Susceptibility of invasive mosquitoes (Diptera: Culicidae) for infection with Japanese encephalitis virus, an emerging zoonosis

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Abstract

Background: Japanese encephalitis virus (JEV) is the principal cause of mosquito-borne encephalitis in human populations within Asia. If introduced into new geographic areas, it could have implications for public and animal health. However, potential mosquito vectors for virus transmission have not been fully investigated. The Asian tiger mosquito, *Aedes albopictus*, has emerged into Europe and is now expanding its geographical range into more northerly latitudes. *Culex quinquefasciatus*, although absent from Europe, has been detected in Turkey, a country with territory in Europe, and could act as a vector for JEV in other regions. To assess the risk of these invasive species acting as vectors for JEV, we have investigated the vector competence of *Ae. albopictus* and *Cx. quinquefasciatus*

Methods: Two colonised lines of *Ae. albopictus* (Italy and Spain), and a line of *Cx. quinquefasciatus* (Tanzania) were compared for susceptibility to infection by oral feeding with JEV strain SA-14, genotype III, at 10^6 PFU/ml and maintained at 25°C. Specimens were processed at 7 and 14 days post-infection (dpi). Rates of infection, dissemination and transmission were assessed through detection of viral RNA by real-time polymerase chain reaction (RT-PCR) in mosquito body, legs and saliva, respectively, at each time point. Where possible, infection and dissemination were confirmed by immunohistochemical (IHC) detection of the JEV envelope protein.

Results: *Aedes albopictus* from Italy showed no susceptibility to infection with JEV strain SA-14. Conversely, *Ae. albopictus* colonised in Spain was susceptible and 100% of infected mosquitoes expressed viral RNA in saliva at 14 dpi. *Culex quinquefasciatus* was highly susceptible to infection as early as 7 dpi and 50% of infected mosquitoes expressed viral RNA in saliva at 14 dpi. Infection and dissemination were confirmed in *Cx. quinquefasciatus* by IHC detection of JEV envelope protein in both the mid-gut and salivary glands.

Conclusions: *Aedes albopictus* from two different locations in Europe range from being fully susceptible to JEV and capable of transmission, through to being totally resistant. *Culex quinquefasciatus*, also appears highly susceptible, therefore both species could potentially act as competent vectors for JEV and facilitate the emergence of JEV into new regions.

Background

Flaviviruses have a global distribution and many species can be transmitted by mosquitoes, ticks and sandflies [1, 2]. Japanese encephalitis virus (JEV) (Family Flaviviridae, Genus *Flavivirus*) is the main aetiological agent for human viral encephalitis in Far East and South East Asia. Five genotypes are currently recognised, all endemic in Asia [3, 4].

Japanese encephalitis virus is maintained in an enzootic cycle between mosquito vectors and avian hosts, especially wading birds [5], although recent infection studies have demonstrated that domestic birds may act as JEV reservoirs [6]. Some mosquito species can act as bridge vectors transmitting the virus to livestock animals such as pigs, which are considered an amplifying host. Humans and other

mammals such as horses are considered dead-end hosts, and recent studies have shown that pig-to-pig transmission can occur by the oronasal infectious route [5, 7]. Due to a combination of climate change, movement of people and livestock, and the introduction of invasive vectors into new areas, geographic expansion of JEV could occur in the future [8, 9].

Culex tritaeniorhynchus is the main mosquito vector of JEV transmission in regions where JEV is endemic, but other species, particularly within the genus *Culex*, can act as vectors [3, 10]. In Europe, *Cx. tritaeniorhynchus* has only been reported in Greece [11], and JEV RNA has only been detected in a pool of *Cx. pipiens* in northern Italy [12]. Recent experimental studies have demonstrated the competence of European populations of mosquitoes to transmit JEV, which include *Cx. pipiens* from the UK and France, as well as *Culiseta annulata* and *Aedes detritus* from the UK [13–15]. Vector competence studies carried out by de Wispelaere et al. [16] demonstrated that a French population of the invasive species *Ae. albopictus* was competent for JEV genotypes III and V, and Huber et al. [17] showed that *Ae. japonicus japonicus* from Germany is also a competent JEV vector. As a consequence, it is likely that there are many unassessed mosquito vectors with endemic European populations that may be able to transmit JEV and facilitate its emergence. In addition, non-native species to Europe have the potential to act as JEV vectors. These include *Cx. quinquefasciatus*, which has been found in Turkey [18], and *Ae. albopictus* and *Ae. japonicus japonicus*, which are now widely distributed in countries around the Mediterranean basin [19, 20]. The detection of *Cx. quinquefasciatus* in Turkey could enable this mosquito species to expand its geographical range into continental Europe in the near future. In other regions, different populations of *Ae. albopictus* have been identified as competent vectors of JEV such as Australia and Taiwan [21–23], and *Cx. quinquefasciatus* in North America [24], Brazil [13], India [25] and a colony from Queensland in Australia [26] were also competent to transmit JEV. However, other studies have shown that a wild caught population of *Cx. quinquefasciatus* from Australia and New Zealand were not competent to transmit JEV [26, 27], and two strains of *Ae. albopictus* (Yungho and Liyang, Taichung County) were less efficient vectors in comparison with a strain originating from Sanhsia (Taipei County) from Taiwan.

