

## Vector Competence of *Culex neavei* (Diptera: Culicidae) for Usutu Virus

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**Abstract.** Usutu virus (USUV), a flavivirus belonging to the Japanese encephalitis serocomplex, was isolated for the first time from a *Culex neavei* mosquito in 1959 in South Africa. Despite multiple isolations of USUV from *Cx. neavei* in Africa, its vector competence remains unproven. Therefore, we infected *Cx. neavei* orally with the USUV reference strain and used reverse transcription–polymerase chain reaction and an indirect immunofluorescence assay to detect virus in bodies, legs, wings, and saliva of mosquitoes. We demonstrated the susceptibility of *Cx. neavei* mosquitoes for the USUV reference strain, its potential to be transmitted, and infection, dissemination, and transmission rates of 90.9%, 40.0%, and 81.3%, respectively. Also, we showed that infection rates are dependent on the virus titer of the blood meal. Given the bionomics of *Cx. neavei*, its role as enzootic vector for USUV in Africa in a mosquito–bird transmission cycle or as bridge vector for USUV transmission to humans is discussed.

### INTRODUCTION

Usutu virus (USUV), a member of the Japanese encephalitis serocomplex of the genus *Flavivirus* (family *Flaviviridae*), was isolated for the first time from *Culex neavei* mosquitoes (Theobald, 1906) in the Natal region of South Africa.<sup>1,2</sup> Since then, USUV has been isolated from mosquitoes or detected by serologic analysis in birds in Africa (Senegal, Central African Republic, Burkina Faso, Côte d'Ivoire, Morocco, Nigeria, and Uganda) and Europe (Austria, Hungary, Switzerland, Italy, Spain, Germany, Poland, Czech Republic, and the United Kingdom).<sup>3–12</sup>

In 2001, USUV emerged for the first time outside Africa, in Austria, and caused severe disease and high mortality rates among blackbirds and great gray owls.<sup>13</sup> Human infections with USUV reported in Central African Republic, Burkina Faso, and Italy in 1981, 2004, and 2009, respectively, have been associated with fever, rash, and jaundice among immunocompetent patients or a severe neurologic syndrome among immunodeficient patients, which emphasizes its potential threat to human health.<sup>3,14–16</sup> In addition, a serosurvey of neutralizing antibodies against USUV among patients with fever and rash in Austria showed a prevalence of 25%.<sup>17</sup> These findings suggest that USUV is transmitted by mosquitoes and its transmission cycle involves birds and humans, although their specific roles remain to be investigated.

Usutu virus has been isolated from numerous mosquito species. However, the vector competence has not been determined for any of them. In Europe, USUV isolations have been reported from *Cx. pipiens*, an urban mosquito known as a bridge vector between birds and humans for West Nile virus, but also from *Aedes albopictus* mosquitoes.<sup>18,19</sup> In Africa, USUV has mostly been isolated from *Cx. neavei* and to a lesser extent from *Cx. perfuscus*, *Cx. quinquefasciatus*, *Aedes minutus*, *Mansonia africana*, and *Coquillettidia aurites*.<sup>3</sup> Given the numerous isolations of USUV from *Cx. neavei*, we re-investigated its vector competence for USUV because a previous limited study showed only its susceptibility to USUV but no transmission.<sup>1</sup> We report the susceptibility and ability

to transmit USUV of *Cx. neavei* by determining infection and dissemination rates and demonstrating the presence of USUV in the mosquito saliva as an indicator of virus transmission.

### MATERIALS AND METHODS

**Virus stocks used for infections.** Mosquitoes were analyzed for their vector competence by using USUV reference strain SAAR1776.<sup>1</sup> Virus stocks were prepared by infecting AP61 (*Aedes pseudoscutellaris*) cells, intracerebral inoculation of suckling mice, and obtaining virus titers as described, using PS cells (porcine stable kidney cell line; American Type Culture Collection, Manassas, VA).<sup>20</sup> The PS cells were used because plaque formation was necessary for quantification of the virus and cannot be observed in AP61 cells. The viral stock titer was  $6 \times 10^7$  plaque-forming units (PFU)/mL when prepared on AP61 cells. A higher virus titer of  $5.5 \times 10^8$  PFU/mL could only be obtained by intracerebral inoculation of suckling mice. Although data on USUV viremia in birds are not available, studies on closely related West Nile virus showed that several bird species develop titers of 5–10 logs PFU/mL, which justifies the choice of 7–8 logs PFU/mL as an initial infective titer for our experiments.<sup>21</sup>

**Mosquitoes.** *Culex neavei* larvae were collected from a ground pool in Barkedji (15°17'N, 14°53'W), a village in the northern Sahelian region of Senegal. For the infection experiments, F<sub>1</sub> generation adult mosquitoes were reared in the laboratory by using standard methods with a temperature of  $27 \pm 1^\circ\text{C}$ , a relative humidity of 70–75%, and a 12-hour photoperiod.<sup>22</sup>

