The Common European Mosquitoes Culex Pipiens and Aedes Albopictus Are Unable to Transmit SARS-CoV-2, After a Natural-mimicking Challenge With Infected Blood

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Short report

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Abstract

Background

On March 11 2020, the World Health Organization (WHO) declared COVID-19 outbreak a pandemic. As the mosquito season progressed, the understandable concern that such insects could transmit the virus began to spread. In response to this request, the vector competence for SARS-CoV-2 of *Culex pipiens* and *Aedes albopictus*, the two most common species of vector mosquitoes in Europe, was investigated. Due to the peculiar feeding behaviour, the role of *Aedes albopictus* in a potential mechanical transmission of the virus was also evaluated.

Methods

For the vector competence study, mosquitoes were allowed to take up an infectious blood meal. Mosquitoes were collected and analysed at 0, 3, 7 and 10 days post-feeding. For the mechanical transmission test *Ae. albopictus* females were allowed to feed for a short time on a feeder containing infectious blood and then on a feeder containing virus-free blood. Mosquitoes and blood were tested for viral presence.

Results

Cx. pipiens and *Ae. albopictus* tested resulted not competent for SARS-CoV-2 and *Ae. albopictus* was unable to mechanically transmit the virus.

Conclusions

This study shows that the most common species of vector mosquitoes in Europe don’t transmit SARS-CoV-2 and, for the first time, that *Ae. albopictus* is unable to mechanically transmit the virus by feeding from a positive host to a healthy host.

Background

On 31 December 2019, Chinese health authorities reported a cluster of severe pneumonia cases of unknown etiology in the city of Wuhan (Hubei province, China). On 11 March 2020, the World Health Organization (WHO) declared COVID-19 a pandemic [1].

SARS-CoV-2 replicates primarily in the respiratory tract of infected patients, and, less efficiently, in a variety of other cell types. In addition, in some cases, especially in those with severe disease, the virus has been detected in the peripheral blood, [2–3–4] raising concern about the risk of vector-born transmission. In this regard, several previous published studies showed the possible role of blood sucking arthropods, including mosquitoes, in the transmission of viruses other than arboviruses for which they are known vectors [5–6–7]. However, the possibility that mosquitoes can transmit SARS-CoV-2 was declared unlikely by the WHO since the beginning of the epidemics [8].

This was confirmed later by experimental studies, which showed that SARS-CoV-2 is unable to replicate in *Aedes* mosquito cells in vitro [9] and in intrathoracically-infected *Aedes aegypti, Aedes albopictus* and *Culex quinquefasciatus* in USA [10]. No data are available yet for the species *Culex pipiens*, one of the most common human biter mosquito in Europe, where it is responsible for the transmission of West Nile and Usutu viruses [11]. In addition, a possible role of mosquitoes as mechanical vectors of viral etiological agents, including respiratory viruses, has already been observed [12–13]. Moreover, the propensity of some mosquito species such as *Ae. albopictus*, to take several meals on different hosts, even over a short period of time, can increase the risk of infectious disease transmission by increasing the frequency of contact with hosts [14].

Based on these assumptions, the vector competence for SARS-CoV-2 was assessed by oral infections for the two most common mosquito species in Europe, *Cx. pipiens* and *Ae. albopictus*. In addition, for the latter species the potential mechanical transmission of the virus was also evaluated. The study was carried out by the Istituto Superiore di Sanità (ISS) and the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe).

