West Nile Virus Transmission by Ticks: An Oddity or Unexplored Reality?

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1 Introduction

West Nile virus (WNV) was first identified in 1937 in Uganda in eastern Africa (Smithburn, et al., 1940), and subsequently associated with major outbreaks of the disease in Africa, Eurasia, Australia and the Middle East. In the 1990s outbreaks began to be reported in both Southern and Northern Europe often associated with more severe disease than seen previously including viral encephalitis and neurological symptoms (Hubalek, et al., 1999). The first report of an outbreak within the Western hemisphere occurred in 1999 in New York City and resulted in human, equine and avian mortalities (Lanciotti, et al., 1999). Since this time WNV outbreaks have been reported in all the states of the US, as well as Canada, Central and South America, and parts of the Caribbean (Aiken, 2003; 2002a; Kramer, et al., 2008). The latest statistics on US WNV infections can be found at the dedicated Center for Disease Control website: (http://www.cdc.gov/ncidod/dvbid/westnile/index.htm).

WNV is a member of the genus Flavivirus that contains over seventy identified viruses, most of which are vectored by mosquitoes or ticks, although a few have no known vectors (Monath, et al., 1996). The virus has been isolated from 60 species of mosquito in the US (Centers for Disease Control and Prevention, 2002b; Hayes, et al., 2005), Culex pipiens being the most important (Campbell, et al., 2002; Donaldson, 1966). However, WNV has also been repeatedly isolated from hard (ixodid) and soft (argasid) tick species in endemic regions of Europe, Africa and Asia (Hoogstraal, 1972; Hoogstraal, et al., 1976; Iakimenko, et al., 1991; L’Vov, et al., 2002; Lvov, et al., 1975; Mathiot, et al., 1990; Platonov, 2001). Ticks rank second only to mosquitoes in their importance as vectors of human pathogens and transmit a greater variety of infectious agents than any other arthropod group (Sonenshine, 1991). Current strategies to control WNV in the US are largely based upon measures to avoid exposure and to control vector species, but at present only mosquito species are targeted by government surveillance and preventative control programmes (http://www.cdc.gov/ncidod/dvbid/westnile/index.htm).

The epidemiology of arthropod-borne diseases is a function of both ecological and physiological parameters, of which vector competency is arguably the most important (Sonenshine, et al., 1994). Vector competency, the ability of biting arthropods to acquire, maintain and transmit infectious agents from reservoir hosts to susceptible hosts, is determined by extrinsic and intrinsic factors including fundamentally, the physiological ability of vector tissue to become infected and to maintain a particular infectious agent (Lane, 1994).

We therefore set out to test whether or not ticks were competent vectors for WNV firstly by testing the physiological ability of tick tissue to become infected with WNV and to maintain the virus. The susceptibility of a cell line derived from a particular arthropod vector to infection by a specific infectious agent can provide information about the determinants of virus transmission and viral persistence in the natural environment (Mussgay, et al., 1975). We undertook a comparative study of the susceptibility of mammalian Vero cells, a clonal mosquito cell line (C6/36) and cell lines derived from the ticks Ixodes ricinus (L.) (IRE/CTVM18), I. scapularis (Say) (ISE6), Rhipicephalus appendiculatus (Neumann) (RAE/CTVM1) and Amblyomma variegatum (Fabricius) (AVL/CTVM17) to infection with twelve flaviviruses, including WNV, using immunofluorescence microscopy and plaque assay techniques.

Secondly we assessed the ability of both ixodid (I. ricinus) and argasid tick species (Ornithodoros moubata) ticks to acquire WNV through feeding, maintain the virus through molting (transstadial transmission) and egg production (transovarial transmission), and finally the ability to infect hosts and other ticks.
In this chapter we describe the results of these experiments and discuss some of the consequential experimental data that has arisen since, within the context of assessing whether or not ticks really are important vectors of WNV. The following experimental details were taken in part from previously published research (Lawrie, et al., 2004a; Lawrie, et al., 2004b).

