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REVIEW

The new European invader *Aedes* (*Finlaya*) *koreicus*: a potential vector of chikungunya virus

Silvia Ciocchetta[a,b], Natalie A. Prow[a], Jonathan M. Darbro[a], Francesca D. Frentiu[b], Sandro Savino[d], Fabrizio Montarsi[c], Gioia Capelli[c], John G. Aaskov[b] and Gregor J. Devine[a]

[a]QIMR Berghofer Medical Research Institute, Royal Brisbane Hospital, Brisbane, Australia; [b]Institute for Health and Biomedical Innovation, and School of Biomedical Science, Queensland University of Technology, Brisbane, Australia; [c]Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy; [d]Geographic Information Systems, Department of Information Engineering, University of Padua, Padua, Italy

ABSTRACT

Arthropod-borne disease outbreaks, facilitated by the introduction of exotic mosquitoes, pose a significant public health threat. Recent chikungunya virus (CHIKV) epidemics in Europe highlight the importance of understanding the vector potential of invading mosquitoes. In this paper we explore the potential of *Aedes koreicus*, a mosquito new to Europe, to transmit CHIKV. Mosquitoes were challenged with CHIKV and maintained at two temperatures: 23 °C and a fluctuating temperature. Total CHIKV infection rates at 3, 10 and 14 days post-feeding were low for both temperature treatments (13.8% at 23 °C; 6.2% at fluctuating T). A low percentage (6.1%, $n = 65$) of mosquitoes maintained at a constant 23 °C showed dissemination of the virus to the wings and legs. Infection of mosquito saliva, with live virus, occurred in 2 mosquitoes. No dissemination was noted under the fluctuating temperature regime. Based on these results we conclude that CHIKV transmission by this species is possible.

KEYWORDS

Aedes koreicus; invasive mosquito species; fluctuating temperature; chikungunya; arbovirus; vector competence; arthropod-borne disease; public health

Introduction

Globalization of trade and travel often results in the introduction of new species into non-native territories [1–4]. Arthropod-borne virus (arbovirus) outbreaks of public health significance have occurred as a consequence [5–12]. In 2012, an *Aedes aegypti* population that had established in Madeira (Portugal) in 2004 [13,14] was responsible for the largest outbreak of dengue in Europe since 1928 [15]. More than 2000 cases were recorded [16] (Figure 1). Similarly, the continuing expansion of *Aedes albopictus* and *Ae. aegypti* [17–19] might aggravate the ongoing pandemic of Zika fever through South and Central America and the Caribbean [20]. A mutation in the chikungunya virus (CHIKV) that facilitates enhanced transmission by *Ae. albopictus* was introduced from India to Ravenna Province, Italy in 2007. The local presence of *Ae. albopictus* set off an epidemic of over 200 human cases [9]. The establishment of *Ae. albopictus* across southern Europe has also led to autochthonous outbreaks of dengue and chikungunya in France in 2010 and 2014 (Figure 1). Further chikungunya outbreaks were reported in France and Italy during 2017 [10–12]. In recent years, a new invader: *Aedes koreicus* has entered Europe, with the largest populations found in Italy [21–24].

Invasive mosquito species in Europe: an overview

Europe has suffered a number of mosquito invasions in recent decades. The first report of *Ae. albopictus* in Europe came from Albania in 1979 [25]. It was then reported from Italy: first at the Genoa docks in 1990 and then, one year later, in Padua. The species is presumed to have been introduced through the import of used tyres from the United States [26,27] and has now established over almost all of the Italian peninsula [28] and in 22 other European countries (www.ecdc.europa.eu).

The commercial trade in tiers was also the most likely cause of the establishment of *Aedes japonicus* in Europe [29]. This species has colonized most of Switzerland, large regions in Austria and Germany and is also present in Belgium, France, Netherlands, Hungary, Slovenia, Croatia and Lichtenstein [30–32]. In 2016, it was also discovered in Italy [33].

Ae. aegypti, the most important arbovirus vector of all [34] was present from the late 1700s in many southern European countries. Reasons for its subsequent disappearance from the region during the 1900s are unclear but it has since re-invaded Madeira (Portugal), European Russia, Georgia and North East Turkey [35,36]. Figure 1 is an updated representation of extent of exotic mosquito introductions in Europe.

