Vector competence of *Aedes aegypti*, *Aedes albopictus*, and *Culex quinquefasciatus* mosquitoes for Mayaro virus

Thiago Nunes Pereira, Fabiano Duarte Carvalho, Silvana Faria De Mendonça, Marcele Neves Rocha, Luciano Andrade Moreira

Grupo Mosquitos Vetores: Endossimbiôntes e Interação Patógeno-Vetor, Instituto René Rachou—Fiocruz, Belo Horizonte, MG, Brazil

* luciano.andrade@fiocruz.br

Abstract

Newly emerging or re-emerging arthropod-borne viruses (arboviruses) are important causes of human morbidity and mortality worldwide. Arboviruses such as Dengue (DENV), Zika (ZIKV), Chikungunya (CHIKV), and West Nile virus (WNV) have undergone extensive geographic expansion in the tropical and sub-tropical regions of the world. In the Americas the main vectors of DENV, ZIKV, and CHIKV are mosquito species adapted to urban environments, namely *Aedes aegypti* and *Aedes albopictus*, whereas the main vector of WNV is *Culex quinquefasciatus*. Given the widespread distribution in the Americas and high permissiveness to arbovirus infection, these mosquito species may play a key role in the epidemiology of other arboviruses normally associated with sylvatic vectors. Here, we test this hypothesis by determining the vector competence of *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* to Mayaro (MAYV) virus, a sylvatic arbovirus transmitted mainly by *Haemagogus janthinomys* that has been causing an increasing number of outbreaks in South America, namely in Brazil. Using field mosquitoes from Brazil, female mosquitoes were experimentally infected, and their competence for infection and transmission rates of MAYV was evaluated. We found consistent infection rate for MAYV in *Ae. aegypti* (57.5%) and *Ae. albopictus* (61.6%), whereas very low rates were obtained for *Cx. quinquefasciatus* (2.5%). Concordantly, we observed high potential transmission ability in *Ae. aegypti* and *Ae. albopictus* (69.5% and 71.1% respectively), in contrast to *Cx. quinquefasciatus*, which could not transmit the MAYV. Notably, we found that very low quantities of virus present in the saliva (undetectable by RT-qPCR) were sufficiently virulent to guarantee transmission. Although *Ae. aegypti* and *Ae. albopictus* mosquitoes are not the main vectors for MAYV, our studies suggest that these mosquitoes could play a significant role in the transmission of this arbovirus, since both species showed significant vector competence for MAYV (Genotype D), under laboratory conditions.

Author summary

The present study demonstrated that *Ae. aegypti* and *Ae. albopictus* mosquitoes can be competent laboratory vectors for MAYV. In contrast, *Cx. quinquefasciatus* mosquitoes
were refractory to MAYV. Regarding the viral dilution and nanoinjection, a higher detection sensitivity was observed after virus nanoinjection into naïve mosquitoes, indicating that only a few viral particles are required to infect mosquitoes, and these particles may not be detected by RT-qPCR before the nanoinjection procedure.

Introduction

The mosquitoes *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* are widely distributed throughout the world, especially in tropical and subtropical regions [1–3]. They are considered a serious concern to public health as vectors of several arboviruses such as DENV, ZIKV, CHIKV, and WNV [4–13]. Studies have shown that *Ae. aegypti* and *Ae. albopictus* mosquitoes exhibit laboratory vector competence for the infection and transmission of MAYV, and *Cx. quinquefasciatus* mosquitoes infected with MAYV have been found in Cuiabá [14–16].

The MAYV was first isolated in 1954 from rural workers in Mayaro, on the island of Trinidad [17] and, like CHIKV, it is an arbovirus of the genus *Alphavirus*, belonging to the family *Togaviridae* [18–20]. To date, three MAYV genotypes (D, L and N) have been identified. The D genotype has a wide geographical distribution, occurring in Brazil, Bolivia, Peru, Suriname, Trinidad, Tobago, Argentina, Colombia, and Venezuela [21–25]. The L genotype was isolated in Brazil and Haiti [24] and contains strains detected only in Brazil [26]. On the other hand, the N genotype, was discovered in an outbreak in 2015 in Venezuela [27].

The MAYV is transmitted primarily by the bite of female mosquitoes of the genus *Hemagogus*, and in nature several vertebrates can host this virus, which is detected in non-human primates, rodents, birds, sloths, and other small mammals [28].

In humans, MAYV infection usually occurs in people with a history of activities in forested areas [18–21]. Long et al. [14], described, in febrile humans, a high load of viral RNA, determined by real-time polymerase chain reaction, ranging from 5.01\times10^2 to 2.18\times10^5 (log_{10}/PFU equivalents/mL). This disease has similar symptomatology to DENV and/or CHIKV, causing an acute and self-limiting febrile illness, which may be accompanied by hemorrhagic phenomena such as petechiae and gingival bleeding. Usually the wrist, ankle, hands, and feet joints are significantly affected, and symptoms may persist for several months, incapacitating the infected person [22,23,29]. In recent years in Brazil, several cases of MAYV have been registered in Pará (2008), Mato Grosso (2012), and Goiás (2014–2016) [20–22,30–34]. Although there is still no evidence of the transmission efficiency of MAYV in an urban cycle, it has the potential to establish an epidemic scenario in the Americas, similar to what occurred with ZIKV and CHIKV [35]. Using mathematical models taking into account outbreaks since 1960 and increasing global temperature, Lorenz et al. [36] predicted that MAYV would expand its area of coverage in the coming years. Thus, the importance of MAYV as a human pathogen with the potential to emerge in urban areas is strong. Therefore, the main objective of this study was to evaluate the vector competence of *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* for MAYV, since these mosquitoes may be involved in the dispersion of this virus.

