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EDITOR'S CHOICE

Experimental study of the susceptibility of a European *Aedes albopictus* strain to dengue virus under a simulated Mediterranean temperature regime

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Abstract. Dengue virus (DENV) has re-emerged in Europe driven by the geographic expansion of the mosquito species *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae) and the introduction of the virus by viraemic travellers. In the present study, the vector competence (VC) of *Ae. albopictus* collected in Catalonia (northeast Spain) was evaluated for two different DENV strains, DENV-1 and DENV-2, the serotypes responsible for all outbreaks of dengue that have occurred in Europe. Mosquitoes were reared under environmental conditions mimicking the mean temperature and humidity recorded in July on the Mediterranean coast of Catalonia. Mosquitoes were fed on an artificial infectious bloodmeal and, at 14 days post-exposure, infection, disseminated infection and transmission rates (IR, DIR, TR) and transmission efficiency (TE) were determined by testing the virus in the body, legs and saliva. The tested *Ae. albopictus* strain was found to be susceptible to both DENV-1 and DENV-2 and to be able to transmit DENV-1. This is the first time that the VC of *Ae. albopictus* for DENV has been tested in Europe in this specific context (i.e. mimicking the Mediterranean temperature and humidity recorded in Catalonia in July). This study confirms the potential of *Ae. albopictus* to start autochthonous DENV transmission cycles in the Mediterranean basin.

Key words. *Aedes albopictus*, dengue virus, Mediterranean climate, transmission, vector competence, Europe.

Introduction

Dengue virus (DENV) is a vector-borne *Flavivirus* of the family *Flaviviridae* and is mainly transmitted by *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). Dengue virus is the most widespread of the arboviruses that affect humans, with more than 390 million cases of infection estimated per year (Bhatt *et al*., 2013). Infection with any of the DENV serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) can either be asymptomatic (in 75% of cases) or result in one of the three clinical forms of (in increasing severity) dengue fever, dengue haemorrhagic fever and dengue shock syndrome (de la Cruz-Hernández *et al*., 2013).

Imported cases of DENV infection have been reported in several European countries in recent years (Neumayr *et al*., 2017). In 2010, two different autochthonous outbreaks occurred in, respectively, France (La Ruche *et al*., 2010) and Croatia (Gjenero-Margan *et al*., 2011). In 2012–2013, a large epidemic was reported in the Portuguese island of Madeira, which has been recolonized by *Ae. aegypti* (Wilder-Smith *et al*., 2014).

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Further autochthonous outbreaks were reported in France in 2013, 2014 and 2015 (Marchand *et al*., 2013; Giron *et al*., 2015; Succo *et al*., 2016). *Aedes albopictus* was indicated as the vector involved in all local transmissions in continental Europe, and the DENV strains belonged to either serotype 1 or serotype 2. *Aedes albopictus* was first introduced into Europe in 1979 (Adhami & Reiter, 1998). Since then, it has spread rapidly to several European countries, especially in the Mediterranean basin, and was first identified in Spain in 2004 (Aranda *et al*., 2006).

To assess the risk for local transmission events, it is essential to evaluate the vector competence (VC) of local mosquito populations. This is particularly important for *Ae. albopictus* as this species' VC for DENV has been shown to vary substantially among populations of different geographic origins (Gubler & Rosen, 1976; Boromisa *et al*., 1987). Previous studies in European *Ae. albopictus* populations have been performed at a constant temperature (28 °C) to simulate the mean temperature in tropical countries where DENV is endemic. Only a few studies have addressed the effects of realistic temperatures on VC for DENV, and all of them were carried out in *Ae. aegypti* (Lambrechts *et al*., 2011; Carrington *et al*., 2013a, 2013b; Gloria-Soria *et al*., 2017). These studies support the notion that local temperatures may alter VC. Therefore, measuring VC while simulating environmental conditions at the mosquito collection site is the best approach to estimating the risk for an outbreak in the case of DENV introduction. Consequently, this study was performed using the mean temperature and humidity recorded in July on the Mediterranean coast of the Iberian peninsula to estimate the risk for autochthonous transmission of DENV.

