Journal of the American Mosquito Control Association, 18(4):284–289, 2002

VECTOR COMPETENCE OF THREE NORTH AMERICAN STRAINS OF *AEDES ALBOPICTUS* FOR WEST NILE VIRUS[1]

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ABSTRACT. To evaluate the potential for North American (NA) *Aedes albopictus* to transmit West Nile virus (WN), mosquito strains derived from 3 NA sources (Frederick County, Maryland, FRED strain; Cheverly, MD, CHEV strain; Chambers and Liberty counties, Texas, TAMU strain) were tested. These strains were tested along with a previously tested strain from a Hawaiian source (OAHU strain). Mosquitoes were fed on 2- to 3-day-old chickens previously inoculated with a New York strain (Crow 397-99) of WN. All of the NA strains were competent laboratory vectors of WN, with transmission rates of 36, 50, 83, and 92% for the FRED, CHEV, OAHU, and TAMU strains, respectively. The extrinsic incubation period for WN in *Ae. albopictus* held at 26°C was estimated to be 10 days. Based on efficiency of viral transmission, evidence of natural infection, bionomics, and distribution, *Ae. albopictus* could be an important bridge vector of WN in the southeastern USA.

KEY WORDS *Aedes albopictus,* West Nile virus, vector competence, extrinsic incubation

INTRODUCTION

West Nile virus (WN) was reported for the 1st time in the Western Hemisphere in 1999 and caused encephalitis in humans in New York City and an epizootic in native and exotic avian populations (Centers for Disease Control and Prevention [CDC] 1999a, 1999b; Lanciotti et al. 2000). Testing field-collected mosquitoes for evidence of WN infection during 1999 and 2000 indicated that *Culex pipiens* L. is the primary vector of this virus and that a number of additional species may be secondary or bridge vectors (CDC 1999b, 2000). Potential secondary or bridge vector species from which multiple instances of WN infection were reported include *Culex restuans* Theobald, *Culex salinarius* Coquillett, *Aedes vexans* (Meigan), *Ochlerotatus japonicus japonicus* (Theobald), *Ochlerotatus triseriatus* (Say), *Ochlerotatus trivittatus* (Coquillett), and *Culiseta melanura* (Coquillett). In September 2000, a single pool of *Aedes albopictus* (Skuse) collected in southeastern Pennsylvania showed evidence of WN infection (CDC 2000).

Since discovery of *Ae. albopictus* in Memphis, TN (Reiter and Darsie 1984), and Houston, TX (Sprenger and Wuithiranyagool 1986), in the 1980s, this species has become established throughout most of the southeastern USA, extending as far north as New Jersey (Moore and Mitchell 1997). Throughout its range in the USA, *Ae. albopictus* is an important human pest, aggressively biting during daylight hours close to its breeding sites. Experimental transmission studies indicate that it is a competent vector of several flaviviruses (Mitchell 1991). Although it is relatively refractory to infection with St. Louis encephalitis virus (Savage et al. 1994), a study with a strain of WN from the outbreak of WN in New York in 1999 indicated that *Ae. albopictus* is a highly efficient laboratory vector of WN (Turell et al. 2001). However, the latter study used a long-colonized strain of *Ae. albopictus* from Hawaii, and therefore may not represent what occurs in nature in regions of the USA where WN is now enzootic.

To elucidate the potential role of *Ae. albopictus* in the epidemiology of WN in the eastern USA, we conducted laboratory studies of the vector competence of 4 strains: 2 newly colonized ones from Maryland where WN is considered enzootic, a long-colonized Texan strain, and the Hawaiian strain (OAHU) used by Turell et al. (2001). Additionally, studies were done to evaluate viral replication and dissemination in these mosquitoes over time.

MATERIALS AND METHODS

Mosquitoes: Three North American (NA) strains of *Ae. albopictus* were evaluated for their ability to transmit WN. These included the Frederick (FRED) strain, derived from eggs collected in Frederick County, Maryland, in July 1999 (Sardelis and Turell 2001); the Cheverly (CHEV) strain, derived from larvae collected from discarded tires in Cheverly, Prince George's County, Maryland, in June

[1] The views of the authors do not necessarily reflect the position of the Department of Defense or the Department of the Army. In conducting research with animals, the investigators adhered to the *Guide for the care and use of laboratory animals,* as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication 86-23, Revised 1996). The facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

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1999; and the Texas A&M University (TAMU) strain, derived from specimens collected in Chambers and Liberty counties, Texas, in 1987. Additionally, the OAHU strain of *Ae. albopictus,* derived from specimens collected in Honolulu, HI, in 1971, was evaluated. The generation tested was F_3 for FRED and CHEV, and $>F_{30}$ for TAMU and OAHU. Larvae were reared at 26 ± 1°C and a 16-h photoperiod and fed ground catfish chow (AquaMax Pond Plus 3000, Purina Mills, Inc., St. Louis, MO).

