

# Are *Aedes albopictus* or other mosquito species from northern Italy competent to sustain new arboviral outbreaks?

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**Abstract.** The Asian tiger mosquito *Aedes albopictus* (Skuse) (Diptera: Culicidae), native to Southeast Asia, has extended its geographical distribution to invade new temperate and tropical regions. This species was introduced in 1990 to Italy and has since become the main pest in urban settings. It was incriminated as a principal vector in the first European outbreak of chikungunya virus (CHIKV) in the province of Ravenna (Italy) in 2007. This outbreak was associated with CHIKV E1-226V, efficiently transmitted by *Ae. albopictus*. The occurrence of this outbreak in a temperate country led us to estimate the potential of *Ae. albopictus* to transmit CHIKV and dengue virus (DENV), and to determine the susceptibility to CHIKV of other mosquito species collected in northern Italy. Experimental infections showed that *Ae. albopictus* exhibited high disseminated infection rates for CHIKV (75.0% in Alessandria; 90.3% in San Lazzaro) and low disseminated infection rates for DENV-2 (14.3% in San Lazzaro; 38.5% in Alessandria). Moreover, *Ae. albopictus* was able to attain a high level of viral replication, with CHIKV detectable in the salivary glands at day 2 after infection. In addition, the other three mosquito species, *Anopheles maculipennis* Meigen, *Aedes vexans vexans* (Meigen) and *Culex pipiens* L., showed variable susceptibilities to infection with CHIKV, of 0%, 7.7% and 0–33%, respectively. This information on vector competence is crucial in assessing the risk for an outbreak of CHIKV or DENV in Italy.

**Key words.** *Aedes albopictus*, chikungunya, dengue, vector competence, Italy.

## Introduction

Since its establishment in Genova in 1990, the Asian tiger mosquito, *Aedes albopictus* (Skuse), has invaded most Italian regions, becoming the major human biting pest (Knudsen *et al.*, 1996). This species was accidentally introduced by trade in used tyres shipped from the U.S.A. (Dalla Pozza *et al.*, 1994). Its high ecological plasticity leads this species to colonize various small containers filled with clean fresh water or organic wastes (Romi & Majori, 2008). Moreover, its ability to feed on different hosts favours its rapid establishment in populated,

as well as in remote, areas (Valerio *et al.*, 2008). Mosquito adults are mostly observed from February to December and peak in density during the summer; they contributed in part to the first European outbreak of chikungunya virus (CHIKV) in 2007.

Chikungunya virus is endemic to Africa, Southeast Asia and the Indian subcontinent, but large CHIKV outbreaks were reported in 2005–2006 in the islands of the Indian Ocean (Renault *et al.*, 2007) and the Indian subcontinent (Yergolkar *et al.*, 2006). Travellers returning from areas affected by CHIKV have been diagnosed in several European

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Fig. 1. Sites of mosquito collections in northern Italy in 2008.

countries (Beltrame *et al.*, 2007). In 2007 a CHIKV outbreak occurred in northern Italy (Rezza *et al.*, 2007). The index case, who had travelled from India in June 2007, developed symptoms while visiting relatives in a northeastern village. A total of 204 cases were recorded between July and September 2007. The virus was detected in local populations of *Ae. albopictus*, confirming the mosquito's role as a main vector. This situation reflects a convergence of several factors favouring the emergence of CHIKV, including: rapid transportation; successful establishment of exotic mosquito species; inadequate mosquito control, and climatic conditions. The high viral loads of patients [e.g.  $10^{8-9}$  RNA copies/mL (Parola *et al.*, 2006)] in an environment highly infested with *Ae. albopictus* allowed the initiation of an inter-human cycle.

The Italian CHIKV strains isolated in 2007 carried a single amino acid substitution from an alanine to a valine at position 226 in the E1 glycoprotein (Rezza *et al.*, 2007; Bordi *et al.*, 2008). This A226V mutation, which was first identified during the outbreak in La Réunion Island, favours transmission by *Ae. albopictus* (Tsetsarkin *et al.*, 2007; Vazeille *et al.*, 2007). Thus, this mutant promotes outbreaks in regions from which the typical vector, *Aedes aegypti* (L), is absent.

