RESEARCH ARTICLE

# Zika Virus, a New Threat for Europe?

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## Abstract

### Background

Since its emergence in 2007 in Micronesia and Polynesia, the arthropod-borne flavivirus Zika virus (ZIKV) has spread in the Americas and the Caribbean, following first detection in Brazil in May 2015. The risk of ZIKV emergence in Europe increases as imported cases are repeatedly reported. Together with chikungunya virus (CHIKV) and dengue virus (DENV), ZIKV is transmitted by *Aedes* mosquitoes. Any countries where these mosquitoes are present could be potential sites for future ZIKV outbreak. We assessed the vector competence of European *Aedes* mosquitoes (*Aedes aegypti* and *Aedes albopictus*) for the currently circulating Asian genotype of ZIKV.

### Methodology/Principal Findings

Two populations of *Ae. aegypti* from the island of Madeira (Funchal and Paul do Mar) and two populations of *Ae. albopictus* from France (Nice and Bar-sur-Loup) were challenged with an Asian genotype of ZIKV isolated from a patient in April 2014 in New Caledonia. Fully engorged mosquitoes were then maintained in insectary conditions (28°±1°C, 16h:8h light: dark cycle and 80% humidity). 16–24 mosquitoes from each population were examined at 3, 6, 9 and 14 days post-infection to estimate the infection rate, disseminated infection rate and transmission efficiency. Based on these experimental infections, we demonstrated that *Ae. albopictus* from France were not very susceptible to ZIKV.

### Conclusions/Significance

In combination with the restricted distribution of European *Ae. albopictus*, our results on vector competence corroborate the low risk for ZIKV to expand into most parts of Europe with the possible exception of the warmest regions bordering the Mediterranean coastline.

## Author Summary

In May 2015, local transmission of Zika virus (ZIKV) was reported in Brazil and since then, more than 1.5 million human cases have been reported in Latin America and the Caribbean. This arbovirus, primarily found in Africa and Asia, is mainly transmitted by *Aedes* mosquitoes, *Aedes aegypti* and *Aedes albopictus*. Viremic travelers returning from America to European countries where *Ae. albopictus* is established could become the source for local transmission of ZIKV. In order to estimate the risk of seeding ZIKV into local mosquito populations, the susceptibility of European *Ae. aegypti* and *Ae. albopictus* to ZIKV was measured using experimental infections. We demonstrated that *Ae. albopictus* and *Ae. aegypti* from Europe were not very susceptible to ZIKV. The threat for a Zika outbreak in Europe should be limited.

## Introduction

Zika virus (ZIKV) (genus *Flavivirus*, family *Flaviviridae*) is an emerging arthropod-borne virus transmitted to humans by *Aedes* mosquitoes. ZIKV infection in humans was first observed in Africa in 1952 [1], and can cause a broad range of clinical symptoms presenting as a "dengue-like" syndrome: headache, rash, fever, and arthralgia. In 2007, an outbreak of ZIKV on Yap Island resulted in 73% of the total population becoming infected [2]. Following this, ZIKV continued to spread rapidly with outbreaks in French Polynesia in October 2013 [3], New Caledonia in 2015 [4], and subsequently, Brazil in May 2015 [5, 6]. During this expansion period, the primary transmission vector is considered to have been *Aedes aegypti*, although *Aedes albopictus* could potentially serve as a secondary transmission vector [7] as ZIKV detection has been reported in field-collected *Ae. albopictus* in Central Africa [8]. As Musso et al. [9] observed, the pattern of ZIKV emergence from Africa, throughout Asia, to its subsequent arrival in South America and the Caribbean closely resembles the emergence of Chikungunya virus (CHIKV). In Europe, returning ZIKV-viremic travelers may become a source of local transmission in the presence of *Aedes* mosquitoes, *Ae. albopictus* in Continental Europe and *Ae. aegypti* in the Portuguese island of Madeira. *Ae. albopictus* originated from Asia was recorded for the first time in Europe in Albania in 1979 [10], then in Italy in 1990 [11]. It is now present in all European countries around the Mediterranean Sea [12]. This mosquito was implicated as a vector of CHIKV and DENV in Europe [13]. On the other hand, *Ae. aegypti* disappeared after the 1950s with the improvement of hygiene and anti-malaria vector control. This mosquito reinvaded European territory, Madeira island, in 2005 [14], and around the Black Sea in southern Russia, Abkhazia, and Georgia in 2004 [12]. The species was responsible for outbreaks of yellow fever in Italy in 1804 [15] and dengue in Greece in 1927–1928 [16]. To assess the possible risk of ZIKV transmission in Europe, we compared the relative vector competence of European *Ae. aegypti* and *Ae. albopictus* populations to the Asian genotype of ZIKV.