Due to the continued risk of invasive mosquito species globally, and the potential emergence of JEV from regions in which it is endemic, this study assessed the vector competence of two populations of *Ae. albopictus* (originating from Italy and Spain), and *Cx. quinquefasciatus* (originating from Tanzania) for JEV genotype III.

Methods

Colonization of mosquitoes

Laboratory colonies of *Ae. albopictus* (Padua, Italy) (year of colonization unknown and donated by Entostudio, Italy), *Ae. albopictus* (Barcelona, year of colonization 2009 and donated by Universidad de Zaragoza, Spain) (Figure 1), and *Cx. quinquefasciatus* (established at the Tropical Pesticides Research Institute (TPRI), Arusha, East Tanzania)(year of colonization at London School of Hygiene and Tropical

Medicine 2010 and donated by London School of Hygiene and Tropical Medicine, UK). Maintenance of *Culex* and *Aedes* mosquitoes in biosecurity level 3 laboratories followed previously published protocols [19,28].

Virus stocks

Japanese Encephalitis virus genotype III (strain SA-14, isolated from *Cx. pipiens* larvae, China 1954) was donated by Dr. Jonas Schmidt-Chanasit, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany). Virus stocks were propagated in Vero cells as previously described [15,19,28].

Assessment of vector competence

Adult females of *Ae. albopictus* (Italy and Spain) and *Cx. quinquefasciatus* (Tanzania) were tested for their vector competence for JEV genotype III at 25°C. Mosquitoes were provided with 1.5 ml of an infectious blood meal composed of defibrinated horse blood, adenosine 5'-triphosphate (final concentration 0.02 mM) and virus stock to give a final virus concentration of 3.3×10^6 PFU/ml, using a Hemotek membrane feeding system (Hemotek Ltd Accrington, Lancashire, UK). Five to ten-day-old adult female mosquitoes of both species were first starved of sucrose for five hours and then allowed to feed on the infectious bloodmeal (as described above) in Bugdorm insect cages of 22 x 22 x 22 cm (bugzarre.co.uk) from 16:00, continuing throughout the night for a minimum of 16 h. The following day they were anaesthetized with Triethylamine (TEA) FlyNap® (Blades Biological Limited, Edenbridge, UK) and separated into groups of blood-fed and non-blood-fed specimens. For the processing of specimens and assessment of vector competence (infection, dissemination and transmission rates), a modified protocol adapted from [15,19,28] was followed. Briefly, at 7 and 14 dpi, mosquitoes were immobilized at -80°C for 2 mins and held in a plastic pot embedded in ice, to ensure that they remained immobile during processing. Legs and wings were removed, saliva samples were taken and the bodies, legs, wings and saliva retained for downstream analysis.

Processing of samples for molecular detection of JEV RNA

The protocol of [15] was used for detection of JEV RNA in tissues using previously published primers [29], which target and amplify a fragment of the NS1 gene. A sample was considered positive for JEV RNA at a Cycle threshold (*ct*) value of 39 or lower.

Immunohistochemistry

The presence of JEV antigen in mosquitoes was determined by immunohistochemistry (IHC) in histological sections. Briefly, twelve infected blood-fed and three non-infected control female mosquitoes were placed in 10% neutral buffered formalin for fixation for 48h. After fixation the wings and extremities