Materials and methods

Mosquito strain

The strain of *Ae. albopictus* used in this study was collected in Sant Cugat del Vallès in 2005 and reared in the laboratory to obtain a stable colony. Mosquitoes were reared under conditions that mimicked the environmental field conditions in their natural habitats in Catalonia during the month of July. Climatological data were provided by the Meteorological Service of Catalonia (www.meteo.cat). Mean day and night temperatures were calculated with respect to the summer photoperiod in Catalonia [26 °C for 14 h (light) and 22 °C for 10 h (dark)]. An average relative humidity of 86% was calculated.

Virus strains

Two DENV strains were tested: (a) strain BE 56 (hereafter named DENV-1), a human isolate that belongs to the DENV-1 serotype, collected during the dengue epidemic in Madeira (2012–2013), and (b) strain 20 112 953 (hereafter named DENV-2), a human isolate belonging to the DENV-2 serotype and collected in 2015 in Thailand.

Virus stocks of DENV-1 were produced following two passages on C6/36 cells, whereas DENV-2 was obtained after one passage on C6/36 cells. Supernatants were collected and stored at − 80 °C prior to their use for mosquito oral feeding.

Vector competence assay design

To investigate VC, four different rates were considered: infection rate (IR); disseminated infection rate (DIR); transmission rate (TR), and transmission efficiency (TE). The IR was defined as the proportion of mosquitoes with virus-positive bodies (abdomen, thorax and head) among the tested mosquitoes. The DIR was defined as the proportion of mosquitoes with infected legs among those with infected bodies. The TR was defined as the proportion of mosquitoes with DENV RNA in saliva among the number of mosquitoes with disseminated infection. Transmission efficiency was defined according to the proportion of mosquitoes with DENV RNA in saliva among the total number of mosquitoes tested.

Female mosquitoes aged 7–10 days and not previously blood-fed were fed using the Hemotek feeding system (Discovery Workshops, Accrington, U.K.) with a pathogen-free chicken skin as a membrane. The bloodmeal was prepared by mixing DENV-infected bovine blood with heparin and ATP (5×10^{-3} M) as phagostimulant (Sigma-Aldrich Corp., St Louis, MO, U.S.A.). The viral load was 5.5 $\log_{10}$ $TCID_{50}$/mL. The viral titre was chosen based on the reported viraemia level reached in human patients infected with DENV (Tang *et al*., 2010). After blood feeding, 10% of fully engorged females were randomly collected and killed to confirm virus exposure. Then, fully engorged females were selected under carbon dioxide (CO_2) anaesthesia and individually transferred to cardboard cages (Watkins & Doncaster Ltd, Leominster, U.K.). Females were kept for an extrinsic incubation period (EIP) of 14 days inside the climatic cabinet. Sucrose solution (10%) was administered on cotton pledgets placed on the mesh screen. At 14 days post-exposure (d.p.e.), all mosquitoes were anaesthetized using CO_2 and dissected. The legs and wings were detached from the body, and both parts were separately homogenized in 0.5 mL of Dulbecco's modified Eagle's medium (DMEM) (Lonza Group AG, Basel, Switzerland). The samples were homogenized at 30 Hz for 1 min using TissueLyser II (Qiagen GmbH, Hilden, Germany) and stored at − 80 °C until tested for DENV. The same protocol was used in all assays aimed at estimating the mortality rate, as well as in those designed to obtain IR and DIR data.

