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Effects of Virus Dose and Extrinsic Incubation Temperature on Vector Competence of *Culex nigripalpus* (Diptera: Culicidae) for St. Louis Encephalitis Virus

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Abstract

Culex nigripalpus Theobald is a primary vector of St. Louis encephalitis virus in the southeastern United States. *Cx. nigripalpus* females were fed blood containing a low (4.0 ± 0.01 $\log_{10}$ plaque-forming unit equivalents (PFUeq)/ml) or high (4.7 ± 0.1 $\log_{10}$ PFUeq/ml) St. Louis encephalitis virus dose and maintained at extrinsic incubation temperatures (EIT) of 25 or 28°C for 12 d. Vector competence was measured via quantitative real-time reverse transcriptase polymerase chain reaction to estimate PFUeq using rates of infection, dissemination, and transmission. There were no differences in infection rates between the two EITs at either dose. The low dose had higher infection rates at both EITs. Dissemination rates were significantly higher at 28°C compared with 25°C at both doses. Virus transmission was observed (<7%) only at 28°C for both doses. The virus titer in body tissues was greater at 28°C compared with 25°C at both doses. The difference between the EITs was greater at the low dose, resulting in a higher titer for the low dose than the high dose at 28°C. Virus titers in leg tissues were greater in mosquitoes fed the high versus low dose, but were not influenced by EIT. Further investigations using a variety of environmental and biological factors would be useful in exploring the complexity of vector competence.

Keywords

St. Louis encephalitis virus; *Culex nigripalpus*; vector competence

Characterization of vector competence for arboviruses is essential in assessing risk from mosquitoes. Mosquitoes exhibit variation in vector competence because of biological, genetic, and/or environmental conditions (reviewed by Hardy et al. 1983). How these factors interact with one another to influence vector competence remains largely unexplored. Laboratory studies are only beginning to explore the complexity of factors controlling vector competence.

Before the introduction of West Nile virus (WNV, family *Flaviviridae:* genus *Flavivirus*) into the United States in the late-1990s, St. Louis encephalitis virus (SLEV, family *Flaviviridae:* genus *Flavivirus*) was the primary flavivirus causing vector borne disease outbreaks in the United States. Both viruses have similar transmission cycles, with enzootic maintenance by *Culex* spp. mosquitoes in wild birds and incidental epidemic transmission to

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humans (Day 2001, Turell et al. 2005). *Culex nigripalpus* Theobald is likely an important enzootic and epidemic vector of SLEV throughout its range in the southern United States because of its propensity to feed on both avian and mammalian hosts (Edman and Taylor 1968, Provost 1969, Mackay et al. 2010).

SLEV has been isolated from field-collected *Cx. nigripalpus* (e.g., Belle et al. 1964, Chamberlain et al. 1964, Dow et al. 1964) and this mosquito is a competent laboratory vector (Sudia and Chamberlain 1964). The same study and Richards et al. (2011) have encountered difficulty in getting *Cx. nigripalpus* to blood feed under laboratory conditions. Sudia and Chamberlain (1964) tested SLEV transmission by allowing infected *Cx. nigripalpus* to feed on uninfected chicks. Although wild birds have been found that were positive for SLEV (Gainer et al. 1964) or SLEV antibodies (Milby and Reeves 1990, Day and Stark 1999), knowledge about bird viremias comes primarily from laboratory investigations (e.g., Chamberlain et al. 1959, Hardy and Reeves 1990). Chamberlain et al. (1959) used chickens to blood feed *Cx. pipiens* and *Cx. p. quinquefasciatus* Say (viremia range 2.7– 4.8 $\log_{10}$) and Gainer et al. (1964) isolated SLEV from wild pigeons (viremia range 3.25–3.75 $\log_{10}$). In one study, increasing virus dose and extrinsic incubation temperature (EIT) increased mosquito susceptibility to SLEV infection and transmission capability (Hardy and Reeves 1990). However, few studies have considered how environmental factors may interact with one another to influence vector competence. Richards et al. (2009, 2010) demonstrated that environmental and biological factors such as EIT, virus dose, and mosquito age do influence each other's effects on *Cx. pipiens quinquefasciatus* vector competence for SLEV and WNV in complex and unpredictable ways. The relationship between infection, dissemination, and transmission of WNV by *Cx. p. quinquefasciatus* was also complex and not predictable (Richards et al. 2012). The current study explores how EIT and virus dose may influence the effect each has on *Cx. nigripalpus* vector competence for a Florida isolate of SLEV.

