One Health 2 (2016) 88–94

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

Culex pipiens and *Culex torrentium* populations from Central Europe are susceptible to West Nile virus infection

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ARTICLE INFO

Article history:
Received 26 November 2015
Received in revised form 14 April 2016
Accepted 19 April 2016
Available online 20 April 2016

Keywords:
Culex mosquitoes
West Nile virus
Infection assay

ABSTRACT

West Nile virus (WNV), a *Flavivirus* with an avian primary host, is already widespread in Europe and might also pose an infection risk to Germany, should competent mosquito vectors be present. Therefore, we analysed the ability of WNV to infect German *Culex* mosquitoes with special emphasis on field collected specimens of *Culex torrentium* and *Culex pipiens* biotype *pipiens*. We collected egg rafts of *Culex* mosquitoes over two subsequent seasons at two geographically distinct sampling areas in Germany and differentiated the samples by molecular methods. Adult females, reared from the various egg rafts, were challenged with WNV by feeding of artificial blood meals. WNV infection was confirmed by real-time RT-PCR and virus titration. The results showed that field collected *C. pipiens* biotype *pipiens* and *C. torrentium* mosquitoes native to Germany are susceptible to WNV infection at 25 °C as well as 18 °C incubation temperature. *C. torrentium* mosquitoes, which have not been established as WNV vector so far, were the most permissive species tested with maximum infection rates of 96% at 25 °C. Furthermore, a disseminating infection was found in up to 94% of tested *C. pipiens* biotype *pipiens* and 100% of *C. torrentium*. Considering geographical variation of susceptibility, *C. pipiens* biotype *pipiens* mosquitoes from Southern Germany were more susceptible to WNV infection than corresponding populations from Northern Germany. All in all, we observed high infection and dissemination rates even at a low average ambient temperature of 18 °C. The high susceptibility of German *Culex* populations for WNV indicates that an enzootic transmission cycle in Germany could be possible.

1. Introduction

West Nile virus [(WNV); family *Flaviviridae*, genus *Flavivirus*] infections are a growing concern to Europe as illustrated by repeated outbreaks of West Nile fever (WNF) and West Nile neuroinvasive disease (WNND) in south-eastern parts of Europe [1]. Further, the increase of imported human WNV infections into Germany [2,3] raises concerns that the virus may also become established here. Since the emergence of arboviruses is closely linked to the presence of suitable vectors and susceptible hosts, the knowledge of principle vector species is essential for selection of adequate control measurements [4].

WNV is maintained in nature within an enzootic cycle involving ornithophilic mosquitoes and birds, but it can infect humans, equines and other vertebrates as illustrated by WNF and WNND in humans [5–7]. Since its first isolation in Uganda in 1937 [8], WNV has been isolated from mosquitoes in Eurasia [1] and Australia [9,10]. Moreover, following a single introduction to New York City in 1999, WNV has also spread throughout the Americas [11,12]. Members of the *Culex* (*C.*) *pipiens* complex (Linnaeus 1758), especially *C. pipiens*, *Culex tarsalis* and *Culex quinquefasciatus*, have been described as enzootic and bridge

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http://dx.doi.org/10.1016/j.onehlt.2016.04.001

vectors for WNV in the United States of America and other WNV endemic regions [4,13,14]. The *C. pipiens* complex members *C. pipiens* biotypes *pipiens* and *molestus* as well as *Culex torrentium* are abundant in Central Europe [15,16]. The *C. pipiens* biotype *pipiens* and *C. torrentium* preferentially take blood meals from birds, rendering them potential enzootic vectors for WNV in Central Europe [17]. Recently, two studies have demonstrated the potential of a Dutch laboratory colony of *C. pipiens* to serve as a vector for WNV [18,19]. Up to now, there are no data available on the vector competence of *C. torrentium* for WNV. In contrast, *C. torrentium* is a proven enzootic vector of another arbovirus, the Sindbis virus, in Sweden [20–22].

Species identification within the *Culex* genus is difficult using classical morphological methods. Differentiation of *C. pipiens* and *C. torrentium* females relies on the occurrence of pre-alar scales, or measurement of the wing veins [23] but both methods are difficult to apply on large number of samples. The difficulties of correct assignment of the *C. pipiens* biotypes and *C. torrentium* might lead to misinterpretations of their vector potential, especially since virus isolation of field collected mosquitoes is a main marker for involvement in transmission.

Here we analyse the ability of Central European populations of *C. pipiens* biotype *pipiens* and *C. torrentium* to become infected with WNV and, in doing so, deliver a proxy for the vector competence estimations of these *Culex* populations for WNV. To avoid culturing effects, we used field collected samples from two geographically distinct regions in Germany and first separated the species and/or biotypes by multiplex qPCR [16]. Secondly, we analysed infection and dissemination rates after experimental feeding of WNV lineage 1 strain NY99 in *C. pipiens* biotype *pipiens* and *C. torrentium* females.

2. Material and methods

2.1. Mosquito strains and field collected mosquito samples

The *C. quinquefasciatus* (Malaysia) laboratory colony was obtained from Bayer (Bayer, Leverkusen, Germany). The *C. pipiens* biotype *molestus* colonies were established in our laboratory and originated from Heidelberg (Mol S), Wendland (Mol W) and Langenlehsten (Mol LL). The Mol S colony was maintained for 3 years in the laboratory prior to infection experiments. The Mol W colony was established from blood fed gravid females collected in 2012 in Wendland/Germany and the Mol LL colony was established from egg rafts collected in 2013 in Langenlehsten/Germany.