2 Materials and Methods

2.1 Viruses, Cell Lines and Infection Procedures

Twelve flaviviruses (Table 1) were used to assess the susceptibility of mammalian (Vero), mosquito (C6/36) and tick cells (IRE/CTVM18; ISE6; RAE/CTVM1; AVL/CTVM17) to infection and measure persistence of infection. The origin of these viruses, and of the cell lines used in these experiments are described in detail in reference (Lawrie, et al., 2004a). In particular, WNV (tick strain), was isolated from ticks feeding on an unidentified bird in the Astrakhan region of the former Soviet Union and had undergone four passes in newborn mouse brain (Lvov, et al., 1975), and Dr Robert Shope of the University of Texas kindly provided WNV (NY99 strain), that had undergone three passes in newborn mouse brain (Lanciotti, et al., 1999). High titer mouse brain suspension stocks of WNV (NY99 strain) (2.9 x 10^7 pfu ml^-1) were diluted in PBS (pH 7.2) to a concentration of 10^5 pfu ml^-1 before use in animal transmission studies.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Virus</th>
<th>Vero</th>
<th>C6/36</th>
<th>RAE/CTVM1</th>
<th>ISE6</th>
<th>IRE/CTVM18</th>
<th>AVL/CTVM17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mosquito</td>
<td>Yellow fever virus</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dengue-2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>St. Louis encephalitis virus</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>West Nile Virus (NY99 strain)</td>
<td>+</td>
<td>+</td>
<td>+^a</td>
<td>+^a</td>
<td>+^a</td>
<td>+^a</td>
</tr>
<tr>
<td></td>
<td>West Nile Virus (tick strain)</td>
<td>+</td>
<td>+</td>
<td>+^a</td>
<td>+^a</td>
<td>+^a</td>
<td>+^a</td>
</tr>
<tr>
<td>Tick</td>
<td>Tick-borne encephalitis virus</td>
<td>+</td>
<td>-</td>
<td>+^a</td>
<td>+^a</td>
<td>+^a</td>
<td>+^a</td>
</tr>
<tr>
<td></td>
<td>Powassan virus</td>
<td>+</td>
<td>-</td>
<td>+^a</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Louping-ill virus (Inverness strain)</td>
<td>+</td>
<td>-</td>
<td>+^a</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Louping-ill virus (Loch strain)</td>
<td>+</td>
<td>-</td>
<td>+^a</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Negishi virus</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>No known vector</td>
<td>Apoi virus</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Modoc virus</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

^aViruses were subcultured three times. ND, not done.

Table 1: Susceptibility of Vero, C6/36 (mosquito) and the tick cell lines RAE/CTVM1, ISE6, IRE/CTVM18 and AVL/CTVM17 to flavivirus infection as measured by immunofluorescence assay (IFA). Viruses were subcultured at least once in the appropriate cell line before IFA unless indicated.
Table 2: Titration of WNV viruses by plaque assay in PS cells after culture in C6/36 or RAE/CTVM1 cells. Titers are expressed as the mean (n=3) pfu ml⁻¹ of recovered media (4 ml total).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Subculture 1</th>
<th>Subculture 2</th>
<th>Subculture 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6/36</td>
<td>7.1 x 10⁴</td>
<td>8.2 x 10⁴</td>
<td>1.4 x 10⁵</td>
</tr>
<tr>
<td>RAE/CTVM1</td>
<td>2.4 x 10⁴</td>
<td>3.0 x 10⁴</td>
<td>6.0 x 10⁴</td>
</tr>
</tbody>
</table>

All cells were grown in L-15 (Leibovitz) medium supplemented with fetal calf serum. Vero cells and AVL/CTVM17 cells were grown at 37°C; IRE/CTVM18, RAE/CTVM1 and C6/36 cell lines were grown at 28°C; and the ISE6 cell line was grown at 30°C. Approximately 4 x 10⁵ cells were infected with 10⁴ pfu (MOI of 0.03) of a particular virus or 100 µl of harvested infectious cell-free culture supernatant. For transmission studies, samples of tick (or mouse brain) homogenate (100 µl) were used to infect 2 x 10⁶ C6/36 cells. The cultures were incubated at the appropriate temperature for 3 days, after which coverslips were removed and fixed in cold acetone before being examined by immunofluorescence microscopy (IFA).

