Is Nonviremic Transmission of West Nile Virus by Culex Mosquitoes (Diptera: Culicidae) Nonviremic?

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ABSTRACT Inter- and intraspecific transfer of West Nile virus (family Flaviviridae, genus Flavivirus, WNV) occurred infrequently when donor Culex tarsalis Coquillett fed concurrently on house finches with recipient Culex quinquefasciatus Say and Cx. tarsalis. Five of six of these house finches had WNV in blood samples collected by jugular venipuncture 30–45 min postfeeding, with titers ranging from 2.3 to 4.2 log_{10} plaque-forming units (PFU)/ml. After 2-wk incubation at 26°C, three Cx. quinquefasciatus and one Cx. tarsalis of 230 blood-fed recipients were infected, of which one Cx. quinquefasciatus was capable of transmission. Our data indicated that infectious female mosquitoes feeding on small vertebrates create a nonpropagative viremia capable of infecting concurrently cofeeding females. The proportion of recipients infected is likely related to the amount of virus expectorated by donor females, the blood volume of the vertebrate host, and the susceptibility of the cofeeding mosquitoes.

KEY WORDS nonviremic transmission, Culex tarsalis, Culex quinquefasciatus, West Nile virus

Nonviremic transmission (NVT) of West Nile virus (family Flaviviridae, genus Flavivirus, WNV) was demonstrated recently for mosquitoes by using a Culex quinquefasciatus Say–laboratory mouse (Mus musculus) experimental model (Higgs et al. 2005). Blood samples from experimental mice lacked detectable virus, leading Higgs et al. (2005) to the conclusion that transmission was nonviremic, i.e., by their definition “before its propagation in the host and its appearance in the circulatory system.” Previously, NVT had been shown among cofeeding ticks with Thogoto (Orthomyxoviridae) and tick-borne encephalitis viruses (Flaviviridae) (Jones et al. 1990; Nutter et al. 1994; Labuda et al. 1997a,b) and among cofeeding blackflies with vesicular stomatitis virus (Rhabdoviridae) (Mead et al. 2000).

The purpose of our experiment was to confirm the recent results of Higgs et al. (2005) by using a natural Culex vector–WNV–avian host system by demonstrating intra- and interspecific transfer of WNV among cofeeding Culex and to assess the transient level of WNV in the vertebrate host circulatory system.

Materials and Methods

Mosquitoes. Cx. tarsalis were used as donor and recipient mosquitoes, and Cx. quinquefasciatus were used as recipient mosquitoes. Both species are susceptible to WNV infection (Goddard et al. 2002, Reisen et al. 2005), important in transmission in the western United States (Reisen et al. 2004, Hom et al. 2005), and feed frequently upon passeriform birds (Reisen and Reeves 1990). Mosquitoes were from the Kern National Wildlife Refuge (KNWR) colony of Cx. tarsalis and the Bakersfield colony of Cx. quinquefasciatus.

Birds. House finches, Carpodacus mexicanus Müller, were used as the avian host, because they are competent hosts for WNV (Reisen et al. 2005), frequently fed upon in nature by Cx. tarsalis (Tempelis et al. 1976), and easy to maintain and handle in captivity. Birds were collected from traps used in a removal program in vineyards near Bakersfield, Kern County, CA, bled to determine previous arbovirus exposure, banded, and maintained in a screened outdoor avairy to observe general health. Birds were fed mixed wild bird seed throughout.

The collection and infection of wild birds with encephalitis viruses was done under Protocols 11184 and 11187, respectively, approved by the Institutional Animal Care and Use Committee of the University of California, Davis, CA, Resident Scientific Collection Permit 80149-02 by the State of California Department of Fish and Game, and Federal Fish and Wildlife Permit No. MB082812.

Virus. We used a NY99 strain of WNV (strain 35211 AAF) and that previously was passaged twice in Vero cells before experimentation (Reisen et al. 2005).

Use of WNV was approved under Biological Use Authorization 0554 by the Office of Environmental

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Health and Safety of the University of California, Davis, and USDA Permit 47901.

Experimental Protocol. Approximately 120 3–5-d-old KNWR females were intrathoracically (i.t.) inoculated with 1,000 PFU of WNV and maintained on 10% sucrose for 1 wk at 26°C, after which 5, 10, or 15 of these donor females were transferred to 1-pt (0.47-L) screened cages containing 50–100 3–5-d-old Cx. quinquefasciatus or Cx. tarsalis recipient mosquitoes (Table 1). Recipient Cx. tarsalis previously were marked with fluorescent dust to enable separation from the i.t.-infected females housed in the same cage. All mosquitoes were starved for 24 h and then exposed to a single restrained house finch for 30 min.

