

Failure to demonstrate experimental vertical transmission of the epidemic strain of Chikungunya virus in *Aedes albopictus* from La Réunion Island, Indian Ocean

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Aedes albopictus was responsible for transmission in the first outbreak of chikungunya (CHIK) on La Réunion Island, Indian Ocean, in 2005-2006. The magnitude of the outbreak on this island, which had been free of arboviral diseases for over 30 years, as well as the efficiency of *Ae. albopictus* as the main vector, raises questions about the maintenance of the CHIK virus (CHIKV) through vertical transmission mechanisms. Few specimens collected from the field as larvae were found to be infected. In this study, *Ae. albopictus* originating from La Réunion were orally infected with a blood-meal containing 10^8 pfu/mL of the CHIKV epidemic strain (CHIKV 06.21). Eggs from the first and second gonotrophic cycles were collected and raised to the adult stage. The infectious status of the progeny was checked (i) by immunofluorescence on head squashes of individual mosquitoes to detect the presence of viral particles or (ii) by quantitative RT-PCR on mosquito pools to detect viral RNA. We analysed a total of 1,675 specimens from the first gonotrophic cycle and 1,709 from the second gonotrophic cycle without detecting any viral particles or viral RNA. These laboratory results are compared to field records.

Key words: vertical transmission - chikungunya virus - *Aedes albopictus* - La Réunion Island - Indian Ocean

Chikungunya virus (CHIKV) is a vector-borne alphavirus transmitted to humans by mosquitoes belonging to the genus *Aedes*. It was first isolated in 1953 in Tanzania (Ross 1956) and until recently outbreaks had only been observed in Africa, Asia and India. In Africa, CHIKV is maintained within a sylvatic cycle with wild mosquitoes as vectors (Jupp & Kemp 1996, Diallo et al. 1999). In Asia and India, a sylvatic cycle has never been demonstrated and CHIKV is mainly transmitted within an urban cycle primarily involving the human-biting *Aedes aegypti* and the less anthropophilic *Aedes albopictus* (Jupp & McIntosh 1988). Re-emergence of CHIKV outbreaks is unpredictable and occurs frequently after 7-8 years of silence. In March 2005, CHIKV emerged for the first time in the southwestern Indian Ocean region in the Comoro Islands, probably introduced from Kenya, where the outbreak started in 2004. The CHIKV rapidly spread to other islands in the region, including Mayotte, the Seychelles, Mauritius and La Réunion, with a major outbreak responsible for at least 266,000 cases (1/3 of the population) from November 2005-June 2006 (Renault et al. 2007). Usually considered to be a secondary vector, *Ae. albopictus* was proven to be responsible for the transmission of CHIKV in La Réunion. *Ae. aegypti* was only present in a few remote areas and was not anthropophilic

(Salvan & Mouchet 1994). Interestingly, the sequencing of viral strains from La Réunion demonstrated the emergence of a mutation in the course of the outbreak and the mutation was detected in the majority of the viral population (Schuffenecker et al. 2006). This mutation was shown to be associated with a better transmission rate by *Ae. albopictus* (Tsetdatkin et al. 2007, Vazeille et al. 2007). Furthermore, viral particles could be detected in the salivary glands at day 2 post-infection and high densities of viral particles were observed in eggs at day 6 post-infection. This last observation raised concerns of possible maintenance of the virus on the island through vertical transmission, a mechanism by which arboviruses can be maintained in nature when climate conditions are unfavourable to the activity of adults: infected females die but the virus can survive in their desiccated eggs. The frequency of this phenomenon is extremely variable depending on the mosquito species and the virus. Vertical transmission of CHIKV has not yet been demonstrated in the laboratory (Jupp et al. 1981, Mourya 1987), but has been proven to occur in the field in *Ae. albopictus*, in La Réunion (Delatte et al. 2008) and in Madagascar (Ratsitorahina et al. 2008). However, the transmission rate was low and only viral RNA was detected, with no virus isolated. Therefore, we attempted to demonstrate vertical transmission under laboratory conditions with *Ae. albopictus* from La Réunion and the epidemic viral strain in order to assess the potential risk of maintenance of CHIKV on the island.

MATERIALS AND METHODS

Mosquitoes - The Providence *Ae. albopictus* strain used in this study originated from a field sample of eggs collected on La Réunion Island in March 2007 (F0). This strain has since been maintained in the laboratory at 28

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± 1°C with 80% relative humidity and a 16:8 h photoperiod. Adults were given 10% sucrose solution and females fed on a mouse to get eggs. To produce enough females of the same physiological age for oral infections, batches of eggs were hatched and larvae reared to the adult stage in pans with tap water and yeast tablets. For vertical transmission experiments, females from the second generation (F2) were used for the first assay (assay A) and F5 generation females were used for the second assay (B).