**Oral infection of mosquitoes.** Oral infections were performed as described with minor changes.<sup>23</sup> Five to 6 day-old F<sub>1</sub> female mosquitoes were sucrose-starved for 24–48 hours before being exposed for 1 hour to an infectious blood meal. The infectious meal consisted of 33% rabbit erythrocytes washed with  $1 \times$  phosphate-buffered saline, 33% virus stock, 20% volume of fetal bovine serum (FBS), 1% (w/v) sucrose, and 5 mM ATP. The blood meals were administered in glass membrane feeders by using chicken skin as membranes. After each feeding, the remainder was titrated. Engorged mosquitoes were incubated at 27°C, a relative humidity of 70–80% and fed with 10% sucrose solution. After 14 days of incubation, each mosquito was processed separately. First, their legs

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and wings were removed and combined in a tube. Mosquitoes were allowed to salivate for 20 minutes into capillary tubes containing pure FBS and bodies were collected in a tube. All samples were stored at  $-80^{\circ}\text{C}$  before testing.

**Analysis of mosquito samples.** Each mosquito has been tested for the presence of USUV by reverse transcription–polymerase chain reaction (RT-PCR) and indirect immunofluorescence assay (IFA) by using a staged procedure starting with screening of all bodies, screening of legs and wings of mosquitoes with virus-positive bodies, and screening of saliva from mosquitoes with virus-positive legs and wings. Bodies of mosquitoes and their legs and wings were homogenized separately in  $450\ \mu\text{L}$  of cell culture medium containing 10% FBS. Samples were centrifuged for 5 minutes at  $7,500\ \text{rpm}$  at  $4^{\circ}\text{C}$ . Fetal bovine serum containing saliva was removed from capillaries and diluted with  $450\ \mu\text{L}$  of cell culture medium containing 10% FBS. Supernatants and medium containing saliva were filtered by using a 1-mL syringe (Artsana, Como, Italy) and sterilized  $0.20\text{-}\mu\text{m}$  filters (Sartorius, Göttingen, Germany).

For each specimen, RNA was extracted by using the QiaAmp Viral RNA Extraction Kit (Qiagen, Heiden, Germany), and reverse transcribed with avian myeloblastosis virus RTase (Promega, Madison, WI) and 382\_NS3R1 primer ( $5'\text{-TATCCTCCTGGTCTTTCCCG-3}'$ ) (TIB Molbiol, Berlin, Germany) according to the manufacturer's instructions. The resulting complementary DNA was amplified by using the Go-Taq PCR Kit (Promega) and 13\_NS3F1 ( $5'\text{-GATGGTGACTTCACCTTAT-3}$ ) and 382\_NS3R1 primers (TIB Molbiol). Cycling conditions were 5 minutes at  $95^{\circ}\text{C}$ ; 45 cycles of 1 minute at  $95^{\circ}\text{C}$ , 1 minute at  $52^{\circ}\text{C}$ , and 1 minute at  $72^{\circ}\text{C}$ ; and 10 minutes at  $72^{\circ}\text{C}$ . The RT-PCR was performed directly for homogenized samples and for cell culture supernatants to confirm a positive IFA result. Before the IFA, 2 mL of delta cell culture tubes (NUNC, Langensfeld, Germany) containing 80% confluent AP61 cells were incubated for 1 hour at  $27^{\circ}\text{C}$  with  $100\ \mu\text{L}$  of filtered supernatant. Two milliliters of cell culture medium containing 5% FBS were added and cells were incubated for 14 days. The medium was changed after 10 days. The IFA was performed by using USUV-specific hyperimmune ascitic fluid as described.<sup>24</sup> Samples positive by IFA were confirmed by RT-PCR and negative samples were passaged up to 4 times to confirm negativity.

**Interpretation of results.** Samples were considered positive once found positive either by RT-PCR, IFA, or both. Mosquitoes with only a virus-positive body indicated infection limited to the midgut, whereas virus in the mosquito body, legs, and wings confirmed disseminated infection. Virus in saliva indicated possible transmission of virus. Infection (no. infected mosquito bodies/no. mosquitoes tested), dissemina-

tion (no. mosquitoes with infected wings and legs/no. infected mosquitoes), and transmission (no. mosquitoes with virus in saliva/no. disseminated mosquitoes) rates were calculated and compared by using the two-tailed Fisher's exact test or chi-square test (Epi-Info Software; Centers for Disease Control and Prevention, Atlanta, GA). *P* values  $< 0.05$  were considered significant.