were removed and placed in sagittal plane prior to routine processing to paraffin blocks. Serial 3- μ m-thick sections of mosquitoes were cut and placed on silane-coated slides (3-trietoxysilyl-propylamine). Proteinase enzyme buffer (DAKO, Ely, Cambridgeshire, UK) applied for 15 min at 20°C was used as antigen retrieval method. A mouse monoclonal anti-Flavivirus E-glycoprotein antibody (ab155882, Abcam, Cambridge, UK) applied at 1 in 50 dilution in Tris Buffered saline with 0.05% Tween 20 (TBST, VWR, Leicestershire, UK) at 4°C for 18–20 h (overnight) was used as primary antibody to detect JEV. Parallel sections were tested with a protein concentration matched mouse immunoglobulin G class 2a (Abcam, Cambridge, UK), as isotype controls in order to identify any non-specific immunolabelling. Slides were then washed in purified water and assembled into coverplates for immunolabelling. DAKO mouse EnVision™+ System, HRP Peroxidase (DAKO, Ely, Cambridgeshire, UK) was used as a secondary antibody and incubated for 30 min at 20°C combined with swine and goat immune serum (Vector Laboratories, Peterborough, UK). Antibody binding was visualized using the chromogen 3,3'-diaminobenzidine (DAB) + substrate-chromogen which results in a brown-colored precipitate at the antigen site after 10 min incubation. Finally, sections were counterstained with Mayer's haematoxylin (HE) and mounted in Distyrene Plasticiser Xylene (DPX) mounting medium (TCS Bioscience, Buckingham, UK) for light microscopy. Sections of West Nile virus-infected mouse brain and JEV-infected Vero cells were used as positive controls for flavivirus immunostaining.

Virus titration

Titration of both stock virus and virus in the infected blood meal were performed by plaque assay as previously described [19,28].

Statistical analysis

The graphical output was carried in the R programme (<http://www.R-project.org>). A *t*-test was performed comparing the *ct* values for RT-PCR between each species.

Results

We assessed the potential to vector JEV in different mosquito populations, *Ae. albopictus* originating from Italy and Spain, and *Cx. quinquefasciatus* from Tanzania, to which it was provided an infectious blood meal containing JEV. A total of 81 females from *Ae. albopictus* originating from Italy successfully fed (28.9%), while 106 females of *Ae. albopictus* originating from Spain fed (52.7%) (Table 1). Conversely, only 79 females of *Cx. quinquefasciatus* fed (37.4%) (Table 2). Mortality was observed between 1 and 2 dpi, then the survival of mosquitoes was relatively stable until 6 dpi before declining towards 13–14 dpi, which is typical for vector competence experiments (Additional file: Figure S1).

Table 1

Infection, dissemination and transmission of *Aedes albopictus* (Italy and Spain) following consumption of a blood meal containing Japanese encephalitis virus genotype III. Groups of mosquitoes were maintained at 25°C for the indicated time periods

Species	Blood meal titre (PFU/ml)	Blood feeding rate (%)		Dpi 7 (%)	Dpi 14 (%)
<i>Aedes albopictus</i> Padua, Italy	1.8 x 10 ⁶	81/281 (29)	Infection (Body)	0/19 (0)	0/28 (0)
			Dissemination (Legs)	0	0
			Transmission (Saliva)	0	0
<i>Aedes albopictus</i> Barcelona, Spain	1.8 x 10 ⁶	106/201 (53)	Infection (Body)	1/18 (6)	4/24 (17)
			Dissemination (Legs)	0/1 (0)	3/4 (75)
			Transmission (Saliva)	0	3/3 (100)
Dpi, days post-infection					

Table 2

Infection, dissemination and transmission of and *Culex quinquefasciatus* following consumption of a blood meal containing Japanese encephalitis virus genotype III. Groups of mosquitoes were maintained at 25°C for the indicated time periods

Species	Blood meal titre (PFU/ml)	Blood feeding rate (%)		Dpi 7 (%)	Dpi 14 (%)
<i>Culex quinquefasciatus</i> Tanzania	1.8 x 10 ⁶	79/211 (37)	Infection (Body)	5/10 (50)	23/29 = (79)
			Dissemination (Legs)	1/5 (20)	4/23 (17)
			Transmission (Saliva)	1/1 (100)	2/4 (50)
Dpi, days post-infection					

Virus infection, dissemination and transmission was initially determined by RT-PCR. *Aedes albopictus* originating from Italy did not show evidence for infection, dissemination or transmission at either 7 or 14 dpi (Table 1). However, *Ae. albopictus* originating from Spain did show infection at 7 dpi (6%), but neither dissemination nor transmission were detected. However, at 14 dpi infection (17%), dissemination (75%) and transmission (100%, n = 3) were detected (Table 1).

