Susceptibility of Fox Squirrels (Sciurus niger) to West Nile Virus by Oral Exposure

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Abstract

Fox squirrels (*Sciurus niger*) (five of eight) were infected with West Nile virus (WNV) when challenged by the oral route with $10^{2.3}$ or $10^{3.4}$ plaque forming units (PFU). The mean maximum serum WNV titer of infected fox squirrels was $10^{5.1}$ PFU/mL and ranged from $10^{4.6}$ to $10^{5.6}$ PFU/mL. These levels of viremia are infectious for several mosquito vectors of WNV. This virus was also isolated from swabs of the oral and rectal cavities, and urine swabs between day 5 and 9 postexposure (p.e.) in amounts as high as $10^{2.0}$, $10^{2.8}$, and $10^{2.0}$ PFU, respectively. WNV RNA was detected in salivary gland and/or kidney tissue of three squirrels between day 65 and 72 p.e. in the presence of WNV neutralizing antibody, suggesting that long-term persistent infection occurs in fox squirrels. These observations justify further studies to determine if nonarthropod transmission and long-term persistent infection occur naturally in fox squirrels and contribute to trans-seasonal maintenance of WNV.

Key Words: Fox squirrels (*Sciurus niger*)—Oral exposure—West Nile virus.

Fox squirrels (*Sciurus niger*) can develop West Nile virus (WNV) serum titers sufficient to infect several species of mosquitoes that subsequently have the potential to transmit the virus to birds, humans, and other vertebrates (Root et al. 2006, Fadgett et al. 2007, Platt et al. 2008). Thus, fox squirrels could have a role in the epidemiology of WNV, especially in peri-domestic settings. Observations made by Root et al. (2005) in areas of high WNV activity revealed that fox squirrels are commonly exposed to WNV. For example 49% of 53 fox squirrels representing six different locations were seropositive for WNV. This observation is consistent with the fact that fox squirrels share common habitats with birds and are exposed to both Aedes and Culex WNV vectors. Our previous study (Platt et al. 2008) also suggested that fox squirrels can be persistently infected with WNV, as the virus was recovered on days 17 and 22 post exposure (p.e.) from urine and oral swabs of two different squirrels with WNV-neutralizing antibody. WNV RNA was also detected in kidney tissue collected from a third squirrel on day 29 p.e. Persistent infection (PI) with WNV has been demonstrated in golden hamsters (*Mesocricetus auratus*) and rhesus macaques (*Macaca mulatta*). Tesh et al. (2005) demonstrated WNV in hamster urine for up to 247 days p.e. Pogodina et al. (1983) isolated WNV from organs of rhesus monkeys, including the kidney for up to 161 days p.e. As such, it is conceivable that similar long-term PI might occur in fox squirrels. If so, long-term PI of squirrels could be a mechanism for trans-seasonal WNV maintenance if periodic WNV shedding occurred in amounts sufficient to infect WNV-antibody-free squirrels by close contact, including the oral route. While fox squirrels are not considered gregarious mammals, their normal behavior would facilitate nonarthropod transmission of shed virus. For example, it is not uncommon for fox squirrels to share the same tree-hole cavity during winter months. There is also extensive interaction among males during the breeding season which in northern temperate latitudes generally occurs between the

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middle of December through June with peak activity in December/January and June. Females generally produce two litters of two to five individuals a year with the majority of births occurring in early spring and mid-summer (Flyger and Gates 1982). Thus, the purpose of this study was to establish whether fox squirrels are susceptible to WNV infection per os.

Two separate experiments utilizing five squirrels each were conducted to determine the infection rates, shedding patterns, and viremia profiles after oral exposure to WNV. Fox squirrels were live-trapped in Story County, IA, in the spring of 2007 and housed in Biosafety Level 3 animal facilities at Iowa State University (ISU). Squirrels were chemically restrained with ketamine/acepromazine given intramuscularly, initially at 0.44:0.44 mg/kg. This dose was subsequently adjusted for individual squirrels to provide restraint without abolishing the pharyngeal reflex. Virus inoculum was WNV strain IA02-crow (Erickson et al. 2006) in 0.5 mL of CO₂-independent medium (Invitrogen, Carlsbad, CA) containing 1% fetal bovine serum. The inoculum was delivered slowly to permit swallowing. Five squirrels were challenged with $10^{2.3}$ plaque forming units (PFU) of WNV, and the other five were challenged with $10^{2.4}$ PFU of WNV. These virus doses were chosen because these amounts of WNV are frequently recovered from swabs of the oral cavity, rectum, and urine swabs of acutely infected squirrels (Root et al. 2006, Platt et al. 2008). Blood and swabs of the oral cavity, rectum, and urine, when possible, were collected as previously described (Platt et al. 2008) from all squirrels before inoculation and on alternate days beginning on day 1 or 2 p.e., continuing through day 9 p.e., and intermittently thereafter including the day of death. All samples were maintained at 4°C after collection until stored at −70°C. Swab and serum samples were tested for WNV by virus isolation in African Green Monkey Kidney (Vero) cells. Virus titers were measured by plaque assay, and serum antibody titers to WNV were determined by the plaque reduction neutralization test 90 (PRNT90) expressed as the reciprocal of the final serum dilution that reduced plaque counts by 90%; mean maximum serum WNV titer for the five viremic squirrels was $10^{3.3}$ PFU/mL and ranged from $10^{2.8}$ to $10^{5.6}$ PFU/mL.

