 2. WNV and SLEV Infection and Dissemination Rates

Group	*WNV infection rate (%)*	*WNV dissemination rate (%)*	*SLEV infection rate (%)*	*SLEV dissemination rate (%)*	*Dual infection rate (%)*	*Dual dissemination rate (%)*	*Number tested*
SLEV[a]-WNV[b]	56	4[c]	88	80	52	0	$n = 50$
WNV[a]-SLEV[b]	100	100	36	0	36	0	$n = 50$
SLEV[a]-X	0	0	100	57	NA	NA	$n = 30$
X-SLEV[b]	0	0	93	21	NA	NA	$n = 30$
WNV[a]-X	100	93	0	0	NA	NA	$n = 30$
X-WNV[b]	100	26	0	0	NA	NA	$n = 19$

[a]Data shown follow 14 days extrinsic incubation at 28°C.
[b]Data shown follow 10 days extrinsic incubation at 28°C.
[c]Mosquito was negative for first exposure virus (SLEV).

Effects of prior SLEV exposure on WNV superinfection

The proportion of mosquitoes infected and disseminated with WNV after prior exposure to SLEV was lower than the second WNV control group (Table 2). The 56% WNV infection rate after prior exposure to SLEV was significantly lower for mosquitoes compared with the 100% infection rate in controls ($\chi^2 = 12.27$, df = 1, $p < 0.001$; Table 2). Significantly fewer mosquitoes had disseminated WNV infections in the WNV superinfection group, 4% compared with 26% in the control ($\chi^2 = 5.26$, df = 1, $p = 0.022$; Table 2).

Effects of WNV superinfection on SLEV infection

Although the SLEV infection rate was significantly lower for mosquitoes exposed first to SLEV and then superinfected with WNV, this group had a higher SLEV dissemination rate than controls (Table 2). SLEV infection rates of 88% for mosquitoes exposed to SLEV before WNV were significantly lower than the 100% infection rate of controls ($\chi^2 = 3.89$, df = 1, $p = 0.048$; Table 2). SLEV dissemination rates were significantly higher at 80% in the treatment group that received SLEV followed by WNV, compared to the 57% first SLEV control ($\chi^2 = 4.47$, df = 1, $p = 0.035$; Table 2).

Effects of SLEV superinfection on WNV infection

WNV infection rates for mosquitoes exposed to WNV before SLEV were not significantly different from control infection rates (Table 2). WNV dissemination rates showed a trend toward statistical significance for mosquitoes exposed to WNV before SLEV compared with controls ($\chi^2 = 3.42$, df = 1, $p = 0.064$; Table 2).

Dual infection

Dually infected mosquitoes were found under all treatment conditions. Thirty-six percent (18/50) of mosquitoes initially exposed to WNV that developed midgut infections, and were subsequently exposed to SLEV were infected with both viruses (Table 2). Similarly, 52% (23/44) of mosquitoes initially exposed to SLEV that developed midgut infections, and were subsequently exposed to WNV exhibited dual infection. Despite the 100-fold excess difference in WNV titer used over SLEV, there was no significant difference in dual infection rate depending upon whether WNV was the primary or secondary infecting virus ($\chi^2 = 2.52$, df = 1, $p = 0.1124$). No mosquitoes exhibited dual dissemination. The only superinfected mosquito to have a disseminated infection with the second virus did not have a disseminated infection with the first virus.

Discussion

These results indicate that prior exposure to SLEV or WNV inhibits the replication of either subsequent virus in *Cx. quinquefasciatus*. When mosquitoes were superinfected with either WNV or SLEV, infection rates and dissemination rates were lower than mosquitoes exposed to only one virus. Initial exposure to one virus appeared to inhibit growth of the subsequent virus. SLEV infection rates were slightly lower in mosquitoes exposed to SLEV followed by WNV, although dissemination rates were slightly higher. Our strain of SLEV replicated more slowly and reaches lower titers than the WNV strain in cell culture and mosquitoes, a difference noted by other researchers for these two viruses (Payne et al. 2006). This difference in growth dynamics could lead SLEV to be more susceptible to interference. Also, the 100-fold lower titers of SLEV blood meals could have affected the outcome for SLEV infection rates in both prior- and subsequent-exposure groups, although SLEV reaches lower peak viremias than WNV in infected hosts such as the brown-headed cowbird and red-winged blackbird (Reisen and Hahn 2007); thus, the viral doses used in this experiment are representative of possible field encounters. Control mosquitoes exposed to a single virus during the second blood meal versus the first blood meal exhibited lower dissemination rates, possibly due to the shorter extrinsic incubation period caused by the experimental setup (10 days vs. 14 days, respectively; see Table 1).