CONTACT Silvia Ciocchetta ✉ silvia.ciocchetta@qimrberghofer.edu.au

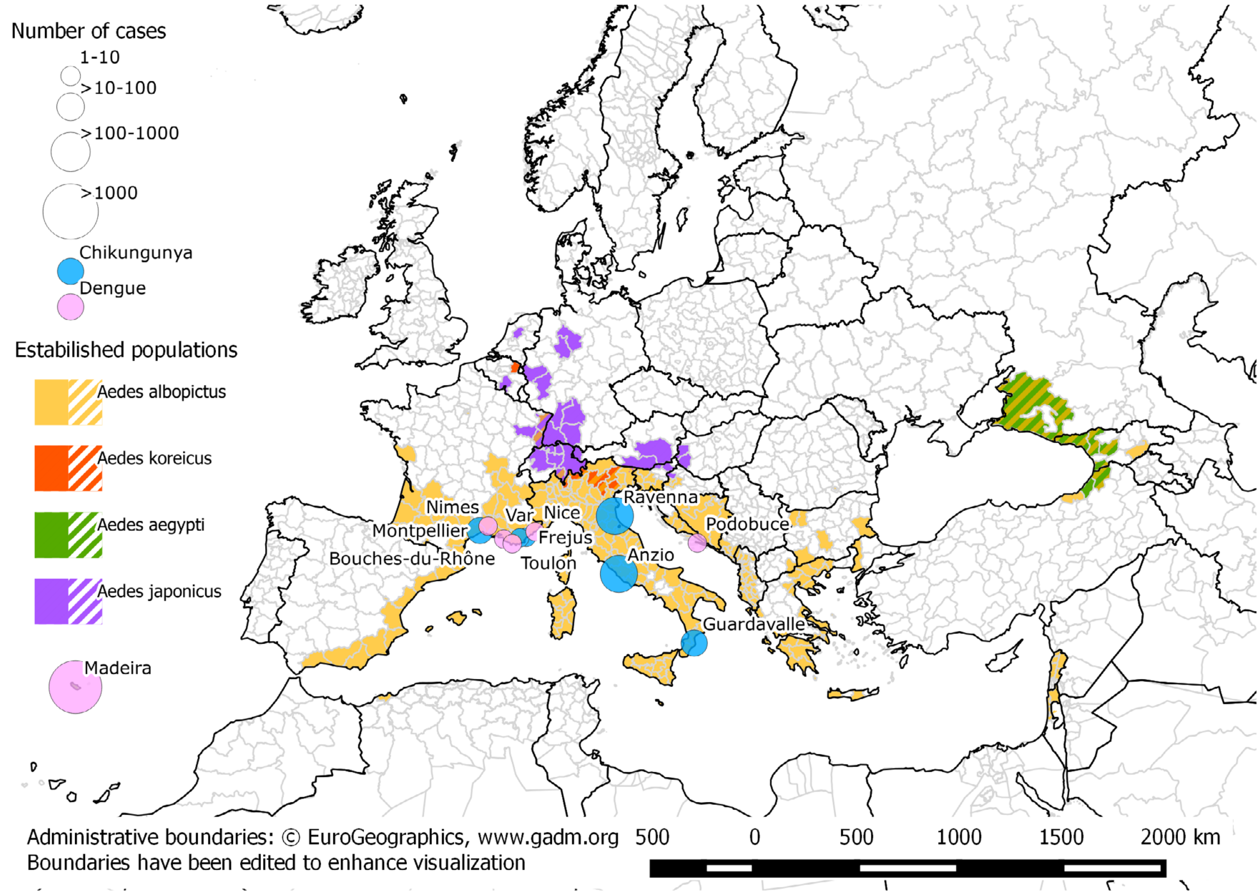

Figure 1. Distribution of invasive mosquito species in Europe (data collated from ECDC maps https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps) and locations and severity of autochthonous dengue and chikungunya outbreaks in Europe from 2007 [9,11,12,16,53–55,83–87]. Map created by Sandro Savino (University of Padua) and Silvia Ciocchetta (QIMR Berghofer).

