FIRST FIELD EVIDENCE FOR NATURAL VERTICAL TRANSMISSION OF WEST NILE VIRUS IN CULEX UNIVITTATUS COMPLEX MOSQUITOES FROM RIFT VALLEY PROVINCE, KENYA

BARRY R. MILLER, ROGER S. NASCI, MARVIN S. GODSEY, HARRY M. SAVAGE, JULIUS J. LUTWAMA, ROBERT S. LANCIOITTI, AND CLARENCE J. PETERS

Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado; Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; Uganda Virus Research Institute, Entebbe, Uganda

Abstract. West Nile virus is a mosquito borne flavivirus endemic over a large geographic area including Africa, Asia, and the Middle East. Although the virus generally causes a mild, self-limiting febrile illness in humans, it has sporadically caused central nervous system infections during epidemics. An isolate of West Nile virus was obtained from a pool of four male Culex univittatus complex mosquitoes while we were conducting an investigation of Rift Valley fever along the Kenya-Uganda border in February–March 1998. This represents the first field isolation of West Nile virus from male mosquitoes and strongly suggests that vertical transmission of the virus occurs in the primary maintenance mosquito vector in Kenya. A phylogenetic analysis of the complete amino acid sequence of the viral envelope glycoprotein demonstrated a sister relationship with a Culex pipiens mosquito isolate from Romania made in 1996. This unexpected finding probably reflects the role of migratory birds in disseminating West Nile virus between Africa and Europe.

West Nile (WN) virus was originally isolated from the blood of a woman from the West Nile Province of Uganda (currently Nile Province) presenting with a febrile illness in 1937.1 In subsequent serologic surveys, neutralizing antibodies were discovered in humans in central and East Africa.2-4 In regions where WN virus is endemic, many infections are inapparent or cause mild disease;5 however, human epidemics with severe disease have occurred in Israel and France.5 A study of an epidemic in Romania in 1996 identified 393 confirmed or probable human cases with 352 central nervous system infections.6

West Nile virus is a member of the Japanese encephalitis group, one of nine serologically defined groups in the genus Flavivirus, family Flaviviridae, listed in the Sixth Report of the International Committee on Taxonomy of Viruses, 1995.7 Although the transmission cycle of WN virus in East Africa has not been precisely defined, it probably involves Culex univittatus complex mosquitoes as the primary enzootic vector as well as the principal vector transmitting virus to humans. Extensive field and laboratory studies in South Africa have documented the importance of this species in the transmission of WN virus.8-12 Avian species are an important bloodmeal source for Cx. univittatus complex mosquitoes and they also serve as a significant virus-amplifying host.9,12

In this report we document the isolation of WN virus from male Cx. univittatus complex mosquitoes, implying that vertical virus transmission (transgenerational) is a natural component to WN virus maintenance in East Africa. Also, molecular genetic analyses of the amino acid sequence comprising the envelope glycoprotein of the virus in comparison with other geographic virus isolates from Africa and Europe are presented.

MATERIALS AND METHODS

Site description. An outbreak of Rift Valley fever struck East Africa in 1997–1998, probably the result of exceptionally heavy rains that produced the worst flooding since 1961.13 An international investigative team was assembled to monitor the westward spread of the virus along the Kenya-Uganda border and to obtain human and livestock blood samples and arthropod specimens for serologic and virologic analyses. This report is based on virus isolation from mosquitoes collected at a single site during the course of this investigation.

Turkwel Gorge Hydroelectric Project is located in north-west Kenya on the border between the West Pokot and Turkana districts (35°5′ E, 1°21′ N, elevation = 780 m), which lie in the Great Rift Valley (Figure 1). The site is bordered by the Karasuk Hills to the northeast and the Cherangani Hills to the southwest (elevation = 3,000 m); the intervening arid and semiarid plains stretch northward along the Turkwel River toward Lake Turkana. Vegetation in West Pokot is composed chiefly of Acacia woodland on the plains and forest at higher altitudes in the surrounding hills. Vegetation at the site of the dam and compound is composed of trees and shrubs of the Acacia commiphora community while vegetation along the riverine community consists of Ficus, Terminalia, Tamarindus, Combretium, and Acacia.14 Acacia tortilis and an understory of woody and herbaceous plants dominate floodplains on either side of the Turkwel River. Pokot and Turkana herdsmen and their livestock traditionally inhabit the area, although the reservoir and irrigation project are attracting settlers (fishermen and farmers) from outside the districts.14 Yearly rainfall in this semi-arid region ranges from 300 to 550 mm a year; daytime temperatures vary from 26°C in the wet season to 32°C in the dry season. A detailed description of the dam site and surrounding area is provided by Renshaw and others.14

