Vector Competence of Culex neavei and Culex quinquefasciatus (Diptera: Culicidae) from Senegal for Lineages 1, 2, Koutango and a Putative New Lineage of West Nile virus

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Abstract. West Nile virus (WN virus) is one of the most widespread arbovirus and exhibits a great genetic diversity with 8 lineages, at least 4 (1, 2, Koutango, and putative new) are present in Africa. In West Africa, Culex neavei and Culex quinquefasciatus are considered as potential vectors for WN virus transmission in sylvatic or urban context. We analyzed the vector competence of these Culex species from Senegal for African lineages and envelope proteins sequences of viral strains used. We showed that lineage 1 is transmitted by both Culex mosquitoes, whereas the putative new lineage 8 is transmitted only by Cx. neavei. Our findings suggest that genetic variability can affect vector competence and depend on mosquito. However, when considering the infective life rate, the mosquito population seems to be inefficient for WN virus transmission in the field and could explain the low impact of WN virus in Africa.

INTRODUCTION

West Nile fever (WN fever) is an emerging disease caused by West Nile virus (WN virus), a neurotropic arthropod-borne virus belonging to the Japanese encephalitis complex of the Flaviviridae family, genus Flavivirus.1–3 The WN virus was first isolated and identified in the West Nile region of Uganda in 1937 from a febrile female adult.4 The WN virus is maintained in nature through an enzootic transmission cycle between ornithophilic mosquitoes and birds, and WN virus outbreaks concern essentially humans and horses as dead-end hosts.3,5–7 The outcome of human infection with WN virus ranges from asymptomatic or mild influenza-like illness to severe neurological and meningoencephalitis syndromes.7 The WN virus is a single-stranded, positive-sense RNA virus. The genomic RNA of about 11 kb contains one long open reading frame flanked by 2 non-coding regions. This open reading frame encodes for a polyprotein, which is processed to individual structural (Capsid, pre-Membrane, Envelope), and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins.1,2 The WN virus is recognized as one of the most widespread flaviviruses worldwide. The WN virus was endemic only in the Eastern Hemisphere, in Europe, Africa, Australia, and Asia until 1999 when the virus unexpectedly emerged in New York City and spread over the last decade throughout north, central, and southern America causing 16,196 meningitis/encephalitis and 1,443 deaths in humans and high mortality among birds.3,5,8–13

Currently, no specific therapy or vaccine is available for use against WN virus infections in humans, despite active research by many laboratories. However, vaccines are available for use in horses.13,14 One of the most striking features of WN virus is its great genetic diversity expressed in 7 lineages and one newly identified (putative new lineage) in Senegal (Dupressoir and others, personal communication), although no specific biological properties have been associated with them.15 In Africa, only lineages 1, 2, Koutango, and this putative new one were reported.16–18 Lineage 1 is subdivided into three clades. Clade 1a consists of strains from Europe, Africa, and America. Clade 1b consists of Australian Kunjin virus, and clade 1c consists of strains from India.19 Lineage 1 is the only one widely distributed. In addition, neuroinvasive diseases and all the major outbreaks are mostly caused by strains belonging to this lineage, although strains of clade 1b are rarely associated with neuroinvasion.20 Lineage 2 was exclusively present in Africa up to 2004 when it was reported in humans and bird populations in Europe, namely in Hungary, Greece, and Italy.21–23 Lineage 2 was also believed not as pathogenic as lineage 1 until it caused severe disease in South Africa and encephalitis among birds and humans in Europe.21–24 Therefore, viruses with high and low neuroinvasiveness phenotype exist in both lineages. Koutango virus initially classified as a distinct virus, was later proven to be a variant of WN virus following phylogenetic studies.18,25 Interestingly, Koutango was mostly isolated from ticks and rodents, a rare feature among WN virus lineages. Finally, a putative new lineage (putative lineage 8) of WN virus was isolated in Kedougou, southeastern Senegal in 1992 (Dupressoir A, personal communication). Phylogenetic studies based on complete genomes are currently in progress to better characterize this new WN virus lineage.