A new European invader: Aedes koreicus

In 2011 a new aedine mosquito species, *Aedes* (*Finlaya*) *koreicus* (Edwards, 1917), was detected in Italy [21]. It has since established in Belgium, Russia, Switzerland, Germany, Hungary and Slovenia [37–42]. The species is well adapted to the temperate Italian climate and, in northern Italy, its distribution overlaps with *Ae. albopictus*. It has also colonized mountainous districts where *Ae. albopictus* remains absent [22,43] (Figure 2). Both mosquitoes breed in artificial containers, although *Ae. koreicus* is seldom found in the same containers as *Ae. albopictus* [44]. *Ae. albopictus* is a highly competitive mosquito and its displacement by *Ae. koreicus* seems unlikely [44]. The distribution of *Ae. koreicus* in Italy suggests that the area infested by the species represents only a small part of its potential European range. Dispersal models predict that parts of the Po and Adige valleys will be colonised over the next decade [24] and that it may establish in areas 400–1500 m above sea level, well above the range of *Ae. albopictus* (which remains below 600–800 m [45]). Despite this ongoing spread, the vector competence of *Ae. koreicus* for human arboviruses is under-explored. There are some limited observations on Japanese Encephalitis virus transmission [46–48] but nothing about its potential to mediate outbreaks of chikungunya.

Chikungunya virus: European outbreaks caused by invasive mosquitoes

Chikungunya is an arboviral disease caused by an alphavirus of the family Togaviridae. CHIKV is transmitted by *Aedes* mosquitoes and characterized by acute febrile arthralgia in symptomatic human patients [49]. Phylogenetic analysis has identified three different genotypes of the virus: West African, Asian and East/Central/South African (ECSA) [50].

The first outbreak of CHIKV in Europe occurred in 2007 in Italy and was mediated by the invasive vector *Ae. albopictus*, and the introduction of a virus with the E1–226 V mutation belonging to the ECSA genotype (Indian Ocean lineage) by a viraemic traveler returning from India to Ravenna province. The E1–226 V mutation increases virus infectivity in *Ae. albopictus* [9,51,52]. The high density of *Ae. albopictus* in the outbreak area facilitated an epidemic involving more than 200 symptomatic human cases [9]. Three years later, autochthonous transmission of CHIKV occurred in Fréjus in southeastern France, and involved two people, *Ae. albopictus* and an ECSA CHIKV strain lacking the adaptive mutation for *Ae. albopictus* [53]. In 2014, *Ae. albopictus* was responsible for transmitting CHIKV (E1–226 V) which resulted in 11 chikungunya cases in