Mosquito collection and virus isolation. Centers for Disease Control and Prevention (CDC) light traps provisioned with carbon dioxide (dry ice) were placed in the Turkwel Gorge compound (35°22′ E, 1°55′ N, elevation = 780 m) near the river and near a temporary campsite of construction workers. A number of the laborers were prostrate with a febrile illness at the time of the collection (see Discussion). The following morning, light traps were removed to a field laboratory and the mosquitoes were counted and placed in
cryovials that were then stored in liquid nitrogen. Mosquitoes were transported to CDC in Fort Collins, Colorado where they were identiﬁed to species and sex when possible and grouped in pools of 50 individuals or less. The collection was processed in 56 pools for virus isolation in Vero cells. The pooled mosquitoes were placed into chilled mortars and triturated in 1.6 ml of BA-1 diluent (1/100 M-199 medium with Hanks’ salts, 0.05 M Tris, pH 7.6, 1% bovine albumin, 0.35 g/L of sodium bicarbonate, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1 μl/ml of Fungizone; E. R. Squibb and Sons, Princeton, NJ). Mosquito suspensions were clariﬁed by centrifugation. One hundred microliters of each suspension was inoculated onto two wells of confluent Vero cells in six-well plastic plates. The plates were incubated for 1 hr at 37°C and were overlaid with a nutrient medium containing 1% agarose (SeaKem LE agarose; FMC BioProducts, Rockland, ME). After 5 days of incubation at 37°C in a 5% carbon dioxide incubator, a second overlay of nutrient agarose medium containing 1% neutral red dye was added over the ﬁrst overlay. The plates were observed daily for 14 days for plaque formation. Infected cells and agarose plugs over plaques were harvested with Pasteur pipettes and inoculated into 25-cm² ﬂasks of confluent Vero cells. Viral antigen in the infected cells was broadly identiﬁed using National Institutes of Health and CDC grouping hyper-immune mouse antisera in an indirect ﬂuorescent assay. ¹⁵ Viral antigen was speciﬁcally identiﬁed with virus-speciﬁc murine antibodies as indicated by results of the grouping ﬂuids. The WN virus isolate was initially identiﬁed with a WN virus-speciﬁc monoclonal antibody. ¹⁶ The alphavirus and flavivirus isolates were identiﬁed by reverse transcriptase–polymerase chain reaction (RT-PCR) and sequencing of the DNA products (see below) using primers that anneal to conserved regions of the viral genomes (Lanciotti R and others, unpublished data). ¹⁷

**Amplification by RT-PCR and sequencing of DNA.** The RNA was extracted from cell culture supernatants with the QIAamp Viral RNA kit (Qiagen, Valencia, CA) using the manufacturer’s instructions. The RT-PCR was performed on the flaviviruses by using primers that amplify an 800-base-pair product at the 3’ end of the nonstructural protein 5’ gene. ¹⁷ An alphavirus RT-PCR was accomplished using the forward primers AJUN [5’-CT(CG)TACGG(CT)(TG)(GA)-(AT)CCTAAAT-3’] and VJUN [5’-CTCTACGGCT(GA)-ACCTGAATGGA-3’] in the junction region of the genome and the reverse primer CCAP [5’-(AG)TA(CT)TG(CG)-AC(AT)GC(TG)CC(GA)TG(GA)TGCCA-3’] in the capsid gene, producing a DNA fragment of about 650 basepairs. The WN virus envelope glycoprotein gene was ampliﬁed with primers KUN109 (5’-CCGGGCTGTCAATATGCTAAAACG-3’) and KUN2505C (5’-ACTCCGCTTCCACA-CGGCAGCTTCTTGC-3’), producing a DNA product of about 2.4 kb. All RT-PCRs were performed using the TITAN One Tube RT-PCR (Boehringer Mannheim Biochemicals, Indianapolis, IN) kit following the manufacturer’s instructions. Ampliﬁed DNA fragments were puriﬁed by electro-
TABLE 1