Materials and Methods

Mosquitoes and Virus

Cx. nigripalpus (11–12 d postemergence) from a colony established from Alachua County, FL, in 1995 (generation = F_{112}) were maintained under a photoperiod 14:10 (L:D) h cycle (Richards et al. 2011). Older mosquitoes were used because we have found this increases feeding success in this colony. Adult mosquitoes were housed in 0.5 liter cardboard cages with mesh screening and provided 10% sucrose and water ad libitum. We used the TBH28 SLEV isolate, obtained from a human patient in 1962 and passaged three times in Vero cells, for all mosquito infections. All experiments were carried out in a biosafety level three laboratory.

Mosquito Infection

The methods used here are described elsewhere (Richards et al. 2009, 2011). Female *Cx. nigripalpus* were deprived of sugar for 48 h and water for 24 h before they fed on bloodmeals at 28°C. The experiment was conducted once and all mosquitoes were fed simultaneously on the same day to ensure the same dose was fed. Bloodmeals were offered to mosquitoes via cotton pledgets soaked in warm (35°C) defibrinated bovine blood (Hemostat, Dixon, CA) mixed with freshly propagated virus stock. The virus stock was determined by plaque assay to have 5.3 $\log_{10}$ plaque-forming unit equivalents (PFUeq) SLEV/milliliter and different volumes of virus stock were added to blood to create different doses. Two doses were used (4.0 ± 0.1 or 4.7 ± 0.1 $\log_{10}$ PFUeq SLEV/milliliter) and two 0.1 ml samples of the bloodmeal were each added to separate tubes containing 1.0 ml BA-1 diluent (Richards et al. 2012) to confirm different doses. Bloodmeal samples were frozen for

subsequent viral assay. The high virus dose was the maximum titer of the virus isolate we could deliver to mosquitoes. After 45 min, mosquitoes were chilled and fully engorged specimens were transferred to cages, provided 10% sucrose ad libitum, and held in incubators at 25 or 28°C (EIT) for 12 d.

Mosquito Processing

After 12 d, saliva was collected as previously described (Anderson et al. 2010), that is, live mosquitoes were forced to salivate for 45 min into capillary tubes containing immersion oil. All surviving mosquitoes from each treatment group were assayed for virus ($N \geq 26$ per group). Mosquito bodies and legs were transferred to tubes coded for each mosquito containing 1.0 ml BA-1 diluent and two 4.5 mm zinc-plated beads.

Virus Assay

Nucleic acids were extracted and PFUeq of viral RNA were determined by quantitative real-time Taqman reverse transcriptase PCR (qRT-PCR) (Richards et al. 2009). The infection rate was the percentage of all mosquitoes tested having infected bodies. The dissemination rate was the percentage of mosquitoes with infected bodies that also had infected legs. The transmission rate was the percentage of mosquitoes with infected legs that also had infected saliva.

Statistical Analysis

Statistical analyses used SAS (SAS Institute 2002). Chi square tests were used to analyze differences in rates of infection and dissemination between doses and EITs. Fisher exact tests were used to analyze transmission rates. Virus titers were log-transformed [log ($x + 1$)] to improve normality before analysis. Analysis of variance (ANOVA, PROC GLM) was used to analyze differences in titers of bodies and legs. Saliva titers were not analyzed further because of small sample sizes. If significant differences were observed, a Duncan multiple comparison test was used to determine which means were significantly different. For all tests, $P < 0.05$ was used to determine significance.

Results

Viral Titer of Bloodmeal

Mosquitoes were fed bloodmeals containing significantly different ($F = 43.73$; df = 1, 4; $P = 0.003$) viral titers (mean ± SE) of either 4.0 ± 0.1 (low dose) or 4.7 ± 0.1 (high dose) $\log_{10}$ PFUeq SLEV/ml.