## Materials and Methods

### Ethics statement

The Institut Pasteur animal facility has received accreditation from the French Ministry of Agriculture to perform experiments on live animals in compliance with the French and European regulations on care and protection of laboratory animals. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Institut Pasteur. No specific

permits were required for the described field studies in locations that are not protected in any way and did not involve endangered or protected species.

## Mosquitoes

Four populations of mosquitoes (two populations of *Ae. aegypti*: Funchal (32°40'N, 16°55'W) and Paul do Mar (32°45'N, 17°13'W), collected on island of Madeira and two populations of *Ae. albopictus*: Nice (43°42'N, 7°15'E) and Bar-sur-Loup (43°42'N, 6°59'E) in France) were collected using ovitraps. Eggs were immersed in dechlorinated tap water for hatching. Larvae were distributed in pans of 150–200 individuals and supplied with 1 yeast tablet dissolved in 1L of water every 48 hours. All immature stages were maintained at 28°C ± 1°C. After emergence, adults were given free access to a 10% sucrose solution and maintained at 28°C ± 1°C with 70% relative humidity and a 16:8 light/dark cycle. The F1 generation of *Ae. aegypti* from Madeira and F7-8 generation of *Ae. albopictus* from France were used for experimental infections.

## Viral strain

The ZIKV strain (NC-2014-5132) originally isolated from a patient in April 2014 in New Caledonia was used to infect mosquitoes. The viral stock used was subcultured five times on Vero cells prior to the infectious blood-meal. The NC-2014-5132 strain is phylogenetically closely related to the ZIKV strains circulating in the South Pacific region, Brazil [5] and French Guiana [17].

## Oral infection of mosquitoes

Infectious blood-meals were provided using a titer of $10^7$ $TCID_{50}$/mL. Seven-day old mosquitoes were fed on blood-meals containing two parts washed rabbit erythrocytes to one part viral suspension supplemented with ATP at a final concentration of 5 mM. Rabbit arterial blood was collected and erythrocytes were washed five times with Phosphate buffered saline (PBS) 24 h before the infectious blood-meal. Engorged females were transferred to cardboard containers with free access to 10% sucrose solution and maintained at 28°C and 70% relative humidity with a 16:8 light/dark cycle. 16–24 female mosquitoes from each population were analyzed at 3, 6, 9, and 14 days post-infection (dpi) to estimate the infection rate, disseminated infection rate and transmission efficiency. Briefly, legs and wings were removed from each mosquito followed by insertion of the proboscis into a 20 µL tip containing 5 µL FBS for 20 minutes. The saliva-containing FBS was expelled into 45 µµL serum free L-15 media (Gibco), and stored at -80°C. Following salivation, mosquitoes were decapitated and head and body (thorax and abdomen) were homogenized separately in 300 µL L-15 media supplemented with 3% FBS using a Precellys homogenizer (Bertin Technologies) then stored at -80°C. Infection rate was measured as the percentage of mosquitoes with infected bodies among the total number of analyzed mosquitoes. Disseminated infection rate was estimated as the percentage of mosquitoes with infected heads (i.e., the virus had successfully crossed the midgut barrier to reach the mosquito hemocoel) among the total number of mosquitoes with infected bodies. Transmission efficiency was calculated as the overall proportion of females with infectious saliva among the total number of tested mosquitoes. Samples were titrated by plaque assay in Vero cells.

## Virus quantification

For head/body homogenates and saliva samples, Vero E6 cell monolayers were inoculated with serial 10-fold dilutions of virus-containing samples and incubated for 1 hour at 37°C followed by an overlay consisting of DMEM 2X, 2% FBS, antibiotics and 1% agarose. At 7 dpi, overlay

was removed and cells were fixed with crystal violet (0.2% Crystal Violet, 10% Formaldehyde, 20% ethanol) and positive/negative screening was performed for cytopathic effect (body and head homogenates) or plaques were enumerated (head and saliva samples). Vero E6 cells (ATCC CRL-1586) were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Eurobio), Penicillin and Streptomycin, and 0.29 mg/mL l-glutamine.

### Statistical analysis

All statistical tests were conducted with the STATA software (StataCorp LP, Texas, USA) using 1-sided Fisher's exact test and *P*-values>0·05 were considered non-significant.