To estimate transmission, two separate assays were designed. In the first assay, transmission was tested using FTA™ cards at two different time-points: 9 d.p.e. and 14 d.p.e. On those days, cotton pledgets were replaced by FTA™ cards (GE Healthcare, Little Chalfont, U.K.). The FTA™ cards were soaked with Manuka honey (Manuka Health New Zealand, Te Awamutu, New Zealand) mixed with a blue alimentary colorant. After collection, FTA™ cards were resuspended in 0.3 mL of phosphate-buffered saline (PBS) and stored at − 80 °C until tested. In the second assay, transmission was tested at 14 d.p.e. with two different methods: (a) using FTA™ cards, as described above, and (b) performing a direct salivary extraction using a capillary technique, as previously described (Dubrulle

et al., 2009). Briefly, after the dissection of the legs and wings, the proboscis was inserted into a P20 pipette tip filled with 7 μL of a 1 : 1 solution of fetal bovine serum (FBS) and 50% sucrose solution. To stimulate salivation, 1 μL of 1% pilocarpine (Sigma-Aldrich Corp.) prepared in PBS at 0.1% Tween 80, was applied to the thorax of each mosquito. After a period of 60 min, the solution containing the saliva was expelled into 1.5-mL tubes containing 193 μL of DMEM; 150 μL were used for viral RNA extraction and the remaining 50 μL were used for DENV isolation.

Virus detection

Viral RNA was extracted from bodies, legs, FTA™ cards and saliva samples with the NucleoSpin® RNA Virus Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's recommendations. The viral RNA was detected by real-time reverse transcription polymerase chain reaction (RT-PCR) as previously described (Leparc-Goffart *et al*., 2009) with minor modifications. A fragment of 88 bp from the 3′ UTR region was amplified using primers DF (AGGACYAGAGGTTAG AGGAGA), DR (CGYTCTGTGCCTGGAWTGAT) and probe DP (6FAM-ACAGCAT ATTGACGCTGGGARAGACC-TAMRA). Amplification was performed using the AgPath One-Step ID RTPCR Kit (Ambion, Applied Biosystems, Inc., Foster City, CA, U.S.A.), and a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc.) programmed as follows: 45 °C for 10 min; 95 °C for 10 min, and 40 cycles of 97 °C for 15 s and 55 °C for 30 s.

Virus isolation and titration

Virus isolation was performed in a monolayer of C6/36 cells. Cells were incubated for 6–7 days (28 °C, 5% CO_2). As a cytopathic effect was not observed, DENV replication was detected in the supernatant using the real-time RT-PCR.

Both DENV stocks were titrated in a monolayer of C6/36 cells. Routinely, eight wells were infected for each 10-fold dilution and 20-μL quantities of inoculum were spiked into each well. After that, 150 μL MEM (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.) supplemented with 2% FBS (Life Science Co., London, U.K.), 2 mM L-glutamine, non-essential amino acids, 1000 U/mL of penicillin, 10 mg/mL of streptomycin and 500 U/mL of nystatin (all from Sigma Corp.) were added per well and the plates were incubated at 28 °C and 5% CO_2 for 7 days. Calculation of the viral titre was performed by virus detection in each well using the real-time RT-PCR described above. Cycle threshold (Ct) values ranged from 18.38 to 22.45 in those wells in which the virus replicated. The $TCID_{50}$/mL was calculated using the method of Reed and Muench (Villegas, 1998).

Estimations of IR and DIR

Assays to estimate IR and DIR were conducted using mosquitoes from the same colony and an identical protocol (i.e. they were two replicates of the same experiment). Thus, the results from the first and second assays could be combined and the differences between DENV-1 and DENV-2 evaluated using a chi-squared test.

In addition, the ability of each strain of DENV to replicate in different mosquito tissues (body and legs) was also evaluated using retrotranscriptase quantitative PCR (qPCR). Mean Ct values of the bodies and legs infected with DENV-1 and DENV-2, respectively, were compared. Briefly, the normality of the data was assessed using the Shapiro–Wilk test, and then data were compared using the *t*-test (in the case of normally distributed data) or the Wilcoxon rank sum test (in the case of non-normally distributed data). All calculations were carried out using R statistical software (http://cran.r-project.org/).

Results

Mosquito infection

The results of the assays for the estimations of mortality, IR and DIR are shown in Table 1.