## RESULTS

*Culex neavei* mosquitoes were orally infected with USUV reference strain SAAR1776 in blood with initial titers of  $2 \times 10^7$  PFU/mL or  $1.8 \times 10^8$  PFU/mL. Infection, dissemination, and transmission rates are summarized in Table 1. Virus titers of blood meals after the infection experiment (post-infection) ranged from  $2 \times 10^4$  to  $4.5 \times 10^6$  PFU/mL, respectively. The decrease in virus titer during feeding experiments might indicate a higher sensitivity of USUV to temperature or other environmental factors.

The average infection rate of mosquitoes was 23.1% without observable virus dissemination and viral titers were  $2 \times 10^4$ – $9 \times 10^4$  PFU/mL post-infection, whereas USUV infected 90.9% of mosquitoes and showed dissemination and transmission rates of 40.0% and 81.3%, respectively, when the viral titer was  $4.5 \times 10^6$  PFU/mL post-infection. The infection rates with blood meals containing viral titers of  $2 \times 10^4$ – $9 \times 10^4$  PFU/mL post-infection were not significantly different among replicates A, B, and C (Table 1) ( $P = 1$ , by Fisher's exact test). However, the mean infection rate of the three replicates (23.1%) was significantly different from that of the replicate with a viral titer of  $4.5 \times 10^6$  PFU/mL post-infection (90.9%) ( $P < 0.0001$ , by Fisher's exact test). Dissemination rates of 0% and 40.0% for oral infections with viral titers of  $2 \times 10^4$ – $9 \times 10^4$  PFU/mL post-infection and  $4.5 \times 10^6$  PFU/mL post-infection, respectively, were not statistically different ( $P = 0.2824$ , by Fisher's exact test).

The combination of RT-PCR and IFA enabled detection of USUV in 43 mosquito bodies, 16 legs and wings, and 13 saliva samples. The RT-PCR performed directly on homogenized samples enabled detection of virus in 39 (90.7%) of 43 mosquito bodies, 12 (75%) of 16 legs and wings, and 2 (15.4%) of 13 saliva samples, which were virus positive after being passaged in AP61 cells. However, it seems that the quantity of virus in several mosquito samples was too low to be detected only by RT-PCR and led to the use of serial passage on AP61 cells for amplification of virus and more efficient detection. Among samples found negative by RT-PCR, USUV could be detected after first, second, or third

TABLE 1

Oral infection experiments with *Culex neavei* mosquitoes fed with blood meals initially containing  $2 \times 10^7$  PFU/mL and  $1.8 \times 10^8$  PFU/mL of Usutu virus reference strain SAAR1776\*

Blood meal titer before infection (PFU/mL)	Replicate	Blood meal titer post-infection (PFU/mL)	Infection rate†	Dissemination rate‡	Transmission rate§
$2 \times 10^7$	A	$2 \times 10^4$	1/3 (33.3%)	0/1 (0%)	–
	B	$4 \times 10^4$	2/9 (22.2%)	0/2 (0%)	–
	C	$9 \times 10^4$	0/1 (0%)	–	–
$1.8 \times 10^8$	A'	$4.5 \times 10^6$	40/44 (90.9%)	16/40 (40.0%)	13/16 (81.3%)

\*PFU = plaque-forming unit. Virus titer of the blood meal was additionally quantified after the feeding procedure (post-infection). After 14 days incubation, fed mosquitoes were analyzed for infection of their bodies (infection), of their legs and wings (dissemination), and the presence of virus in the saliva (transmission).

†No. infected mosquito bodies/no. mosquitoes tested.

‡No. mosquitoes with infected wings and legs/no. infected mosquitoes.

§No. mosquitoes with virus in saliva/no. disseminated mosquitoes.

passage in AP61 cells for 68.4%, 21.1%, and 10.5% of samples, respectively. The fourth passage did not show additional positive samples.

Furthermore, our experiments showed significant differences in the percentage of feeding success (no. engorged mosquitoes/no. mosquitoes exposed to blood meal  $\times$  100) and depended on the time of day when experiments were performed. The feeding success in experiments obtained between 3:00 PM and 4:00 PM ranged from 1% to 9%, and the feeding success obtained between 9:30 PM and 10:30 PM was 45% ( $P < 0.0001$ , by chi-square test).

## DISCUSSION

Repeated USUV isolations from *Cx. neavei* suggested its role as a potential vector species. Although, susceptibility of *Cx. neavei* to USUV could be demonstrated earlier with an infection rate of 20%, USUV transmission to hamsters failed.<sup>1</sup> In our experimental infections, USUV could infect *Cx. neavei* and disseminate in the mosquito bodies. Furthermore, the presence of virus in the mosquito saliva (81.3% for the blood meal with  $4.5 \times 10^6$  PFU/mL post-infection) is an indicator for virus transmission, and thereby strongly suggests the potential of *Cx. neavei* mosquitoes to transmit the USUV reference strain.