Virus and virus assay: The WN strain (Crow 397-99) was isolated from a dead crow found in the Bronx, New York City, NY, during an epizootic in 1999 (Turell et al. 2000) and had been passaged once in Vero cell culture. Viral stock suspensions, triturated mosquito suspensions, and chicken blood samples were tested for infectious virus by plaque assay on African green monkey kidney (Vero) cells as described by Gargan et al. (1983), except that the 2nd overlay, containing neutral red, was added 2 days after the 1st overlay.

Vector competence studies: Mosquitoes, 4 to 5 days old, were allowed to feed on a chicken (*Gallus gallus* L.) that had been inoculated subcutaneously 24 or 48 h earlier with 0.1 ml of a suspension containing $10^{4.2}$ plaque-forming units (PFU) of WN. When most of the mosquitoes in a carton had fed (≈15 min), the chicken was transferred to a 2nd carton containing a different strain of mosquitoes. This was repeated until all 4 strains of mosquitoes had an opportunity to feed upon the same chicken. Immediately after mosquito feeding, a 0.1-ml blood sample was obtained from the jugular vein of each chicken and diluted in 0.9 ml of diluent (10% heat-inactivated fetal bovine serum in Medium 199 (Invitrogen, Carlsbad, CA) with Earle's salts, $NaHCO_3$, and antibiotics) plus 10 units of heparin per milliliter to determine the viremia at the time of mosquito feeding. Engorged mosquitoes were transferred to 3.8-liter cartons with netting over the open end and maintained in an incubator as described above. Four days after the infectious blood meal, an oviposition substrate was added to each cage.

To determine transmission rates, some of the mosquitoes that had taken an infectious blood meal were individually allowed to refeed on a 2-day-old chicken 13 days after the initial infectious blood meal. Immediately after the transmission attempt, the mosquitoes were killed by freezing at −20°C for ≈5 min and their legs and bodies were triturated separately in 1 ml of diluent and frozen at −70°C until assayed for virus. Presence of virus in a mosquito's body indicated infection, whereas virus in the legs indicated that the mosquito had a disseminated infection (Turell et al. 1984). Mosquitoes not used in the transmission attempts were killed and ground as described above on day 14 after the infectious blood meal.

Viral replication and dissemination studies: To evaluate viral replication and dissemination over time, each strain of mosquito was fed on a single chicken that had been inoculated with WN 48 h earlier. Immediately after ingesting the infectious blood meal, 5 mosquitoes of each strain were killed, triturated, frozen, and assayed as described above. Handling and maintenance after bloodfeeding for the remaining mosquitoes was as described above, except that samples of 10 mosquitoes of each strain were killed, ground, and frozen for later assay on days 1, 4, 7, 10, 14, 21, and 28 after bloodfeeding.

Statistics: The infection rate was calculated as the number of infected mosquitoes/total tested ×100. The dissemination rate was calculated as the number of mosquitoes with positive legs/total tested ×100. The transmission rate was calculated as the number of mosquitoes transmitting virus by bite (chickens were viremic when bled the day after the transmission attempt)/the number of mosquitoes that fed on these chickens. Infection, dissemination, and transmission rates were compared by chi-square or Fisher exact tests as appropriate and significant differences were determined at the 95% confidence level (SAS Institute Inc. 1999). A 1-way analysis of variance (ANOVA) was used to test for differences in the mean titer (log transformed) of WN ingested among the strains. The means (log transformed) from the study of viral replication in the bodies of infected mosquitoes were subjected to 2-way ANOVA to evaluate the main effects for strain and day after oral exposure and the interaction term. For terms found to be significant ($\alpha = 0.05$) by ANOVA, the means were separated by Duncan's multiple range test (SAS Institute Inc. 1999).