## Results

### *Aedes aegypti* from Madeira transmit ZIKV efficiently

To test whether *Ae. aegypti* from a European territory were able to transmit ZIKV, we analyzed the vector competence of two *Ae. aegypti* populations collected on the island of Madeira based on three parameters: viral infection of the mosquito midgut, viral dissemination to secondary organs, and transmission potential, analyzed at 3, 6, 9, and 14 dpi. Only mosquitoes presenting an infection (i.e. infected midgut) were analyzed for viral dissemination. The two populations presented similar infection ($P$ = 0.50 (3 dpi), 0.17 (6), 0.36 (9), 0.50 (14); Fig 1) and disseminated infection ($P$ = 0.59 (3 dpi), 0.63 (6), 0.43 (9), 0.06 (14); Fig 1) with the highest rates measured at 9 dpi and 9–14 dpi, respectively. When examining transmission efficiency, only *Ae. aegypti* Funchal were able to transmit ZIKV at 9 (1 individual among 20 tested) and 14 dpi (1 among 20) (Fig 1). When considering the number of viral particles in heads, no significant difference was detected between *Ae. aegypti* Funchal and *Ae. aegypti* Paul do Mar ($P$ = 1 (3 dpi), 0.22 (6), 0.60 (9), 0.38 (14); Fig 2). When examining viral loads in saliva, only *Ae. aegypti* Funchal exhibited 1550 particles at 9 dpi and 50 at 14 dpi (Fig 2).

### French *Ae*. *albopictus* showed significantly reduced competence to transmit ZIKV

To determine if *Ae. albopictus* present in continental Europe were able to sustain local transmission of ZIKV as previously observed with CHIKV and DENV, we evaluated the vector competence of two *Ae. albopictus* populations collected in Nice and Bar-sur-Loup in the South of France. When compared with *Ae. aegypti*, the two *Ae. albopictus* populations showed similar infection rates at 3 dpi ($P$ = 0.08) and 6 dpi ($P$ = 0.11) and disseminated infection rates at 9 dpi ($P$ = 0.62) and 14 dpi ($P$ = 0.10) (Fig 1). Only one individual among 24 *Ae. albopictus* Bar-sur-Loup tested at 14 dpi was able to transmit ZIKV (Fig 1). When analyzing the number of viral particles in heads, only few mosquitoes were infected (Fig 2). When examining saliva, one *Ae. albopictus* Bar-sur-Loup exhibited 2 viral particles at 14 dpi (Fig 2).

In summary, ZIKV dissemination through *Ae. aegypti* was noticeably superior and the virus in saliva was detected earlier in *Ae. aegypti* than in *Ae. albopictus*. However both mosquito species showed similar transmission efficiencies at 9–14 dpi.

## Discussion

ZIKV could be transmitted, spread and maintained in Europe either via (i) Madeira where the main vector *Ae. aegypti* has been established since 2005 or (ii) Continental Europe where *Ae. albopictus* is known to have been present since 1979 [12]. We demonstrated that ZIKV was amplified and expectorated efficiently in saliva by European *Ae. aegypti* from Madeira. This contrasts with the lower vector competence for ZIKV of French *Ae. albopictus*. Taking these
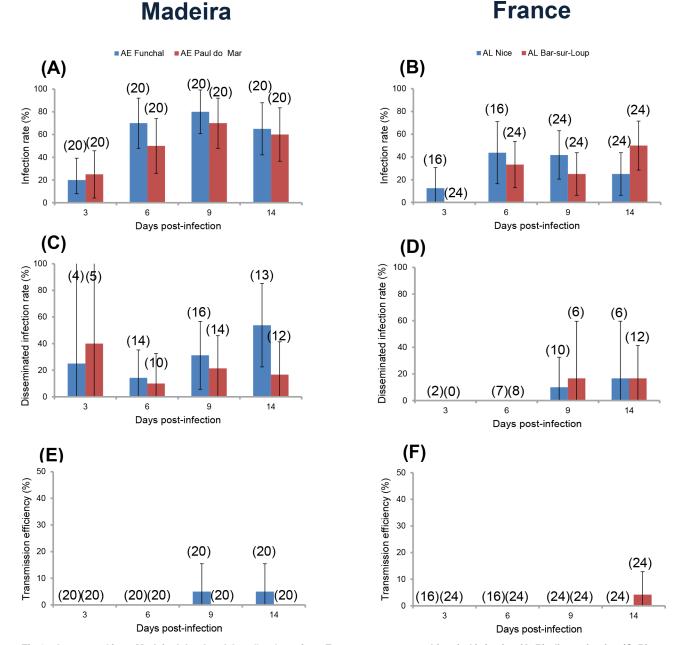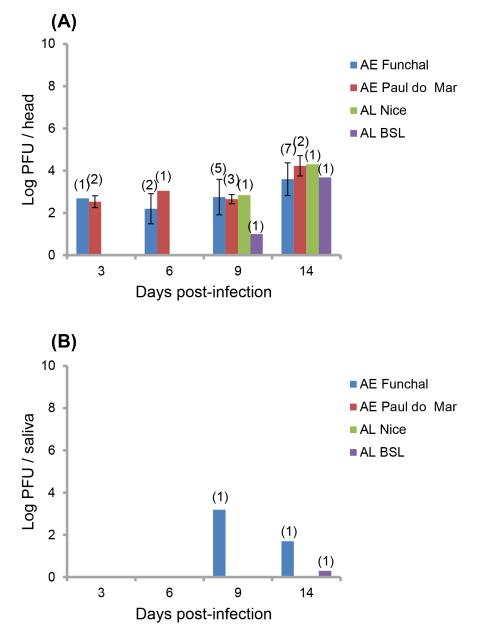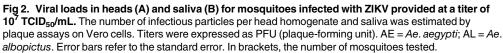
**Fig 1.** *Ae. aegypti* **from Madeira Island and** *Ae. albopictus* **from France were assessed for viral infection (A, B), dissemination (C, D), and transmission (E, F) at days 3, 6, 9, 14 after infection with ZIKV provided at a titer of $10^7$ TCID$_{50}$/mL.** 16–24 mosquitoes were sampled each day. Infection rates were measured as the percentage of mosquitoes with infected bodies among the total number of analyzed mosquitoes. Disseminated infection rates were estimated as the percentage of mosquitoes with infected heads (i.e., the virus has successfully crossed the midgut barrier to reach the hemocoel) among the total number of mosquitoes with infected bodies. The transmission efficiency was calculated as the overall proportion of females with infectious saliva among the total number of tested mosquitoes. AE = *Ae. aegypti*; AL = *Ae. albopictus*. In red, countries where ZIKV has been isolated. Error bars show the confidence intervals (95%). In brackets, the number of mosquitoes tested.