The spread of *Ae. albopictus* in other European countries, as well as the increase of imported cases of *Aedes*-borne viruses such as CHIKV and dengue (DENV), indicates an increase in the risk for recurrent arboviral outbreaks in western countries. Here we report an evaluation of the vector competence for CHIKV and DENV of *Ae. albopictus* and other mosquito species, *Aedes vexans vexans* (Meigen), *Anopheles maculipennis* Meigen and *Culex pipiens* L., from northern Italy.

## Materials and methods

### Mosquitoes

Seven mosquito samples were collected in northern Italy as larvae and eggs (Fig. 1). The F0 females were fed with a 10% sucrose solution *ad libitum* and maintained in insectaries until infection. Four species were studied: *Ae. albopictus*, *Ae. vexans*, *An. maculipennis* and *Cx pipiens*.

### Virus

Tests were performed for two viruses, CHIKV and DENV; these represent the two main arboviruses associated with *Ae. albopictus*. The CHIKV strain isolated in November 2005 from a patient in La Réunion Island presented an amino acid change (A226V) in the envelope glycoprotein E1 (Schuffenecker *et al.*, 2006). This strain is phylogenetically close to isolates from the Italian strain of CHIKV (Bordi *et al.*, 2008; Kumar *et al.*, 2008). The dengue 2 (DENV-2) strain was isolated in 1974 from a patient in Thailand (Vazeille-Falcoz *et al.*, 1999). Both viral stocks were produced on *Ae. albopictus* C6/36 cells and stored at  $-80^{\circ}\text{C}$ .

### Oral infection of mosquitoes

Females deprived of sucrose solution 24 h before feeding were exposed to a bloodmeal composed of 67% washed rabbit erythrocytes and 33% viral suspension. The procedure is described in Vazeille-Falcoz *et al.* (1999). The bloodmeal had a final titre of  $10^{7.0}$  PFU (plaque-forming units)/mL for CHIKV and  $10^{8.0}$  MID<sub>50</sub> (mosquito infectious doses)/mL for DENV-2. These titres were selected according to data obtained from previous experiments (Vazeille-Falcoz *et al.*, 1999; Vazeille *et al.*, 2007). We confirmed that female mosquitoes ingested an infectious bloodmeal by titrations performed on randomly chosen females 1 h after ingestion; all were positive, confirming the ingestion of infectious viral particles (data not shown). To estimate the disseminated infection rate and vector competence, blood-fed females were maintained at  $28^{\circ}\text{C}$  for 14 days. Surviving females were killed at  $-80^{\circ}\text{C}$  and beheaded on a slide. CHIKV and DENV antigens were detected in head squashes by immunofluorescence assay (IFA). Mosquito samples were compared according to the disseminated infection rate which corresponds to the proportion of females positive by IFA on head squashes among surviving females 14 days post-infection (p.i.).

### Viral replication in *Ae. albopictus*

Samples from Alessandria and San Lazzaro were examined. Every 2 days from day 0 to day 14 p.i., five females were killed and dissected to collect the midgut and the salivary glands. The midgut controls the entry of the virus into the mosquito's haemocoel, which is followed by the infection of the salivary glands. The virus must then be secreted into the

saliva, in which it is transmitted the next time the mosquito attempts to feed. Midguts and salivary glands were prepared for RNA extraction using the Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany) followed by a one-step quantitative reverse transcription polymerase chain reaction (qRT-PCR) to detect the number of viral RNA copies. Protocols have been described in detail in Vazeille *et al.* (2007).

## Results

*Aedes albopictus* from Alessandria and San Lazzaro were highly susceptible to CHIKV, with high and not significantly different disseminated infection rates (Fisher's exact test:  $P = 0.19$ ) of 90.3% in San Lazzaro and 75.0% in Alessandria (Table 1). These mosquitoes were less susceptible to DENV-2, with lower and significantly different rates (Fisher's exact test:  $P = 0.01$ ) of 14.3% in San Lazzaro and 38.5% in Alessandria. For the other three species, disseminated infection rates for CHIKV ranged from 0% to 33.3%. Specimens of *An. maculipennis* and two samples of *Cx pipiens* (from Novi Ligure and Predosa) were not susceptible. *Aedes vexans* from Bosco Marengo showed a rate of 7.7%.