The wide distribution of *Cx. neavei* in Africa indicates a possible key role in USUV dispersal and endemic transmission.<sup>25</sup> *Culex neavei* was also found associated with several other flaviviruses (Bagaza, Yaounde, West Nile, and Koutango), alphaviruses (Sindbis and Babanki), and orbivirus (ArD66707, a tentatively new orbivirus regularly isolated in Senegal and provisionally named Sanar virus).<sup>15,26</sup> The prevalence of USUV among birds and the ornithophilic behavior of *Cx. neavei* suggest that these mosquitoes may play a major role as endemic vectors in a mosquito-bird transmission cycle in Africa.<sup>27,28</sup> This hypothesis is further supported by the observed peak biting activity of *Cx. neavei* at night, and its high abundance in the tree canopy, which is consistent with roost and nest behavior of many bird species.<sup>29,30</sup>

Oral infection rates for *Cx. neavei* with USUV were 23.1% and 90.9%, respectively, for infective blood meals with virus titers of  $2 \times 10^4$ – $9 \times 10^4$  PFU/mL and  $4.5 \times 10^6$  PFU/mL post-infection. The dose dependence of vector susceptibility on infecting titer has been demonstrated for other arboviruses, such as West Nile virus and St. Louis encephalitis virus.<sup>31,32</sup> Although in experimental infections a viral titer of  $4.5 \times 10^6$  PFU/mL post-infection was necessary for high infection rates and detectable virus dissemination, low-titer viremia might still contribute to USUV transmission in nature.

In Senegal, 141 USUV isolations from *Cx. neavei* mosquitoes have been reported.<sup>3</sup> A recent study of the temporal distribution of *Cx. neavei* mosquitoes in Barkedji showed highest mosquito abundances during September–November, and showed a positive correlation with rainfall after a lag time of one month.<sup>33</sup> It is noteworthy that this period of high vector abundance, and therefore potentially high USUV transmission activity, coincides with the time when resident and migratory birds meet in Senegal.<sup>34</sup> Therefore, infection of migratory bird species with USUV might lead to its further dispersal in Africa, or even provide an explanation for USUV introduction into Europe.

Furthermore, in Senegal, *Cx. neavei* is present in all biogeographic zones, ranging from the Sahelian to the Sudan-Guinean zones.<sup>3,23,33</sup> Analysis of the spatial distribution of *Cx. neavei* in Barkedji showed its high abundance in sylvatic environment but low abundance in villages.<sup>33</sup> Therefore, mosquitoes are likely to encounter birds and other mammals gathering around water places, facilitating an enzootic transmission cycle of USUV. The low abundance of this vector species in inhabited areas, in combination with the low anthropophily, may explain the rarity of reported human USUV infections in Africa.<sup>27,35</sup> This observation has been further strengthened by a serosurvey among the human populations in an area of reported USUV activity in Senegal that did not show any evidence for USUV infections (Nikolay B and others, unpublished data). These findings suggest that in Africa, USUV transmission by *Cx. neavei* occurs mainly in a sylvatic cycle with minor impact on the human population.

An often-encountered problem during experimental oral infections is to obtain a sufficient number of fed mosquitoes necessary to draw conclusions about infection, dissemination, and transmission rates. Interestingly, we have observed that the shift of feeding time from 3:00 PM to 9:30 PM has led to a significant higher feeding success for *Cx. neavei*, which is consistent with the observed peak biting activity in nature.<sup>29</sup> This observation should be taken into account in the design of experimental infection studies on *Cx. neavei* and other closely related species.

Regarding the methods used for virus detection, we observed that analysis of homogenized samples by RT-PCR is not sufficient to detect all positive samples. Therefore, we recommend at least three serial passages in AP61 cells, followed by IFA and RT-PCR analysis to increase the sensitivity of detection.

We have demonstrated the potential of *Cx. neavei* to transmit USUV and suggest involvement of this mosquito species in sylvatic transmission in Africa. However, this study was based on one population of *Cx. neavei* mosquitoes, and the susceptibility and ability to transmit virus may vary significantly among different populations of the same species. Therefore, further studies on *Cx. neavei* populations from different countries or bioclimatic zones might provide better understanding of its vector competence.

We are still far from understanding completely the USUV transmission cycle. Besides the documented infections of birds and humans, the range of vertebrate species serving as USUV reservoir hosts is largely unknown. Furthermore, identification of other wild or domestic mosquito vector species is necessary for a better understanding of the modes of USUV transmission. In Europe, USUV has been isolated from *Cx. pipiens*, suggesting its role as a vector.<sup>17</sup> A member of the *Cx. pipiens* complex, *Cx. quinquefasciatus*, is abundant in the domestic environment in Africa. Therefore, investigation of its vector competence might also bring insights to understand the USUV transmission cycle in the domestic environment in Africa.

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