In addition, WN virus diversity has also been described for an N-linked glycosylation site of the envelope protein, which plays a role in WN virus neuroinvasiveness in mice, viral replication, and transmission of WN virus in mosquitoes.26–29 The WN fever appears to have only a minor impact on human health in Africa. Indeed, WN virus was associated with sporadic human outbreaks in Africa, particularly in Tunisia, Algeria, Egypt, Sudan, Congo, and South Africa.30–32 However, WN virus epidemiology in Africa differs from that seen in the United States or Romania where about one-fifth (20%) of WN virus human infections are symptomatic, and 1 in 150 (0.7%) develops neurological disease.29,33–35 These rates are much higher than those reported in Egypt and South Africa, where neurological disease is rare.30,35 In addition, in countries like Senegal, seroprevalence of WN virus antibodies can be up to 80% in humans, horses, birds, and virus repeatedly isolated from mosquitoes and wild vertebrates hosts, however no WN virus outbreak has ever been reported.36–40 These observations can be explained by the lack of appropriate WN surveillance, low pathogenicity of circulating isolates, low competence of local vectors, or exposure (and subsequent development of immunity) of a large proportion of the human
population to WN virus before an age in which neuroinvasive disease risk increases.

To address those questions, we analyzed in this work, the vector competence of the *Culex neavei* group (in reference to morphological variation within *Cx. neavei*) and *Culex quinquefasciatus*, for lineages 1, 2, Koutango, and a putative new lineage of WN virus in Senegal. Indeed, numerous mosquitoes have been found naturally infected by WN virus in Africa, particularly in Senegal, mostly belonging to the genus *Culex*. Among them, the *Cx. neavei* group, *Culex pociilipes*, and *Culex antennatus* were considered as the main potential vectors for WN virus transmission in Senegal regarding their bionomic and their regular infection during the virus amplification. A study on spatial pattern and feeding behavior of potential WN vectors in Senegal showed that *Cx. neavei* group populations are present in the different land use and land cover classes including barren, temporary ponds, and wooded savannah, and are attracted by both horses and birds. Therefore, the *Cx. neavei* group could be good bridge vectors for the sylvatic transmission of WN virus between birds and horses in Senegal. Considering the urban transmission, and the human impact of the virus circulation, *Culex quinquefasciatus* seems to be the main candidate in the West African context regarding its bionomic. Indeed, this mosquito species is present all year round, in domestic environment and in interaction with human populations. Members of the *Cx. quinquefasciatus* complex are known to be the main WN vectors in Europe and America regarding their proven vector competence. Furthermore, in Europe and America, the complex has been implicated in the urban transmission of WN virus, with outbreaks impacting human and animal health.

All these considerations justify the inclusion of the *Cx. neavei* group and *Cx. quinquefasciatus*, which are good candidate vectors for the transmission of WN virus between birds, horses, and humans in sylvatic and urban contexts, respectively.

**MATERIALS AND METHODS**

**Mosquitoes.** The *Cx. quinquefasciatus* and *Cx. neavei* groups were collected respectively from latrine and ground pool in Barkédi village (15°17 North, 14°53 West), in the Ferlo region of Senegal. The F1 generation adult mosquitoes used for the experimental infection were reared in the laboratory region of Senegal. The F1 generation adult mosquitoes used of 70°C, for the experimental infection were reared in the laboratory.

**Virus stocks.** The WN virus isolates ArD76986, B956, ArD96655, and ArD94343 corresponding to lineages 1, 2, Koutango, and a putative new lineage, respectively, were used and described in Table 1. These strains were named ArD76986-L1, B956-L2, ArD96655-Kout, and ArD94343-Put8, referring to the strains and lineages. The virus stocks were prepared by inoculating *Aedes pseudoscutellaris* (AP61) continuous cell lines for 4 days and immunofluorescence assay (IFA) was performed as previously described to assess the cell infection with WN virus. The supernatant of infected cells were aliquoted, frozen at −80°C, and used as viral stocks for mosquito infection. Virus stocks were titrated as previously described, using PS cells (Porcine stable kidney cell line, American Type Culture Collection, Manassas, VA).