RESULTS

All strains of *Ae. albopictus* were susceptible to infection with WN at both viral titers tested (Table 1). Susceptibility to infection for each strain significantly increased with viral titer ($\chi^2 > 5.4$, df = 1, $P < 0.02$). Additionally, within viral titer levels, susceptibility to infection was associated with strain tested ($\chi^2 \geq 11.6$, df = 3, $P \leq 0.009$). At the higher viral titer, infection rates of the TAMU (96%) and OAHU (93%) strains were significantly higher ($\chi^2 \geq 10.0$, df = 1, $P \leq 0.002$) than infection rates of the FRED (56%) and CHEV (67%) strains. Dissemination rates for all strains and at both viral doses were 0–11% lower than the corresponding infection rate. Transmission rates for mosquitoes exposed to a chicken with a viremia of $10^{6.8}$ were significantly affected by strain ($\chi^2 = 10.80$, df = 3, $P = 0.013$), ranging from 36% for the FRED strain to 92% for the TAMU strain. However, the reduced efficiency was due to a reduced infection rate because the percentage of infected mosquitoes that developed a disseminated infection and the percentage of mosquitoes with a disseminated infec-

Table 1. Infection, dissemination, and transmission rates for 4 strains of *Aedes albopictus* orally exposed to West Nile virus (WN).

Strain (collection location)	Infectious dose[1]	Number tested	Infection rate[2]	Dissemination rate[3]	Transmission rate[4]	Transmission (dissemination) rate[5]
FRED (Frederick County, MD)	5.7	45	27 (15–42)a	24 (13–40)a	NT	NT
CHEV (Cheverly, MD)	5.7	45	42 (28–58)ab	38 (24–53)ab	NT	NT
TAMU (Chambers and Liberty counties, TX)	5.7	45	53 (38–68)ab	49 (34–64)ab	NT	NT
OAHU (Honolulu, HI)	5.7	45	60 (44–74)b	58 (42–72)b	NT	NT
FRED (Frederick County, MD)	6.8	45	56 (40–70)x	49 (34–64)x	36 ([11–69], 11)x	80 ([28–99], 5)
CHEV (Cheverly, MD)	6.8	45	67 (51–81)x	64 (49–78)xy	50 ([21–79], 12)xy	67 ([30–93], 9)
TAMU (Chambers and Liberty counties, TX)	6.8	45	96 (85–99)y	87 (73–95)y	92 ([62–99], 12)y	100 ([62–100], 11)
OAHU (Honolulu, HI)	6.8	45	93 (82–99)y	93 (82–99)y	83 ([52–99], 12)y	91 ([59–99], 11)

[1] Viremia titer at time of feeding in $\log_{10}$ plaque-forming units per milliliter of blood.

[2] Percentage of mosquitoes containing virus in their bodies (95% CI). By infectious dose, infection rates followed by the same lowercase letter are not significantly different at $\alpha = 0.05$ after adjusting for multiple comparisons.

[3] Percentage of mosquitoes containing virus in their legs (95% CI). By infectious dose, dissemination rates followed by the same lowercase letter are not significantly different at $\alpha = 0.05$ after adjusting for multiple comparisons.

[4] Percentage of mosquitoes that transmitted virus by bite 13 days after ingesting a WN-infected blood meal ([95% CI], number fed). Transmission rates followed by the same lowercase letter are not significantly different at $\alpha = 0.05$ after adjusting for multiple comparisons. NT, not tested.

[5] Percentage of mosquitoes with a disseminated infection that transmitted virus by bite 13 days after ingesting a WN-infected blood meal ([95% CI], number disseminated fed). All transmission rates were not significantly different at = 0.05. NT, not tested.