Materials and methods

Ethics statement

The human blood used in all experiments was obtained from a blood bank (Fundação Hemominas), according to the terms of an agreement with the Instituto René Rachou, Fiocruz/MG (OF.GPO/CCO agreement—Nr 224/16).
Mosquito species and rearing

For this study, three mosquito species were used: *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*. *Ae. aegypti* and *Ae. albopictus* mosquitoes were collected from ovitraps, whereas *Cx. quinquefasciatus* were collected using an entomological ladle. All collections occurred in the neighborhood of Pampulha (19.8527° S, 43.9560° W), in the city of Belo Horizonte, Brazil, in the first half of 2017. The *Ae. aegypti* and *Ae. albopictus* populations were initiated from at least 2,000 eggs for each species, whereas for *Cx. quinquefasciatus*, the colony was obtained with more than 3,000 larvae.

Eggs/larvae were transported to the insectary of the René Rachou Institute, Fiocruz/MG, and were kept in a controlled environment, at 27 ± 2°C and ~82% RH with a 12:12 h light/dark cycle. Larvae were maintained with fish food pellets, Tetramin tropical and GoldFish Colour. After emergence and identification, adults were kept on a 10% sucrose solution regimen, *ad libitum*. Adult females were fed with human blood in an artificial feeder for egg production. To simulate field conditions and minimize the effects of inbreeding and population colonization, all experiments were conducted with mosquitoes from the same geographic area and up to the third generation.

Mayaro virus culture

MAYV isolated from the serum of an infected human in Trinidad (strain TRVL 4675) in 1954 and belonging to the D genotype was originally acquired from the American Type Culture Collection (ATCC). Full-length sequences are available on GenBank [37]. To prepare the MAYV for oral feeding, a frozen virus stock (an aliquot of MAYV was kindly supplied by the Flavivirus Laboratory of the Oswaldo Cruz Institute—IOC / Fiocruz) was passaged once through C6/36 cells (approximately 2 million cells) in Leibowitz L-15 medium supplemented with 10% fetal calf serum and maintained at 28°C. The supernatant viral stock was harvested after day 4 (for the second experiment and at 5 days for the first experiment). Both viral titers were quantified after a freeze-thaw cycle and two replicates (A and B) were used to evaluate the mosquito infection rates. For the first replicate, the viral titer was $1 \times 10^9$ PFU/mL, and for the second, $6 \times 10^9$ PFU/mL.

Mosquito infection and transmission analysis

In order to analyze infection rates and transmission rates of MAYV, batches of mosquitoes were analyzed 7 and 14 days after infection (dpi). From each blood-fed mosquito we collected and assayed two samples for MAYV: head + thorax and saliva. Mixed head and thorax samples were assayed for virus to discriminate infection, and the infection rate was defined as the number of samples with detectable virus divided by all samples tested. Samples of nanoinjected saliva were assayed for virus to discriminate transmission, and the transmission rate was defined as the number of saliva samples with detectable virus divided by all samples from mosquitoes with detectably virus in the body (head + thorax). For infection, 5-day-old adult females were allowed to ingest a mixture of viral supernatant and human blood (2:1). On the first replicate, the viral titer was $1 \times 10^5$ PFU/mL, and for the second, $6 \times 10^5$ PFU/mL, which was offered for 45 minutes through glass feeders using pig intestine as the membrane and a water jacket system with the temperature maintained at 38 °C. Immediately after feeding, fully engorged females were separated and maintained with a 10% sucrose solution until the end of the experiment.

Mosquitoes were anesthetized with CO$_2$ and kept on an ice plate while the legs and wings were removed. Each mosquito proboscis was inserted in a 10 μL pipette tip containing a 1:1 solution of 10 μL of 30% sucrose and sterile fetal calf serum. After 30 minutes, the contents of the tips were individually collected in 0.6 mL tubes and stored at ~80°C until processing.
In order to test the arboviruses absence in the blood, prior to the experiments, we performed tests for Yellow Fever (YFV), DENV, ZIKV, CHIKV, and MAYV. A group of mosquitoes was fed on the blood sample and, after 10 days post-feeding, these mosquitoes were checked by RT-qPCR. This blood test is a routine procedure in our laboratory to ensure the absence of virus in the blood.

**Confirmation of infectious particles in saliva by nanoinjection**

To confirm infectivity in saliva, individual samples of undiluted saliva from each mosquito species were nanoinjected into 10–15 naïve *Ae. aegypti* (mosquitoes that had never had contact with any viruses), using a Nanoject II (Drummond Sci) portable injector. In each mosquito, a 276 nL dose of saliva was nanoinjected intrathoracically (pleural membrane) with a pulled glass capillary. Nanoinjected mosquitoes were collected at 5 days post nanoinjection and, on average, 6 whole mosquitoes, per injected saliva, were processed and analyzed by RT-qPCR.

**Virus serial dilution and nanoinjection**

To try understanding our findings about the fact some negative mosquito produced positive mosquitoes after saliva nanoinjection (see previous section), we tested the sensitivity of virus detection particles through RT-qPCR. Serial dilutions (10-fold) of known virus stocks were nanoinjected into naïve mosquitoes. Replicates A and B had initial concentrations of $2.09 \times 10^4$ and $5.59 \times 10^4$ viral copies for virus stocks, respectively, and were further diluted 6 times. As a control, uninfected cell supernatant was used. Nanoinjected mosquitoes were collected at 5 days post-nanoinjection and only 5 mosquitoes (whole body), per dilution, were processed and analyzed by RT-qPCR.

**Analysis of MAYV by RT-qPCR**

Total RNA from mosquitoes was extracted to verify the infection rates (mosquito head+thorax), transmission rates (through saliva nanoinjection into naïve mosquitoes) or serial virus dilution and nanoinjection. The extraction of RNA viral was performed with the High Pure Viral Nucleic Acid Kit (Roche), following the manufacturer’s instructions. The thermocycling conditions were as follows: reverse transcription at 50°C for 10 min, RT inactivation/initial denaturation at 95°C for 30s, 40 cycles of 95°C for 5 s and 60°C for 30s, followed by cooling at 37°C for 30s. The volume of the reaction was 10 μL (5x LightCycler Multiplex RNA Virus Master (Roche), with 1 μM primers, 0.2 μM probe, and 125 ng RNA.