observations and the overall average lower temperatures of most regions of Europe into account, the risk of major outbreaks of Zika fever in most areas of Europe, at least for the immediate future, appears to be relatively low.

**(A)**



**(B)**



**Fig 2. Viral loads in heads (A) and saliva (B) for mosquitoes infected with ZIKV provided at a titer of $10^7$ TCID$_{50}$/mL.** The number of infectious particles per head homogenate and saliva was estimated by plaque assays on Vero cells. Titers were expressed as PFU (plaque-forming unit). AE = *Ae. aegypti*; AL = *Ae. albopictus*. Error bars refer to the standard error. In brackets, the number of mosquitoes tested.

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Our results highlight the potential risk for ZIKV transmission on Madeira where two main factors are present: the presence of the main vector, *Ae. aegypti* introduced in 2005 [18] and imported cases from Brazil with which Madeira, an autonomous region of Portugal, maintains active exchanges of goods and people sharing the same language. Thus Madeira Island could be considered as a stepping stone for an introduction of ZIKV into Europe.

Autochthonous cases of CHIKV and DENV have been reported in Europe since 2007: CHIKV in Italy in 2007, South France in 2010, 2014, and DENV in South France in 2010, 2013, 2015, and Croatia in 2010 [19]. The invasive species *Ae. albopictus* first detected in

Europe in 1979 [10] has played a central role in this transmission [19]. Thus, there might be a risk of a similar establishment of ZIKV in Europe upon the return of viremic travelers [20, 21]. We showed that *Ae. albopictus* from South France were less competent for ZIKV infection requiring 14 days to be expectorated in the mosquito saliva after infection. Therefore, we can suggest that the Asian tiger mosquito from Southern France and more widely, Europe, are less suitable to sustain local transmission of ZIKV compared to CHIKV and perhaps, DENV. *Ae. albopictus* Nice were not able to expectorate ZIKV in saliva at day 14 post-infection like *Ae. albopictus* Bar-sur-Loup suggesting two populations genetically differentiated.

Considering the extensive airline travel between Latin America and Europe, the risk for local transmission of ZIKV in the European area where the mosquito *Ae. albopictus* is widely distributed, is assumed to be minimal based on our studies of vector competence. Nevertheless, reinforcement of surveillance and control of mosquitoes should remain a strong priority in Europe since *Aedes* mosquitoes also transmit DENV and CHIKV and virus adaptation to new vectors cannot be excluded, as previously observed with CHIKV in La Reunion [22, 23].

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## Author Contributions

**Conceived and designed the experiments:** HJ ABF.

**Performed the experiments:** HJ GS LM.

**Analyzed the data:** GS CAS ABF.

**Contributed reagents/materials/analysis tools:** LM CAS.

**Wrote the paper:** HJ ABF.

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