Culex quinquefasciatus showed higher prevalence of infection (50%), dissemination (20%) and transmission (100%, $n = 1$) at 7 dpi. In addition, higher prevalence of infection (79%), dissemination (17%) and transmission (50%, $n = 2$) were observed at 14 dpi (Table 2). Of the mosquito populations assessed, *Cx. quinquefasciatus* appeared the more competent vector for JEV, demonstrating the highest levels of dissemination and transmission, despite bloodfeeding at a lower rate than *Ae. albopictus* originating from Spain. However, these results demonstrated that a population of *Ae. albopictus* originating from Spain, may act as a competent vector for JEV.

To compare relative amounts of virus genome in the tissue we analysed, the threshold values from each amplification cycle (ct) were evaluated, in particular at 14 dpi (Fig. 2). The ct values detected from *Cx. quinquefasciatus* were significantly lower compared to *Ae. albopictus*, suggesting a higher level of viral RNA detection in these samples ($t = 2,163$, $p = 0.019$).

Cellular distribution of JEV infection in *Cx. quinquefasciatus* was determined by IHC on sections of FFPE whole mosquitoes to detect JEV envelope antigen. All segments of the mosquito, head, thorax and abdomen, were examined by light microscopy. JEV-immunolabeled cells were observed in the posterior midgut of seven infected females of *Cx. quinquefasciatus* (Fig. 3; Additional file, Figure S2). Immunolabelling was present in clusters of epithelial cells, predominantly ciliated pseudostratified intestinal cells, located in the posterior midgut region, as characterised by dark brown pigment deposition within the cytoplasm. The levels of midgut epithelial cells infection were highly variable with labelling ranging from a continuous row of cells to single cells (Additional file, Figure S2). Positive intracytoplasmatic immunolabelling was observed in the the salivary gland of one specimen of *Cx. quinquefasciatus*, defined by the presence of secretory masses corroborating the detection of virus by this and other methods such as PCR (Table 2). Isotype control immunolabelled sections did not show any non-specific staining on infected mosquitoes.

Discussion

Our results show that *Ae. albopictus* originating from Spain and *Cx. quinquefasciatus* originating from Tanzania were susceptible to infection by JEV genotype III. In addition, our study demonstrates that after fourteen days at 25°C, JEV virus was able to disseminate throughout *Ae. albopictus* originating from Spain and *Cx. quinquefasciatus* from Tanzania with viral RNA being detected in their saliva by RT-PCR, and also in the salivary glands of *Cx. quinquefasciatus* by IHC. This suggests that the studied populations may be competent vectors for JEV genotype III under our experimental conditions, corroborating previous findings from populations of *Ae. albopictus* and *Cx. quinquefasciatus* [13, 16, 21, 23, 24, 26, 30].

Both *Ae. albopictus* and *Cx. quinquefasciatus* are indigenous in tropical areas, where temperatures above 30°C can be encountered, and they are well known vectors for many arboviruses [24, 31–34]. Although

Cx. quinquefasciatus has not yet been detected in Europe, its morphological, ecological and phylogenetic similarity with *Cx. pipiens*, and its ability to colonize new areas via ship and airline vessels [35], makes it a potentially important invasive species for studying the transmission of re-emerging zoonotic viruses such as JEV. The origin of *Cx. quinquefasciatus* is thought to be West Africa from where it colonized other regions due to trade and migration. The species reached the Americas during the 1800s, spreading to Asia and the Pacific via whaling and merchant vessels [36, 37]. It is a common species in Africa, thus our finding that the population from Tanzania is a highly competent vector for JEV is epidemiologically relevant in the event of JEV incurring in the African continent.

Conversely, *Ae. albopictus* is now widely distributed in many countries in continental Europe and the Mediterranean basin, with a few sporadic recorded incursions into more temperate regions such as the United Kingdom [38]. The species is widely distributed in Italy, Spain, and Germany, and several populations of this species are considered the main drivers for outbreaks of dengue and chikungunya in Europe [39]; it is also a known secondary vector of Zika virus in Latin America [28]. The species has spread rapidly throughout Europe, mainly due to being transported by road vehicles, where it is now a well-known biting pest together with *Ae. japonicus japonicus* [40]. In our study, the Italian population of *Ae. albopictus* from Padua, Northern Italy, was not a competent vector for JEV genotype III, although it is an efficient vector of other arboviruses such as chikungunya virus [41]. Previous reports have suggested that the different origins of the Italian *Ae. albopictus* populations which were introduced separately from different tropical and subtropical areas over the past three decades [41], could be the basis for differences in their vector competence. It is worth noting that the experimental conditions in this study maintained constant heat and humidity with a 24-hour day night photoperiod, which are standard conditions during vector competence studies [15, 19]. However, these conditions are not representative of natural conditions. Given that this and other studies suggest that temperature appears to be critical for both vector competence and vector mortality in this experimental system, future studies would need to incorporate local variation between minimum and maximum temperature/humidity means, to relate it with what occurs in nature. Infection experiments carried out in *Cx. pipiens* have shown that a limiting factor at which this species becomes unable to transmit JEV genotype III is temperature, with higher temperatures (25°C) causing increased mortality in infected mosquitoes, when compared to mosquitoes held at 20°C [15]. However, there was no increased mortality observed for *Ae. albopictus* or *Cx. quinquefasciatus* at 25°C in the present study, suggesting that under our experimental conditions an elevated temperature and infection with JEV strain SA-14 did not cause additional mosquito mortality. This may be a consequence of the mosquito species and virus strain used [3].