Several studies have shown that the timing of secondary oral infection of *Ae. triseriatus* with various bunyaviruses affects whether coinfection or interference will occur (Beaty et al. 1985, Sundin and Beaty 1988). Mosquitoes exposed to the second virus within 24 h of the first virus exposure become readily infected with both viruses. However, if >7 days elapsed between virus exposures, mosquitoes became refractory to secondary infection. Further, studies have found that mosquitoes transovarially infected with La Crosse virus (a bunyavirus) were much less susceptible to superinfection with another strain of La Crosse virus, or a related bunyavirus, snowshoe hare virus, than uninfected controls (Borucki et al. 1999), although complete interference did not occur. Although the current study tested the same amount of time between viral introductions, perhaps by varying the elapsed time between virus exposures, the effects on the interference

of secondary infection would have changed. With an elapsed time of 4 days between viral introductions, mosquitoes were not completely refractory to infection with the second virus, but there was evidence for inhibition.

The current study found that dissemination of SLEV was enhanced in *Cx. quinquefasciatus* due to exposure to a secondary virus. In a previous study, *Culex tarsalis* mosquitoes exposed to blood meals containing eastern equine encephalitis virus and, 1 week later, WEEV (Togaviridae: Alphavirus) became infected with both viruses, but transmission of eastern equine encephalitis virus was diminished in the dually infected mosquitoes, while transmission of WEEV by dually infected mosquitoes was enhanced (Chamberlain and Sudia 1957). Interestingly, *Cx. tarsalis* was able to simultaneously transmit both of these closely related viruses, a finding that differed from the complete interference of viral dissemination of either secondarily infecting virus shown in the current study.

Within the mosquito, there are certain physiological barriers to infection, dissemination, and transmission (Hardy et al. 1983, Mellor 2000). These barriers include the midgut infection barrier (a barrier against infection of the mesenteronal epithelial cells), the midgut escape barrier (a barrier against passage through the basal lamina of the mesenteron to enter the hemocoel, where virions can undergo secondary amplification in hemocoel-associated cells and tissues), and salivary gland infection barrier (a barrier against entrance into the salivary gland or exit into the salivary gland lumen) (Hardy et al. 1983). Our use of 100-fold greater titer WNV than SLEV in this study did not appear to have affected the observed rates of dual infection in the midguts of sequentially exposed mosquitoes. Therefore, the difference in virus titer that these mosquitoes were exposed to is unlikely to confound our interpretations of the biological relevance of sequential infection, particularly because our finding of dually infected, but not dually disseminated, mosquitoes suggests a midgut escape barrier to dissemination of the superinfecting virus, rather than a midgut infection barrier. In addition, the body titers of mosquitoes sequentially exposed to virus were not different from those exposed to a single virus, again suggesting that those exposure titer differences did not, ultimately, impact virus replication. Our finding supports previous research that indicates that a superinfecting virus in a flavivirus–flavivirus system will not be transmitted (Altman 1963). It may be that prior infection and dissemination with one virus blocks the dissemination of another, perhaps by blocking movement out of the mosquito midgut by inducing a host response against related viruses.

The effect of sequential blood meals upon arbovirus infection dynamics in mosquitoes has primarily been examined within Orthobunyavirus literature as an experimental tool to study reassortment. In some of those experiments that dealt with vertical and venereal transmission, an enhancement of those transmission modes was observed after a noninfectious blood feeding (as compared to rates among nonblood-fed mosquitoes) (Chandler et al. 1990). Our experimental design did not control for the possibility that prior blood feeding could alter virus infection/dissemination rates. However, previous research suggests that infection rates do not differ significantly between prefed and unfed populations (Beaty and Thompson 1978). Ultimately, the influence of prior blood feeding on infection rates is a potentially important question and should be addressed in future research.

Mosquito immune responses to fungal, bacterial, and protozoan infections such as melanization and innate immune responses are well known (Lowenberger 2001, Christensen et al. 2005). Less attention has been given to possible immune responses to viral infection, as mosquitoes do not have antibody-dependent immunity, but a recent study indicates that an RNA interference (RNAi)–mediated response may counter viral infection (Keene et al. 2004). Researchers used RNAi to block the RNAi pathway in *Anopheles gambiae* mosquitoes, and in this way enhanced replication of an alphavirus, O'nyong nyong virus (Keene et al. 2004). The presence of one arbovirus may activate the RNAi pathway, and thus diminish the dissemination of a superinfecting, related arbovirus out of the midgut. This phenomenon could lead to infection and dissemination rates similar to those seen in the current study, as well as previously documented interference of superinfection between related arboviruses (Altman 1963, Beaty et al. 1983).