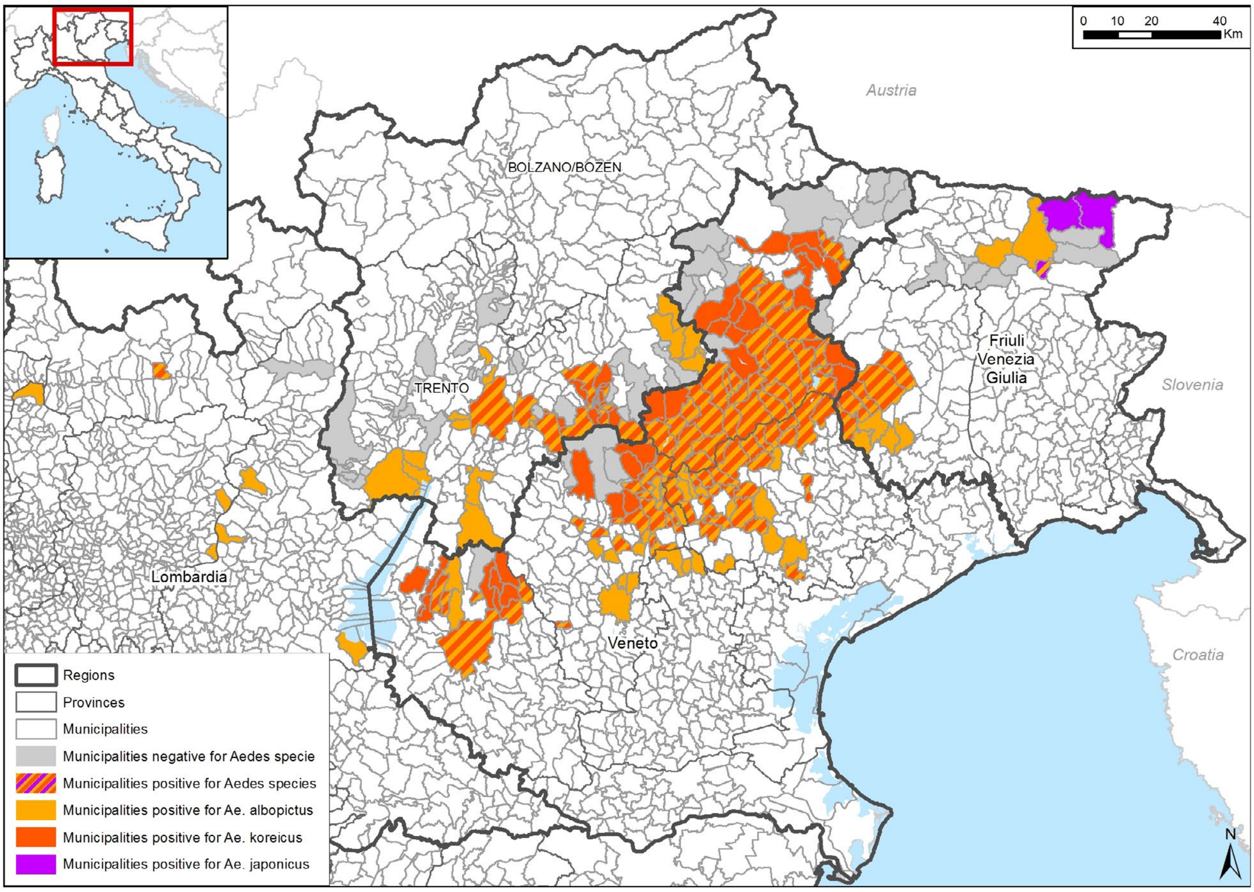

Figure 2. Distribution of *Ae. koreicus*, *Ae. albopictus* and *Ae. japonicus* in northeastern Italy (data and map provided by Geographic Information Systems office, IZSVe).

Montpellier, southern France [54]. In August 2017 eight autochthonous cases of chikungunya were diagnosed in the Var department, south-eastern France, an area where *Ae. albopictus* is established [55]. In the same month, an outbreak of CHIKV (without the E1–226 V mutation) in the Lazio and Calabria Regions of Italy caused more than 300 cases (Figure 1). *Ae. albopictus* is the only potential vector in the area [10–12].

Despite the rapid spread and anthropophilic habits of *Ae. koreicus* [56] and the now common transmission of CHIKV in some parts of Europe, the risk of transmission of human disease agents by this species is largely unknown. This manuscript presents preliminary data on the potential of *Ae koreicus* to transmit CHIKV and provides the first indication of the susceptibility of *Ae. koreicus* to infection with CHIKV *'La Reunion'* strain.

Materials and methods

We tested the potential of *Ae. koreicus* to transmit CHIKV *'La Reunion'* (carrying the mutation E1-A226 V) under constant and fluctuating temperature regimes in the laboratory. The fluctuating temperatures mimicked those occurring during a typical summer in Belluno, Italy, an area where there are established and thriving populations of *Ae. koreicus*. Mosquitoes, sourced from Belluno and kept in colony for 3 years, were reared in the quarantine insectary at QIMR Berghofer Medical Research Institute (QIMR Berghofer) as per Ciocchetta *et al.* [57]. Infected *Ae. albopictus* mosquitoes, sourced from Hammond Island, Torres Strait (Australia) and kept in colony for 3 years (also reared at QIMR Berghofer: Hugo *et al.* [70]), were used as a validation of our infection technique. Mosquito infection and sample processing were performed in a biosafety level 3 quarantine facility at QIMR Berghofer.

After a 24 h starvation period (in which the standard 10% sucrose solution was substituted with distilled water only) 342 adult female mosquitoes, 3–5 days old, were transferred to 750 mL plastic containers (ca. 100 individuals per container) covered with gauze lids. The mosquitoes were allowed to feed for 1 h through glass membrane feeders (37 °C) covered by a porcine intestinal membrane filled with an infectious blood meal [58]. The infectious blood meal was obtained by adding 1 mL of stock virus CHIKV *'La Reunion'* strain (LR2006-OPY1; GenBank KT449801 [59]) to 24 mL of defibrinated sheep blood (Life Technologies, Mulgrave, VIC, Australia) at a final titre of 10^8 $TCID_{50}$/mL ($TCID_{50}$ is the dilution ratio of the virus required to cause 50% mortality of cells used as a substrate for inoculation: in this experiment these were C6/36 *Ae. albopictus* cells). The infectious blood meal was sampled before and after feeding to determine that there was no degradation of virus titre over

Table 1. The daily fluctuating temperature regime under which *Ae. koreicus* was maintained (75 ± 5% relative humidity, 12 h light: 12 h dark cycle).