**Oral infection of mosquitoes.** Five to 7-day-old female mosquitoes were starved for 48 hours before their exposition to an infectious blood meal for 1 h. The blood meal consists of 33% volume of rabbit washed erythrocytes, 33% volume of viral stock, 20% volume of fetal bovine serum (FBS), 10% volume of sucrose 5 mM ATP, and ~150 to 250 µg of sodium bicarbonate. The blood meal was administered in glass membrane feeders by using chicken skin as membranes.

After feeding, a sample of remaining blood meal was stored at −80°C for titration. The mosquitoes were then cold anesthetized and the engorged mosquitoes were incubated at 27°C, with relative humidity of 70–80% and fed with sucrose at 10% for 15 days. A second oral infection was done when ≤ 30 mosquitoes were engorged, and when blood meal titer was similar the two independent experiments were pooled. To follow the evolution of infection and dissemination over time, 2–8 specimens were collected and killed, frozen at 4, 8, and 12 days post-feeding (dpf). For each mosquito, legs and wings were put together in a tube, the body in another separate tube. For individuals incubated up to 15 days, their saliva was collected by placing their proboscis into a capillary tube containing pure FBS for 20 min and collecting the content of the capillary tube in a separate tube. All the samples collected at different stages were kept at −80°C until their analysis.

**Mosquito sample analysis.** The samples were screened by reverse transcription-polymerase chain reaction (RT-PCR) and confirmed by IFA for detection of WN virus. Mosquito body samples were first screened, followed by legs and wings when bodies were found positive and saliva when WN virus was detected in legs and wings. Each mosquito’s body, legs, and wings were homogenized in 500 µL cell culture medium containing 10% FBS and clarified by centrifugation at 1,500 × g, 4°C in 10 min. The supernatant was then filtered using a 1 mL syringe (Artsana, Como, Italy) and sterilized with 0.2 µm filters (Sartorius, Göttingen, Germany). The saliva samples were diluted in 500 µL cell culture medium containing 10% FBS and then filtered.

The RNA was extracted from each sample using the QiaAmp Viral RNA Extraction Kit (Qiagen, Heiden, Germany), according to the manufacturer’s instructions. The RNA samples were screened for WN virus presence by RT-PCR using the following primers (NS3F1 5' TGATTG 3', NS3R1 5' GATGARGTSCARA 3' at position 4888-4907, and NS3R1 5' AGTYYYTTCYTCTTCCY 3' at position 6193-6211), AMV RT (Promega, Madison, WI) for the transformation in cDNA, and Go-Taq PCR Kit (Promega) for amplification.

**Table 1**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Lineage</th>
<th>Place of isolation</th>
<th>Year of isolation</th>
<th>Species</th>
<th>No. of passages</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
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<td>ArD76986</td>
<td>1</td>
<td>Senegal</td>
<td>1990</td>
<td><em>Culex pociilipes</em></td>
<td>10</td>
<td>KJ131500</td>
</tr>
<tr>
<td>B956</td>
<td>2</td>
<td>Uganda</td>
<td>1937</td>
<td>Human</td>
<td>11</td>
<td>AY532665.1</td>
</tr>
<tr>
<td>ArD96655</td>
<td>Koutango</td>
<td>Senegal</td>
<td>1993</td>
<td><em>Rhipicephalus gulhoni</em></td>
<td>8</td>
<td>KJ131501</td>
</tr>
<tr>
<td>ArD94343</td>
<td>Putative</td>
<td>Senegal</td>
<td>1992</td>
<td><em>Culex perfuscus</em></td>
<td>12</td>
<td>KJ131502</td>
</tr>
</tbody>
</table>

*Four strains belonging to lineages 1, 2, Koutango, and a putative new one were used. The places, years of isolation, species, numbers of passages, and accession numbers were mentioned.*
according to the manufacturer’s instructions. The reverse transcription reaction was performed according to the manufacturer’s instructions, and the PCR conditions were as follows: 5 min 95°C, 40 cycles 1 min 95°C, 1 min 53°C, 1:30 min 72°C, and 10 min 72°C.

