Short Communication

Vertical Transmission of Zika Virus (Flaviviridae, Flavivirus) in Amazonian Aedes aegypti (Diptera: Culicidae) Delays Egg Hatching and Larval Development of Progeny

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Abstract

Zika virus (ZIKV) has emerged as a globally important arbovirus and has been reported from all states of Brazil. The virus is primarily transmitted to humans through the bite of an infective Aedes aegypti (Linnaeus, 1762) or Aedes albopictus (Skuse, 1895). However, it is important to know if ZIKV transmission also occurs from Ae. aegypti through infected eggs to her offspring. Therefore, a ZIKV and dengue virus (DENV) free colony was established from eggs collected in Manaus and maintained until the third–fourth generation in order to conduct ZIKV vertical transmission (VT) experiments which used an infectious bloodmeal as the route of virus exposure. The eggs from ZIKV-infected females were allowed to hatch. The resulting F1 progeny (larvae, pupae, and adults) were quantitative polymerase chain reaction (qPCR) assayed for ZIKV. The viability of ZIKV vertically transmitted to F1 progeny was evaluated by cultivation in C6/36 cells. The effects of ZIKV on immature development of Ae. aegypti were assessed and compared with noninfected mosquitoes. Amazonian Ae. aegypti were highly susceptible to ZIKV infection (96.7%), and viable virus passed to their progeny via VT. Moreover, eggs from the ZIKV-infected mosquitoes had a significantly lower hatch rate and the slowest hatching. In addition, the larval development period was slower when compared to noninfected, control mosquitoes. This is the first study to illustrate VT initiated by oral infection of the parental population by using mosquitoes, which originated from the field and a ZIKV strain that is naturally circulating in-country. Additionally, this study suggests that ZIKV present in the Ae. aegypti can modify the mosquito life cycle. The data reported here suggest that VT of ZIKV to progeny from naturally infected females may have a critical epidemiological role in the dissemination and maintenance of the virus circulating in the vector.

Key words: Zika virus, vertical transmission, Aedes aegypti, fitness cost

Zika virus (ZIKV) was first isolated in 1947 and until 2007, the only reported outbreak had been in Micronesia (Kindhauser et al. 2016). In early 2015, Brazil reported the first case of ZIKV in a Northeastern state (Zanluca et al. 2015) with rapid expansion to all regions of the country. By 2017, 3,754 probable cases of ZIKV had been registered with an incidence rate of 1.8 cases/1,000 inhabitants.
VT of ZIKV to the Ae. aegypti Progeny

Four days post infection (4 d.p.i.), 30 mosquitoes were randomly selected from the two experimental groups. Each mosquito was placed into a small individual cage and allowed to oviposit over the following 3 d. On the 7 d.p.i., the parental infected mosquitoes were individually evaluated for ZIKV by qPCR (Secundino et al. 2017). Four days after the oviposition, eggs from positive females (ZIKV-infected) and control mosquitoes were separated into two groups and placed in small cups to observe individual hatching. The resulting F1 larvae (L3–L4) were randomly placed into sixty groups and placed in small cups to observe individual hatching. The data were compared with the control group. The two groups were kept in the same room, and in the identical controlled conditions (temperature [28°C], humidity [80%], food availability, and breeding container).

Effect of VT-ZIKV on Ae. aegypti Developmental Stages

Thirty parentally ZIKV-infected Ae. aegypti were randomly selected and allowed to oviposit in order for us to evaluate the number of laid eggs, time to hatch, and period of larval development until pupation of the infected F1 progeny. Larvae and pupae were counted every day since the eggs hatched. The data were compared with the control group. The two groups were kept in the same room, and in the identical controlled conditions (temperature [28°C], humidity [80%], food availability, and breeding container).

Viability of VT-ZIKV to the Progeny

Fifteen F1 VT-ZIKV Ae. aegypti adults were grouped into three pools of five individuals and macerated in L-15 medium with 1% Penicillin/Streptomycin (10,000 U/ml), 2.5 g/ml Amphotericin B and 2% FBS, cultivated in C6/36 cells for 5 d as described above for observation of ZIKV growth and cytopathic effect (CPE; Contreras and Arumugaswami 2016). The ZIKV-free mosquitoes were used as negative control and inoculated in cell culture at the same time as test mosquitoes.