2.2 Ticks

We tested a hard tick species, *I. ricinus* and a soft tick species, *O. moubata* for their vector competence with WNV (NY99 strain). These species are not native to the US and were chosen mainly for their availability. *O. moubata* ticks were considered potential vectors for the Eg101 strain of WNV in a study by Whitman and Aitken in 1960 (Whitman, *et al.*, 1960). *I. ricinus* ticks are the primary vectors of *Borrelia burgdorferi*, the aetiological agent of Lyme disease in Europe and important vectors of the flaviviruses *Tick-borne encephalitis virus* (TBEV) and *Louping-ill virus* (LIV) (Sonenshine, *et al.*, 1994).

Ticks were taken from colonies reared and maintained for many generations at CEH Oxford according to standard methods (Jones, *et al.*, 1988). Colony ticks were WNV negative by RT-PCR testing (15 individuals tested of each species).

2.3 Tick Infection and Co-feeding Transmission Experiments

Seven groups of six BALB/c mice (female, 4-6 weeks old) were inoculated by sub-cutaneous injection with 10⁴ pfu of WNV. Three of the mice were bled daily from the tail to follow the course of viremia by plaque assay. Two groups of mice were infested with *I. ricinus* nymphs (twenty per mouse); one group was infested three days prior to inoculation, the other four days after inoculation (Swallow, 1985). The other five groups of mice were infested with 2nd instar *O. moubata* ticks (ten per mouse) on either the same day (day 0), or 1, 2, 3, or 4 days after inoculation (Table 3). After the initial experiment, and in order to increase the number of positive ticks available for experimentation, a further twelve mice were infested with *O. moubata* two days after inoculation with WNV.

Ticks housed in gauze-covered neoprene feeding chambers on mice (Jones, *et al.*, 1988) were removed when fully engorged; 24 hours after infestation in the case of *O. moubata* ticks and six days after infestation in the case of *I. ricinus* nymphs. The engorged ticks were then stored at 20°C in KCl saturated desiccators until testing for the presence of WNV or until ready for a further bloodmeal (duration of storage indicated in Table 3). After storage, the ticks (pools and individuals) were homogenised in 500 µl of PBS using plastic homogenisers under sterile conditions. The homogenates were frozen and stored
<table>
<thead>
<tr>
<th>Species</th>
<th>Developmental stage</th>
<th>Days from inoculation to infestation</th>
<th>Days after engorgement&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IFA&lt;sup&gt;b&lt;/sup&gt; (±/--)</th>
<th>RT-PCR&lt;sup&gt;c&lt;/sup&gt; (no. positive/no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;i&gt;O. moubata&lt;/i&gt;</td>
<td>0</td>
<td>1, 2, 7</td>
<td>- (8)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; bloodmeal (infected mice)</td>
<td>1</td>
<td>1, 2, 3, 4, 5, 6, 7</td>
<td>- (5)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1, 2, 3, 4, 5, 6, 7, 14</td>
<td>+ (5)</td>
<td>+ (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1, 2, 3, 4, 5, 6, 7, 14</td>
<td>+ (5)</td>
<td>+ (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1, 3, 7</td>
<td>- (5)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; instar</td>
<td>2</td>
<td>22</td>
<td>+ (5)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>22</td>
<td>+ (5)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; bloodmeal (uninfected mice)</td>
<td>2</td>
<td>132</td>
<td>+ (5)</td>
<td>7/14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; instar</td>
<td>2</td>
<td>60 (3)</td>
<td>+ (5)</td>
<td>+ (5)</td>
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<td></td>
<td>4&lt;sup&gt;th&lt;/sup&gt; instar</td>
<td>2</td>
<td>75 (25)</td>
<td>+ (5)</td>
<td>+ (5)</td>
</tr>
<tr>
<td>Uninfected co-fed</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; instar</td>
<td>N/A</td>
<td>5</td>
<td>ND</td>
<td>15/66</td>
</tr>
<tr>
<td>&lt;i&gt;O. moubata&lt;/i&gt;  ticks</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; instar</td>
<td>N/A</td>
<td>45</td>
<td>ND</td>
<td>4/15</td>
</tr>
<tr>
<td>&lt;i&gt;I. ricinus&lt;/i&gt;</td>
<td>Nymph</td>
<td>4</td>
<td>2</td>
<td>ND</td>
<td>0/12</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; bloodmeal (infected mice)</td>
<td>-3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>2</td>
<td>ND</td>
<td>0/25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>30</td>
<td>ND</td>
<td>0/25</td>
<td></td>
</tr>
<tr>
<td>BALB/c mice&lt;sup&gt;e&lt;/sup&gt;</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>- (1)</td>
<td>1/17</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of days after the ticks had completed feeding on inoculated mice at which the ticks were tested for virus infection. Where indicated brackets depict those ticks that had fed a 2<sup>nd</sup> time and the number of days after which the ticks were tested.