Viral isolation was attempted from samples using AP61 cells, and the presence of virus was detected by IFA. All RT-PCR positive samples were confirmed by IFA, and negative samples were serially passaged on AP61 cells up to four times to amplify any low titered virus and detect additional positive samples or to confirm their negativity. The additional positive samples detected during amplification on AP61 cells were also confirmed by RT-PCR.

**Interpretation of results.** Mosquito samples were considered as positive when they were detected by RT-PCR and IFA. The rates of infection (i.e., number of positive bodies/number of tested mosquitoes), dissemination (i.e., number of mosquito with positive legs and wings/number of positive mosquitoes), and transmission (i.e., number of mosquito with positive saliva/number of mosquito with positive legs and wings) were compared using Epi-Info software (Centers for Disease Control and Prevention, Atlanta, GA). These data were analyzed with the 2×2 contingency table using the Fischer exact test. For multiple comparisons, Bonferroni corrected P values (denoted p*) are provided and used for statistical significance. The degree of freedom (df) is always 1 with the contingency table and p or p* value < 0.05 was considered as significant.

**Sequencing and analysis of envelope protein coding region.** The RNA was extracted from each viral stock with the QiaAmp Viral RNA extraction Kit (Qiagen), according to the manufacturer’s instructions. The envelope protein’s coding region was amplified by RT-PCR using specific primers (E1F 5’ TGRTTDATCTGTGTGWTTCTCCT 3’ at position 977-996 and E1R 5’ TGRGAAATGAGYAACAGRGAC 3’ at position 2130-2150) using the same conditions as for RT-PCR of mosquito samples analysis. The PCR products were purified from the agarose gel using the Gel extraction kit (Qiagen) and sequenced by Cogenics (Beckman Coulter Genomics, Essex, UK). Alignment was performed using MEGA 5.05 software. GenBank accession numbers of the isolates sequenced are given in Table 1.

**RESULTS**

**Vector competence of Cx. quinquefasciatus.** The blood meal titers post-feeding, infection, dissemination, and transmission rates for ArD76986-L1, ArD96655-Kout, B956-L2, and ArD94343-Put8 are presented in Table 2.

For ArD76986-L1, the blood meal titer post-feeding was 6×10⁶ plaque-forming units/mL (pfu/mL). The infection rates were 25% (1 of 4 bodies) from Day 4 to 12 post-feeding and no dissemination was obtained. At 15 dpf, the infection, dissemination, and transmission rates were 75.9% (22 of 29 bodies), 18.2% (4 of 22 legs and wings), and 25% (1 of 4 saliva).

For B956-L2, the blood meal titer was 10⁶ pfu/mL, and no infection was obtained from Day 4 to 12 post-feeding. At 15 dpf, the infection rate was 5.3% (1 of 19 bodies) but no dissemination in legs and wings was observed.

For ArD96655-Kout, and ArD94343-Put8, 2 oral infections were done to get more than 30 engorged mosquitoes. The blood meal titers for ArD96655-Kout and the ArD94343-Put8 were 10⁶ and 3×10⁶ pfu/mL, respectively, for the first infection, and 1.5×10⁷ and 5×10⁶ pfu/mL for the second infection. However, no mosquito infection was obtained.

Comparisons of the different strains at 15 dpf using statistical analysis showed that infection rates obtained for B956-L2, ArD96655-Kout, and ArD94343-Put8 were comparable (p* value = 1), whereas they were significantly lower than ArD76986-L1 (p* value from 5.2×10⁻⁸ to 2.7×10⁻⁵). For dissemination rates, ArD76986-L1 and B956-L2 were comparable (P value = 0.8). However, only ArD76986-L1 was present in Cx. quinquefasciatus saliva.

**Vector competence of Cx. neavei.** The blood meal titers post-feeding, infection, dissemination, and transmission rates for ArD76986-L1, B956-L2, ArD96655-Kout, and ArD94343-Put8 are presented in Table 3.