The finding of positive bodies indicated that the virus was able to cross the midgut infection barrier successfully at 14 d.p.e. Positive legs showed mosquitoes in which the virus was also able to cross the midgut escape barrier. Assessment of infection showed that the cumulative IRs for DENV-1 and DENV-2 were 53% (49/93) and 33% (26/78), respectively. The statistical analysis revealed the IR was significantly higher for DENV-1 than for DENV-2 ($P = 0.041$). The cumulative DIR values were also higher for DENV-1 than for DENV-2 at 53% (26/49) and 35% (9/26), respectively (Table 1). However, the difference was not statistically significant ($P = 0.12$).

Viral loads of DENV-1 and DENV-2 were analysed in bodies and legs. The mean ± standard deviation (SD) Ct values of the bodies infected with DENV-1 and DENV-2 were 23.0 ± 2.8 and 25.6 ± 3.5, respectively (Fig. 1).

The result of the Wilcoxon rank sum test indicated that the difference in mean Ct values between DENV-1 and DENV-2 was statistically significant ($P = 0.00016$). This result suggested that DENV-1 was able to replicate more efficiently than DENV-2 in the body of *Ae. albopictus*. By contrast, the mean ± SD Ct values of the legs infected with DENV-1 and DENV-2 were 32.1 ± 2.0 and 34.0 ± 3.4, respectively (Fig. 1). The result of the *t*-test indicated that the difference in mean Ct values between DENV-1 and DENV-2 was not statistically significant ($P = 0.151$). Therefore, although DENV-1 was able to replicate more efficiently than DENV-2 in mosquito bodies, females with disseminated infection had similar amounts of virus in the legs regardless of viral strain.

Transmission of DENV

In the first assay, the FTA™ cards were collected at 9 d.p.e. and at 14 d.p.e. The FTA™ cards collected at 9 d.p.e from both groups (DENV-1 and DENV-2) showed negative findings (Table 2). However, two FTA™ cards collected at 14 d.p.e. from two mosquitoes exposed to DENV-1 were positive. These

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Table 1. Mortality, infection rates (IRs) and disseminated infection rates (DIRs).

	Mortality		IR		DIR	
	DENV-1	DENV-2	DENV-1	DENV-2	DENV-1	DENV-2
First assay	7/50 (14%)	15/50 (30%)	27/43 (63%)	9/35 (26%)	10/27 (37%)	5/9 (56%)
Second assay	0/50 (0%)	6/49 (12%)	22/50 (44%)	17/43 (40%)	16/22 (73%)	4/17 (24%)
Total	7/100 (7%)	21/99 (21%)	49/93 (53%)	26/78 (33%)	26/49 (53%)	9/26 (35%)

Rates were obtained at 14 days post-exposure for dengue virus (DENV) serotypes 1 (DENV-1) and 2 (DENV-2) in the first and second assays.

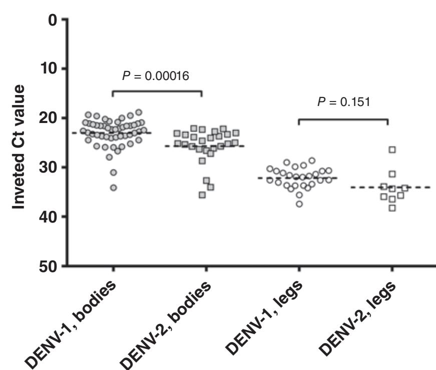

Fig. 1. Viral loads of dengue virus (DENV) serotypes 1 (DENV-1) and 2 (DENV-2) in bodies and legs of infected mosquitoes. The mean Ct value for each group is represented by a discontinuous line. *P*-values indicate a statistically significantly higher viral replication for the DENV-1 strain than for the DENV-2 strain in bodies ($P = 0.00016$). No statistical significant difference ($P = 0.153$) between viral replications in legs was evidenced.

specimens also showed positive legs, indicating the presence of a disseminated infection. Therefore, the TR for DENV-1 was 20% (2/10). Conversely, at the same time-point (14 d.p.e.), all the FTA™ cards from mosquitoes exposed to blood infected with DENV-2 were negative.