<sup>b</sup> Tick homogenate samples were scored positive if >10% of inoculated C6/36 cells showed specific fluorescence with both 813 and 546 monoclonal antibodies. Numbers of ticks in each pool are shown in parenthesis.

<sup>c</sup> Where indicated by +, pools of ticks were tested; numbers of ticks in each pool are shown in parenthesis. ND, not done.

<sup>d</sup> <i>I. ricinus</i> ticks depicted -3 were attached to hosts 3 days prior to inoculation with WNV.

<sup>e</sup> Mice were infested with infected <i>O. moubata</i> ticks and after 14 days were sacrificed and the brain homogenates tested by IFA and RT-PCR. N/A, not applicable.

**Table 3:** Results of immunofluorescence assay (IFA) and/or nested RT-PCR from <i>O. moubata</i> and <i>I. ricinus</i> ticks fed either on WNV inoculated BALB/c mice or non-infected mice (co-fed ticks).

at -70°C until analysed. Tick homogenates were assayed for infectious virus antigen (by immunofluorescence assay) and/or viral RNA (by RT-PCR).

Co-feeding transmission experiments were carried out by infesting clean BALB/c mice (n=7 (Harlan, UK)) with ten 3rd instar <i>O. moubata</i> ticks 57 days after they had taken an infectious bloodmeal, and ten uninfected ticks (2nd instar) in separate feeding chambers. The two feeding chambers were separated by at least 1 cm. Co-feeding experiments lasted 15 days.

To investigate tick to host transmission ten BALB/c mice were infested with cohorts of five, ten, fifteen or twenty 3rd instar <i>O. moubata</i> ticks 57 days after an infectious bloodmeal. Fifteen days after
infestation, the mice (including those used for co-feeding) were euthanised and the brains removed. The brains were homogenised in 1 ml of sterile PBS and stored at -70°C until they were tested for WNV.

2.4 Immunofluorescence Assays (IFA)

Indirect immunofluorescence assays (IFA) were carried out on fixed cells as described previously (E. A. Gould, et al., 1985a). Cells were treated with a monoclonal antibody (mAb813) that is specific for the E-protein of flaviviruses and is broadly cross-reactive (E. A. Gould, et al., 1985a). WNV infected cells were additionally probed with WNV-specific monoclonal antibody (mAb 546) (E.A. Gould, et al., 1990). Goat anti-mouse polyvalent antiserum conjugated with fluorescein-isothiocyanate (Sigma) was used as a secondary antibody. Incubation times for each antibody were 1 hr at 37°C. Washes (x3) were carried out in PBS at room temperature. Infected cells were visualized using an Olympus epifluorescence microscope and photographs taken using a Nikon epifluorescence microscope and camera. Uninfected cultures of each cell line were used as negative controls and appropriately infected Vero cells were used as positive controls. Examples of typically infected cells are shown in Figure 1. Cell cultures were designated infected if more than 5% of cells showed specific fluorescence (Table 1). Unless indicated, IFA were carried out on cells from the second sub-culture. Tick samples were deemed positive when more than 10% of the cells showed specific fluorescence with both monoclonal antibodies (Table 3).