Phase	Degrees (°C)	Light step (Illuminance)
1 (0.15 h)	12	1 (1667 Lx)
2 (0.15 h)	12	2 (3334 Lx)
3 (2.30 h)	12	3 (5000 Lx)
4 (3 h)	17	3 (5000 Lx)
5 (3 h)	22	3 (5000 Lx)
6 (2.30 h)	27	3 (5000 Lx)
7 (0.15 h)	27	2 (3334 Lx)
8 (0.15 h)	27	1 (1667 Lx)
9 (3 h)	27	0 (0 Lx)
10 (3 h)	22	0 (0 Lx)
11 (3 h)	17	0 (0 Lx)
12 (3 h)	12	0 (0 Lx)

the feeding period. During feeding, a tube containing dry ice generated a small amount of CO$_2$ that encouraged feeding activity (Ciocchetta, unpublished observations). After feeding, mosquitoes were anesthetized with CO$_2$ and sorted in a cold Petri dish. Males and non-engorged females were discarded. Engorged females were transferred to a fresh container and maintained for 14 days in environmental chambers (MLR-352-PE Climate Chamber, Panasonic, Osaka, Japan) at two temperature regimes: (1) constant temperature of 23 °C, with a 12 h light: 12 h dark cycle and 75 ± 5% relative humidity; (2) fluctuating temperature based on the average temperatures registered during summer in Belluno (Italy) (Table 1) (data from Belluno Airport Meteorological Station, code 264, 46°42′00″N-12°07′48″E, Regional Agency for Environmental Prevention and Protection in Veneto, http://www.arpa.veneto.it/bollettini/storico, May to October 2011–2014), with a 12 h light: 12 h dark cycle and 75 ± 5% relative humidity. During the holding period mosquitoes were provided with 10% sucrose *ad libitum*.

At 3, 10 and 14 days post-feeding, *Ae. koreicus* females were anesthetized using CO$_2$, and dissected (25 mosquitoes on each day). The *Ae. albopictus* controls were included to validate our infection technique and were maintained at the constant temperature regimen. Dissection occurred at day 10 only. Legs and wings were removed from each mosquito and transferred to separate 1.5 mL screw cap microfuge tubes containing 4 zirconium silica beads stored at −80 °C. Live mosquitoes deprived of wings and legs were stuck to a glass plate with double-sided sticky tape. These mosquitoes were permitted to salivate for 20 min by inserting their proboscis into a P200 tip loaded with 50 μL of collecting medium (RPMI 1640, 2% Foetal Bovine Serum (FBS), 1% Penicillin-streptomycin, 0.25 μg mL^{-1} Amphotericin B) (Gibco; Thermo Scientific, Waltham, MA, USA). Peristaltic movements of the abdomen and labellae were observed for each individual mosquito under a stereoscope, confirming that saliva was expectorated. After 20 min, the contents of the P200 tips were emptied into a 1.5 mL microfuge tube. Each mosquito body was placed in a separate tube. All samples were stored at −80 °C until processing.

Each mosquito body was placed in a tube with collecting medium (as above) buffered with 1:100 10 mM HEPES, homogenized and inoculated onto C6/36 cells cultured in 5% CO$_2$ atmosphere at 27 °C [60]. Wings and legs were combined and processed in the same way as the body. Inoculations with saliva and infected blood followed the same procedure except for the initial homogenization step: 10μL of collecting medium with the mosquito saliva were directly inoculated to the plates after thawing. After a 3-day incubation period, all plates were assayed using an established ELISA technique [61] in which the conjugate solution (horseradish peroxidase labelled affinity purified goat-antimouse immunoglobulin G: DAKO Corporation, Carpinteria, CA, USA) was diluted at 1:1000. Non-neutralizing monoclonal antibodies (Hybridoma clone D7) were diluted 1:200 in blocking buffer and 50 μL were added to each well. Cells infected with CHIKV *'La Reunion'* provided a positive control for the assay. The final chromogenic substrate added to the plates consisted of 50μL/well of TMB (3,3′,5,5′-Tetramethylbenzidine Substrate System, Sigma-Aldrich®). The plates were then incubated in the dark for 30 min. A 50μL/well stop solution (Stop Reagent for TMB Substrate, Sigma-Aldrich®) was added and the absorbency at 450 nm was measured in a microplate reader (BioTek™ Synergy™ H4 Hybrid Microplate Reader). Wells were scored as positive for virus when the optical density (OD) was greater than twice the mean OD of the uninfected control wells [62]. The virus titres of individual mosquitoes were determined by calculating 50% end points [63] expressed as the log10 TCID$_{50}$ mL^{-1}. We first tested for CHIKV *'La Reunion'* infection in mosquito bodies. Wings, legs and saliva were processed only if body samples were positive for that particular mosquito.