MAYV infection/transmission rates in mosquitoes was quantified by RT-qPCR using a LightCycler 96 (Roche). A multiplex assay was performed according to previous studies, with the MAYV primers MAYVF 5’-GTG GTG TGT GCA CAG TGA ATC TTT C-3’ and MAYVR 5’-CAA ATG TCC ACC AGG CGA AG-3 and the May-Probe 5’-FAM/ATG GTG GTA GGC TAT CCA GGT C/3lABkFQ-3’ (14). For the mosquito control we used the ribosomal gene S17 (RPS17) primers 17S-F 5’-TCC GTG GTA TCT CCA TCA AGC T-3 and 17S-R 5’-CAC TTC CGG CAC GTA GTT GTC-3 and the probe 5’-HEX/CAG GAG GAG CGT GGT GAG CGC AG/3BHQ2-3’ (33). All samples were tested in duplicate for MAYV, and the viral genome was determined by comparison with a standard curve using serial dilutions of the target gene cloned into the pGEMT-Easy plasmid (Promega).

**Data analysis**

Mosquito infection rate was analyzed with both Person omnibus normality and D’Agostino tests. Fisher’s exact test was then used to assess differences in viral prevalence. Comparisons
were significant for \( P \) values lower than 0.05, and viral load data were compared through the Mann-Whitney U test. All analyses were performed by using Prism V 7.4 (GraphPad).

Results

MAYV infection in *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus*

Our analysis of different post-infection time points showed that *Ae. aegypti* and *Ae. albopictus* became positive for MAYV. In addition, in both replicates (Fig 1A and 1B) there was increased

![Graph showing viral load in different species](https://doi.org/10.1371/journal.pntd.0007518.g001)
Table 1. *Aedes aegypti, Aedes albopictus, and Culex quinquefasciatus* orally infected with Mayaro virus. The initial viral titer was determined by plaque-forming units (PFU/mL). Infected/total mosquito numbers are shown parenthesis.

| MAYV         | Titer (PFU/mL) | Days post- infection | Infection rate % |
|--------------|----------------|----------------------|------------------|
| Replicate A  | 1×10⁹          | 7                    | 60 (12/20)       |
|              |                | 14                   | 85 (17/20)       |
| Replicate B  | 6×10⁹          | 7                    | 25 (5/20)        |
|              |                | 14                   | 60 (12/20)       |

https://doi.org/10.1371/journal.pntd.0007518.t001

infection rate at 14 dpi compared with 7 dpi in *Ae. aegypti* and *Ae. albopictus*. On the other hand, *Cx. quinquefasciatus* mosquitoes were shown to be refractory to MAYV.

In replicate A (Fig 1) at 7 dpi, we observed differences in the infection rate between *Cx. quinquefasciatus* and *Ae. aegypti* (*p* < 0.0001) or *Ae. albopictus* (*p* = 0.0010), but no difference was observed between *Ae. aegypti* and *Ae. albopictus*. Considering the infection rate at 14 dpi, the same pattern was observed. *Cx. quinquefasciatus* was significantly different from *Ae. aegypti* (*p* < 0.0001) and *Ae. albopictus* (*p* < 0.0001). Comparisons at 7 and 14 dpi for the same mosquito species showed differences for *Ae. aegypti* (*p* = 0.0043) and *Ae. albopictus* (*p* = 0.0230), clearly showing increasing infection over time.

For replicate B (Fig 1) at 7 dpi, we only observed differences in the infection rate between *Cx. quinquefasciatus* and *Ae. albopictus* (*p* = 0.0207). At 14 dpi, we observed significantly different infection rate between *Cx. quinquefasciatus* and both *Ae. aegypti* (*p* < 0.0001) and *Ae. albopictus* (*p* < 0.0001). When we compared infection rates at the two time points (7 and 14 dpi), the only mosquito species that showed a difference was *Ae. aegypti* (*p* = 0.0262); however, nonsignificant increasing infection rate trend was observed for *Ae. albopictus*.

In general, our combined results from both 7 and 14 dpi show higher susceptibility to MAYV in *Ae. albopictus* (61.6%), followed by *Ae. aegypti* (57.5%). However, *Cx. quinquefasciatus* mosquitoes may be considered refractory to MAYV infection (with only a 2.5% infection rate). The infection rates of MAYV, in each species, in both experiments, and at different dpi are shown in Table 1.

Amplification of MAYV in mosquitoes after nanoinjection

To verify the occurrence of MAYV in mosquito saliva, we added a virus amplification step in live *Ae. aegypti*. Using preamplification by nanoinjection in mosquitoes, MAYV virus was detected in 69.5% (*n* = 69) of the individual saliva samples from orally infected *Ae. aegypti* with detectable amount of virus in their bodies at days 7 and 14 after infection. Likewise, for *Ae. albopictus* 71.1% (*n* = 59) of the mosquitoes with detectable amounts of virus in their bodies also expelled virus in saliva. These results show that orally infected *Ae. aegypti* and *Ae. albopictus* from Brazil could transmit the virus in their saliva and thus are competent laboratory vectors of MAYV.

In contrast, none of the 54 nanoinjected mosquitoes with *Cx. quinquefasciatus* saliva were able to become infected. We also nanoinjected saliva from negative *Ae. aegypti* and *Ae. albopictus* mosquitoes (through RT-qPCR) and, surprisingly, some samples were able to infect naïve mosquitoes. Saliva samples from 3 negative *Ae. aegypti* (Fig 2A) were nanoinjected into 15 mosquitoes, and 6 (40%) became infected with MAYV. Saliva from 3 negative *Ae. albopictus* (Fig 2B) was nanoinjected in 15 mosquitoes, and 9 (60%) became infected with MAYV.