For ArD76986-L1, the blood meal titers for the two oral infections were 1.5×10⁶ and 2.25×10⁶ pfu/mL, respectively. Because the titers were similar, the experiments were pooled. The overall infection rates ranged from 14.3% (1 of 7 bodies) to 25% (2 of 8 bodies) for Days 4, 8, and 12 post-feeding and no dissemination was obtained. At Day 15 post-feeding, the overall infection, dissemination, and transmission rates were 55% (11 of 20), 54.5% (6 of 11 legs and wings), and 83.3% (5 of saliva).

For B956-L2, the blood meal titer was 1.5×10⁵ pfu/mL, and no infection was obtained from Days 4 to 12 post-feeding. At Day 15 post-feeding, the infection and dissemination were 6.7% (2 of 30 bodies) and 50% (1 of 2 legs and wings). However, there was no detection of the virus in the saliva.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Blood meal titer</th>
<th>Days post-feeding</th>
<th>Infection†</th>
<th>Dissemination‡</th>
<th>Transmission§</th>
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<tbody>
<tr>
<td>ArD76986</td>
<td>6×10⁶</td>
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<td>0/1</td>
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</tr>
<tr>
<td>L1</td>
<td>8</td>
<td>1/4</td>
<td>0/1</td>
<td>1/4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1/4</td>
<td>0/1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>22/29</td>
<td>4/22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B956</td>
<td>10⁵</td>
<td>4</td>
<td>0/4</td>
<td>Na</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>8</td>
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<tr>
<td></td>
<td>12</td>
<td>0/4</td>
<td>Na</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>1/19</td>
<td>0/1</td>
<td>Na</td>
<td></td>
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<tr>
<td>ArD96655</td>
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<td>Na</td>
<td></td>
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<tr>
<td>Kout</td>
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<td>0/2</td>
<td>Na</td>
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<td></td>
<td>12</td>
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<td>15</td>
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<td>Put8</td>
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<td>15</td>
<td>0/19</td>
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<tr>
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<td>5×10⁵</td>
<td>15</td>
<td>0/13</td>
<td>Na</td>
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</tbody>
</table>

* Cx. quinquefasciatus mosquitoes have been fed with blood meals containing the different WN virus strains used. The virus titer of the blood meal was quantified after the feeding procedure and mentioned. After incubation, mosquitoes were analyzed for infection (number of mosquitoes containing virus in their bodies among the total), dissemination (number of mosquitoes containing virus in their legs and wings among the infected mosquitoes) and transmission (number of mosquitoes containing virus in their saliva among the mosquitoes with dissemination). Na = not applicable. Exp1 and Exp2 correspond to 2 independent experimental infections.
Table 3

<table>
<thead>
<tr>
<th>Virus</th>
<th>Blood meal titer</th>
<th>Days post-feeding</th>
<th>Infection</th>
<th>Dissemination</th>
<th>Transmission</th>
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<tbody>
<tr>
<td>L1</td>
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<td>1/7</td>
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</tr>
<tr>
<td>L2</td>
<td>1.5 × 10⁵</td>
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<td>1/7</td>
<td>0/1</td>
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<td></td>
<td></td>
<td>15</td>
<td>0/5</td>
<td>0/1</td>
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<td>B956</td>
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<td>1/18</td>
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<td>ArD94343 Put</td>
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</table>

* Cx. neavei mosquitoes have been fed with blood meals containing the different WN virus strains used. The virus titer of the blood meal was quantified after the feeding procedure and mentioned. After incubation, mosquitoes were analyzed for infection (number of mosquitoes containing virus in their bodies among the total), dissemination (number of mosquitoes containing virus in their saliva among the mosquitoes with dissemination) and no infection was obtained. Exp1 and Exp2 correspond to 2 independent experiments.