Effects of Virus Dose and EIT on Vector Competence

Infection, dissemination, and transmission rates are shown in Fig. 1. No transmission was observed at 25°C. Only three mosquitoes (28°C) transmitted SLEV. Infection rates were not significantly different between EITs (low dose: $\chi^2 = 0.021$, df = 1, $P = 0.885$; high dose: $\chi^2 = 0.832$, df = 1, $P = 0.362$). However, at both EITs, infection rates were significantly higher in the low dose group compared with the high dose group (25°C: $\chi^2 = 3.87$, df = 1, $P = 0.049$; 28°C: $\chi^2 = 4.13$, df = 1, $P = 0.042$). Dissemination rates were significantly higher at 28°C compared with 25°C at both doses (low dose: $\chi^2 = 25.77$, df = 1, $P < 0.0001$; high dose: $\chi^2 = 17.39$, df = 1, $P < 0.0001$). There were no differences in dissemination rates because of dose at either EIT (25°C: $\chi^2 = 0.058$, df = 1, $P<0.810$; 28°C: $\chi^2 = 1.55$, df = 1, $P = 0.213$). At 28°C, there was no difference in transmission rates between doses ($P = 0.586$). As has previously been observed in this colony (Richards et al. 2011), low feeding success (5%) and high (38%) mortality occurred, limiting the sample size possible.

Virus titers of bodies, legs, and saliva are shown in Table 1 and the results of ANOVA for leg and body titers are in Table 2. Body titer was significantly greater at 28°C compared with 25°C at both doses. Dose influenced leg titer with significantly greater titers in the high dose than the low dose at both EITs.

Discussion

Significantly higher infection rates were observed at the low dose compared with the high dose at both 25 and 28°C. This showed that the effect of virus dose remained the same at both EITs used here. Others have shown modulation of virus because of EIT, for example, *Culex tarsalis*-Western equine encephalitis virus (Kramer et al. 1998), *Cx. univittatus*-WNV (Reisen et al. 2006), and higher infection rates at low doses (*Cx. p. quinquefasciatus*-WNV; Richards et al. 2010). Although increased virus dose often increases infection rates, our observation with SLEV and *Cx. nigripalpus* shows this is not always the case. This is consistent with the complexity found for various environmental effects on *Cx. p. quinquefasciatus* vector competence for SLEV and WNV (Richards et al. 2009, 2010, 2011). The increase in dissemination accompanying higher temperature here was not influenced by dose, though other mosquito populations may react differently. Unfortunately, the low number (<7%) of mosquitoes transmitting SLEV here precluded assessment of transmission effects. Further studies will require greater sample sizes than we were able to maintain. However, the extreme decrease in numbers transmitting from numbers disseminated is of interest, and may affect interpretation of surveillance data (Bustamante and Lord 2010).

Sudia and Chamberlain (1964) fed mosquitoes on SLEV-infected chicks (viremias 3.8 – 4.6 logs LD_{50}), incubated at 27°C, and refed on uninfected chicks, resulting in 100% infection and 60–100% transmission rates. Transmission rates in this study depended on virus origin (Florida vs. California) and incubation period (15–26 d). We observed infection rates similar to Sudia and Chamberlain (1964). The transmission rates we observed differ from Sudia and Chamberlain (1964), likely because of differences in mosquito populations, virus strains, virus doses, incubation periods, transmission assay, and other factors. The complexity of the contributing factors is a major impediment to characterizing vector competence in either nature or in the laboratory under varying conditions. Future studies should explore which factors are most important and the dynamics of their influence on one another.