Virus serial dilution and nanoinjection

In order to try to understand why negative mosquitoes (through RT-qPCR) are able to produce infectious saliva, we performed a series of virus dilutions and injections into naïve
mosquitoes, followed by RT-qPCR detection. We found that some viral samples, upon dilution, are not detected through RT-qPCR but are able to infect naïve mosquitoes, using the nanoinjection methodology. Our results showed that RT-qPCR had a detection limit of around 10 copies of the MAYV genome.

Discussion

To our knowledge, this is the first study to examine the vector competence of Ae. aegypti, Ae. albopictus, and Cx. quinquefasciatus mosquitoes for MAYV (genotype D) in Brazil. Our results show that Ae. aegypti mosquitoes can become infected with MAYV, and in general, these mosquitoes present high infection rates at 14 dpi, averaging $9.6 \times 10^4$ to $4.7 \times 10^4$ viral genome copies per mosquito for replicates A and B, respectively (Fig 1).

Burstolin et al., 2018 [38] evaluated two strains of MAYV; the genotype L strain isolated from Hg. janthinomys mosquitoes in Para, Brazil, in March 1991, and the genotype D strain originally isolated from a monkey in Para, Brazil, in May 1978. These authors observed in Ae. aegypti that the genotype L exhibited significantly higher infection rates (86.2% at 7 dpi and 51.7% at 14 dpi) when compared with the genotype D strain (7.1% at 7 dpi and 0% at 14 dpi). In the present study, using the genotype D strain, we observed higher infection rates (Fig 1 replicates A and B together): 42.5% at 7 dpi and 72.5% at 14 dpi. However, we have to take into consideration that the genetic background of the vector can strongly influence its susceptibility to the virus [39], as well as the relationship between viral titer and infection rate. When we try to
look at the relationship between viral titer and percentage of infection rate, we see that Diop et al., 2019 [40] using MAYV genotype L with a viral titer of $1 \times 10^6$ focus forming unit (FFU/mL) obtained 53.8% infection rate in Ae. aegypti, which was like our infection rate (57.5%). Burstolin et al., 2018 [38] with a viral titer of $1 \times 10^7$ FFU/mL obtained an infection rate of 86.2% (on 7dpi) and 51.7% (14 dpi) in Ae. aegypti. Yet, in our experiments, even with a viral titer 2 logs above, we obtained 60% infection rate at 7dpi and 85% at 14dpi for replicate A and 25% at 7dpi and 60% at 14dpi for replicate B (Fig 1).

Our results showed an increasing infection rate over time and overall lower infection rate compared to Burstolin et al., 2018 [38], which even with lower viral titer obtained more infected mosquitoes, but with decreasing infection rate over time. Furthermore, these authors noted significantly higher titers in mosquito bodies (7 and 14 dpi), which in most cases were greater than $1 \times 10^6$ viral genome copies per mosquito.

Yet, our results show that although our viral titer is high, fewer mosquitoes passed the range of $1 \times 10^6$ viral genome copies per mosquito. However, we would like to point out that such comparisons are difficult to make since the genotype/isolate and evaluation used by Burstolin et al., 2018 [38] and Diop et al., 2019 [40] were different from what we used.

A previous study by Pereira et al. [41] showed that Ae. aegypti mosquitoes from Rio de Janeiro were highly susceptible to MAYV, presenting a higher number of viral genome copies per mosquito than those obtained in this study. However, this difference may be related to the viral input at the time of infection or even the genetics of the mosquitoes used in our study. Regarding genetic variability, Gokhale et al. [42] suggested that for CHIKV, which is a similar virus to MAYV, the vector genetic background strongly influence the susceptibility to the virus [43].

MAYV cases have been reported in the North, Northeast and Center-West regions of Brazil [21,22,30–33]. Ae. aegypti mosquitoes naturally infected with MAYV have been found in Cuiabá, Mato Grosso [15], but, at this time, they cannot be incriminated as mosquito species for this virus. Thus, to evaluate viral transmission rate, MAYV-infected saliva of Ae. aegypti was nanoinjected into naïve mosquitoes, with 69% of mosquitoes becoming infected. Other laboratory studies also confirmed the ability of this vector to transmit MAYV [14,16,41]. Therefore, our results confirm that this mosquito species has great potential for infection/transmission rates of MAYV and, therefore, could play an important role in the transmission of this virus, if it becomes urbanized.

Ae. albopictus mosquitoes were found to have a similar infection rate to Ae. aegypti, showing high susceptibility to MAYV. At 14 dpi, significant numbers of viral particles were observed in this species. So far, there are few studies showing the relationship between MAYV and Ae. albopictus.

Smith and Francy [44], evaluated the vector efficiency of a Brazilian Ae. albopictus mosquito line fed on viremic hamster blood for MAYV and found that the infection rate ranged from 9% to 16%. The authors classified this strain as being relatively refractory to MAYV infection but suggested that it may become more susceptible, serving as a secondary vector in an outbreak or as a bridge vector between MAYV transmission cycles. Wiggins et al. [16] observed in an oral infection experiment that Ae. albopictus mosquitoes had a significantly higher infection rate than Ae. aegypti mosquitoes, a similar pattern observed in our study.

Diop et al., 2019 [40] using a viral titer of $1 \times 10^6$ FFU/mL (MAYV genotype L) obtained in mosquitoes Ae. albopictus 76.6% of infection rate. The authors also report the increased expression levels of thioester containing protein 22 (TEP22) and Niemann–Pick type C1 (NPC1 - gene responsible for facilitating mosquito infection when infected with Dengue) gene transcripts were observed in infected Ae. albopictus. In our results, although with a different genotype, we had less infected mosquitoes when fed high viral titer (61.6%—MAYV genotype
D), this fact may indicate that for MAYV the viral titer may not be primarily responsible for the success of the infection, but secondary factors such as the viral genotype, the expression of genes related to the mosquito immune system or even the genetic background of the vector.