For ArD96655-Kout, the blood meals titers for the two oral infections were 2 × 10⁷ and 1.5 × 10⁸ pfu/mL, respectively. For the first experiment at Day 15 post-feeding, the infection rate was 18.2% (2 of 11 bodies), the dissemination rate was 50% (1/2 legs and wings), however there was no detection of the virus in the saliva. For the second one with a higher titer (1.5 × 10⁸ pfu/mL), no mosquito infection was obtained. The infection rates are comparable between these two infections at Day 15 post-feeding ($P$ value = 0.16).

For ArD94343-Put8, the blood meal titer was 5 × 10⁶ pfu/mL and no infection was obtained from Days 4 to 12 post-feeding. At Day 15 post-feeding, the infection, dissemination, and transmission rates were 5.6% (1 of 18 bodies), 100% (1 of 1 legs and wings), and 100% (1 of 1 saliva), respectively.

Statistical analysis of infection rates at Day 15 post-feeding, showed that B956-L2, ArD96655-Kout (Exp1 and Exp2), and ArD94343-Put8 are comparable ($p^*$-value = 1). They were significantly lower than ArD76986-L1 ($p^*$-value from 4.02 × 10⁻³ to 1.16 × 10⁻²) except ArD96655-Kout (Exp1), which is comparable with ArD76986-L1 ($p^*$-value = 0.51). No significant difference was observed for dissemination or transmission rates among the different isolates ($p^*$-value = 1). However, only ArD76986-L1 and ArD94343-Put8 were detected in mosquito saliva of Cx. neavei.

Comparison of ArD76986-L1 on Cx. quinquefasciatus and Cx. neavei showed no significant differences of infection, and transmission ($P$ value = 0.11), however the dissemination rates were significantly different ($P$ value = 0.04).

For B956-L2, ArD96655-Kout, and ArD94343-Put8, comparisons of Cx. quinquefasciatus and Cx. neavei showed no significant differences of infection and dissemination ($p$ or $p^*$-value ≥ 0.10).

For both mosquito species infections, positive bodies were detected from Day 4 post-feeding, whereas all dissemination were obtained at 15 dpf (Tables 2 and 3).

**Envelope protein N-linked glycosylation site.** Sequences of the envelope (E) coding region of the WN virus strains used in this study were analyzed in Figure 1. The results showed several variable regions from a lineage to another one throughout the envelope protein (Figure 1, in frame), among them, the N-linked glycosylation site, which plays a role in the vector competence of the mosquito. The glycosylation site at position 154 of WN virus NY99 strain, the ArD76986-L1, and the ArD94343-Put8 strains have similar amino acid composition (NYS) unlike the ArD96655-Kout (NFS) and B956-L2 strain, which has a deletion of the entire site.

**DISCUSSION**

We have shown the potential of mosquito populations in Senegal to transmit the lineages 1 and the putative new lineage 8 in saliva, and infection/dissemination of lineages 2, and Koutango of WN virus circulating in Senegal and Africa. Our findings showed that the WN virus strains ArD76986-L1 and ArD94343-Put8 infect mosquitoes, and proceed to dissemination in the legs/wings and saliva. The presence of virus in saliva suggests that the virus transmission is possible and proves the vector competence of Cx. quinquefasciatus for lineage 1, and Cx. neavei for lineages 1 and the putative new lineage 8.

Our data confirmed results obtained for both species or their counterpart in South Africa, New Zealand, United States, France, and Kenya. The transmission rates reported in those studies ranged from 7% to 100% and are consistent with the transmission rate of 25% observed for Cx. quinquefasciatus in this study.

The low infection rates obtained in our study could not be explained by the virus titers. Indeed, using 6 × 10⁶ pfu/mL of the lineage 1 strain in Cx. quinquefasciatus we had 75% of the infection, although no infection was obtained with a titer of 10⁶ or 1.5 × 10⁵ pfu/mL of the Koutango lineage, or 3 × 10⁵ pfu/mL of the putative new lineage 8 with the same mosquito species. In addition, using a lower titer of 10⁶ pfu/mL of lineage 2 or the putative new lineage, we obtained more than 5% of the infection in Cx. quinquefasciatus and Cx. neavei, respectively. All these data suggest that infection rates depend on viral strains or mosquito species rather than virus titer. These suggestions are reinforced with previous experiments. Indeed, it has been shown that lineage 1 with a similar virus titer of 10⁶ pfu/mL generated the highest infection rate up to 98% positive. However, Cx. quinquefasciatus exhibited an infection rate of 39–55% using a titer of 10⁷ pfu/mL, which is comparable to or lower than the rates obtained in this study.