2.5 Virus Titration by Plaque Assay

The porcine kidney cell line PS was grown in Eagles Minimal Essential Medium containing 7% fetal calf serum and plaque assays were carried out in 24-well plates using a 1.5% CMC overlay as described previously (E.A. Gould, et al., 1985b). Cells were incubated at 37°C (+5% CO₂) for 4 days and the plates were then stained with naphthalene black.

2.6 Nested RT-PCR Assay

RNA was extracted from homogenised samples (100 µl) using RNAGents total RNA extraction kit in accordance with the manufacturer’s instructions (Promega). cDNA synthesis was carried out with SuperScript II reverse transcriptase (Invitrogen) and 3'(1) primer (primer sequences can be found at (Lawrie, et al., 2004a)) for 50 minutes at 42°C, in a total volume of 20 µl. PCR was carried out on the cDNA (1µl) using 5'(1) and 3'(1) primers. Nested PCR was carried out on 1 µl of the first-round PCR product using the nested primers 5'(2) and 3'(2). All PCR reactions were carried out in a 50 µl volume with REDTaq DNA polymerase (Sigma). A Hybaid Touchdown thermal cycler was used with the following programme: 94.5 °C for 1min, thirty cycles of 94 °C for 40s, 56°C for 1 min and 72°C for 1 min followed by a final extension step of 72°C for 10 mins. Viral stock, RNA extracted from uninfected ticks and PBS only containing samples were used as control reactions. Positive samples gave a PCR product of about 1.2 kbp. This method could detect RNA from a viral stock equivalent of 9 pfu (data not shown).

To confirm the identity of RT-PCR products, PCR products were gel purified using QIAquick (Qiagen) columns in accordance with manufacturer’s instructions. The purified DNA was sequenced with an ABI automatic sequencer and the nested primers 5'(2) and 3'(1), and a primer based upon the internal sequence of the E gene of WNV (not shown).
Results

3.1 Physiological Competence of Tick Cells for WNV infection

The mosquito cell line C6/36 showed no signs of infection by the tick-borne viruses tested, or by Apoi virus or Modoc virus, that have no known vector. By contrast, C6/36 cells were positive for viral antigen after infection with the mosquito-borne flaviviruses (Table 1). The tick cell lines RAE/CTVM1, IRE/CTVM18, AVL/CTVM17 and ISE6 were tested for their susceptibility to infection with the viruses indicated in Table 1. All the different tick cell lines tested were susceptible to infection by the same viruses. Moreover, there was no appreciable difference in the cytoplasmic distribution of viral antigen in infected cells between either individual cell lines or the different viruses tested (Figure 1).

Figure 1: Examples of mosquito and tick cells infected with WNV (NY-99 strain). Cells were visualized using a Nikon epifluorescence microscope with FITC filter. Magnification 20x. Reproduced from Lawrie et al (2004) Med. Vet. Entomol., 18(3), 268-274.
All tick cell lines were susceptible to infection by the tick-borne flaviviruses but not the mosquito-borne flaviviruses, with the exception of WNV (both strains). The mean titer of recovered WNV (NY99) sub-cultured three times in RAE/CTVM1 cells was $4.1 \times 10^4$ pfu ml$^{-1}$ (Table 2). Examples of RAE/CTVM1, IRE/CTVM18, AVL/CTVM17 and ISE6 cells infected with WNV (NY99) and immunoprobed with mAb813 are shown in Figure 1.

3.2 Host to Tick Transmission

BALB/c mice inoculated with WNV were weakly viremic, two and three days after injection, with mean titers of $6 \times 10^3$ and $3 \times 10^3$ pfu ml$^{-1}$ blood respectively. After four days, viremia was no longer detectable by plaque assay although the mice developed severe neurological symptoms after five or six days and were humanely killed. *O. moubata* ticks that had fed on mice on days corresponding to the viremic period (i.e. days 2 and 3 after inoculation), but not those fed outside this period, contained viral antigen as measured by IFA (Table 3). Two days after engorgement, 17% (n=12) *I. ricinus* ticks that started to feed on hosts three days prior to WNV inoculation, but not those that had started to feed four days after inoculation, were positive for WNV RNA. When the former group of ticks was tested 28 days later no evidence of infection was found. Infected *O. moubata* ticks in contrast maintained the virus after having molted into the next instar (i.e. 3rd instar), following a second non-infectious bloodmeal and after having molted for a second time into 4th instars. Fifty percent of the individual ticks (n=14) tested by RT-PCR were positive for WNV RNA when examined 132 days after the initial infectious bloodmeal.