House finches were bled immediately after the mosquito feeding period and then daily for 4 d to detect circulating virus to ensure that one or more donor females expectorated virus. One hundred microliters of blood was collected from the jugular vein by using a syringe with a 28-gauge needle, and the blood was expelled immediately into 400 µL of diluent containing phosphate-buffered saline, 10% fetal bovine serum and antibiotics. Blood samples were allowed to clot for 15 min, clarified by centrifugation, and then frozen at −80°C until tested.

Immediately after blood feeding, mosquitoes were anesthetized with CO2 and engorged females were counted and transferred to clean cages. After being held for 2 wk on 10% sucrose at 26°C, females were anesthetized with triethylamine; identified to species, dust status, or both; and their ability to transmit virus was evaluated using an in vitro capillary tube method (Aitken 1977). Dusted and undusted Cx. tarsalis were sorted under a UV lamp, and undusted females were examined at 30X magnification to verify the presence of a scar from previous i.t. inoculation. Because many of the i.t.-inoculated donor females did not survive the 2-wk incubation period, transmission also was evaluated for an additional 15 i.t.-infected KNWR females from the same cohort to measure the quantity of virus expectorated. After a 10–15-min transmission period, capillary tube contents were expelled into 300 µL of diluent, and the mosquito body and expectorate were frozen at −80°C until tested.

Virus Detection. House finch sera collected before experimentation were negative for antibodies to WNV, St. Louis encephalitis, and western equine encephalomyelitis viruses when tested using an enzyme immunoassay (Chiles and Reisen 1998). Initial experimental finch blood samples and all mosquito bodies were screened for WNV by real-time reverse transcription-polymerase chain reaction (RT-PCR) by using primers published previously (Lanciotti et al. 2000). Initial and subsequent finch blood samples and bodies and expectorant samples from mosquitoes with positive TaqMan scores (Ct < 40 cycles) also were

Table 1. Number of donor and recipient mosquitoes in each group blood feeding, infected, and transmitting West Nile virus

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor Finch viremia* at 30–45 min</th>
<th>Culex species</th>
<th>Recipient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. added</td>
<td>No. fed</td>
<td>Alive at 2 wk</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0</td>
<td>No transm</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>25</td>
<td>10</td>
</tr>
</tbody>
</table>

Trans, transmission or transmitting; quinque., quinquefasciatus; *log10 plaque forming units/mL.

Fig. 1. Viremia profile in log10 PFU/ml for six house finches fed upon by zero to eight intrathoracically inoculated Cx. tarsalis females. Day 0 samples were taken 30–45 min after females blood fed. Females represented by solid lines passed virus to recipient mosquitoes. Virus detection threshold was 1.7 log10 PFU/ml.
tested for infectious virus by plaque assay on Vero cell culture.

Results and Discussion

Overall, 25 (42%) of the 60 i.t.-infected donor Cx. tarsalis blood fed and were detected either dead or alive after the 2-wk holding period (Table 1). Of the 10 living donor females, six expectorated from 1.1 to 3.8 log10 PFU of WNV. These females plus the extra i.t.-infected females with positive salvia samples (n = 16) expectorated 2.5 ± 0.53 (mean ± 95% CI; range 1.3–4.4) log10 PFU of WNV.

When bled 30–45 min after the mosquito blood-feeding period, five of six pinches had RNA (32.1 ± 0.86 \[mean ± SE Ct\]; range 30.7–32.2) and infectious virus (2.9 ± 0.93 \[mean ± SE viremia\]; range 2.3–4.2, log10 PFU/ml) in blood samples collected by jugular venipuncture. Only these five pinches subsequently became viremic through virus propagation (Fig. 1). No engorged i.t.-infected donor Cx. tarsalis were recovered from the cage with the negative house finch, and all recipient mosquitoes tested negative. Although pinches were not weighed, field-collected pinches in California reportedly weigh from 20 to 23 g (Partin 1933). If blood volume is similar to other animals their size (≈5.5 ml/100 g), then total blood volume would be <1.2 ml per finch. Adjusting for volume, pinches may have contained from 240 to 19,020 PFU of WNV immediately after mosquitoes fed on them. One of two mosquitoes tested in group 2 expectorated 4.4 log10 PFU (25,118 PFU), which was greater than the estimated virus concentration in the pinches. Therefore, highly infectious mosquitoes transmitting virus to small hosts seemed able to create an immediate “viremia” similar to the artificial bloodmeals created by inoculating virus directly into the circulatory system (Weaver et al. 1991, Mahmood et al. 2004). This mechanism also may have resulted in the “nsviremic transmission” reported previously using the laboratory mouse host system (Higgs et al. 2005), because the Sebring colony of Cx. quinquefasciatus they used expectorated on average 4.5 log10 PFU of WNV at 14 d postinfection (Vanlindingham et al. 2004).

