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Comparative Usutu and West Nile virus transmission potential by local *Culex pipiens* mosquitoes in north-western Europe

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ABSTRACT

Originating from Africa, Usutu virus (USUV) first emerged in Europe in 2001. This mosquito-borne flavivirus caused high mortality rates in its bird reservoirs, which strongly resembled the introduction of West Nile virus (WNV) in 1999 in the United States. Mosquitoes infected with USUV incidentally transmit the virus to other vertebrates, including humans, which can result in neuroinvasive disease. USUV and WNV co-circulate in parts of southern Europe, but the distribution of USUV extends into central and northwestern Europe. In the field, both viruses have been detected in the northern house mosquito *Culex pipiens*, of which the potential for USUV transmission is unknown. To understand the transmission dynamics and assess the potential spread of USUV, we determined the vector competence of *C. pipiens* for USUV and compared it with the well characterized WNV. We show for the first time that northwestern European mosquitoes are highly effective vectors for USUV, with infection rates of 11% at 18 °C and 53% at 23 °C, which are comparable with values obtained for WNV. Interestingly, at a high temperature of 28 °C, mosquitoes became more effectively infected with USUV (90%) than with WNV (58%), which could be attributed to barriers in the mosquito midgut. Small RNA deep sequencing of infected mosquitoes showed for both viruses a strong bias for 21-nucleotide small interfering (si)RNAs, which map across the entire viral genome both on the sense and antisense strand. No evidence for viral PIWI-associated RNA (piRNA) was found, suggesting that the siRNA pathway is the major small RNA pathway that targets USUV and WNV infection in *C. pipiens* mosquitoes.

Introduction

In the last two decades a number of clinically significant arthropod-borne viruses (arboviruses) have emerged and re-emerged in continental Europe. Autochthonous transmission of dengue virus has occurred in France in 2014 [1] and chikungunya virus transmission has been recorded in Italy (2007) [2] and France (2010, 2014) [3,4]. Both of these viruses are transmitted by the invasive Asian tiger mosquito *Aedes albopictus*, which has colonized parts of Europe [5]. Native *Culex* mosquitoes are the main vectors for two pathogenic lineages of another arbovirus, West Nile virus (WNV), which are now endemic in southern Europe [6]. Mosquitoes and birds maintain the enzootic transmission cycle of WNV. Infected mosquitoes, however, may also feed on other vertebrates resulting in frequent infections in humans and horses [7]. In 1999, WNV was introduced in the United States. The outbreak that followed was characterized by high mortality rates in various American bird species and resulted in the largest outbreak of human neuroinvasive disease to date [8].

In Austria (2001), a sudden and substantial die-off occurred in Eurasian blackbirds (*Turdus merula*), closely resembling the 1999 WNV outbreak in the United States. Not WNV, but a related flavivirus (family *Flaviviridae*), Usutu virus (USUV), was identified in infected birds. This was the first isolation of USUV on the European continent [9]. The virus was first discovered in South Africa in 1959 and since then it has been identified in a number of African countries [10]. After the initial outbreak in Austria, USUV activity has been detected in birds from Spain, Italy, Switzerland, the Czech Republic, Hungary, United Kingdom, Poland, Croatia, Germany and Belgium [11,12]. In some southern European countries USUV co-circulates with WNV [13]. The

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high mortality in a large number of avian species enabled the spread of USUV to be monitored via the surveillance of dead birds [14]. Most of the USUV-positive bird species were blackbirds (*T. merula*), which belong to the same genus as the suspected WNV reservoir in the United States, the American robin (*Turdus migratorius*) [15]. USUV infected mosquitoes may also feed on other vertebrates, and the virus has been detected in horses [16] and bats [17]. Infections in humans have resulted in two diagnosed clinical cases in Africa [10]. In Europe, two Italian and three Croatian patients with neuroinvasive disease have been reported [18–20], attributed to USUV. However, serological evidence suggests that less severe and subclinical cases of human USUV infections occur regularly in endemic areas [21–23].

Similar to WNV, USUV is mostly transmitted by *Culex* mosquitoes. In Africa USUV has been isolated from *Culex neavei*, *Culex perfuscus*, *Culex univitattus* and *Culex quinquefasciatus*. Additionally, USUV has also been detected in a number of mosquito species from other genera [10]. Among European mosquito species, USUV is mostly found in the northern house mosquito (*Culex pipiens*), which is abundant throughout the northern hemisphere [13].

The presence of competent mosquitoes dictates the potential spread of arthropod-borne pathogens. Vectors are considered competent when they can transmit the pathogen from one vertebrate host to the next. Arboviruses, like USUV, are ingested by the mosquito via a blood meal of an infected vertebrate host, infect the epithelial cells that line the mosquito midgut, escape to the hemolymph, and finally accumulate in the saliva to be transmitted during the next blood meal [24]. Determining vector competence provides an insight into the viral transmission dynamics and is essential to assess the risk for future outbreaks. The only laboratory experiments with USUV were done with the African mosquito, *C. neavei* [25]. To better understand, predict, and assess the potential spread of USUV in Europe we investigated the vector competence of the northern house mosquito *C. pipiens* for USUV. In addition, we investigated the activity of RNA interference (RNAi), which is a major antiviral defense system of mosquitoes and other insects [34, 35]. The RNAi response against USUV has never been studied.