3.3 Co-feeding Transmission

Five days post-engorgement, 23% (n=66) of uninfected 2nd instar *O. moubata* ticks that had co-fed with infected cohorts of 3rd instar ticks on non-infected mice were positive for WNV RNA (Table 3). The remaining unfed ticks (n=15) were tested after they had molted into 3rd instars, 45-days after co-feeding. Four of these ticks (27%) were positive for WNV RNA. The identities of the PCR products obtained from three positive samples were confirmed by sequence analysis.

3.4 Tick to Host Transmission

Infected cohorts of *O. moubata* ticks (3rd instar) were fed on uninfected mice in order to investigate tick to host transmission. Of the seventeen uninfected mice used (including mice used in co-feeding experiments), none showed clinical signs of infection. One of the brains tested, from a mouse infested with an infected cohort of 20 ticks, was positive by RT-PCR but negative when tested by IFA (Table 3). The PCR product was sequenced to confirm the identity of WNV.

4 Discussion

The distinction between mosquito-borne and tick-borne flaviviruses was initially based upon immunological and ecological relationships (Gould *et al.*, 1985; Gould *et al.*, 1990). More recently, phylogenetic analysis has confirmed this classification and revealed the existence of mosquito-borne, tick-borne and NKV clades within the Flavivirus genus (Gaunt *et al.*, 2001). Arthropod primary cell and tissue cultures, and subsequently continuous cell lines have proven to be valuable tools in the study of arboviruses (Mussgay *et al.*, 1975; Pudney, 1987; Singh, 1972). However, relatively little is known of the susceptibil-
ility of tick cell lines to arbovirus infection as until recently few tick cell lines were available. This study and others show that in general the ability of a particular flavivirus to infect either mosquito or tick cells, or in the case of NKV viruses neither cell types, reflects this classification. Nevertheless, despite the evolutionary distance between the mosquito-borne and tick-borne virus lineages, it is clear that some flaviviruses such as WNV can infect and replicate in both mosquito and tick cell lines.

Although WNV is phylogenetically and serologically defined as a mosquito-borne flavivirus (Gaunt et al., 2001), our results demonstrated that WNV can infect and maintain infection in tick-derived cell lines. These results are consistent with previous studies of R. appendiculatus, Boophilus microplus (Canestrini) and D. parumapertus (Neumann) cell lines (Pudney, 1987; Varma, et al., 1975), although a Haemaphysalis spinigera (Neumann) derived cell line tested by Banerjee et al was not susceptible to WNV infection (Banerjee, et al., 1977). So in other words it appears that unlike other mosquito-borne flaviviruses, there is no intrinsic cellular barrier to infection of tick-derived tissue by WNV.

Next we tested the transmission ability of ticks as vectors for WNV. Our study demonstrated that both I. ricinus and O. moubata ticks become infected with WNV (NY99 strain) through feeding upon virus-inoculated rodent hosts, but only when these hosts were viremic (i.e. systemic transmission). Thirty days after engorgement, however, we no longer found any evidence of WNV infection in the I. ricinus ticks. This suggests that this tick species does not support replication of the virus, and therefore is not a competent vector of WNV. Consequent research has shown similar results with other ixodid species. I. scapularis (the main US Lyme disease vector), A. americanum (L.), Dermacentor andersoni, and D. variabilis ticks were demonstrated to acquire WNV, and retain the virus transtadially, but were not able to transmit WNV back to the host (J.F. Anderson, et al., 2003b). More recently I. pacificus ticks fed on viremic song sparrows were also able to acquire WNV but unable to effectively transmit the virus to either birds or western fence lizards, the natural host species of this tick (Reisen, et al., 2007). Hyalomma marginatum ticks were fed on rabbits infected with WNV and all three developmental stages were found to acquire the virus, and to maintain it transstadially but not trans-ovarially (Formosinho, et al., 2006).