VT-ZIKV Detection and Quantification by qPCR

Samples were processed for RNA extraction (QIAamp viral RNA Mini kit, Qiagen, Hilden, Germany) (Lanciotti et al. 2008). Reverse transcription with cDNA production was carried out using a random primer and M-MLV enzyme, and subsequent TaqMan-based qPCR assay for ZIKV (primers set ZIKV 1086 and ZIKV 1162c) (Lanciotti et al. 2008, Secundino et al. 2017). Standard curve used was from gBlocks Gene Fragments dilutions, range 30,000 to 3 copies.

Statistical Analyses

Shapiro Wilk and two-tailed Student’s t-tests were used to evaluate significance among the groups in relation to mean numbers of laid eggs. Differences in numbers of hatched eggs among groups were evaluated using two-tailed χ² or Fisher’s tests. Period of time to egg hatching and development time of the larvae until pupation were
analyzed using a Kaplan-Meier (log-rank) analysis. P values ≤ 0.05 were considered significant. Statistical analysis was performed in GraphPad Prism, version 5.00 (San Diego, CA) and Stata (Stata Statistical Software, version 13.0).

Results

ZIKV Infection of the Amazonian Ae. aegypti and Vertical and Trans-stadial Transmission in the Progeny

The Amazonian Ae. aegypti were very susceptible to ZIKV infection. Individual qPCR analysis of the 7 d.p.i mosquitoes revealed an infection rate (IR) of 96.7% (29/30) with a median (md) = 1.3 x 10^6 ZIKV cDNA copies/mosquito. The analysis of a total of 600 F1 individuals in 20 pools per life stage demonstrated high filial infection rates (FIR) due to VT of ZIKV as follows: 55% in larvae (11 out of 20 pools) md = 2.5 x 10^2 ZIKV cDNA copies, 50% in pupae (10 out 20 pools) md = 1.9 x 10^2 ZIKV cDNA copies and 70% in adults (14 out of 20 pools) md = 1.7 x 10^2 ZIKV cDNA copies. Trans-stadial transmission (i.e., presence of ZIKV from one life stage to the next), showed the minimum filial infection rates (MFIR) to be 1:18 in larvae, 1:20 in pupae, and 1:14.3 in adults (Table 1 and Fig. 1).

Effect of VT-ZIKV and Trans-stadial Infection in the Development of Amazonian Ae. aegypti

Out of the 30 selected parental ZIKV-infected Ae. aegypti, 29 of these laid 2,853 eggs while the same number of the control mosquitoes laid 2,672 eggs (one female in each group died before laying eggs), of these 2,215 and 2,214 hatched, respectively. The mean number of laid eggs by each ZIKV-infected and control mosquitoes were 98.36 (±22.20) and 92.14 (±26.44), respectively, with no significant difference in oviposition rates (Fig. 2a). The eclosion rates (number of eggs hatched/number of eggs laid) were 82.9% for control and 77.6% for VT-ZIKV-infected mosquitoes, which was statistically significant (P < 0.001) (Fig. 2b).

Fitness Costs of VT-ZIKV-infected Ae. aegypti Progeny

The fitness costs of the VT-ZIKV-infected F1 Ae. aegypti progeny were measured using survivorship curves following the Kaplan-Meier method and tested for significance by Log-rank test (P < 0.05). The number of eggs hatched daily and the period of larval development to pupae was compared between the VT-ZIKV-infected and control mosquitoes. Differences in the hatch rate occurred in the first 10 d. The VT-ZIKV group had the slowest hatching and the larval development period (eclosion to pupation) compared with control group (P < 0.001; Fig. 3a and b).

Viability of VT-ZIKV of the Ae. aegypti Progeny

The supernatant of macerated F1 adult mosquitoes was evaluated for viable ZIKV by cell cultures. Orally infected VT-ZIKV Ae. aegypti F1 of the progeny was able to infect C6/36 cell cultures and CPE was observed at 5 d.p.i. (Fig. 4).

Discussion

The first two cases of ZIKV were confirmed in Manaus, State of Amazonas, in January 2016. In 2017, 1 yr later, the city confirmed 4,418 Zika cases including 500 pregnant women and five microcephaly cases in newborns (Semsa 2017). It is astonishing how quickly ZIKV spread through the city and improving our understanding of ZIKV dispersal and transmission is critical. Today, it is

Table 1. ZIKV infection of Aedes aegypti and F1 progeny

| Parental | F1 progeny |
|----------|------------|
| IR (%)   | Median cDNA copies ± SD | Stage | N° pools (10 individuals) | FIR (%) | MFIR b | Median cDNA copies ± SD |
| 29/30 (96.7) | 1.3 x 10^6 ± 1.4 x 10^7 | Larvae | 20 | 11/20 (55) | 1:18.2 | 2.5 x 10^2 ± 2.1 x 10^3 |
|          |             | Pupae  | 20 | 10/20 (50) | 1:20.0 | 1.9 x 10^2 ± 8.3 x 10^2 |
|          |             | Adult  | 20 | 14/20 (70) | 1:14.3 | 1.7 x 10^2 ± 2.9 x 10^2 |

a Filial infection rate.
b Minimum filial infection rate.