Data analysis

CHIKV dissemination in *Ae. koreicus* under constant and fluctuating temperature regimes was compared using Fishers exact test (Prism GraphPad 6®, GraphPad Software, San Diego, CA, USA).

Results and discussion

All *Ae. albopictus* ($n = 4$) were positive for CHIKV at values consistent with previous experiments (10^6 TCID$_{50}$ mL^{-1}, $n = 1$; $10^{6.5}$ TCID$_{50}$ mL^{-1}, $n = 3$) [60] (Figure 3). This expected result validated our infection protocols. *Ae. koreicus* demonstrated a high survivorship after 14 days at constant and fluctuating temperatures (95.8%, $n = 71$; 98.1%, $n = 53$) and high feeding rates (65.5%, $n = 342$). Virus titres in the blood before and after the feeding period (1 h) were: 10^8 TCID$_{50}$ mL^{-1} and $10^{6.5}$ TCID$_{50}$ mL^{-1}, respectively. Despite these very favourable infection conditions, CHIKV *'La Reunion'* was subsequently detected in a very small percentage of mosquito bodies (13.8%, $n = 65$, T = 23 °C; 6.2%, $n = 64$, T = fluctuating). Titres

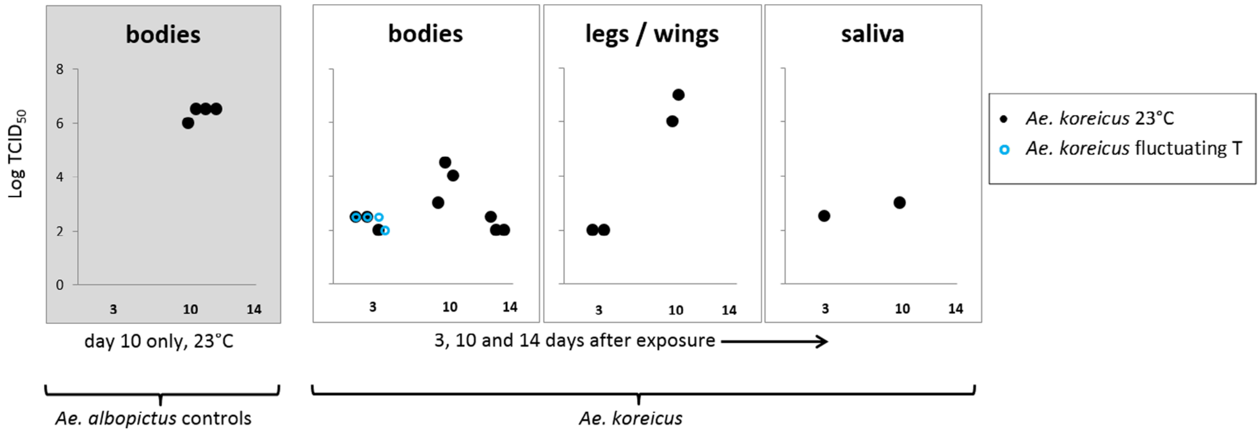

Figure 3. CHIKV *'La Reunion'* titres in *Ae. koreicus* measured 3, 10 and 14 days post-infection in mosquitoes at 23 °C and at fluctuating temperature (75 ± 5% relative humidity, 12 h light: 12 h dark cycle).
The validation of the technique, using a small number of *Ae. albopictus*, is also shown.

Table 2. CHIKV *'La Reunion'* infection and dissemination to the wings/legs and saliva in *Ae. koreicus* mosquitoes maintained at 23 °C and fluctuating temperature (75 ± 5% relative humidity, 12 h light: 12 h dark cycle).