In the second assay, all the FTA™ cards and saliva samples were collected at 14 d.p.e. The FTA™ cards and saliva samples from mosquitoes exposed to both viral strains tested negative by RT-qPCR. Moreover, direct isolation in Vero cells from saliva samples gave negative results.

Given the results obtained using molecular techniques with FTA™ cards, the estimated TE for DENV-1 was 4.7% (2/43).

Discussion

For the first time in Europe, the VC of *Ae. albopictus* for DENV was estimated under a protocol that mimicked the temperature conditions present in a Mediterranean area during the summer month of July. Recent data suggest that the accurate assessment of the risk for DENV transmission in a mosquito population requires the incorporation of local temperature in the VC experiments (Gloria-Soria *et al.*, 2017). The results of the present study provide evidence that the *Ae. albopictus* strain tested is susceptible to oral infection with both DENV-1 and DENV-2 at a viral load ($5.5 \log_{10}$ $TCID_{50}$/mL) within the range of viraemia ($3–8 \log_{10}$ $TCID_{50}$/mL) observed in humans (Salazar *et al.*, 2007). The IR was statistically higher for DENV-1 than for DENV-2, which indicates that susceptibility is dependent on the serotype of DENV, as observed by Gubler & Rosen (1976).

The *Ae. albopictus* strain tested in the present study was more susceptible to DENV-1 infection in terms of disseminated infection (DIR: 53%) than the French population of *Ae. albopictus* (DIR: 28–45%) exposed to $10^{5.3}$ focus-forming units (FFU)/mL of bloodmeal and maintained at a constant temperature (28 °C) (Vega-Rua *et al.*, 2013). Such variation may be explained by the different environmental conditions assayed, but geographical differences in the vector population and the virus strain cannot be ruled out.

Previous studies examining VC in Mediterranean populations of *Ae. albopictus* (from France, Italy and Lebanon) (Moutailler *et al.*, 2009; Talbalaghi *et al.*, 2010; Haddad *et al.*, 2012) for the same Asian DENV-2 genotype and conducted under identical environmental conditions (constant temperature of 28 °C) showed a wide range of DIRs of 12–69%, 14–38% and 32–47%, respectively. The fluctuating conditions of the present study (daytime temperature of 26 °C and night-time temperature of 22 °C) produced a DIR of 35% for the DENV-2 strain, which is within the range of DIRs reported in the earlier works (Moutailler *et al.*, 2009; Talbalaghi *et al.*, 2010; Haddad *et al.*, 2012).

In addition to differences in IRs between DENV strains, the present study found a significant difference in viral infectivity. The comparison of mean Ct values in the body for both DENV strains indicated a statistically significantly higher viral replication for DENV-1 than for DENV-2, which may reflect differences in the immune response to viral infection (Sánchez-Vargas *et al.*, 2009). By contrast, there were no statistically significant differences between the strains tested in the mean Ct values for the legs. This suggests that the efficiency of viral replication in the midgut did not determine the amount of virus in other tissues after dissemination, as reported in previous studies (Bosio *et al.*, 1998).

The use of FTA™ cards was originally developed for field studies (Hall-Mendelin *et al.*, 2010, 2017). However, FTA™ cards may also be used in VC assays and allow the collection of saliva from a single mosquito at different time-points without requiring the specimen to be killed. The technique does not require trained personnel, whereas the capillary technique does, and reduces the operative time required to collect the sample. The principal disadvantage of the technique is that the saliva cannot be used for direct virus isolation in cell culture. Thus, although the use of FTA™ cards cannot replace virus isolation, it represents a good strategy for evaluating transmission capacity

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Table 2. Transmission rates of dengue virus (DENV) serotypes 1 (DENV-1) and 2 (DENV-2) in the first and second assays.