Figures 2 and 3 present the amount of viral RNA estimated in midguts and salivary glands of females from Alessandria and San Lazzaro analysed at different days p.i. For *Ae. albopictus* from Alessandria, the number of viral RNA copies in midguts increased from day 0 ( $10^{5.7} \pm 10^{0.1}$  copies/midgut) to day 4 p.i. ( $10^{7.5} \pm 10^{0.4}$  copies/midgut) and decreased slightly at day 6 p.i. to  $10^7 \pm 10^{0.5}$  copies/midgut. From day 8 to day 14 p.i., the number of viral RNA copies fluctuated at around  $10^8$  copies/midgut. In salivary glands, viral RNA was detectable at day 2 p.i. ( $10^{1.7} \pm 10^{0.6}$  copies/salivary glands) and decreased slowly to reach a minimum at day 6 p.i. ( $10^{1.3} \pm 10^{0.5}$  copies/salivary glands). Subsequently, viral RNA varied from  $10^{2.2} \pm 10^{0.7}$  copies/salivary glands at day 8 p.i. to  $10^{1.3} \pm 10^{1.2}$  copies/salivary glands at day 14 p.i.

For *Ae. albopictus* from San Lazzaro, the number of viral RNA copies in midguts increased from day 0 ( $10^{5.6} \pm 10^{0.2}$  copies/midgut) to day 2 p.i. ( $10^8 \pm 10^{0.5}$  copies/midgut)

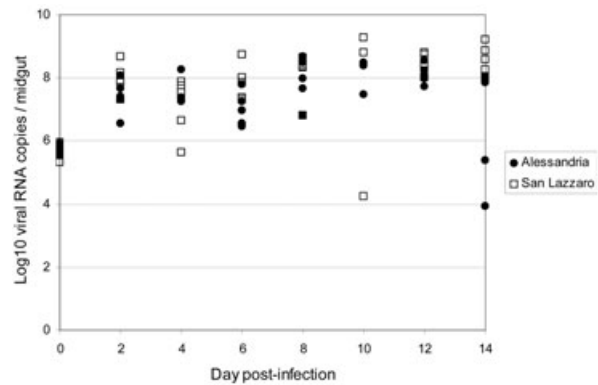
**Table 1.** Disseminated infection rates to chikungunya and dengue viruses of different mosquito species sampled in northern Italy in 2008.

Species	Site	Disseminated infection rate ( <i>n</i> )	
		CHIKV*	DENV-2†
<i>Aedes albopictus</i>	San Lazzaro	90 (31)	14 (42)
	Alessandria	75 (32)	38 (65)
<i>Aedes vexans</i>	Bosco Marengo	8 (26)	–
<i>Anopheles maculipennis</i>	Ovada	0 (10)	–
<i>Culex pipiens</i>	Novi Ligure	0 (1)	–
	Predosa	0 (45)	–

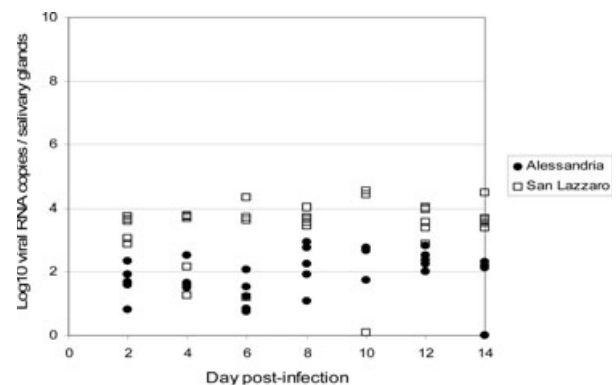
\*CHIKV at a titre of  $10^{7.5}$  PFU/mL;

†DENV-2 at a titre of  $10^{8.5}$  MID<sub>50</sub>/mL.