Concerning the lineages 2 and Koutango, Cx. quinquefasciatus, exhibited no or low susceptibility, respectively, whereas Cx. neavei were susceptible and capable to disseminate the infection. However, no virus could be recovered from the saliva of both species raising the question of the competence of these vectors for lineages 2 and Koutango, and transmission and maintenance mechanisms of these WN virus lineages in nature. Recently, it has been shown that Rabensburg, a European WN virus lineage, should be considered as an intermediate between the mosquito-specific and horizontally
transmitted WN virus in light of replication in cells and vector competences studies. Therefore, it would be relevant to investigate vertical transmission and vector competence of other mosquito species, namely *Culex, Aedes* spp., or ticks found infected with WN virus in nature to fully understand the transmission dynamics of these lineages in nature.

These differences in the vector competence patterns could be explained by the genetic variability of the virus strains used in our experiment, particularly in the envelope protein. Indeed, our results showed several variable regions in the envelope protein. Among them, the only one described is the N-linked glycosylation site. This site is present in many lineage 1 strains, although it is present only in some lineage 2 strains, including neuroinvasive South African strains (Figure 1). Interestingly, many of the WN virus neuroinvasive in mice, or associated with significant outbreaks, including the American epidemics, contained the N-linked glycosylation site NYS in the E protein. Previous studies on mosquito vector competence have shown that glycosylation of WN virus envelope protein can affect the efficiency of viral replication and transmission and is mosquito colony or species dependent.

In our study, only strains with NYS were detected in mosquito saliva, whereas Koutango and lineage 2, which contain NFS or a deletion of the entire motif, respectively, were never detected in saliva. These data suggest that the presence or absence of the glycosylation site, and also its amino acid composition, could play an important role in vector competence and reinforce previous data. However, the absence from *Cx. quinquefasciatus* saliva of the putative new lineage 8, which contained also NYS motif and the presence of other variable regions through the envelope protein, showed that other viral determinants are also implicated in the vector competence. Taking into account the *p*-values, the impact of all these viral determinants on vector competence was more pronounced in *Cx. quinquefasciatus* (effects from 5.2 × 10⁻⁸ to 2.7 × 10⁻⁵) where the lineage 1 is significantly different from all the others lineages, compared with *Cx. neavei* (effects from 4.02 × 10⁻³ to 1.16 × 10⁻²) where Koutango and lineage 1 were comparable. This suggests that genetic variability impact on vector competence in a manner mosquito-species dependent. Phylogenetic analysis of these strains, based on complete genome is in progress in our laboratory to better characterize this genetic variability and further investigations should be dedicated to the role and nature of glycosylation site and other variable regions to mosquito vector competence by using infectious clones.

These observations and the bionomics of *Cx. quinquefasciatus* and *Cx. neavei* give insights into their respective role in the transmission of WN virus in nature and may also explain the low impact on humans. The wide distribution of *Cx. neavei* in Africa, its adaptation in sylvatic environment, and its vector competence for WN virus suggest that this mosquito species is a good candidate in the propagation and transmission of WN virus in Africa. However, taking into account the extrinsic incubation period (EIP) of 15 days obtained in our study and the parity rate obtained from previous studies, we have estimated a survival of infective life rate between (0.75)¹⁵ and (0.88)¹⁵ for *Cx. neavei*. This means that only 1.3% to 10.4% of the *Cx. neavei* population will survive at 15 dpf when infection to transmission occurs. This low survival infective life rate coupled with the low infection rates (5–55%) obtained in our study could suggest an inefficient WN virus transmission.