	FTA™ at 9 d.p.e.		FTA™ at 14 d.p.e.		Saliva	
Assay	DENV-1	DENV-2	DENV-1	DENV-2	DENV-1	DENV-2
1	0/43	0/35	2/10	0/5	NA	NA
2	NA	NA	0/16	0/4	0/50	0/43

Positive samples of FTA™ cards (9 d.p.e. and 14 d.p.e.) and saliva (14 d.p.e.) by retrotranscriptase quantitative polymerase chain reaction. d.p.e., days post-exposure; NA, not applicable.

at intermediate time-points without the need to create subgroups of specimens and sacrifice them periodically.

The transmission capacity of each mosquito was tested by using FTA™ cards at two different time-points (9 d.p.e. and 14 d.p.e.). At 9 d.p.e., no mosquitoes were able to transmit either strain. However, at 14 d.p.e., the mosquito strain tested was able to successfully transmit DENV-1. By contrast, DENV-2 was not detected in mosquito saliva at 14 d.p.e. at the conditions used in the present work. These negative results do not imply that DENV-2 transmission will not occur under different conditions. Thus, the extended period needed to complete the EIP of DENV (from 9 d.p.e. to 14 d.p.e.) may have an impact on the VC of this Spanish mosquito strain, potentially reducing the risk for outbreak and providing a larger time window in which to implement surveillance and vector control measures. In previous VC studies using Mediterranean mosquito populations (Haddad *et al*., 2012; Vega-Rua *et al*., 2013), EIP varied although the mosquitoes were held at a constant temperature of 28 °C. The DENV-1 strain presented a shorter EIP and could be transmitted by a French population of *Ae. albopictus* at 9 d.p.e. (67%) (Vega-Rua *et al*., 2013). By contrast, DENV-2 seemed to require a longer EIP to achieve transmission (Vega-Rua *et al*., 2013). The same strain was not detected in saliva of a Lebanese *Ae. albopictus* population at 10 d.p.e., but achieved a 38% TR at 21 days (Haddad *et al*., 2012). It is important to note that, by contrast with the previous studies, the present experimental infections were performed under a protocol that mimicked fluctuating temperature conditions, which strengthens the findings of this work. The average temperature within the current profile (22–26 °C) is 24 °C, which is 4 °C lower than temperatures used in the comparable studies mentioned above, and which may potentially result in lower infection and transmission rates, and also lengthen the EIP (Poole-Smith *et al*., 2015). The results of previous studies in *Ae. aegypti* VC for DENV under realistic temperature regimes indicate that fluctuations around low temperatures (20 °C) enhanced the mosquito's VC (Carrington *et al*., 2013a). This was not reported with fluctuations around high temperatures (26 °C) (Lambrechts *et al*., 2011; Carrington *et al*., 2013b). In Europe, DENV epidemics have occurred in the Mediterranean area during the summer. These environmental conditions allowed *Ae. albopictus* mosquitoes to acquire and subsequently transmit DENV from viraemic travellers to local populations (Semenza *et al*. 2014). The results of the present study confirm that the *Ae. albopictus* strain from Catalonia is competent for DENV under the temperature conditions that prevail on the Mediterranean coast of Catalonia during the summer. Although the present results point to a low TE (4.6%) for DENV-1, this parameter is only one of the multiple components used to determine VC. Additional ecological (mosquito population densities, feeding behaviour, daily mosquito survival) and anthropogenic (frequency of arrival of viraemic hosts, population density) factors must be considered to determine the real risk for DENV transmission in the Mediterranean area.

Conclusions

The present results indicate that the tested *Ae. albopictus* strain was susceptible to both DENV-1 and DENV-2. The mosquito strain tested was also able to transmit DENV-1, demonstrating that a local transmission event is possible in the Mediterranean region. This study contributes to knowledge of the VC of *Ae. albopictus* for DENV, which may be useful in the development of DENV risk models and surveillance programmes.

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The authors declare no conflicts of interest.

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