Fig. 1. Schematic representation of the experimental design.
well known that ZIKV behaves differently from other Flaviviruses since it is capable of being transmitted sexually between humans via its presence in body fluids such as semen and urine, and in the female genital tract (Baud et al. 2017).

In cities like Manaus, several factors such as unplanned urban expansion, irregular or absence of waste collection, and poor socioeconomic conditions contribute to the development of breeding sites and subsequent increases in the \textit{Ae. aegypti} populations. Previously, only two studies, using mosquitoes from Rio de Janeiro, a Southeastern city, described the susceptibility of Brazilian \textit{Ae. aegypti} to ZIKV. The first study reports a high infection and disseminated IR (Dutra et al. 2016), which is similar to our findings. The second study reports a high IR, but with low levels of disseminated infection in recently colonized \textit{Ae. aegypti} (Chouin-Carneiro et al. 2016). Since we worked with a mosquito population from Manaus, a Northern city, these differences may reflect the genetic or physiological variability in the permissiveness of these geographically different \textit{Ae. aegypti} populations.

The Amazonian \textit{Ae. aegypti} mosquitoes are highly susceptible to oral ZIKV infection and we verified the VT to their progeny. The MFIR of the larvae and adults of the \textit{Ae. aegypti} was greater than other studies describing VT in other populations (Thangamani et al. 2016, Ciota et al. 2017). It is worth noting that, in the previous VT studies, the infections were artificially initiated via intra-thoracic infection.
injection by syringe, however, our study was the first one to demonstrate the VT-ZIKV by natural oral infection of the parental mosquitoes. Additionally, this study used third and fourth generations of *Ae. aegypti* from eggs collected in the field, infected with a ZIKV strain that is naturally circulating in the country. In 2016, a study in Rio de Janeiro Brazil showed for the first time that among 963 *Ae. aegypti* males only one was naturally infected with ZIKV by venereal and/or VT (Ferreira-de-Brito et al. 2016). Certainly, this experimental model is a more accurate reflection of how efficiently VT-ZIKV mosquitoes occur in an endemic region.

Many studies have shown that pathogens, including arboviruses transmitted by insects, can affect vector fitness mainly related to their physiology (Power 1992, Keating et al. 2013). Decreased fecundity and fertility of DENV infected parenteral *Ae. aegypti* and lengthening of the larval development period of VT-DENV infected progeny have been reported (Joshi et al. 2002, Maciel-de-Freitas et al. 2011). In addition, our study shows that ZIKV infection also alters *Ae. aegypti*’s physiology, e.g., by delaying hatching of the eggs and by changing the larvae stage. Essentially, there is no significant difference in the number of laid eggs by ZIKV-infected parenteral *Ae. aegypti* compared to the noninfected mosquitoes, but a decrease in egg eclosion rate occurred, and the larval development period of the VT-ZIKV-infected progeny increased.

Our results show that ZIKV infection in *Ae. aegypti* has the potential to modify the mosquito life cycle by increasing the time of two major life stages: egg eclosion and larval development. The data reported here suggest that VT of ZIKV to progeny from naturally infected females may have a critical role in the dissemination and maintenance of the virus circulating in the vector.

VT occurs, but with a small number of cDNA viral copies in VT-ZIKV-infected progeny, similar to a study with VT-DENV-1 in *Ae. aegypti* and *Ae. albopictus* (Buckner et al. 2013). These are important aspects that need to be explored further in the context of urban vector-borne disease dispersal since they can have a significant epidemiological impact. The developmental delays in immature vector mosquitoes and the decrease in the eggs hatching rate induced by ZIKV infection may make the mosquito more vulnerable to vector control of immature stages. This kind of information about vector biology is important for directing interventions in vector control; and this can influence the dispersion and incidence of the vector biology is important for directing interventions in vector control of immature stages. This kind of information about vector biology is important for directing interventions in vector control; and this can influence the dispersion and incidence of the vector.

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