Methods

For the vector competence analysis, *Aedes albopictus* and *Cx. pipiens* colonies were fed on an infectious blood meal by an artificial membrane feeding system [15]. SARS-CoV-2 used for the experiments was isolated from patients infected in Italy during the Covid-19 pandemic [16]. For the infectious blood meal performed by ISS the virus was diluted 1:3 in rabbit blood (final virus concentration: 1.20×10^6 plaque forming units (PFU)/mL. For the experimental infections Italian *Cx. pipiens* and *Ae. albopictus* long-established laboratory colonies from ISS Insectary, were used. Five-eight-day-old mosquitoes were selected and cages containing 80 females for each species, were set up. To stimulate the blood sucking, the mosquitoes were starved 12 hours before the experimental infection, by depriving them of the sucrose solution. Infection experiment was performed, in a Biosafety Level 3 Laboratory (BSL3) cabinet at a temperature of 28 °C and a relative humidity of about 70%. Female mosquitoes were allowed to feed for 120 min through a pig intestine membrane covering a glass feeder containing the blood, maintained at 37 °C by a warm water circulation system. During the experiment two cages of *Cx. pipiens* and *Ae. albopictus* were subjected in parallel to an uninfected blood meal and subsequently monitored during the study, as control, to verify the survival of mosquito populations under experimental conditions. Experiments at IZSVe were performed on *Ae. albopictus* and followed the same protocol adopted by ISS with slight modifications, as the virus was diluted 1:20 to achieve a final concentration of 10^6 PFU/mL in defibrinated sheep blood and a different artificial feeder device was used (Hemotek®) for blood meal. Two cages of 80 females were set up using an Italian long-established laboratory colonies of *Ae. albopictus* from Entostudio (srl) Insectary. After the meal only fully engorged females were selected in a glove box and then transferred and maintained in a climatic chamber (26 ± 1 °C; 70% relative humidity; 14 h:10 h light/dark cycle) with a 10% sucrose solution. About 6–10 mosquitoes of both species were individually analysed at 0, 3, 7 and 10 dpi. At day 0, specimens of each mosquito species were tested to

confirm the ingestion of viral particles; at the following times (3, 7 and 10 dpi), each mosquito was individually examined by separating the body from the legs and wings. Mosquito bodies were investigated to evaluate the infection rate (IR), calculated as the number of SARS-CoV-2 RNA-positive bodies compared to the total number of females tested. Legs and wings were tested to assess the dissemination rate (DR), calculated as the number of samples with SARS-CoV-2 RNA-positive legs and wings (pooled together) among infected mosquitoes. SARS-CoV-2 RNA load was assessed by quantitative reverse transcription PCR (qRT-PCR). According to the procedure adopted by ISS, viral RNA was extracted by using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany). Five µL of extracted RNA were analysed for N2 gene by qRT-PCR, using protocol from the Centers for Disease Control and Prevention (CDC) [17]. SARS-CoV-2 titer was assessed by crossing point values compared with a standard curve obtained from 10-fold serial dilutions of virus stock of known concentration (3.6×10^6PFU/mL). According to the procedure adopted by IZSVe, viral RNA was extracted by using MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific) with automated extraction instrument KingFisher Flex (Thermo Fisher Scientific). Five µL of extracted RNA were analysed for E gene by Real-time RT-PCR (rRT-PCR) [18]. The samples were exchanged between the two Institutes for cross-analysis, to strengthen the molecular analysis and make the results more reliable.

For the mechanical transmission study, only the *Ae. albopictus* species was selected for its behaviour during the blood meal. This diurnal species does not make a complete meal with a single bite but, if disturbed, it can make short and frequent meals on the same or different hosts. The mechanical transmission test was performed by using the same membrane feeding system, at the same conditions. Eighty *Ae. albopictus* females, previously starved, were allowed to feed for a short time, being constantly disturbed, on a feeder containing infectious blood at a concentration of $1{,}2 \times 10^6$ PFU/mL. After 2 minutes, the feeder was removed and replaced immediately afterwards with a feeder containing 3 mL of a virus-free blood on which the mosquitoes completed the meal until they became replete. After meal, the blood was collected and analysed for the viral presence. The engorged female mosquitoes were killed and stored at -80 °C for subsequent analysis. The blood was subjected to RNA extraction and amplification by qRT-PCR, using both protocols.

Results

The vector competence analysis showed that immediately after the infectious blood meal, at 0 days post infection (dpi), all tested *Ae. albop*ictus and *Cx. pipiens* mosquitoes were positive in qRT-PCR, confirming the ingestion of viral particles. Viral titers were variable especially for *Ae. albopictus* with a minimum value of $1{,}97 \times 10^2$ PFU equivalents up to a maximum value of 2.23×10^3 PFU equivalents, depending on the blood meal taken by mosquitoes (Fig. 1). At 3 dpi 3 out of 22 *Ae. albopictus* and 2 out of 10 *Cx. pipiens* analysed resulted infected showing an IR value of 13.6% and 20%, respectively (Table 1). In *Ae. albopictus* bodies, the viral titers were 7.94×10^1, 1.01×10^2 and 8.18×10^2 PFU equivalents. In the two positive *Cx. pipiens* bodies the titers were considerably lower, 8.10 and 0.22 PFU equivalents. At 7 dpi only 1 out of 20 *Ae. albopictus* showed a positive body with a viral titer of 7.1 PFU equivalents. On the