The current study expands knowledge of an interference phenomenon that can occur between related arboviruses, by showing infection dynamics for SLEV and WNV in *Cx. quinquefasciatus*, an interaction not previously reported. Although previous studies have shown interference to occur between related arboviruses in mosquito vectors, many of these studies used less-sensitive techniques for determining infection, dissemination, and titer of the coinfecting viruses. Effects of arboviral coinfection need to be better understood to characterize the mechanisms behind interference. Future research should describe the influences of time of exposure and initial titer exposure on superinfection, dissemination, and transmission.

Acknowledgments

We, respectively, thank Aissa Doumboya and Lillian Stark for providing strains of WNV and SLEV. We thank Drs. Stephanie Richards, Michael Reiskind, Chelsea Smartt, and L. Philip Lounibos as well as Catherine Westbrook and two anonymous reviewers for assistance in reviewing this manuscript, and Jessie Dyer for assistance in developing the dual probe assay. This research was supported by an NIH Grant (AI-042164). Support for K.P. was from the University of Florida Alumni Fellowship.

Disclosure Statement

No competing financial interests exist.

References

Altman, RM. The behavior of Murray Valley encephalitis virus in *Culex tritaeniorhynchus* Giles and *Culex pipiens quinquefasciatus* Say. Am J Trop Med Hyg 1963; 12:425–434.

Araujo, FMDC, Nogueira, RMR, De Araujo, JMG, Ramalho, ILC, et al. Concurrent infection with dengue virus type-2 and DENV-3 in a patient from Ceara, Brazil. Mem Inst Oswaldo Cruz 2006; 101:925–928.

Beaty, BJ, Thompson, WH. Tropisms of La Crosse virus in *Aedes triseriatus* (Diptera: Culicidae) following infective blood meals. J Med Entomol 1978; 14:499–503.

Beaty, BJ, Bishop, DH, Gay, M, Fuller, F. Interference between bunyaviruses in *Aedes triseriatus* mosquitoes. Virology 1983; 127:83–90.

Beaty, BJ, Sundin, DR, Chandler, LJ, Bishop, DH. Evolution of bunyaviruses by genome reassortment in dually infected mosquitoes (*Aedes triseriatus*). Science 1985; 230:548–550.

Borucki, MK, Chandler, LJ, Parker, BM, Blair, CD, et al. Bunyavirus superinfection and segment reassortment in transovarially infected mosquitoes. J Gen Virol 1999; 80 (Pt 12):3173–3179.

Bustin, S. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000; 25:169–193.

Butler, JF, Hess, WR, Endris, RG, Holscher, KH. *In vitro* feeding of Omithodoros ticks for rearing and assessment of disease transmission. In: Griffiths, DA, Bowman, CE, (eds). *Acarology VI*. Ellis Horwood Limited: West Sussex, UK; 1984:1075–1081.

Calheiros, CML, Fontes, G, Williams, P, Rocha, EMM. Experimental infection of *Culex* (Culex) *quinquefasciatus* and *Aedes* (Stegomyia) *aegypti* with *Wuchereria bancrofti*. Mem Inst Oswaldo Cruz 1998; 93:855–860.

Chamberlain, RW, Sudia, WD. Dual infections of eastern and western equine encephalitis viruses in *Culex tarsalis*. J Infect Dis 1957; 101:233–236.

Chandler, LJ, Beaty, BJ, Baldridge, GD, Bishop, DH, et al. Heterologous reassortment of bunyaviruses in *Aedes triseriatus* mosquitoes and transovarial and oral transmission of newly evolved genotypes. J Gen Virol 1990; 71 (Pt 5):1045–1050.

Christensen, BM, Li, J, Chen, CC, Nappi, AJ. Melanization immune responses in mosquito vectors. Trends Parasitol 2005; 21:192–199.

Derouich, M, Boutayeb, A, Twizell, EH. A model of dengue fever. Biomed Eng Online 2003; 2:4.

Eaton, BT. Heterologous interference in *Aedes albopictus* cells infected with alphaviruses. J Virol 1979; 30:45–55.

Gargan, TP, Bailey, CL, Higbee, GA, Gad, A, et al. The effect of laboratory colonization on the vector-pathogen interactions of Egyptian *Culex pipiens* and Rift Valley fever virus. Am J Trop Med Hyg 1983; 32:1154–1163.

Gerhardt, R. West Nile virus in the United States (1999–2005). J Am Anim Hosp Assoc 2006; 42:170–177.

Hardy, JL, Houk, EJ, Kramer, LD, Reeves, WC. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annu Rev Entomol 1983; 28:229–262.

Karpf, AR, Blake, JM, Brown, DT. Characterization of the infection of *Aedes albopictus* cell clones by Sindbis virus. Virus Res 1997; 50:1–13.