<table>
<thead>
<tr>
<th>Taxa*</th>
<th>Number collected (number of pools)</th>
<th>Virus isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>An. (Cel.) funestus</td>
<td>20 (3)</td>
<td>–</td>
</tr>
<tr>
<td>An. (Cel.) gambiae</td>
<td>7 (1)</td>
<td>–</td>
</tr>
<tr>
<td>An. (Cel.) pretoriensis</td>
<td>11 (2)</td>
<td>–</td>
</tr>
<tr>
<td>An. (Cel.) squamosus/cydiptidis</td>
<td>4 (1)</td>
<td>–</td>
</tr>
<tr>
<td>An. (Cel.) rivalorum</td>
<td>41 (1)</td>
<td>–</td>
</tr>
<tr>
<td>An. sp.</td>
<td>6 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ae. (Adm.) culmennis</td>
<td>5 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ae. (Stg.) metallicus</td>
<td>11 (3)</td>
<td>–</td>
</tr>
<tr>
<td>Ae. (Stg.) simpsoni complex</td>
<td>5 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ae. (Stg.) vittatus</td>
<td>1 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ae. (Och.) sp.</td>
<td>1 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ae. (Stg.) sp.</td>
<td>4 (3)</td>
<td>–</td>
</tr>
<tr>
<td>Cq. (Coq.) cristata</td>
<td>24 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Cq. (Coq.) cristata (males)</td>
<td>3 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Cq. (Coq.) microanulata</td>
<td>1 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Cx. (Cux.) duttoni</td>
<td>6 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Cx. (Cux.) ethiopicus</td>
<td>1 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Cx. (Cux.) p. quinquefasciatus</td>
<td>2 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Cx. (Lat.) tigripes</td>
<td>1 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Cx. (Cux.) univittatus complex</td>
<td>86 (6)</td>
<td>Sindbis, Bagaza</td>
</tr>
<tr>
<td>Cx. (Cux.) univittatus complex (males)</td>
<td>12 (3)</td>
<td>West Nile</td>
</tr>
<tr>
<td>Cx. (Cux.) sp.</td>
<td>20 (2)</td>
<td>–</td>
</tr>
<tr>
<td>Cx. (Cux.) sp. (males)</td>
<td>3 (2)</td>
<td>–</td>
</tr>
<tr>
<td>Cs. sp. (males)</td>
<td>2 (2)</td>
<td>–</td>
</tr>
<tr>
<td>Er. silvestris</td>
<td>1 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ma. (Mnl.) africana/uniformis</td>
<td>14 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ma. (Mnl.) sp. (males)</td>
<td>4 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ma. sp. (male)</td>
<td>1 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Ur. sp.</td>
<td>3 (2)</td>
<td>–</td>
</tr>
<tr>
<td>Ur. sp. (male)</td>
<td>1 (1)</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>301 (56)</td>
<td>3</td>
</tr>
</tbody>
</table>


phoresis on 1% agarose gels with the QIAquick gel extraction kit (Qiagen) and eluted with water (50 ng/µl).

Positive and negative strands of the purified DNA fragments were sequenced with the Taq DyeDeoxy Terminator Cycle sequencing kit (Perkin-Elmer/Applied Biosystems, Foster City, CA) using various internal primers (list available from the authors) according to the manufacturer’s directions. Sequence assembly and editing were accomplished with the SeqMan II module of Lasergene (DNASTAR, Madison, WI). The DNA sequences were translated by using the EditSeq module of Lasergene and the resulting amino acid sequences were aligned with the MegAlign module of Lasergene.

Phylogenetic analyses. The deduced amino acids of the complete envelope gene of WN virus, isolated from male Cx. univittatus complex mosquitoes from Kenya (GenBank Accession No. AF146082), were analyzed by distance and parsimony algorithms (see below). The other taxa in the alignment came from geographic strains of WN virus where complete gene envelope sequences were available from publications or from the GenBank database (Nigeria, GenBank Accession No. M12294, unknown source; Israel, Chambers, human, no GenBank Accession No. given); Romania, human, GenBank Accession No. AF130362; and Romania, Cx. pipiens mosquito, GenBank Accession No. AF130362).