WNV was recovered between day 5 and 9 p.e. from swabs of the oral cavity, and rectum of four squirrels, and from urine swabs of two of these four squirrels. The maximum amount of WNV recovered from these swabs was $10^{2.0}, 10^{2.8},$ and $10^{7.3}$ PFU, respectively. This period of shedding was consistent with the shedding pattern of fox squirrels infected with WNV by needle or mosquito bite observed earlier (Platt et al. 2008), except that the onset of shedding in orally infected squirrels occurred approximately 2 days later. WNV RNA was detected by real-time RT-PCR in tissues of the kidney (two squirrels) and salivary gland (three squirrels) between day 65 and 72 p.e. Histological lesions or WNV antigen were not detected in any salivary gland tissue from WNV-infected squirrels with the exception of squirrel 4, which died on day 17 p.e. Fresh salivary gland and kidney tissues from these squirrels were also assayed for WNV RNA, because salivary gland and kidney tissue are likely sources of potential virus shedding. These tissues were stored at −70°C in RNAlater (Ambion, Austin, TX) until processed for total RNA extraction using TRIzol LS Reagent (Invitrogen) following the manufacturer’s protocol. Real-time RT-PCR was conducted by the Veterinary Diagnostic Laboratory on these tissues using a standardized protocol based on the method described by Lanciotti et al. (2000). All protocols were performed in accordance with the ISU Institutional Animal Care and Use Committee. The response of fox squirrels to oral exposure to WNV is summarized in Table 1. Briefly, five squirrels developed WNV viremias and three failed to become viremic. Two squirrels died without a detectable viremia on day 4 p.e. during sampling and were subsequently excluded from the study. The typical viremia pattern of fox squirrel WNV infection was $10^{3.1}$ PFU/mL and ranged from $10^{2.8}$ to $10^{5.6}$ PFU/mL. WNV was recovered between days 5 and 9 p.e. from swabs of the oral cavity, and rectum of four squirrels, and from urine swabs of two of these four squirrels. The maximum amount of WNV recovered from these swabs was $10^{2.0}, 10^{2.8},$ and $10^{7.3}$ PFU, respectively. This period of shedding was consistent with the shedding pattern of fox squirrels infected with WNV by needle or mosquito bite observed earlier (Platt et al. 2008), except that the onset of shedding in orally infected squirrels occurred approximately 2 days later. WNV RNA was detected by real-time RT-PCR in tissues of the kidney (two squirrels) and salivary gland (three squirrels) between days 65 and 72 p.e. Histological lesions or WNV antigen were not detected in any salivary gland tissue from WNV-infected squirrels, which died on day 17 p.e. (Table 1). WNV was not isolated from internal organs of any squirrel.

Fox squirrels are susceptible to oral infection by amounts of WNV that are commonly recovered from the oral cavity, rectum, and urine of squirrels with active infections. More importantly, the WNV serum titers observed in orally infected squirrels (Table 1) are sufficient to infect several competent

**Table 1. Response of Fox Squirrels (Sciurus niger) to Per Os Exposure to WNV**

<table>
<thead>
<tr>
<th>Squirrel no.</th>
<th>WNV challenge dose*</th>
<th>Day of death</th>
<th>PRNT90 titer on death day</th>
<th>Detection of WNV RNA</th>
<th>Serum WNV titer by day postexposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kidney</td>
<td>1 2 3 4 5 6 7 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Salivary gland</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.4</td>
<td>78</td>
<td>0</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>3.4</td>
<td>72</td>
<td>60</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>3.4</td>
<td>65</td>
<td>320</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>4a</td>
<td>3.4</td>
<td>17</td>
<td>&lt;10</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>3.4</td>
<td>58</td>
<td>160</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>2.3</td>
<td>55</td>
<td>0</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>2.3</td>
<td>70</td>
<td>160</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>2.3</td>
<td>69</td>
<td>0</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

*Log$_{10}$ PFU/mL delivered in 0.5 mL of CO₂-independent cell culture medium.

Histological findings were mild multifocal gliosis and satellitosis in the brain and spinal cord, moderate multifocal lymphoplasmacytic and histiocytic interstitial nephritis, and mild multifocal lymphoplasmacytic hepatitis.

PRNT$_{90}$ plaque reduction neutralization titer$_{90}$ expressed as the reciprocal of the final serum dilution that reduced plaque counts by 90%; _, not done.
species of Culex and Aedes mosquitoes (Sardelis et al. 2001, Turell et al. 2001, Goddard et al. 2002, Tiawsirisup et al. 2008). It is important to note that the levels of WNV viremia that occurred in these orally infected squirrels may have been greater than observed because of sampling constraints. Although the detection of WNV RNA in kidney and salivary gland tissue at 65 to 72 days p.e. in the absence of infectious virus is not proof of PI, it is suggestive and justifies further studies to determine if long-term PI with periodic shedding occurs in fox squirrels.

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Disclosure Statement

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