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**Figure 1.** Alignment of a portion of Envelope protein: mapping of the glycosylation site. Envelope protein from amino acid 126 to 325 were aligned using MEGA 5.05 software. Dots: conserved amino acids. Lines: deleted amino acids. Variable sites are framed, particularly the glycosylation site at position 154, which is in bold. Strains used for this study are in bold. ArD172162 (accession no. KJ131504) is a lineage 2 strain isolated in Senegal in 2003 from *Culex antennatus*, and AnD95153 (accession no. KJ131503) is a Koutango strain isolated in Senegal in 1993 from *Mastomys erythroleucus*. SPU116/89 (accession no. EF429197) and SA381/00 (accession no. EF429199) are South African lineage 2 strains isolated from Humans in 1989 and 2000, respectively, with high and mild neuroinvasiveness, respectively, in mice. 

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Concerning *Cx. quinquefasciatus*, the species is known to be highly associated with habitats close to human dwellings with an anthropophilic and indoor resting behavior. This bionomics related to its vector competence for lineage 1 shown in this study is consistent with the hypothesis that, as in Europe and America, *Cx. quinquefasciatus* might be responsible for domestic/urban transmission of WN virus in Africa. However, the mosquito populations tested in this study seem to be less efficient than their counterpart in the United States or Europe. The EIP obtained in this study was longer than the one reported previously because the dissemination and transmission occurred at Day 15 post-feeding in our experiments, whereas dissemination was obtained earlier at Day 5 and transmission at Day 7, post-feeding during a vector competence analysis of *Culex pipiens* using NY99 strain. Furthermore, based on our data and the daily survival rate obtained in earlier studies, the estimated survival of infective life rate ranged from (0.871) (0.883). In other words, only 12.59 to 15.46% of the populations of *Culex quinquefasciatus* will survive up to Day 15 post-infection, which is the time needed from infection to transmission. Our *Cx. quinquefasciatus* are therefore competent, but its infective life rate does not seem to be high enough to sustain an efficient transmission of WN virus to humans in the field. The data may explain the low impact of WN virus on humans in Africa.

In this work, we showed that worldwide WN virus lineage 1 is transmitted by both *Culex* mosquitoes tested, whereas the African putative new lineage is transmitted only by *Cx. neavei*. These transmitted lineages seem to be more adapted than lineages 2 and Koutango, which need more adaptation to reach mosquito salivary glands and to be transmitted to birds, humans, and others mammals in Africa. The vector competence of only *Cx. neavei* for local putative lineage, suggests that in Senegal, this lineage potentially circulates in the sylvatic context. However, the lineage 1 adaptation to both mosquito species, in sylvatic and domestic environments, is probably caused by intrinsic factors, and leads to its worldwide distribution. The introduction of lineage 1 in the United States and its rapid spread in America reinforces this hypothesis. Interestingly, the majority of human outbreaks in the world were caused by lineage 1 strains despite the presence of local strains, namely putative new lineage 8 and Koutango in Africa, Rabensburg and LEIVKrn88-190 (lineage 4) in Europe. However, a report of severe human cases caused by lineage 2 in Europe, which was a local African lineage, suggests that there is an increased opportunity for local lineages to adapt in new environments and ecological niches, and to be transmitted. Further phylogenetic studies to better understand genetic diversity within and between WN virus lineages will certainly be helpful to gain insights into the impact of the virus variability and its impact on vector competence of candidate vectors.

Recent evidence has suggested that in America, human WN virus incidence increases with urbanization and agriculture, and this is probably a result of the habitats and anthropophilic behavior of WN virus vectors such as *Cx. quinquefasciatus*. In Africa, the increased urbanization and vector competence of *Cx. quinquefasciatus* for WN virus proven in our study, suggest that WN infections in humans may increase in the future. However, the lower transmission efficiency of lineage 1 by *Cx. quinquefasciatus* hypothesized herein because of the infective life rate of mosquito populations in Senegal stands against that argument. In such a perspective, an appropriate surveillance for WN virus infections in humans is needed to better assess the real impact and burden in Africa comparatively to other infections such as malaria, and understand the epidemiology for a better control and response to its emergence.

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