Infection rates were significantly higher in *Ae. albopictus* (85%–100%) than in *Ae. aegypti* (67–82%). The same mosquito species may present differentiated vector competence at different sites, since different genotype/genotype interactions between the virus and vector may occur. As an example, it has been demonstrated that the re-emergence of the CHIKV may have been facilitated by the genetic adaptation of the virus to the *Ae. albopictus* vector [45,46].

In 2016, the first imported case of MAYV in a French citizen was reported in an area where the *Ae. albopictus* mosquito is well established [47], thus highlighting the need to better understand the vector competence of this mosquito, as well as its possible role in the transmission of MAYV.

To evaluate viral transmission rate in our experiments, *Ae. albopictus* mosquito saliva submitted to MAYV infection was nanoinjected into naïve mosquitoes, resulting in a high rate of infectivity. Smith and Francy [44], noted in their study that approximately half (5/11) of the mosquitoes infected with hamster viremic blood were able to transmit MAYV when their saliva was tested in capillary tubes. Wiggins et al. [16] observed that *Ae. albopictus* mosquitoes exhibited low transmission rate of MAYV in saliva expectorates. However, these authors used a different methodology.

We also attempted to study whether saliva originating from negative mosquitoes samples were able to infect naïve mosquitoes. Unexpectedly, negative mosquitoes samples from *Ae. aegypti* and *Ae. albopictus* were able to infect other mosquitoes through their saliva. Previous experiments in our group have shown the same effect for DENV with a negative thorax and positive saliva, but it resulted in a lower infection rate.

Furthermore, some studies have described similar results in *Ae. aegypti* and *Cx. quinquefasciatus* with ZIKV, DENV, and WNV [48–50]. One important hypothesis to consider is that over the course of infection, viral decline occurs in other tissues, but the salivary glands/saliva remains positive for transmission. Furthermore, we suspect that mosquito salivation may, in some cases, deplete the glands of almost all viral particles, which could not be detected (in the head) through RT-qPCR.

To better understand these findings, we investigated the relationship between the amounts of (detectable) particles required for mosquito infection. It was possible to observe that samples classified as negative for RT-qPCR were able to infect other mosquitoes, indicating that only a few viral particles are necessary to initiate infection. In addition, it was observed that regardless of the nanoinjected viral doses they produced between $5.6 \times 10^5$ to $1.4 \times 10^6$ viral genome copies per mosquito respectively for replicates A and B (S1 Fig). Therefore, nanoinjection of the viral saliva/dilution in the mosquito acts as a model that amplifies the particles and facilitates later detection [51]. This may explain why saliva from heads classified as negative produced positive mosquitoes upon injection. We believe that only a few viral particles, which cannot be detected through RT-qPCR, are required to infect a mosquito. We suggest that for a broader understanding of this finding, complementary studies using immunofluorescence assays to detect transmission through saliva originating from PCR-negative heads should be undertaken.

When evaluating the vector competence of *Cx. quinquefasciatus*, only two mosquitoes were found to be positive for MAYV, and no mosquito became infected after injection with saliva from these *Cx. quinquefasciatus* mosquitoes submitted to MAYV infection. Corroborating our data, Brustolin et al. [38], also tested the vector competence of *Cx. quinquefasciatus* and observed that this mosquito had either poor or null infection and transmission rates for MAYV.
*Cx. quinquefasciatus* is quite abundant in Brazil, and to date there is only a single record of this mosquito harboring MAYV in Cuiabá [15]. This mosquito is, however, a vector of *Wuchereria bancrofti* in Brazil, an etiologic agent of lymphatic filariasis in humans [52], and was recently incriminated in ZIKV transmission in the metropolitan region of Pernambuco [8]. Guo et al. [48], have also demonstrated the vector competence of *Cx. quinquefasciatus* for ZIKV in China. In contrast, several other studies have demonstrated the lack of ability of this mosquito to infect and transmit ZIKV [53–55]. These results confirm that the vector competence of the same mosquito species can vary geographically, emphasizing the importance of studying the vector competence of different mosquitoes and from different localities.

In conclusion, our studies show that, under laboratory conditions, *Ae. aegypti* and *Ae. albopictus* can be infected and potentially transmit MAYV (genotype D), although *Cx. quinquefasciatus* exhibited poor vector competence. To our knowledge this is the first report of a study involving the vector competence of a particular MAYV genotype, done simultaneously with three mosquito species. *Ae. aegypti* and *Ae. albopictus* are widely distributed throughout the Americas [3,56], and although they are not the main vectors for MAYV, they can potentially play a significant role in the transmission rate of this virus. We suggest that further studies should be conducted to demonstrate the vector competence of the same species towards other MAYV genotypes and even isolates, since distinct isolates of the same virus can behave differently on the same vector species. Furthermore, studies to demonstrate the co-infections with other arboviruses, as well as ecological studies on the recent YFV outbreaks, considering that *Hg. janthinomys* is a common vector for both viruses.

**Supporting information**

S1 Fig. Sample dilutions and detection of Mayaro virus. A and B represent different replicates. Each viral dilution (represented by green dots) was nanoinjected into naïve mosquitoes, followed by virus detection through RT-qPCR (red dots). Samples 8A and 8B are mock controls. Each red spot represents a single female mosquito.