contrary, no viral genome was found in *Cx. pipiens* at the same collection time. At the last collection time, 10 dpi, no positive bodies were found in both mosquito species. The virus was never detected in legs plus wings of the analysed *Ae. albopictus* and *Cx. pipiens* specimens (Table 1). During the experiment, the potentially infected mosquitoes of both species were allowed to lay eggs (first gonotrophic cycle). Larvae were reared and maintained up to the adulthood following standardized procedures [15]. Two pools of 10 larvae per species were processed for N2 gene by qRT-PCR and for E gene by rRT-PCR and resulted negative for SARS-CoV-2. In addition, mosquito adults (15 *Ae. albopictus* and 13 *Cx. pipiens*) of both species, emerged from the first gonotrophic cycle, were analysed for viral presence by qRT-PCR, resulting negative.

The results of the mechanical transmission analysis did not highlight the presence of viral genome in the virus-free blood on which the *Ae. albopictus* mosquitoes completed the meal immediately after feeding partially on a feeder containing infectious blood. The engorged mosquitoes were individually analysed by qRT-PCR. We analysed 21 mosquitoes, all SARS-CoV-2 positive. The viral titer detected in individual mosquitoes was variable, ranging from 6.32×10^2 to 3.44×10^1 PFU equivalents, depending on ingested viral particles by mosquitoes during the disturbed infectious blood meal.

Table 1
Infection and dissemination rates of *Aedes albopictus* and *Culex pipiens* artificially infected with SARS-CoV-2.

	Aedes albopictus		***Culex pipiens***	
Day post infection	IR* **(%)**	DR** **(%)**	IR* **(%)**	DR** **(%)**
0	12/12 **(100%)**	-	6/6 **(100%)**	-
3	3/22 **(13.6%)**	0/22 **(0%)**	2/10 **(20%)**	0/10 **(0%)**
7	1/20 **(5%)**	0/20 **(0%)**	0/10 **(0%)**	0/10 **(0%)**
10	0/22 **(0%)**	0/22 **(0%)**	0/11 **(0%)**	0/11 **(0%)**
* Infection rate: virus positive bodies/tested				
** Dissemination rate: virus positive legs + wings/tested				

Discussion

Human infection with SARS-CoV-2, a novel coronavirus of probable zoonotic origin [19], is characterized by a spectrum of clinical conditions, ranging from mild upper airway respiratory symptoms to severe life-threatening pneumonia. In Italy, in February 2020, with the approach of the favorable season for the development of mosquitoes, the COVID outbreak has aroused concern in public opinion and health authorities for a potential transmission of the disease through mosquito bites. This concern was also

justified by the detection of SARS-CoV-2 in the blood of some patients with percentages variable of positivity in human sera and/or whole blood from 8% [2] to 40% [20].

Among mosquito species, *Ae. albopictus* and *Cx. pipiens* are widespread in the country and represent efficient vectors of some of the most relevant and well-known arboviruses such as chikungunya and West Nile viruses.

In addition, the *Ae. albopictus*, due to its marked aggressiveness and anthropophilia, the wide diffusion at very high local densities and the trophic diurnal behavior, could represent a powerful mechanical vector of SARS-CoV-2 in an urban environment, especially in presence of a high viral circulation among the population and in absence of mosquito control activities, suspended during the lockdown. Starting from these assumptions, we tested the potential vector competence for SARS-CoV-2 of the most common and widespread mosquito species in Europe, *Ae. albopictus* and *Cx. pipiens*, using a membrane feeding system to simulate a more natural blood meal. SARS-CoV-2 genome was detected in the bodies of both species after 3 dpi, and at 7 dpi in the body of only one *Ae. albopictus* specimen, at a very low viral titer. The decreasing trend of the viral titer in the tested mosquitoes, at the different collection times, shows that the virus is progressively digested. A blood meal is digested by a mosquito on average after three days, but in many species this phase can last longer and exceed 5 days [21], like in one on the mosquitoes of our study, in whose body viral RNA was detected on the seventh day after the infectious meal. In particular, viral RNA was detected at a very low titer in the mosquito body, while it was undetectable in the wings and legs. This result show that SARS-CoV-2 did not spread to the mosquito haemocele, but remained confined to the midgut, indicating that the localization and its decrease are attributable to the digestion process and activation of the mosquito immune system.