Virus - Strain 06.21 CHIKV, provided by the French National Reference Center for Arboviruses of the Institut Pasteur, was isolated on *Ae. albopictus* C6/36 cells (Igarashi 1978) from a serum collected in November 2005 from a new-born male from La Réunion Island. CHIKV 06.21 was the major genotype isolated from patients during the 2005-2006 outbreak and presented an A226V mutation in the *E1* gene (Schuffenecker et al. 2006). Viral production and titration were described by Vazeille et al. (2007).

Vertical transmission experiments - Two experiments were performed. The first one (assay A) allowed us to collect eggs from the first gonotrophic cycle of orally infected females. In the second experiment (assay B), a first non-infectious blood-meal was given to allow the oviposition of eggs from the first gonotrophic cycle before the infectious meal was provided 10 days later. This protocol was chosen since we were unable to get infected females to have a second blood-meal under the level 3 confinement insectarium (L3) conditions.

Assay A - Infection assays were performed with seven-day-old females that were allowed to feed for 15 min through a chicken skin membrane covering the base of a glass feeder containing the blood-virus mixture, which was maintained at 37°C. The infectious meal was composed of a virus suspension diluted (1:3) in washed rabbit erythrocytes isolated from arterial blood collected 24 h before the infectious meal (Vazeille-Falcoz et al. 1999). To enhance the oviposition rate, bovine serum albumin (BSA) was added to the meal, i.e., red cells were washed in PBS then mixed with L-15 medium complemented with 10% BSA. Phagostimulant ATP was added at a final concentration of 5×10^{-6} M. To ensure a high rate of disseminated infection of females, a titre of 10^8 plaque-forming units (pfu)/mL was used in the meal. It has been shown that this titre ensures a disseminated infection rate of 100% in *Ae. albopictus* females from La Réunion (Vazeille et al. 2007).

Fully engorged females were transferred to individual tubes and maintained with 10% sucrose at $28 \pm 1^\circ\text{C}$ until death. Filter papers were wetted two days after the blood-meal to allow oviposition. Eggs were collected and kept in the L3 confinement laboratory, where they were hatched and reared to the adult stage with a 16:10 h photoperiod.

Assay B - Under the L3 confinement conditions in which the infected females were kept, we were unable to get *Ae. albopictus* females to take a second blood-meal after they had laid their eggs from the first gonotrophic cycle and this despite numerous attempts. We therefore

made the choice to offer a first non-infectious meal in a regular insectarium and then, after the females had laid their first batch of eggs (10 days later), to bring them to L3 confinement for the infectious meal and collect the eggs from the second gonotrophic cycle. Provision of infectious blood-meals and collection of progeny were performed as in assay A.

Viral detection in progeny - In assay A, the infected status of the progeny was tested by two means: (i) the presence of CHIKV was checked by indirect immunofluorescence on head squashes (Kubersky & Rosen 1977) using a mouse ascetic fluid provided by the French National Reference Center for Arboviruses of the Institut Pasteur and (ii) bodies were pooled per family and sex, with a maximum of 15 specimens per pool, to check for the presence of viral RNA in these pools by quantitative RT-PCR. In assay B, the presence of viral RNA was checked in whole mosquitoes pooled by family and sex with no more than 10 specimens per pool. A family consisted of the progeny of one infected female.

Quantitative RT-PCR analysis - Total RNA was extracted using the Nucleospin[®] RNA II kit (Macherey-Nagel), following the manufacturer's instructions. All procedures were previously described by Vazeille et al. (2007). The one-step RT-PCR was performed with a Power SYBR[®] Green RNA-to-CT[™] one step kit (Applied Biosystem) in a volume of 25 μL containing 2 μL RNA template, 12.5 μL 2X Power SYBR[®] Green I RT-PCR Mix, 0.25 μL sense (2.5 μM) primer, 0.25 μL anti-sense (2.5 μM) primer and 0.2 μL RT enzyme mix. Primers were selected from the E2 structural protein regions of sequences retrieved from the GenBank database by the Laboratory for Urgent Response to Biological Threats at the Institut Pasteur: sense Chik/E2/9018/+ (CACCGCCGCAACTACCG) and anti-sense Chik/E2/9235/- (GATTGGTGACCGCGGCA). The amplification program in an Applied Biosystem 7700 real-time PCR system consisted of: reverse transcription at 48°C for 30 min, an activation step of the polymerase at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, 72°C for 30 s and 95°C for 20 s and a final ramping step lasting 19 min 59 s. The size of the amplification product was 217 bp. After amplification, a melting curve was acquired to check the specificity of the PCR products. Each pool was analysed in duplicate. Signals were normalised to the negative control, consisting of a pool of non-infected mosquitoes from the same species and generation. Normalised data were used to measure the number of RNA copies in mosquitoes according to the ΔC analysis. A CHIKV RNA synthetic transcript was used as a positive control at two concentrations: 10^2 and 10^5 RNA copies.