CHIKV, chikungunya virus; DENV-2, dengue 2 virus; *n*, number of females tested; PFU, plaque-forming units; MID<sub>50</sub>, mosquito infectious doses.



**Fig. 2.** Chikungunya virus replication in midguts of *Aedes albopictus* from Alessandria and San Lazzaro after oral infection.



**Fig. 3.** Chikungunya virus replication in salivary glands of *Aedes albopictus* from Alessandria and San Lazzaro after oral infection.

and fluctuated around  $10^8$  copies/midgut until day 14 p.i. In salivary glands, viral RNA became detectable at day 2 p.i. at  $10^{3.4} \pm 10^{0.4}$  copies/salivary glands and fluctuated around  $10^{3-4}$  copies/salivary glands. When comparing viral replication profiles of midguts, numbers of viral RNA copies according to day p.i. were quite similar in females from both Alessandria and San Lazzaro. By contrast, the number of viral RNA copies in the salivary glands of females from San Lazzaro was nearly twice as high as that in glands of females from Alessandria, strengthening the higher disseminated infection rate obtained at day 14 p.i. for San Lazzaro females (Table 1).

## Discussion

We showed that samples of *Ae. albopictus* from northern Italy are more susceptible to infection with CHIKV than DENV; CHIKV was detectable in the salivary glands from 2 days after the ingestion of an infectious bloodmeal. Moreover, a correlation between a high replication level in salivary glands and a high disseminated infection rate was found for *Ae. albopictus* from Alessandria when infected with CHIKV. In addition, *Ae. vexans*, *An. maculipennis* and *Cx pipiens*,

which are among the dominant mosquito species in northern Italy, were either not susceptible or were less susceptible to CHIKV.

*Aedes albopictus* developed a disseminated infection from day 2 after infection; salivary glands contained viral RNA detected by quantitative RT-PCR. This confirms the ability of the species to deliver early infectious viral particles through saliva (Dubrulle *et al.*, 2009). These results support our previous data on *Ae. albopictus* (Vazeille *et al.*, 2007, 2008; Moutailler *et al.*, 2009) and demonstrate the likely key role of this species as a vector of CHIKV in the province of Ravenna during the summer of 2007 (Rezza *et al.*, 2007). Among mosquito species usually found in the north of Italy, *Ae. vexans* exhibited a low susceptibility to CHIKV. However, given the very high densities of this species in July and August during the tourist season (Romi *et al.*, 2004), it may act as a potential secondary vector. In addition, *An. maculipennis*, which is widely found during the summer season, was refractory to CHIKV infection. This result supports our previous findings (data not shown), suggesting that most *Anopheles* spp. are refractory to CHIKV. Lastly, the two *Cx pipiens* samples were found to be refractory to the virus, as were those from southern France (Vazeille *et al.*, 2008).

At the viral dose tested ( $10^7$  PFU/mL), more than 70% of *Ae. albopictus* became infected after feeding on the infectious meal. Viral loads in patients can reach  $10^{8-9}$  viral RNA/mL during the first 2–4 days after the onset of symptoms (Parola *et al.*, 2006), allowing *Ae. albopictus* to be infected successfully and to transmit 2 days after infection (Dubrulle *et al.*, 2009). This short delay in transmission may heighten the risk for an imported infection to spread among local *Ae. albopictus*, as occurred in 2007 in Italy. The role of the other species cannot be neglected. Special attention should be given to *Ae. vexans*. The recent occurrence of an autochthonous transmission of CHIKV in Italy raises questions about the persistence of the virus in Italy. Most probably, because vertical transmission has not been demonstrated for *Ae. albopictus* infected with CHIKV (Vazeille *et al.*, 2009), the spread of infection will remain limited. However, numerous cases of imported CHIKV viraemia are reported each year in Europe from regions in which CHIKV is endemic. Therefore, Italy, as well as other parts of southern Europe, are at risk for infection with arboviruses, such as DENV and CHIKV, which have serious effects on public health. Further investigations should be encouraged with mosquitoes from other parts of Italy and even from other European countries in which *Ae. albopictus* has become established.

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