Maximum parsimony analysis was accomplished with the PHYLIP software package, version 3.5c, sequentially running the executable files SEQBOOT to generate 1,000 data sets for bootstrapping; PROTPARS to infer an unrooted phylogeny from amino acid sequences; CONSENSE to build a strict consensus, bootstrapped tree; and DRAWGRAM to generate the phylogram.19

The p-distance algorithm in the software package MEGA was used to estimate distances on the amino acid alignment.20 The topology of an unrooted tree was recovered with the neighbor-joining method proposed by Saitou and Nei,21 also implemented in MEGA. Gapped sites were ignored in the distance estimations.

**RESULTS**

Mosquito collections and virus isolations. The adult mosquito species captured in CDC light traps on February 26, 1998 are listed in Table 1. A total of 301 specimens were collected, representing 18 named species in seven genera.

The dominant taxa were Cx. univittatus complex (33%), Anopheles rivalorum (14%), Coquillettidia cristata (9%), and An. funestus (7%). Three virus isolations were made: Sindbis (SIN) virus (genus Alphavirus, family Togaviridae) and Bagaza (BAG) virus (Ntaya virus group; genus Flaviivirus, family Flaviviridae) from Cx. univittatus complex females (Table 1). West Nile virus (Japanese encephalitis virus group; genus Flaviivirus, family Flaviviridae) was isolated for the first time from male Cx. univittatus complex mosquitoes, indicating that the virus was vertically transmitted from female parent to progeny.

Virus genome sequencing. The entire envelope (E) gene of the WN virus isolate KN3829 was sequenced and translated into deduced amino acid sequences and compared with other geographic isolates of WN virus for which complete E gene sequences were available. The alignment contained a total of 501 amino acid sites; 26 sites (5%) were variable and 3 sites were phylogenetically informative at positions 93, 313, and 413 (Figure 2). Amino acid substitutions were observed throughout the E glycoprotein; however, more than half (15 of 26) were located in the putative flavivirus A domain (approximately amino acids 50–130 and 185–300) or domain II of Rey and others.22,23 or domain II of Rey and others.24,25

Phylogenetic analyses. An unrooted tree (Figure 3) produced from this alignment demonstrated that the Kenyan isolate from male mosquitoes formed a distinct and highly supported lineage with respect to a mosquito isolate and a human isolate from the 1996 WN epidemic in Romania.18 Virus strains from Israeli (human isolate) and Nigeria (unknown isolate) were more distantly related. Both parsimony and distance trees showed identical topologies and the Romanian-Kenyan clade was highly supported by bootstrap analyses.18 The Cx. p. pipiens mosquito isolate from Romania formed a sister relationship with the Kenyan Cx. univittatus complex mosquito isolate, also with high bootstrap support.
Figure 2. Deduced amino acid sequences of the envelope proteins of the five West Nile virus strains: WNI (Israel, human), RO97-50 (Romania, mosquito), RO96-1030 (Romania, human), WNN (Nigeria, unknown), KN3829 (Kenya, mosquito). The single letter code for amino acids is used. Dashes indicate identity between sequences and dots indicate deletions.
in heightened vector populations. Pod vector habitats and creating new ones that would result in increased evidence of vector-borne disease by expanding existing arthropod habitats downstream from it to increase the incidence of Rift Valley fever virus. Irrigation projects are effective in increasing vector populations because they create water sources for mosquitoes. The Nigerian isolate (WNN) was used as the outgroup in the program PROTPARS.

**DISCUSSION**

The isolation of three arboviruses representing three different taxa from only 301 mosquitoes is unusual and demonstrates a very high level of virus activity in this arid environment. The *Cx. univittatus* complex was the most abundant species collected with carbon dioxide–baited CDC light traps on the night of February 26, 1998. We have used the designation of *Cx. univittatus* complex to describe the taxon from which the virus isolates were made. This complex provisionally contains three species: *Cx. univittatus*, *Cx. perexiguus*, and *Cx. neavei*. Based on an examination of male genitalia from reared specimens made at the Turkwel site one year later, *Cx. perexiguus* is probably the species from which the virus isolates were made (Savage HM, unpublished data). We have taken a conservative approach to identification because the genitalia of specimens in the WN virus–positive pool were not examined. Besides WN virus, two other arboviruses were also isolated from females of this species complex, an alphavirus (SIN) and a flavivirus (BAG). The association of WN virus and BAG virus in arbovirus surveys from arthropods has been noted on a number of occasions in Africa. Both are common African viruses associated with *Culex* mosquitoes.