Treatment	Day post feeding	Engorged mosquitoes	Infection (tested)	Dissemination wings/legs (tested)	Dissemination saliva (tested)
23 °C	3	21	3 (21)	2 (3)	1 (3)
	10	19	3 (19)	2 (3)	1 (3)
	14	25	3 (25)	0 (3)	0 (3)
Fluctuating T °C	3	21	4 (21)	0 (4)	0 (4)
	10	21	0 (21)	NT*	NT*
	14	22	0 (22)	NT*	NT*

Note: NT* = Not tested as bodies were negative.

ranged from $10^2 – 10^{4.5}$ TCID50 mL^{-1} (at 23 °C) to $10^2 – 10^{2.5}$ $TCID_{50}$ mL^{-1} (at the fluctuating temperature) (Figure 3).

Dissemination to wings and legs was observed in just four mosquitoes at days 3 and 10 post-feeding (10^2–10^7 $TCID_{50}$ mL^{-1}, Figure 3) and salivary dissemination occurred in two of those four individuals: $10^{2.5}$ $TCID_{50}$ mL^{-1} at day 3 post-feeding and 10^3 $TCID_{50}$ mL^{-1} at day 10 post-feeding (Figure 3). Only when held at a constant temperature did CHIKV *'La Reunion'* disseminate to the wings and legs and reach the saliva of *Ae. koreicus* (Table 2). These low infection rates are unlikely to be a consequence of insufficient incubation periods at low temperature: infection of *Ae. albopictus* salivary glands by CHIKV E1–226 V has been observed to occur 6 days post-exposure at 20 °C [64].

Artificially constant temperatures (23 °C) appeared to encourage greater dissemination of virus (4/65) when compared to 'real world' temperature fluctuations (0/64) but low infection rates meant that these differences were not significant (Fishers exact test, $p = 0.12$). *A posteriori*, assuming similar infection rates, we would need to have tested approximately double the number of mosquitoes (calculated using G*Power 3.1.9.2, $n = 296$, $\alpha = 0.05$, $1–\beta = 0.95$; [65]) to prove the impact of temperature regimes.

Ae. aegypti and *Ae. albopictus* are the main vectors of CHIKV [66–71] with *Ae. albopictus* being responsible for all CHIKV outbreaks in Europe [12,72–75]. The average temperature in European cities experiencing CHIKV outbreaks is 20 °C or above [73] although maximum transmission potential is realised between 26 and 29 °C [76]. A number of other *Aedes* species are effective vectors of CHIKV E1-A226 V under laboratory conditions and this includes *Ae. japonicus* [77] which is phylogenetically close to *Ae. koreicus* [78,79]. Salivary dissemination occurred in 38.5% of engorged *Ae. japonicus* after 14 days at 28 °C [77]. It is therefore unsurprising that *Ae. koreicus* shows vector potential under laboratory conditions, but its increasing geographic range in Italy (Figure 2), its human biting habit [56] and its capacity to expectorate live CHIKV after just three days suggests that CHIKV transmission by this species under field conditions should not be discounted.

Our work indicated that only a small proportion of *Ae. koreicus* from our laboratory colony could vector CHIKV under optimal rearing temperatures and that realistic temperature fluctuations might further mitigate the risks of transmission. This would be consistent with studies on dengue virus in which mosquitoes exposed to constant temperatures showed higher midgut infection levels [80] and higher dissemination [81] rates compared with mosquitoes maintained at fluctuating temperatures.

In our temperature regimens, relative humidity was a constant parameter although it may also be a variable that could affect *Ae. koreicus* vector competence. We recommend that further studies mimic the relative humidity fluctuations that may impact virus dynamics and mosquito survival in the field [82].

Conclusions

Our results suggest that low-level transmission of the CHIKV *'La Reunion'* strain by *Ae. koreicus* is possible in regions with temperatures similar to those used in our experiments. These findings help define the relative public health risks of this new mosquito invasion in comparison with the existing threats posed by *Ae. albopictus* and may assist in prioritising the resources that might be directed towards its surveillance or control.

We encourage further studies on the risks related to *Ae. koreicus* invasion across the range of climates and geographic regions suitable for the establishment of this invader. According to the European Centre for Disease Prevention and Control database (http://atlas.ecdc.europa.eu/public/index.aspx), at least 9332 travel-associated cases of CHIKV were imported to Europe between 2008 and 2015. These viraemic importations present a clear risk of autochthonous viral transmission where competent vectors exist. *Ae. koreicus* may now be added to that list.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

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Disclosure statements

No potential conflict of interest was reported by the authors.

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ORCID

Silvia Ciocchetta http://orcid.org/0000-0001-5007-8541
Francesca D. Frentiu http://orcid.org/0000-0001-8628-4216

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