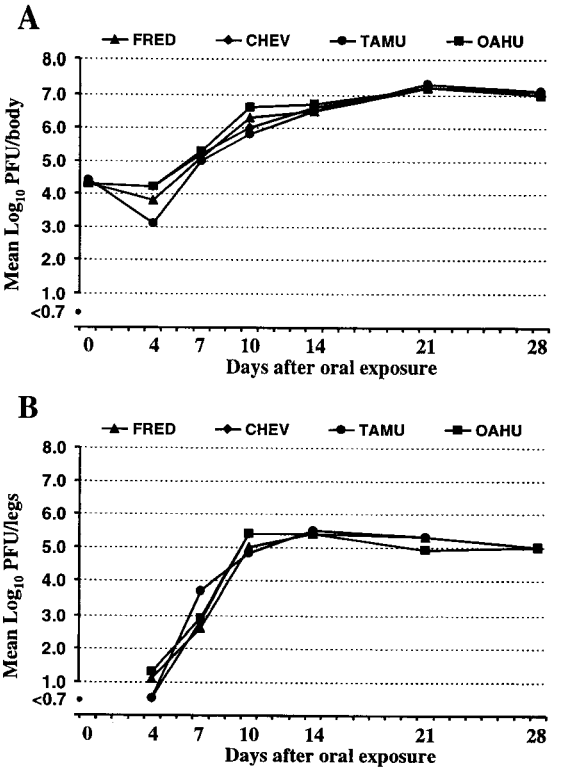

Fig. 1. Mean viral titers over time in the bodies (A) and legs (B) of infected *Aedes albopictus* after oral exposure to a West Nile virus–infected chicken with a viremia of $10^{6.8}$ plaque-forming units per milliliter of blood and held at 26°C. FRED, Frederick County, Maryland, strain; CHEV, Cheverly, MD, strain; TAMU, Texas strain; OAHU, Hawaii strain. $n = 5$ on day 0 and 3–9 on days 4–28. Standard error of the mean was less than ± 0.4 at each data point.

tion that successfully transmitted virus by bite did not differ by strain of *Ae. albopictus* tested ($\chi^2 < 5.7$, df = 3, $P > 0.13$).

From a chicken with a viremia of $10^{6.8}$ PFU/ml of blood, approximately $10^{4.2}$ infectious virions were ingested per mosquito (Fig. 1A). The strain of mosquito had no significant effect on the amount of virus ingested (ANOVA; F = 0.04; df = 3,16; $P = 0.99$). Two-way ANOVA revealed that the main effect for strain proved to be nonsignificant (F = 2.2; df = 3,140; $P = 0.088$) and that the main effect for day after oral exposure was significant (F = 126.7; df = 5,140; $P < 0.001$). The mean titers in the bodies by strain and day after oral exposure are displayed in Figure 1A. Means separation tests showed that viral titers significantly increased between days 4, 7, 10, and 14, but did not significantly increase from day 21 to 28. The interaction between strain and day after oral exposure proved to be nonsignificant (F = 0.5; df = 15,140; $P = 0.95$). During this particular study, infection rates

for the FRED, CHEV, TAMU, and OAHU strains were 42, 59, 88, and 86%, respectively.

Evidence was found of virus escaping the midgut of the FRED and OAHU strains 4 days after oral exposure (Fig. 1B). By 7 days after oral exposure, virus was detected in the legs of all strains. Viral titers in the legs of all strains peaked between 10 and 14 days after the infectious blood meal. For all the strains combined, the percentages of infected mosquitoes that showed evidence of having a disseminated infection (virus in the legs) on days 4, 7, 10, 14, 21, and 28 were 14, 85, 100, 96, 100, and 96%, respectively.

DISCUSSION

This study showed that the NA strains of *Ae. albopictus* have the potential to serve as WN vectors based on their susceptibility to infection and their ability to transmit WN efficiently. This finding is consistent with previous laboratory transmission studies that showed that WN is transmitted by a broad range of NA mosquito species, including a number of *Culex* and *Aedes* species (Turell et al. 2000, 2001; Sardelis and Turell 2001; Sardelis et al. 2001). Additionally, the transmission rate for the OAHU strain in this study (83%) closely agreed with the rate reported by Turell et al. (2001), who estimated the transmission rate for the same strain to be 73% when tested under nearly identical conditions. Because WN-infected wild birds (e.g., crows and house sparrows) can develop viremias $>10^8$ PFU/ml of blood (Work et al. 1955), the viremias of $10^{5.7-6.8}$ PFU/ml used in our study should be representative of what the mosquitoes would be exposed to in nature.

Of the barriers to the biological transmission of arboviruses by bite (midgut infection barrier [MIB], midgut escaper barrier [MEB], and salivary gland barrier [SGB]), the MIB appeared to be the most important determinant of vector competence of the strains tested in this study. However, by increasing the titer of the infectious blood meal, the MIB could largely be overcome. Infection rates were identical or nearly identical to dissemination rates, thus indicating that a MEB was not important. Lastly, because transmission rates were comparable with dissemination rates, only a slight, if any, SGB was apparent. Of the NA mosquito species tested thus far (Turell et al. 2000, 2001; Sardelis and Turell 2001; Sardelis et al. 2001), laboratory vector efficiency is primarily modulated by a MIB. Additionally, these earlier studies indicate that vector competence level ranges widely, from inefficient (<5% transmission rate) through highly efficient (>60% transmission rate), and that a pronounced MEB occurs in a number of common *Culex* species. The *Ae. albopictus* strains tested here were efficient to highly efficient laboratory vectors of WN.