Although it had not rained in the area for weeks prior to our arrival (Chepikiriua M, Supervisor, Kerio Valley Development Authority, unpublished data), the diversity of mosquito species collected (Table 1) was impressive. The effect of the most recent El Niño event on mosquito populations in the area of Turkwel Gorge is unknown; however, the heavy and prolonged rains associated with this El Niño certainly produced large populations of waterfowl mosquitoes in northeastern Kenya, thereby augmenting the transmission of Rift Valley fever virus. Renshaw and others noted the increased flight activity of infected birds during their spring migration. Many of the Paleartic, long-distance avian migrants are channeled into Africa via the Great Rift Valley to their winter quarters; infected birds returning north provide a means for direct and relatively rapid movement of WN virus from enzootic foci in Africa into the Mediterranean region. The mode of infection is not as efficient as ovarian infection demonstrated by members of the California encephalitis virus group of the genus *Bunyavirus* (*Bunyaviridae*), it does provide a source of WN virus persistence in environments where amplifying hosts are temporarily absent or immune. An ancillary consequence of vertical transmission is that human infection is possible in the absence of an active enzootic transmission cycle.

![Figure 3](image-url). A parsimony consensus bootstrap tree based on the deduced amino acid sequences of the West Nile virus envelope gene. Virus strains are WNI (Israel, human), RO97–50 (Romania, mosquito), RO96–1030 (Romania, human), WNN (Nigeria, unknown), KN3829 (Kenya, mosquito). Numbers above branches are bootstrap percentages (1,000 replications). The Nigerian isolate (WNN) was used as the outgroup in the program PROTPARS.
It is difficult to assess the overall importance of vertical transmission of WN virus in Cx. univittatus complex mosqui-
to species because a quantitative model of the complex natural history of this virus in the many habitats in which it is found does not exist. None of the tropical flaviviruses face the selec-
tion pressure of the members of the California encephali-
tsitis virus group, which exist in latitudes where virus can only exist in mosquito ova during winter months. Culex eggs hatch immediately following embryonation, unlike desiccation-
resistant Aedes eggs, where the virus can persist for months during the dry season, making it even more unlikely that vertical infection of WN virus in vectors is important in the natural history of the virus. Indeed, vertical transmission of flaviviruses has not been considered an important component of virus persistence because of its inefficiency relative to certain bunyaviruses and the difficulty of substanti-
ating it in the field, although few comprehensive studies have been undertaken with the objective of directly answer-
ing this question. An investigation into an epidemic of yel-
low fever (YF) in a dry area (Sudanese climatic region) of Senegal in 1995 provided convincing evidence that vertical transmission of YF virus in Aedes aegypti mosquitoes played a major role in the spread of the infection to humans. Broom and others have also demonstrated that vertical trans-
mision of Murray Valley encephalitis virus in Ae. tremulus is an important component of virus persistence in arid re-
gions of western Australia. These studies challenge the par-
adigm of the relative unimportance of vertical virus trans-
mision and they demonstrate that transgenerational infec-
tion of arthropod vectors may be an under-appreciated ele-
ment in flavivirus transmission cycles.

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Authors’ addresses: Barry R. Miller, Roger S. Nasci, Marvin S. God-
seney, Harry M. Savage, and Robert S. Lanciotti, Division of Vector Borne Infectious Disease, National Center for Infectious Diseases, Centers for Disease Control and Prevention, PO Box 2087, Fort Collins, CO 80522-2087. Julius J. Luwama, Uganda Virus Research Institute, Department of Arbovirology, PO Box 49, Entebbe, Uganda. Clarence J. Peters, Division of Virology and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Mailstop A-26, 1600 Clifton Road NE, Atlanta, GA 30333.

Reprint requests: Barry R. Miller, Division of Vector Borne Infectious Disease, National Center for Infectious Diseases, Centers for Disease Control and Prevention, PO Box 2087, Fort Collins, CO 80522-2087.

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