Infection and dissemination rates of *Cx. p. quinquefasciatus* were characterized using the same strain of SLEV used here (Richards et al. 2009). Comparable mosquito age, dose, and EITs to the current study showed ≥two-fold lower infection (24–36%) and dissemination (8–9%) rates at 25°C in *Cx. p. quinquefasciatus* (Richards et al. 2009) than *Cx. nigripalpus.* At 28°C, the study by Richards et al. (2009) showed infection (28–72%) and dissemination (25–27%) rates of *Cx. p. quinquefasciatus* were lower than for *Cx. nigripalpus* here. Though variation in vector competence is expected, more importantly, the species also showed differences in interactions between environmental factors. The body infection rate and body SLEV titers of *Cx p. quinquefasciatus* were affected by EIT, dose, and the EIT × dose interaction (Richards et al. 2009), while only *Cx. nigripalpus* body titers were influenced by EIT here. Dose had a greater effect on *Cx. p. quinquefasciatus* than *Cx. nigripalpus* under similar conditions used here, hence risk assessment of these species based on infection rate would depend on environmental conditions. Conversely, the leg SLEV titers of both *Cx. p. quinquefasciatus* (Richards et al. 2009) and *Cx. nigripalpus* (this study) were affected only by dose, not EIT or the EIT × dose interaction. Dose had a greater effect on leg titers than EIT in both species, showing that the dose is important in virus dissemination out of the midgut under these conditions. These results suggest how the tested colonies reacted to the same virus and not the full range of variation that might be encountered by each species and under different conditions. These comparisons highlight variation in vector competence

occurring within and between species and the essential need to explore this under interacting conditions.

Our studies show that the influence of environmental factors is complex and unfortunately unpredictable. Not all SLEV-infected *Cx. nigripalpus* are infectious and many environmental factors can influence the effects each has on vector competence in complex ways.

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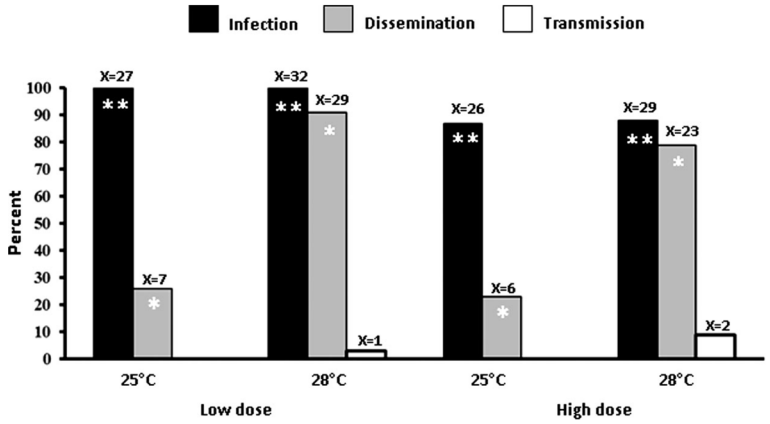

Fig. 1.

Rates of infection, dissemination, and transmission in *Cx. nigripalpus* fed either a low or high dose of SLEV and incubated at 25 or 28°C for 12 d. Sample sizes are as follows: 25°C, low dose (N = 27); 28°C, low dose (N = 32); 25°C, high dose (N = 30); 28°C, high dose (N = 33). The number SLEV-positive (X) is given above the bars in the figure. *Variable is significantly different between EITs. **Variable is significantly different between doses.

Table 1

The mean titers (log_{10} PFUeq SLEV/ml) ± SE for *Cx. nigripalpus* fed a low or high virus dose and incubated at 25 or 28°C for 12 d

EIT	No. tested	Body titer[a]	Leg titer[a]	Saliva titer
Low dose				
25°C	27	2.3 ± 0.2c	1.0 ± 0.3c	—
28°C	32	3.6 ± 0.1a	1.4 ± 0.1bc	0.3
High dose				
25°C	30	2.4 ± 0.2c	1.7 ± 0.4ab	—
28°C	33	3.0 ± 0.2b	2.0 ± 0.1a	0.7 ± 0.6

[a]Treatment groups with the same letter in each column are not significantly different by means comparisons.

Table 2

Analysis of variance showing differences in the mean titers of bodies and legs between doses and EITs

Variable	df (numerator, denominator)	*F*	*p*[a]
Body titer			
EIT	1, 110	31.27	<**0.0001**
Dose	1, 110	1.67	0.199
EIT × dose	1, 110	3.57	0.061
Leg titer			
EIT	1, 61	3.71	0.059
Dose	1, 61	10.09	**0.002**
EIT × dose	1, 61	0.04	0.843

Analyses were not carried out on saliva titer because of low sample size.

[a]Significant *P* values in bold.