C. pipiens biotype *pipiens* and *C. torrentium* mosquitoes were obtained from egg raft collections carried out in Hamburg area/Germany [Altes Land 53°35′N 9°32′E, Langenlehsten 53°30′N 10°44′E and Hamburg City 53°32′N, 9°57′E; hereafter referred to as the North population (N)] and Lake Constance/Germany [Radolfzell-Böhringen 47°44′N, 8°58′E and Mettenau 47°43′N, 8°59′E; hereafter referred to as the South population (S)]. Specimens for infection experiments were collected from August to October in 2012 and 2013. The egg collection was carried out using gravid traps filled with hay infusion placed in proximity to natural breeding sites of *Culex* mosquitoes, i.e. water bodies to attract gravid females and stimulate egg deposition. Traps were checked twice a day for freshly deposited egg rafts, which were retrieved from water surface using a wooden spatula and placed in individual plastic cups for transportation to the laboratory.

2.2. Rearing of larvae and adult mosquitoes

Field collected and laboratory bred mosquitoes were kept at 23 +/− 2 °C with a relative humidity of 80% and a 16 h:8 h light:dark photoperiod. Field collected egg rafts were floated separately in dechlorinated water and hatched larvae were fed on TetraMin flaked fish food (Tetra GmbH, Melle, Germany). From each individual egg raft, 4–5 larvae were used for molecular taxonomic identification as described previously [16]. Once identified, larvae were pooled according to species or biotype and emerging females (4–14 days of age) were distributed into plastic vials at 10–15 females each. Adult mosquitoes were fed on fructose pads (8% D(−)-Fructose, Carl Roth GmbH, Karlsruhe, Germany; 0.02% 4-Aminobenzoic acid, Sigma Aldrich, Seelze, Germany) for maintenance and starved overnight prior to infection. To facilitate egg production of *C. quinquefasciatus* and *C. pipiens* biotype *molestus* laboratory colonies, a blood meal consisting of human erythrocyte concentrate (Blood group 0, Blood bank, University Hospital Hamburg)/50% FCS (PAA/GE Healthcare Life Sciences, Germany)/0.5% fructose (Carl Roth GmbH, Karlsruhe, Germany) was provided weekly.

2.3. Experimental infection and dissemination assays

Mosquitoes used in experiments were kept in incubators at 25 °C or 18 °C/80% humidity. Infection was performed overnight via an artificial blood meal containing 1.0–1.6 × 10^7 PFU WNV lineage 1 strain NY99 [24]/mL blood meal presented on cotton sticks. This method has been shown to lead to efficient WNV infection in *C. pipiens* [25]. Fully engorged mosquitoes were either frozen at −80 °C (day 0) or kept at 25 °C or 18 °C for 14 to 35 days. The two temperatures were chosen to mimic the climatic conditions in Germany with 25 °C representing the mean average temperature in Germany in July/August in the south of Germany and 18 °C representing the maximum average temperature during a minimum of 4 months/year in the north and south of Germany. For WNV RNA purification, mosquitoes were homogenised separately in 500 μl of medium (Schneider's Drosophila Medium, PAN Biotech, Aidenbach, Germany). To calibrate our infection assay, we used laboratory strains of *C. pipiens* biotype *molestus* and *C. quinquefasciatus*. *C. pipiens* biotype *molestus* has been described as a WNV vector in Israel [26], and *C. quinquefasciatus* was described by several studies from North America as a competent vector for WNV [14,27,28]. For the analysis of disseminating infection, frozen mosquitoes were beheaded under a dissection microscope and the body and head were separately homogenized in medium. The virus detection in heads as a method to measure the dissemination rate has been established previously by several other studies [29–32].

2.4. Quantification of viral RNA and infectious particles

WNV RNA purification was performed with QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The quantitative real-time PCR was performed using QuantiTec Probe RT-PCR MasterMix Kit (Qiagen, Hilden, Germany), with Light Cycler 480 II (Roche, Rotkreuz, Switzerland) and using 9 μl reaction mix containing 0.6 μM of the following primers and 0.2 μM of the following probes: OSM_145: GGCAATGGAGTCATAATG; OSM_146: GCATCTCAGG TTCGAATC; OSM_147: -FAM-CCAACGGCTCATACATAAGCG-BHQ1 and 2 μl RNA. For the analysis of virus titres, mosquito organ homogenate was filtered using 0.20 μm filters and inoculated on Vero cells (96-well format) with 10-fold dilutions and indirect immunofluorescent revelation after 3 days. Briefly, inoculated Vero cells were fixed in 4% formaldehyde for 30 min and immunostained using WNV recombinant E protein mouse monoclonal antibody (ABIN782271, antibodies-online GmbH, Germany) diluted 1:100 in PBT for 1–2 h and then with fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG (115-095-003, Jackson ImmunoResearch Laboratories, Inc., USA) diluted 1:200 in PBT for 1 h. Infected wells were counted and viral titres were calculated using the Spearman and Kärber algorithm described by Hierholzer and Killington [33].

2.5. Statistics

Fisher's exact test was applied to assess differences of proportions between the species groups. A p value of less than 0.05 is deemed statistically significant. The statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc., CA, USA).