Keene, KM, Foy, BD, Sanchez-Vargas, I, Beaty, BJ, et al. RNA interference acts as a natural antiviral response to O'nyong-nyong virus (Alphavirus; Togaviridae) infection of *Anopheles gambiae*. Proc Natl Acad Sci USA 2004; 101:17240–17245.

Kimura-Kuroda, J, Yasui, K. Antigenic comparison of envelope protein E between Japanese encephalitis virus and some other flaviviruses using monoclonal antibodies. J Gen Virol 1986; 67 (Pt 12):2663–2672.

Lam, KS, Marshall, ID. Dual infections of *Aedes aegypti* with arboviruses. I. Arboviruses that have no apparent cytopathic effect in the mosquito. Am J Trop Med Hyg 1968; 17:625–636.

Lanciotti, RS, Kerst, AJ. Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. J Clin Microbiol 2001; 39:4506–4513.

Lanciotti, RS, Kerst, AJ, Nasci, RS, Godsey, MS, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000; 38:4066–4071.

Lillibridge, KM, Parsons, R, Randle, Y, Travassos da Rosa, AP, et al. The 2002 introduction of West Nile virus into Harris County, Texas, an area historically endemic for St. Louis encephalitis. Am J Trop Med Hyg 2004; 70:676–681.

Lorono-Pino, MA, Cropp, CB, FarfaÌn, JA, Vorndam, AV, et al. Common occurrence of concurrent infections by multiple dengue virus serotypes. Am J Trop Med Hyg 1999; 61:725–730.

Lowenberger, C. Innate immune response of *Aedes aegypti*. Insect Biochem Mol Biol 2001; 31:219–229.

Mackenzie, JS, Gubler, DJ, Petersen, LR. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 2004; 10:S98–S109.

Mellor, PS. Replication of arboviruses in insect vectors. J Comp Pathol 2000; 123:231–247.

Mondini, A, Bronzoni, RVdM, Cardeal, ILS, Santos, TMILd, et al. Simultaneous infection by DENV-3 and SLEV in Brazil. J Clin Virol 2007; 40:84–86.

Mourya, DT, Thakare, JR, Gokhale, MD, Powers, AM, et al. Isolation of chikungunya virus from *Aedes aegypti* mosquitoes collected in the town of Yawat, Pune District, Maharashtra State, India. Acta Virol 2001; 45:305–309.

Myers, RM, Carey, DE. Concurrent isolation from patient of two arboviruses, chikungunya and dengue type 2. Science 1967; 157:1307–1308.

Payne, AF, Binduga-Gajewska, I, Kauffman, EB, Kramer, LD. Quantitation of flaviviruses by fluorescent focus assay. J Virol Methods 2006; 134:183–189.

Reisen, WK, Hahn, DC. Comparison of immune responses of brown-headed cowbird and related blackbirds to West Nile and other mosquito-borne encephalitis viruses. J Wildl Dis 2007; 43:439–449.

Richards, SL, Pesko, K, Alto, BW, Mores, CN. Reduced infection in mosquitoes exposed to blood meals containing previously frozen flaviviruses. Virus Res 2007; 129:224–227.

Rozeboom, LE, Kassira, EN. Dual infections of mosquitoes with strains of West Nile virus. J Med Entomol 1969; 6:407–411.

Savage, HM, Smith, GC, Moore, CG, Mitchell, CJ, et al. Entomologic investigations of an epidemic of St. Louis encephalitis in Pine Bluff, Arkansas, 1991. Am J Trop Med Hyg 1993; 49:38–45.

Sundin, DR, Beaty, BJ. Interference to oral superinfection of *Aedes triseriatus* infected with La Crosse virus. Am J Trop Med Hyg 1988; 38:428–432.

Thavara, U, Siriyasatien, P, Tawatsin, A, Asavadachanukorn, P, et al. Double infection of heteroserotypes of dengue viruses in field populations of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) and serological features of dengue viruses found in patients in southern Thailand. Southeast Asian J Trop Med Public Health 2006; 37:468–476.

Wang, WK, Chao, DY, Lin, SR, King, CC, et al. Concurrent infections by two dengue virus serotypes among dengue patients in Taiwan. J Microbiol Immunol Inf 2003; 36:89–95.

Wenming, P, Man, Y, Baochang, F, Yongqiang, D, et al. Simultaneous infection with dengue 2 and 3 viruses in a Chinese patient return from Sri Lanka. J Clin Virol 2005; 32:194–198.

Zebovitz, E, Brown, A. Interference among group A arboviruses. J Virol 1968; 2:1283–1289.

Address correspondence to:
Christopher N. Mores
Department of Pathobiological Sciences
School of Veterinary Medicine
Louisiana State University
Skip Bertman Drive
Baton Rouge, LA 70803

E-mail: cmores@lsu.edu