As expected, the analysis of *Ae. albopictus* and *Cx. pipiens* larvae and adults, born from eggs deposited by potentially infected mosquitoes, demonstrated the absence of vertical transmission of the virus.

In addition to biological transmission, mechanical transmission of a pathogen is possible in nature [6]. For this reason, we also investigated the possible mechanical transmission of SARS-CoV-2 by the *Ae. albopictus*, a mosquito species with a diurnal and peculiar trophic activity which, if disturbed, can make short and frequent meals on the same or different hosts. *Ae. albopictus* females, that had partially fed on the infectious blood and, soon afterwards, completed the meal with virus-free blood, were all positive confirming the ingestion of viral particles by the mosquitoes. On the contrary, virus-free blood was analyzed and no viral genome was detected. This suggests that mosquitoes, partially engorged with a first infectious blood, are unable to mechanically release the virus, immediately after, biting an uninfected host.

Conclusions

In conclusion our findings provide further and definitive scientific evidence that SARS-CoV-2 cannot replicate and spread in the mosquito and this was proven naturally, i.e. through direct ingestion of an infectious blood meal. The mosquito's midgut epithelium plays an important role against invading

pathogens by acting as a physical barrier, but also activating local defenses and triggering the systemic immune response.

Finally, this study shows for the first time that *Ae. albopictus* is unable to mechanically transmit the virus by feeding from a SARS-CoV-2 positive host to a healthy host, even in the hypothetical case of very high viremia.

Abbreviations

WHO: World Health Organization; *Ae.: Aedes; Cx.: Culex;* ISS: Istituto Superiore di Sanità; IZSVe: Istituto Zooprofilattico Sperimentale delle Venezie; PFU: plaque forming units; BSL3: Biosafety Level 3 Laboratory; CDC: Centers for Disease Control and Prevention; qRT-PCR: quantitative reverse transcription PCR; IR: infection rate; DR: dissemination rate;

Declarations

Ethics approval

The protocol for routine blood mosquito feeding has been approved by the Service for Biotechnology and Animal Welfare of the Istituto Superiore di Sanità (National Institute of Health) and has been authorised by the Italian Ministry of Health with the Decree 222/2011-B, according to the Legislative Decree 116/92, which implemented in Italy the European Directive 86/609/EEC on laboratory animal protection. In accordance with this legislation the presence and approval of an Ethical Committee is not required. The animals used in this study were housed and treated in strict accordance with the recommendations in the Legislative Decree 116/92 guidelines and animal welfare was routinely checked by veterinarians from the Service for Biotechnology and Animal welfare. Rabbits, used in this study, were from the animal facility of the ISS; blood was collected from the ear vein of the rabbit according to the European legislation for the care and the use of laboratory animals. All efforts were made to minimize animal suffering. For the experiment at IZSVe animals were not handled, a commercial certified defibrinated sheep blood was used (Batch 19420.1). Pig intestine epithelium, used for the experimental infections, is a commercial fresh product, very common in Italy (used for making sausages); it can be found in every butchery or supermarket. We just rinsed it with a saline solution and cut a square portion before use.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

For ISS: CF, MDL, FS, LT conceived and coordinated the study; CF, MDL, FS, LT and AA performed the mosquito infection and subsequent sampling; CF and AA processed the mosquitoes and the blood of the mechanical transmission; GM performed the biomelocular analyses on the mosquitoes; CF wrote the manuscript together with MDL; GR read and revised the manuscript. The authors read and approved the manuscript.

For IZSVe: FM, MB and AM performed the mosquito infection and subsequent sampling; SR performed the biomolecular analyses on the mosquitoes; GC critically revised the manuscript.