Of 400 recipient Cx. quinquefasciatus and Cx. tarsalis, 230 (58%) blood fed and survived the subsequent 2-wk incubation period, of which four (1.7%) were infected and one (0.4%) expectorated virus (Table 1). Recipient infection was detected only in the two groups that fed on pinches with the highest immediate viremias, consistent with a positive relationship between the quantity of virus expectorated by donor mosquitoes and the efficiency of infection of the recipient females. Three of four positive were Cx. quinquefasciatus, thereby documenting interspecific transmission (Table 2). The positive Cx. tarsalis had a high Ct score of 35, and WNV was not detected by plaque assay. Although Cx. tarsalis are more susceptible to oral infection with WNV than Cx. quinquefasciatus (Reisen et al. 2005), few females of either species became infected at doses similar to the “viremias” created by donor females in the current experiment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Culex</th>
<th>Ct</th>
<th>Log10 PFU Body</th>
<th>Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>quinquefasciatus</td>
<td>32</td>
<td>2.6</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>2</td>
<td>quinquefasciatus</td>
<td>29</td>
<td>4.1</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>2</td>
<td>quinquefasciatus</td>
<td>27</td>
<td>4.2</td>
<td>1.6</td>
</tr>
<tr>
<td>5</td>
<td>tarsalis</td>
<td>35</td>
<td>&lt;1.3</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

Threshold of virus detection in bodies and expectorate (Exp.) was 1.5 and 0.5 log10 PFU.

Our experiment confirmed previous observations that a low percentage of uninfected Culex mosquitoes concurrently feeding with infectious females on small vertebrate hosts become infected (Higgs et al. 2005). The percentage of positive recipient females in our experiment was low, partly because we did not determine when the donor females commenced blood feeding. It is likely some of the recipient females engorged before the initiation of blood feeding by the donor females or before virus was distributed throughout the finch circulatory system. Higgs et al. (2005) did not allow recipient females to blood feed until after the donor females commenced feeding, most likely enhancing transmission to recipient mosquitoes. Our experiment also showed that some NVT recipient mosquitoes were capable of transmission. Higgs et al. (2005) did not measure transmission rates, but they inferred this was possible due to the high virus titers in infected females.

In summary, a low proportion of recipient female mosquitoes may become infected when cofeeding with infectious females before virus propagation in the vertebrate host. This nonpropagative transfer through the circulatory system is dependent upon 1) the quantity of virus expectorated by donor mosquitoes, 2) the blood volume of the vertebrate host, and 3) the oral susceptibility of the recipient mosquitoes. Our results suggest that females transferring >4 log10 PFU into a vertebrate host with a limited blood volume (here ≈1 ml) produce a viremia of sufficient titer to infect cofeeding females. The ability to infect recipient females is contingent upon the probability that the recipient female encounter sufficient virions within the bloodmeal to initiate a mesenteronal infection (Lord et al. 2006). Our mosquitoes expectorated WNV directly into the circulatory system (rather than into the skin or pool feeding), and virus was detectible in the blood within 30–45 min after feeding.

If efficiency of recipient infection is related to the volume of virus in the vertebrate host circulatory system, then host blood volume is critical. Both the current and previous (Higgs et al. 2005) studies used small hosts (weight ≈20–25 g) with a total blood volume of ≈1.0–1.5 ml. Smaller hosts such as passeriform nestlings would have a smaller blood volume and potentially a higher concentration of virus, whereas progressively larger hosts such as American crows would circulate progressively less virus per milliliter of blood. The suggestion that large hosts
such as horses that do not produce an elevated viremia or that have been vaccinated would now be important in WNV transmission (Higgs et al. 2005) needs substantiation by further experimentation to assess the significance of both NVT and nonpropagative transfer.

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