The labelling of virus antigen in *Cx. quinquefasciatus* confirmed that at 25°C, JEV was able to infect the posterior midgut epithelial cells such as ciliated pseudostratified intestinal cells, which corroborates detection of virus in the mosquito body by molecular means. This supports a previous study showing that midgut epithelial cells are a major site of viral replication [15]. In addition, viral antigen was observed in mid-gut and salivary glands by IHC, which demonstrated that at 25°C and by 14 dpi, the virus was able to overcome the midgut barrier and to infect secondary organs such as the salivary glands. Previous

studies found that JEV present in the midgut appeared viable by the recovery of live virus *in vitro* from homogenised mosquito bodies [15]. However, it was unclear whether the restriction of JEV to the midgut was a result of active anti-viral control by the mosquitoes or the lower experimental temperature restricting virus replication. The authors suggested that an increase in temperature, or an increase in the duration of the experiment, could potentially trigger further virus replication and escape from the midgut, our results suggest that temperature may be a contributing factor in full viral dissemination.

Conclusions

Of the mosquito populations studied, there was no evidence that the virus could infect or disseminate within the *Ae. albopictus* originating from Italy at 25°C at either 7 or 14 dpi. By contrast, *Ae. albopictus* originating from Spain and *Cx. quinquefasciatus* originating from Tanzania proved to be susceptible to infection as early as 7 dpi. Dissemination occurred in a proportion of infected mosquitoes and JEV was detected in the saliva of these mosquitoes, suggesting the potential of these mosquito populations to transmit JEV genotype III (strain SA-14). Considering that several mosquito species have been shown to be competent vectors for a number of arboviruses, our results contribute to this expanding dataset and indicate that if JEV were to emerge in new areas, there are a number of mosquito populations that could facilitate its transmission and persistence.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated by this study and used is presented within this published article.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

NJ obtained funding for the study. LMHT, AJF, SS, FZXL, SA, AN, conceived and designed experiments. LMHT, AJF, SS, FZXL performed the experiments. LMHT, AJF, SS, FZXL, SA, AN, SB, AD, PV, KLM, and NJ analysed the data. LMHT wrote the first draft. NJ revised the draft and all authors contributed to and approved the final draft.

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Figures

Figure 1

Map of Europe showing distribution *Aedes albopictus* and indicating the sites where the colonised strains were originally trapped (blue circle). Map published by European Centre for Disease Prevention (modified from <https://www.ecdc.europa.eu/en/publications-data/aedes-albopictus-current-known-distribution-march-2021>).

Figure 2

Boxplot comparing the cycle threshold (*ct*) values for *Aedes albopictus* and *Culex quinquefasciatus* from RNA extractions of specimens infected with Japanese encephalitis virus and maintained at 25°C. *Culex quinquefasciatus* *ct* values were significantly lower compared to *Ae. albopictus*, suggesting that quantity of viral RNA was higher in these samples. Significant difference denoted by a double asterisk (**).

Figure 3

Japanese encephalitis virus infection at 25°C of posterior midgut epithelial cells in *Cx. quinquefasciatus*. (a) Head (H), thorax (T), abdomen (Ab). (b) Intracytoplasmic immunolabelling in the distal lobes of salivary gland, defined by the presence of secretory masses (SM); intense antigen labelling particularly in the basal region of the epithelium (red arrow). (c) Antigen labelling in the apical ciliated cells (arrow) and basal epithelial cells (arrowhead) of the posterior midgut; lumen of the midgut (L). Scale bar: 500µm (a); 20µm (b, c).

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