RESULTS

Assay A - Progenies from the first gonotrophic cycle were collected from 107 females (Table). Eggs were collected from days 3-29 post-infection, with a majority of females (50) laying their first eggs on day 6 post-infection; 13 laid their eggs on day 3 and the remainder did so after day 6 post-infection. Immunofluorescence

assays performed on head squashes of all 1,675 descendants were negative. No viral RNA was detected in the bodies of these samples.

Assay B - Progenies from the second gonotrophic cycle were collected from 94 females (Table). Eggs were collected from days 4-21 post-infection. The first eggs were laid between days 4-6 post-infection, i.e., 81 females laid their first eggs on day 4, 7 on day 5 and 6 on day 6 post-infection. No viral RNA was detected in the 1,709 descendants.

TABLE

Progeny of *Aedes albopictus* females (assay A and assay B) infected with chikungunya virus 06.21 from La Réunion Island

	Assay A ^a	Assay B ^b
Mothers (n)	107	94
First eggs	day 3 pi	day 4 pi
Last eggs	day 29 pi	day 21 pi
Descendants per female (n)	1-66	1-78
Males (n/pools)	754 (119)	820 (126)
Females (n/pools)	921 (118)	889 (137)
Total descendants (n)	1,675	1,709

a: gonotrophic cycle 1; b: gonotrophic cycle 2; pi: post-infection.

DISCUSSION

Vertical transmission has long been suspected to be the mechanism by which CHIKV could be maintained in nature, thereby explaining its cyclic re-emergence in the absence of a sylvatic cycle. However, previous studies have failed to demonstrate this phenomenon in *Aedes furcifer* and *Ae. aegypti formosus* from South Africa (Jupp et al. 1981) and in *Ae. aegypti* and *Ae. albopictus* from India (Mourya 1987). Tesh (1984) noted that, in contrast to the other arbovirus groups, little field or experimental evidence suggested the possible vertical transmission of alphaviruses. However, the first emergence of CHIKV in the Indian Ocean, as well as the unusual implication of *Ae. albopictus* as the main vector responsible for the massive outbreak in La Réunion, raised concerns about a possible maintenance of the virus during the dry season. Therefore, specimens were collected in the field in La Réunion in 2006 and a low rate of vertical transmission was detected (Delatte et al. 2008). In the same year in Madagascar, the presence of CHIKV RNA was also detected in *Ae. albopictus* but no infectious viral particle was recovered from these samples (Ratsitorahina et al. 2008). To try to elucidate this situation, we decided to conduct vertical transmission experiments with a strain of *Ae. albopictus* from La Réunion and the CHIKV epidemic strain (CHIKV 06.21). As for previous authors, we were unable to detect vertical transmission despite the use of a very high dose of infectious particles in the infectious blood-meal and the very high load of infectious particles detected in mothers (i.e., infected females)

(Vazeille et al. 2007). However, our negative results are not surprising considering the low rate of vertical transmission described in Delatte et al. (2008), i.e., two positive pools out of 6,853 individuals. Thus the minimum infection rate for field records was then two out of 6,853, not significantly different from the results of our laboratory study performed on 3,384 mosquitoes ($p > 0.05$, Fisher's exact test). In experimental vertical transmission experiments, it is usual to adopt the best conditions allowing the highest survival rate of infected females, such as plenty of food at the larval stage to obtain large size females, maximum humidity and suitable temperature during the incubation period of adult females, appropriate photoperiod. Although they may be favourable to the survival of the vector, these optimal conditions may not be ideal for the transmission of the virus to the progeny. We observed a high load of viral particles in the eggs, but the infection was obviously not transmitted to the next generation or was cleared during the early stages of development.

In conclusion, the negative results we obtained and the low rates observed in the field lead us to think that vertical transmission of CHIKV has not played a key role in the maintenance of the virus in La Réunion. The re-introduction of the virus by infected humans is more likely to be the cause of a putative new outbreak.

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