In contrast to I. ricinus ticks, we found that infected O. moubata ticks maintained infectious virus for at least 132 days (length of experiment) and WNV persisted transtadially through at least two developmental stages. Evidence for tick to host transmission of WNV was found in our study, although the level of infection observed was low (i.e. sub-clinical). Again these data are consistent with previous transmission studies carried out with soft tick species. Whitman & Aitken observed much higher levels of transmission from WNV-infected O. moubata ticks to day-old chicks than in our study, but only when very high feeding densities were used (49 ticks per chick) (Whitman, et al., 1960). O. maritimus and O. erraticus infected ticks were shown to transmit WNV to uninfected mice (Vermeil, et al., 1959, 1960), although O. savignyi and O. erraticus ticks did not (Hurlbut, 1956; Taylor, et al., 1956). An artificial membrane system was used to infect A. arboreus ticks, which were then able to transmit the virus to uninfected hosts (Abbassy, et al., 1994), and more recently Carios capensis ticks were shown to acquire and effectively transmit WNV to pekin ducklings (Anas domesticus) (Hutcheson, et al., 2005).

The transmission of flaviviruses such as Tick-borne encephalitis virus (TBEV) and Louping-ill virus (LIV) from infected to non-infected ixodid ticks through co-feeding on non-viraemic hosts (non-systemic transmission) is a well-established phenomenon (Randolph, et al., 1996). Indeed this mode of transmission is believed to play a significant role in the epidemiology of these diseases (Randolph, et al., 1999). Therefore we tested for co-feeding transmission of WNV between infected and uninfected O. moubata ticks. Over 22% of the uninfected ticks were positive for WNV RNA five days after co-feeding. A similar percentage of ticks were positive forty days later, after having molted to the next developmental
stage. As co-fed ticks were in contact with the mice for less than 24 hours, this strongly suggests that WNV was non-systemically transmitted between infected and uninfected ticks, as there was insufficient time for viremia to develop. Consistent with these findings consequent research by Higgs et al demonstrated that the primary vector of WNV, C. p. p. could also become infected through co-feeding on non-viremic hosts (Higgs, et al., 2005).

In summary, these data suggest that argasid (soft) but not ixodid (hard) tick species can mechanically acquire, maintain and transmit WNV. These data are however somewhat at odds with field collected records that describe the frequent isolation of WNV not only from soft ticks (Hoogstraal, 1972; Hoogstraal, et al., 1976; Lvov, et al., 1975), but also hard ticks including I. ricinus, I. lividus, R. turanicus, A. variegatum, H. marginatum, D. marginatus species (reviewed by Anderson et al., (J. F. Anderson, et al., 2003a). This discrepancy suggests that ixodid ticks are dead-end vectors for WNV transmission but can acquire infection naturally, most likely due to exposure to viremic hosts, although given the results of the experiments mentioned above it would be tempting to believe that co-feeding between ticks and vector-competent mosquito species might also occur.

So what about argasid (soft) ticks, are they likely to be important vectors of WNV? Like other arboviruses, WNV is naturally maintained by an enzootic cycle, consisting of C. p. p., the major vector species and passerine birds as the major amplifying hosts (Komar, et al., 2003). Although WNV infects a number of non-avian vertebrates, most mammals, including humans, appear to be dead-end hosts as they routinely fail to develop sufficient viraemia in order to re-infect a vector and hence continue the transmission cycle. Even though argasid ticks appear to have the ability to be physiologically competent vectors for WNV, in reality the low level of virus infection acquired by these ticks coupled with the fact that tick-to-host transmission appears to be very inefficient when compared to mosquito transmission (Hurlbut, 1956), suggests that ticks are unlikely to be important vectors of WNV. Furthermore, the relative scarcity of WNV isolation from field collected ticks suggest that whilst natural infections can occur, the frequency is insufficient to play a significant role in the natural transmission cycle of WNV. Additionally, in any case it is important to realise that argasid ticks are highly unlikely to represent a danger to the public in the current US epidemic, as although these ticks are common in Northern America, unlike hard ticks, soft ticks only very rarely parasitize land animals or humans (Sonenshine, 1991).

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References