**Acknowledgments**

We wish to thank you Dra. Ana Maria Bispo de Filippis (IOC—FIOCRUZ) for donating the MAYV isolate. We are in debt to Dr. Marco Antônio Silva Campos and Dr. Alexandre de Magalhães Vieira Machado and their team, who provided viral culture infrastructure in the Laboratório de Imunologia de Doenças Virais (IRR—FIOCRUZ). We are grateful to all members from the Group Mosquitos Vetores: Endossimbiontes e Interação Patógeno-Vetor, (IRR—FIOCRUZ), especially for Dr. Alvaro Gil Araujo Ferreira for his critical reading of the manuscript. We are grateful to Belo Horizonte municipality and the Universidade Federal de Minas Gerais who helped to collect mosquito samples.

**Author Contributions**

**Conceptualization:** Thiago Nunes Pereira, Luciano Andrade Moreira.

**Data curation:** Thiago Nunes Pereira.

**Formal analysis:** Thiago Nunes Pereira, Luciano Andrade Moreira.

**Funding acquisition:** Luciano Andrade Moreira.

**Investigation:** Thiago Nunes Pereira, Fabiano Duarte Carvalho.
Methodology: Thiago Nunes Pereira, Fabiano Duarte Carvalho, Silvana Faria De Mendonça, Marcele Neves Rocha.

Project administration: Luciano Andrade Moreira.

Supervision: Luciano Andrade Moreira.

Validation: Fabiano Duarte Carvalho.

Writing – original draft: Thiago Nunes Pereira, Fabiano Duarte Carvalho.

Writing – review & editing: Luciano Andrade Moreira.

References

1. Juliano SA, Lounibos LP. Ecology of invasive mosquitoes: effects on resident species and on human health. Ecol Lett [Internet]. 2005; 8(5):558–74. Available from: https://doi.org/10.1111/j.1461-0248.2005.00755 PMID: 17637849

2. Ministério da Saúde B. Guia de vigilância do Culex quinquefasciatus. 2011. 80 p.

3. Kraemer MUG, Sinka ME, Duda KA, Myline AQN, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors Aedes aegypti and Ae. Albopictus. Elife. 2015; 4(JUNE 2015):1–18.

4. Blitvich BJ. Transmission dynamics and changing epidemiology of West Nile virus. Anim Health Res Rev. 2008; 9(1):71–86. https://doi.org/10.1017/S1466252307001430 PMID: 18348742

5. Ng LC, Hapuarachchi HC. Tracing the path of Chikungunya virus-Evolution and adaptation. Infect Genet Evol [Internet]. 2010; 10(7):876–85. Available from: https://doi.org/10.1016/j.meegid.2010.07.012 PMID: 20654736

6. Dibo MR, Menezes RMT De, Ghirardelli CP, Mendonça AL, Chiaravalloti Neto F. The presence of Culicidae species in medium-sized cities in the State of São Paulo, Brazil and the risk of West Nile fever and other arbovirus infection. Rev Soc Bras Med Trop. 2011; 44(4):496–503. https://doi.org/10.1590/s0037-86822011000400019 PMID: 21860898

7. Staples JE, Fischer M. Chikungunya at the Door—Déjà Vu All Over Again? N Engl J Med [Internet]. 2014; 371(10):885–7. Available from: https://doi.org/10.1056/NEJMp1408509 PMID: 25029435

8. Guedes DRD, Paiva MHS, Donato MMA, Barbosa PP, Krokovsky L, Rocha SWS, et al. Zika virus replication in the mosquito Culex quinquefasciatus in Brazil. Emerg Microbes Infect. 2017; 6(8).

9. Ottesen EA, Ramachandran C. Lymphatic Filariasis Infection and Disease: Control Strategies. Parasitol Today. 1995; 11(4):129–31.

10. Gardner CL, Ryman KD. Genetics M. HHS Public Access. Clin Lab Med. 2015; 30(1):237–60.

11. Grard G, Caron M, Mombo IM, Nkoghe D, Mboui Ond O, Jiolle D, et al. Zika Virus in Gabon (Central Africa) - 2007: A New Threat from Aedes albopictus? PLoS Negl Trop Dis. 2014; 8(2):1–6.

12. Samy AM, Elagip AH, Kenawy MA, Ayres CFJ, Peterson AT, Soliman DE. Climatic Change Influences on the Global Potential Distribution of the Mosquito Culex quinquefasciatus, Vector of West Nile Virus and Lymphatic Filariasis. PLoS One [Internet]. 2016; 11(10):e0163863. Available from: https://doi.org/10.1371/journal.pone.0163863 PMID: 27695107

13. Marabuto E, Rebelo MT. The Asian tiger mosquito, Aedes albopictus (Skuse, 1894), a vector of dengue, chikungunya and zika, reaches Portugal. bioRxiv [Internet]. 2017;192575. Available from: https://www.biorxiv.org/content/early/2017/09/22/192575

14. Long KC, Ziegler SA, Thangamani S, Hauser NL, Kochel TJ, Higgs S, et al. Experimental transmission of Mayaro virus by Aedes aegypti. Am J Trop Med Hyg. 2011; 85(4):750–7. https://doi.org/10.4269/ajtmh.2011.11-0359 PMID: 21976583

15. Pereira Serra O, Fernandes Cardoso B, Maria Ribeiro AL, dos Santos FA, Dezengrini Shessarenko R, Mayaro virus and dengue virus 1 and 4 natural infection in culicids from Cuiabá, state of Mato Grosso, Brazil. Mem Inst Oswaldo Cruz. 2016; 111(1):20–9. https://doi.org/10.1590/0074-02760150270 PMID: 26784852

16. Wiggins K, Eastmond B, Alto BW. Transmission potential of Mayaro virus in Florida Aedes aegypti and Aedes albopictus mosquitoes. Med Vet Entomol. 2018; 1–7.