Here we show for the first time that *C. pipiens* is a highly effective European USUV vector. We provide an insight into the virus replication dynamics and the antiviral RNAi response within the mosquito vector and show how the vector competence of USUV relates to that of WNV at different temperatures.

Materials and methods

Cells and viruses

C6/36 cells were grown in Leibovitz L15 (Life Technologies, The Netherlands) medium, which was supplemented with 10% FBS. Vero E6 cells were cultured with DMEM Hepes (Life Technologies, The Netherlands)-buffered medium supplemented with 10% FBS containing penicillin (100 IU/ml) and streptomycin (100 μg/ml). When Vero E6 cells were infected with mosquito lysates or saliva the growth medium was supplemented with fungizone (2.5 μg/ml) and gentamycin (50 μg/ml). This medium will be referred to as fully supplemented medium. Passage 2 (P2) virus stocks of USUV, Bologna '09 (GenBank accession no. HM569263) [26] and WNV Gr'10 lineage 2 (GenBank accession no. HQ537483.1) [27,28] were grown on C6/36 cells and titrated on Vero E6 cells.

Mosquito rearing

The European *C. pipiens* colony originated from Brummen, The Netherlands (°05′23.2″N 6°09′20.1″E) and was established in 2010 and maintained at 23 °C. The mosquito colony was kept in Bugdorm cages with a 16:8 light:dark (L:D) cycle and 60% relative humidity (RH), and provided with a 6% glucose solution as a food source. Bovine or chicken whole blood was provided through the Hemotek PS5 (Discovery Workshops, UK) for egg production. Egg rafts were allowed to hatch in tap water supplemented with Liquifry No. 1 (Interpet Ltd., UK). Larvae were fed with a 1:1:1 mixture of bovine liver powder, ground rabbit food and ground koi food.

In vivo infections

Two-to-five day old mosquitoes were infected either via ingestion of an infectious blood meal or via intrathoracic injections. Oral infections were performed by mixing whole chicken blood with the respective P2 virus stock to the indicated final concentration. Mosquitoes were allowed to membrane feed, using the Hemotek system, in a dark climate controlled room (24 °C, 70% RH) [29]. After 1 h, mosquitoes were sedated with 100% CO_2 and the fully engorged females were selected. During intrathoracic injections the mosquitoes were sedated with CO_2 by placing them on a semi-permeable pad, attached to 100% CO_2. Mosquitoes were infected by intrathoracic injection using the Drummond nanoject 2 (Drummond Scientific Company, United States). Virus-exposed mosquitoes were incubated at the indicated temperatures with a 16:8 L:D cycle and fed with 6% sugar water during the course of the experiment.

Salivation assay

Transmission was determined using the forced salivation technique [29]. Briefly, mosquitoes were sedated with 100% CO_2 and their legs and wings were removed. Their proboscis was inserted into a 200 μl filter tip containing 5 μl of salivation medium (50% FBS and 50% sugar water) W/V 50%). Mosquitoes were allowed to salivate for 45 min. Mosquito bodies were frozen in individual Eppendorf tubes containing 0.5 mm zirconium beads at −80 °C. The mixture containing the saliva was added to 55 μl of fully supplemented growth medium.

Infectivity assays

Frozen mosquito bodies were homogenized in the bullet blender storm (Next Advance, United States) in 100 μl of fully supplemented medium and centrifuged for 90 s at 14,500 rpm in an Eppendorf Minispin Plus centrifuge (14,000 cf) inside the biosafety cabinet of the Wageningen biosafety level 3 laboratory. Thirty μl of the supernatant from the mosquito homogenate or the saliva-containing mixture was inoculated on a monolayer of Vero cells in a 96-well plate. After 2–4 h incubation the medium was replaced by 100 μl of fresh fully supplemented medium. Wells were scored for virus specific cytopathic effects (CPE) at three days post infection. Viral titers were determined using 10 μl of the supernatant from the mosquito homogenate in an end point dilution assay on Vero E6 cells. Infections were scored by CPE, three days post infection.

Analysis of small RNA libraries

Pools of twelve WNV or USUV infected mosquitoes were lysed in TRIzol (Life Technologies) reagent and total RNA was isolated. The isolation and sequencing of small RNAs were described previously [30]. In short, RNA was size separated by PAGE gel electrophoresis and small RNAs (19–33 nucleotides) were isolated. The small RNA library was prepared with the TruSeq Small RNA Sample Preparation Kit (Illumina) and sequenced on an Illumina HiSeq 2500 by Baseclear (www.baseclear.nl). FASTQ sequence reads were generated with the Illumina Casava pipeline (version 1.8.3) and initial quality assessment was performed by Baseclear using in-house scripts and the FASTQC quality control tool (version 0.10.0). FASTQ sequence reads that passed this quality control were analyzed with Galaxy [31]. Sequence reads were clipped from the adapter sequence (TruSeq 3′ adapter indexes #1 and #5) and mapped with Bowtie (version 1.1.2) [28] to the WNV (GenBank: HQ537483.1) and USUV (GenBank: HM569263.1) genomes. Size profiles of the viral small RNAs were obtained from all reads that mapped to their respective genomes