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Evidence of Limited Laboratory Infection of *Culex Tarsalis* (Diptera: Culicidae) by Usutu Virus

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Abstract

Background: Usutu virus (USUV) is an emerging flavivirus, closely related to West Nile virus (WNV), that has spread into Europe from Africa. Since Culex tarsalis Coquillett is an important vector for WNV transmission in the United States, we tested the ability of USUV to replicate in and be transmitted by these mosquitoes.
Materials and Methods: USUV was used to infect 3-4 day-old Cx. tarsalis with 5.6 to 7.5 $\log_{10}$ pfu/ml in goose bloodmeals. Saliva, heads, and bodies were collected on day 13 or 14 and analyzed by RT-qPCR for detection for USUV vRNA. Blotting paper punches were also collected daily to assess viral transmissibility.
Results: The low and high dose blood meal resulted in 0% and 19.6% of the mosquitoes having established infections, respectively. All of the high dose had a dissemination of USUV RNA to the heads and none of the filter papers had detectable USUV RNA, but five of the capillary saliva collections were positive, representing 45.5% of the infected mosquitoes.
Conclusions: Limited infection of Cx. tarsalis was observed when exposed to bloodmeals with greater than 107 pfu/mL of USUV, indicating this vector is not likely to have a key role in transmission of the virus.

Keywords: Usutu virus, arbovirus, *Culex tarsalis*, vector competence

Usutu virus (USUV) was first isolated in Swaziland (present-day Eswatini) in 1959 and has been detected across sub-Saharan Africa (Roesch et al., 2019). Repeated introductions of USUV into Europe have occurred since the 1950s, where it now considered endemic (Benzarti et al., 2020, Roesch et al., 2019). USUV, like WNV, is a member of the Japanese encephalitis virus antigenic complex of flaviviruses and the enzootic cycles of both utilize birds and mosquitoes of the genus *Culex* (Abbo et al., 2021). One of the major vectors of WNV, particularly in western North America where WNV incidence is high, is *Culex tarsalis*. USUV is associated with mass mortality events in blackbirds (*Turdus merula*), similar to WNV's lethality for American crows (*Corvus brachyrhynchos*) (Benzarti et al., 2020; Roesch et al., 2019,). In humans, USUV is principally asymptomatic, but symptoms have included fever, rash, meningoencephalitis and facial paralysis (Roesch et al., 2019). The similarities between WNV and USUV cause concern that USUV could rapidly spread in North America, as WNV did.

USUV (UG09615: accession MN813491; isolated from *Cx. univittatus* Theobald in Uganda, 2012) (Mossel et al., 2017) was used to infect *Cx. tarsalis* with 5.6 to 7.5 $\log_{10}$ pfu/mL in goose bloodmeals as previously described (Ledermann et al., 2023). The low dose used here (5.6 $\log_{10}$ pfu/mL) was similar to the maximum viremia developed by 2 day old chickens (Kuchinsky et al., 2021), and the high dose (7.2–7.5 $\log_{10}$ pfu/mL) was comparable to the maximum viremia found in canaries (Benzarti et al., 2020). The bloodfed female mosquitoes were individually caged in 12 well plates (Corning 3512) sealed with lids made of acrylonitrile butadiene styrene and organdy netting (Ledermann et al., 2023). The Kern National Wildlife Refuge (KNWR) laboratory strain of *Cx. tarsalis* were fed from 30 μL of 10% sucrose solution in 0.25-inch diameter blotting paper punches changed daily (Whatman GB003), and were maintained at 28°C, 80% humidity, with 12 h photoperiods for 13 or 14 days. Establishment of infection was assayed from macerated bodies, dissemination was assayed using ground heads, and transmissibility was assessed from the final blotting

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Table 1. Infection Results on Day 13/14 Post Infection of *Cx. tarsalis* (KNWR) Mosquitoes with USUV (UG09615) as Determined by qRT-PCR

	Titer	*% infection (number positive/number tested)*			
Replicate	*(log_{10} pfu/mL)*	*Body*	*Head*	*Filter paper pads*	*Capillaries*
1	5.6	0.0 (0/23)	0.0 (0/23)	0.0 (0/23)	ND
2	7.2	21.7 (5/23)	21.7 (5/23)	0.0 (0/23)	60.0 (3/5)
3	7.5	18.2 (6/33)	18.2 (6/33)	0.0 (0/33)	33.3 (2/6)
Combined 2 & 3	7.2–7.5	19.6 (11/56)	19.6 (11/56)	0.0 (0/56)	45.5 (5/11)

ND, not done.

paper and capillary collections of saliva by RT-qPCR. Extracted RNA was tested for the presence of USUV RNA by the TaqMan QuantiTect Probe RT-PCR protocol (Qiagen). A 50 μL total reaction volume consisted of kit components, 10 μL of RNA, 400 nM of primer (USUV 5356 FWD: GAG CAY AGY GGC ACA GAG ATA G, USUV 5467 REV: GAG CCT CAT CCA TCA CAA AGA) and 150 nM of probe (USUV 5394 FWD: FAM TGC CAC TCT AAC CCA CAG ACT CAT BHQ1). The reactions were subjected to 45 cycles of amplification in a CFX-96 Real-Time PCR detection system (BioRad) according to the recommended conditions. The limit of detection was found to be C_t 39.3, using previously described techniques (Ledermann et al, 2011).

No *Cx. tarsalis* had any detectable USUV infection after exposure to the lower dose bloodmeal (Table 1). The higher dose bloodmeals resulted in 19.6% of the mosquitoes having established infections and all of these demonstrated dissemination of USUV RNA to the heads. None of the filter papers had detectable USUV RNA, but five of the capillary saliva collections were positive, representing 45.5% of the infected mosquitoes. Limitations of this study included using a laboratory strain of *Cx. tarsalis*, which may not have the same capacity for infection as all wild strains, and utilizing viral RNA as a proxy for infectious virus.

The infection rates and transmission potential of USUV in *Cx. tarsalis* were lower than that reported for *Culex pipiens* Linnaeus at comparable titers (Abbo et al., 2021, Cook et al., 2018; Fros et al, 2015). The failure for USUV to infect any *Cx. tarsalis* with a low titer bloodmeal, and only 20% of *Cx. tarsalis* becoming infected when provided blood meals of > 7 $\log_{10}$ pfu/mL indicates that USUV has difficulty establishing infection in this species. Unlike WNV, which can exceed viremic titers of 11 $\log_{10}$ pfu/mL in blue jays and common grackles (Komar et al, 2003), the highest USUV viremic titer reported is 8 $\log_{10}$ $TCID_{50}$/mL in canaries (Benzarti et al, 2020), suggesting *Cx. tarsalis* is unlikely to obtain a bloodmeal of sufficient titer to become infected from birds. Even though *Cx. tarsalis* is an effective WNV vector, these findings indicate they are refractory for USUV transmission and spread by these vectors in the US is unlikely, unless USUV viremia in birds is substantially higher than reported thus far (Benzarti et al, 2020; Kuchinsky et al, 2021). Based on our findings and data from other investigators, *Culex quinquefasciatus* Say and *Cx. pipiens* are more likely to spread USUV in the United States than *Cx. tarsalis* (Cook et al, 2018).

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Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Author Disclosure Statement

J.P.L., N.M.B., H.R.H., and A.M.P. declare no conflicts of interest.

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