Although we only evaluated a few strains of *Ae. albopictus,* analysis of the data suggested that some intraspecific variation may occur in vector competence among NA strains of *Ae. albopictus* for WN. Previous studies have shown that vector competence for flaviviruses varies widely among populations of *Aedes* (Gubler and Rosen 1976, Gubler et al. 1979, Kay et al. 1984, Tabachnick et al. 1985, Boromisa et al. 1987, Tran et al. 1999). Further studies with more NA strains of *Ae. albopictus* from a wider range of geographic sources need to be done to elucidate this issue.

Knowledge of the extrinsic incubation period (EIP), along with other factors, is vital for estimating vectorial capacity. The transmission studies showed that *Ae. albopictus* transmitted WN 13 days after taking an infectious blood meal. Additionally, the studies of viral replication and dissemination over time indicated that virus had escaped the midgut by day 7 in >80% of all infected mosquitoes and that the amount of virus circulating in the hemolymph (as estimated by the titer in a mosquitoes legs) peaked between 10 and 14 days after the infectious blood meal. Although the presence of virus in the hemolymph does not indicate that the salivary glands are infected and that infectious virions are present in the saliva, it is probable that, based on the viral replication and dissemination studies, the EIP may be around 10 days. Additional transmission studies are needed to more precisely determine the EIP and to evaluate the effect of temperature on EIP for WN in *Ae. albopictus.* The duration of the EIP in this study is typical of the EIP of 9–12 days reported for other flavivirus–mosquito combinations at comparable incubation temperatures (Watts et al. 1987, Miller et al. 1989, Reisen et al. 1993).

Although *Ae. albopictus* seems to be among the most efficient laboratory vectors of WN, a number of important aspects of the mosquito's bionomics must be considered to properly evaluate whether or not this species will become important in the transmission of WN in nature. *Aedes albopictus* is considered an opportunistic feeder, taking blood meals from birds and mammals, including humans (Tempelis et al. 1970, Sullivan et al. 1971). Relatively recent studies of host-seeking patterns of *Ae. albopictus* in North America found that 3–16% of its blood meals are from birds (Savage et al. 1993, Niebylski et al. 1994). The flight range of *Ae. albopictus* is relatively short, approximately a few hundred meters (Bonnet and Worcester 1946, Rosen et al. 1976), which may limit its role in WN transmission because of the need to become infected at an avian focus of infection and then fly to human habitations. However, the mosquito's container breeding sites are often found associated with human habitation, and the avian reservoir hosts of WN (e.g., crows, blue jays, and house sparrows) are ubiquitous.

Aedes albopictus is established throughout the southeastern USA (Moore and Mitchell 1997) and

is commonly reported to be an important biting pest. *Aedes albopictus* has been found infected with eastern equine encephalomyelitis virus (Mitchell et al. 1992), a virus with a similar enzootic transmission cycle to WN. In 2000, WN was detected in a single pool of *Ae. albopictus* captured in southeastern Pennsylvania (CDC 2000), close to the northernmost established range of this species in the mid-Atlantic region. West Nile virus was detected in 4 Atlantic states (Connecticut, Maryland, New Jersey, and New York) in 1999; in 12 states and the District of Columbia, extending from most of the New England states to North Carolina in 2000 (CDC 2000); and in 27 states and the District of Columbia in 2001 (CDC 2001). As WN continues its southward spread, the probability of contact between this pathogen and *Ae. albopictus* will be greater. As such, personnel involved in the entomological arm of WN surveillance programs should ensure that *Ae. albopictus* is collected and tested. Also, further evidence of WN infection in wild-caught *Ae. albopictus* is needed to more firmly incriminate this mosquito as a vector of WN.

The results of this study, combined with evidence of natural infection in and knowledge of the distribution and bionomics of *Ae. albopictus,* suggest that this species could function as a bridge vector for WN between the enzootic *Culex* spp.–avian cycle and susceptible mammalian hosts, including humans. Because of the possibility of intraspecific variation in vector competence, testing of local strains of *Ae. albopictus* to determine their transmission efficiency may be warranted to best estimate the role this species may play in the epidemiology of WN in a particular area.

ACKNOWLEDGMENTS

We thank C. Bosio, D. Dohm, and K. Kenyon for their critical reading of the manuscript; J. Olson (Department of Entomology, Texas A&M University, College Station, TX) for providing the Texas strain of *Ae. albopictus*; and M. Delgado and D. Schachner for their assistance with the mosquito rearing. This research formed part of a Ph.D. dissertation by the senior author.

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