17. Casals J, Whitman L. Mayaro virus: a new human disease agent. I. Relationship to other arbor viruses. Am J Trop Med Hyg. 1957; 6(6):1004–11. https://doi.org/10.4269/ajtmh.1957.6.1004 PMID: 13487972

18. Vasconcelos PFC, Travassos da Rosa APA, Degalli N, Travassos da Rosa JFS, Pinheiro FP. Clinical and ecoepidemiological situation of human arboviruses in Brazilian Amazonia. Cienc Cult [Internet].
19. Coimbra TL, Santos CL, Suzuki a, Petrella SM, Bisordi I, Nagamori a H, et al. Mayaro virus: imported cases of human infection in Sao Paulo State, Brazil. Rev Inst Med Trop Sao Paulo [Internet]. 2007; 49 (4):221–4. Available from: http://ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17823750 https://doi.org/10.1590/s0036-46652007000400005 PMID: 17823750

20. Azevedo RSS, Silva EVP, Carvalho VL, Rodrigues SG, Nunes Neto JP, Monteiro HAO, et al. Mayaro fever virus, Brazilian amazon. Emerg Infect Dis. 2009; 15(11):1830–2. https://doi.org/10.3201/eid1511.090461 PMID: 19891877

21. Pinheiro F, Freiras R, Rosa -Tracassos J, Yvone G, Wyller M, Leduc J. An outbreak of mayaro virus disease. Am J Trop Med Hyg. 1981; 30(3):674–81. https://doi.org/10.4269/ajtmh.1981.30.674 PMID: 6266263

22. Mourão MP, Bastos M de S, de Figueiredo RP, Gimaqu JBL, dos Santos Galusso E, Kramer VM, et al. Mayaro Fever in the City of Manaus, Brazil, 2007–2008. Vector-Borne Zoonotic Dis [Internet], 2012; 12(1):42–6. Available from: http://www.liebertonline.com/doi/abs/10.1089/vbz.2011.0669 PMID: 21923266

23. Halsey ES, Siles C, Guevara C, Vilcarromero S, Jhonston EJ, Ramal C, et al. Mayaro virus infection, Amazon Basin region, Peru, 2010–2013. Emerg Infect Dis. 2013; 19(11):1839–42. https://doi.org/10.3201/eid1911.130777 PMID: 24210165

24. Lednicky J, Madsen V, Rochars B De, Elbadry M, Loeb J, Telisima T, et al. Mayaro Virus in Child with Acute. 2016; 22(11):2015–7.

25. Forshey BM, Guevara C, Laguna-torres VA, Cespeedes M, Vargas A, Aguayo N, et al. Arboviral Etiologies of Acute Febrile Illnesses in Western South America, 2000–2007. 2010; 4(8):2000–7.

26. Powers Ann M, Russell KL, Olson J, Vasconcelos PFC, Travassos A, Rosa DA, et al. Genetic relationships among mayaro and una viruses suggest distinct patterns of transmission. 2006; 75(3):461–9.

27. Auguste AJ, Liria J, Forrester NL, Giambalvo D, Moncada M, Long KC, et al. Evolutionary and Ecological Characterization of Mayaro Virus Strains Isolated during an. 2015; 21(10):13–15.

28. Thoisy B, Gardon J, Salas R, Morvan J, Kazanjii M. Mayaro Virus in wild mammals, French Guiana. Emerg Infect Dis. 2003; 9(10):12–17.

29. Slegers CAD, Keuter M, Günther S, Schmidt-Chanasit J, van der Ven AJ, de Mast Q. Persisting arthralgia due to Mayaro virus infection in a traveler from Brazil: Is there a risk for attendants to the 2014 FIFA World Cup? J Clin Virol [Internet]. 2014; 60(3):317–9. Available from: https://doi.org/10.1016/j.jcv.2014.04.020 PMID: 24856445

30. Figueiredo RMP, Thatcher BD, De Lima ML, Almeida TC, Alecrim WD, De Farias Guerra MV. Doenças exantemáticas e primeira epidemia de dengue ocorrida em Manaus, Amazonas, no período de 1998–1999. Rev Soc Bras Med Trop. 2004; 37(6):476–9. https://doi.org/10.1590/s0036-46652004000600009 PMID: 15765597

31. Tavares-Neto J, Freitas-Carvalho J, Teixeira Nunes MR, Rocha G, Guerreiro Rodrigues S, Damasceno E, et al. Pesquisa de anticorpos contra arbovírus e o vírus vacinal da febre amarela em uma amostra da população de Rio Branco, antes e três meses após a vacina 17D. Rev Soc Bras Med Trop. 2004; 37(1):1–6. https://doi.org/10.1590/s0036-86822004000100001 PMID: 15042172

32. Silva-Nunes M da, Malafronte R dos S, Luz B de A, Souza EA de, Martins LC, Rodrigues SG, et al. The Acre Project: the epidemiology of malaria and arthropod-borne virus infections in a rural Amazonian population. Cad Saúde Pública [Internet]. 2006; 22(6):1325–34. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-311X2006000600021&lng=en&tlng=en https://doi.org/10.1590/s0102-311x2006000600021 PMID: 16751971

33. Zuchi N, Silva Heinen LB, Santos MAM, Pereira FC, Sihessarenko RD. Molecular detection of Mayaro virus during a dengue outbreak in the state of Mato Grosso, Central-West Brazil. Mem Inst Oswaldo Cruz. 2014; 109(6):820–3. https://doi.org/10.1590/0074-0276140108 PMID: 25141284

34. Brunini S, Fraça D, Silva J, Rezza G. High Frequency of Mayaro Virus IgM among Febrile Patients, Central Brazil—Volume 23, Number 6—June 2017—Emerging Infectious Disease journal—CDC. Emerg Infect Dis [Internet]. 2017; 23(6):1025–6. Available from: http://wwwnc.cdc.gov/eid/article/23/6/16-0929_article.htm https://doi.org/10.3201/eid2306.160929 PMID: 28518022

35. Lucas D, Esposito A, Antonio B. Review article Will Mayaro virus be responsible for the next outbreak of an arthropod-borne virus in Brazil? Brazilian J Infect Dis [Internet]. 2017; 21(5):540–4. Available from: http://dx.doi.org/10.1016/j.bjid.2017.06.002

36. Lorenz C, Azevedo TS, Virgino F, Aguiar BS, Chiara-valioti-Neto F, Suesdek L. Impact of environmental factors on neglected emerging arboviral diseases. PLoS Negl Trop Dis. 2017; 11(9):1–19.
37. Kantor AM, Lin J, Wang A, Thompson DC, Franz AWE. Vector / Pathogen / Host Interaction, Transmission Infection Pattern of Mayaro Virus in Aedes aegypti (Diptera: Culicidae) and Transmission Potential of the Virus in Mixed Infections With Chikungunya Virus. 2019; 56(1):832–43.

38. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, et al. A Wolbachia Symbiont in Aedes aegypti Limits Infection with Dengue, Chikungunya, and Plasmodium. Cell. 2009; 139(7):1268–78. https://doi.org/10.1016/j.cell.2009.11.042 PMID: 20064373

39. Pereira TN, Rocha MN, Sucupira PHF, Carvalho FD, Moreira LA. Wolbachia significantly impacts the vector competence of Aedes aegypti for Mayaro virus. Sci Rep [Internet]. 2018; 8(1):1–9. Available from: https://doi.org/10.1038/s41598-017-17765-5

40. Carrington LB, Chau B, Tran N, Thanh N, Le H, Thi T, et al. Field- and clinically derived estimates of Wolbachia-mediated blocking of dengue virus transmission potential in Aedes aegypti mosquitoes. 2018; 115(2):361–6.

41. Brustolin M, Pujhari S, Henderson CA, Rasgon JL. Anopheles mosquitoes may drive invasion and transmission of Mayaro virus across geographically diverse regions. 2018; 7(11):1–11.

42. Lambrechts L, Chevillon C, Albright RG, Thaisomboonsuk B, Richardson JH, Jarman RG, et al. Genetic specificity and potential for local adaptation between dengue viruses and mosquito vectors. 2009; 11(3) 1–11.

43. Diop F, Alout H, Diagne CT, Baronti C, Vargas REM, De AN. Differential Susceptibility and Innate Immune Response of Aedes aegypti and Aedes albopictus to the Haitian Strain of the Mayaro Virus. 2019; 12 (4):1–11.

44. Gokhale MD, Paingankar MS, Sudeep AB, Parashar D. Chikungunya virus susceptibility & variation in populations of Aedes aegypti (Diptera: Culicidae) mosquito from India. Indian J Med Res. 2015; 142 (12):33–43.

45. Vasconcelos PFC, Calisher CH. Emergence of Human Arboviral Diseases in the Americas, 2000–2016. Vector-Borne Zoonotic Dis [Internet]. 2016; 16(5):295–301. Available from: http://online.liebertpub.com/doi/10.1089/vbz.2016.1952 PMID: 26991057

46. Smith GC, Francy DB. Laboratory studies of a Brazilian strain of Aedes albopictus as a potential vector of Mayaro and Oropouche viruses. J Am Mosq Control Assoc [Internet]. 1991; 7(1):89–93. Available from: http://europemc.org/abstract/MED/1646286 PMID: 1646286

47. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. Chikungunya virus adaptation to Aedes albopictus mosquitoes does not correlate with acquisition of cholesterol dependence or decreased pH threshold for fusion reaction. Virol J. 2011; 8(3):1–12.

48. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in Chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 2007; 3(12):1895–906.

49. Liagonne-Barets M, Icard V, Leparc-Goffart I, Prat C, Perpoint T, André P, et al. A case of Mayaro virus infection imported from French Guiana. J Clin Virol [Internet]. 2016; 77:(12)66–8. Available from: http://dx.doi.org/10.1016/j.jcv.2016.02.013

50. Guo XX, Li CX, Deng YQ, Xing D, Liu QM, Wu Q, et al. Culex pipiens quinquefasciatus: A potential vector to transmit Zika virus. Emerg Microbes Infect. 2016; 5(9):e102–5. https://doi.org/10.1038/emi.2016.102 PMID: 27599470

51. Smartt CT, Richards SL, Anderson SL, Erickson JS. West Nile Virus Infection Alters Midgut Gene Expression in Culex pipiens quinquefasciatus Say (Diptera: Culicidae). 2009; 81(2):258–63.

52. Tsujimoto H, Hanley KA, Sundararajan A, Devitt NP, Schilkey D, Hansen IA. Dengue virus serotype 2 infection alters midgut and carcass gene expression in the Asian tiger mosquito, Aedes albopictus. 2017; 12(3):1–20.

53. Demarquay J-N. Note on a Tumour of the Scrotal Sac Containing a Milky Fluid (Galactocele of Vidal) and Enclosing Small Wormlike Beings That Can be Considered as Hematoid Helminths in the Embryos Stage. Trop Med Parasitology Class Investig. 1863; 11(7):374–7.

54. Fernandes RS, Campos SS, Ribeiro PS, Raphael LMS, Bonaldo MC, Lourenço-de-Oliveira R. Culex quinquefasciatus from areas with the highest incidence of microcephaly associated with Zika virus infections in the northeast region of Brazil are refractory to the virus. Mem Inst Oswaldo Cruz. 2017; 112 (8):577–9. https://doi.org/10.1590/0074-02760170145 PMID: 28767975

55. Arnaoud F, Atayme-Nten C, Vega-Rúa A, Lourenço-De-Oliveira R, Vazeille M, Failoux AB. Culex mosquitoes are experimentally unable to transmit zika virus. Eurosurveillance. 2016; 21(35):1–4.

56. Duchemin JB, Mee PT, Lynch SE, Vedururu R, Trinidad L, Paradkar P. Zika vector transmission risk in temperate Australia: A vector competence study. Virol J. 2017; 14(1):1–10. https://doi.org/10.1186/s12985-016-0669-1