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Figures

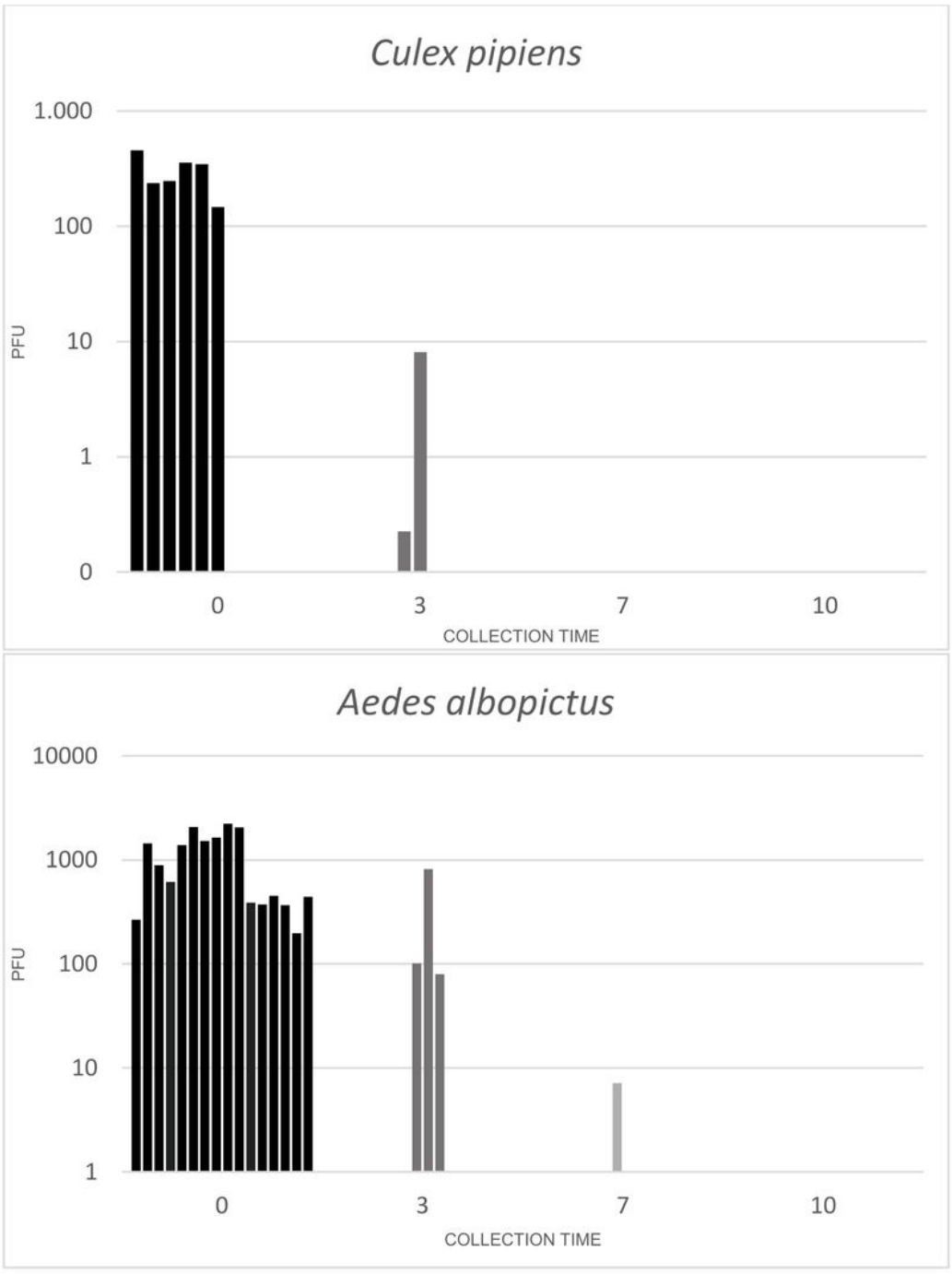

Figure 1

Viral titers in Aedes albopictus and Culex pipiens bodies analysed at different days post-infection with SARS-CoV-2.

Supplementary Files

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- graphicalabstract.pptx