Brief Report

Vector Competence to Zika Virus Changes Depending on the Manaus's Region Origin of the *Aedes aegypti*: A Study of an Endemic Brazilian Amazonian City

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Abstract: Zika virus (ZIKV) is transmitted to humans by the infectious bite of mosquitoes like *Aedes aegypti*. After a viremic blood meal, the virus must infect the midgut, disseminate to tissues, and reach the salivary gland to be transmitted to a vertebrate host. Many factors influence the mosquito's ability to become infected and transmit viruses, such as the mosquito's genetic diversity, intrinsic antiviral barriers, and midgut microbiota. This study evaluated the patterns of ZIKV infection in *Ae. aegypti* field populations of a city. The infection rate, disseminated infection rate, viral transmission rate, and transmission efficiency were measured by quantitative PCR at 14 days post-infection. The results showed that all *Ae. aegypti* populations had individuals susceptible to ZIKV infection and able to transmit the virus. The infection parameters showed the city's geographical area of origin of the *Ae. aegypti* influences their vector competence for ZIKV transmission.

Keywords: Zika virus; field mosquito population; vector competence; transmission efficiency; infection rate; disseminated infection rate

1. Introduction

Zika virus (ZIKV) is an arthropod-borne virus transmitted through the bite of mosquitoes of the genus *Aedes* (*Stegomyia*). *Aedes aegypti* is the primary vector in tropical and subtropical regions of the globe and is highly adapted to urban habitats (9). The worldwide distribution of *Ae. aegypti* (10) and its permissiveness to ZIKV contribute to the broad range of regions at risk of Zika fever outbreaks, with the disease representing a recurring threat to the worldwide health system (7,11). The understanding of the dynamics of the *Ae. aegypti* susceptibility to ZIKV is a necessary step in fighting against the virus circulation and the consequent Zika fever spread.

ZIKV belongs to the genus *Flavivirus*, family Flaviviridae, and is closely related to other health-relevant arboviruses, such as dengue (DENV) and chikungunya (CHIKV) viruses. It was first isolated in the Zika forest of Uganda in 1947 from a sentinel *Rhesus* monkey (1). The first human cases of ZIKV infection were detected in Uganda and Tanzania in 1952, followed by sporadic cases reported only on the African and Asian continents. The first outbreak of Zika outside these continents occurred in 2007 in Micronesia. After that, a large epidemic occurred in French Polynesia in 2013-2014, where symptomatic infections affected approximately 11% of the population (2,3). Following outbreaks in

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Micronesia and French Polynesia, the virus reemerged and spread rapidly across the Americas in 2015 (4), causing a significant outbreak in Brazil, where the virus was a cause of neurological disorders such as Guillain-Barré syndrome in adults, and microcephaly in neonates (5). Despite the apparent decrease in cases reported since the last outbreak, ZIKV infection remains considered a significant global human threat, especially in Latin America (8).

To establish an infection, the arboviruses such as ZIKV must overcome multiple barriers within the mosquito for transmission to occur. After digestion of the blood meal, the virus must infect the midgut, disseminate out of midgut cells, cross the midgut basal lamina to reach the hemolymph, and then infect the salivary glands (SG) to be transmitted to vertebrate hosts through a subsequent blood feeding (12). The time interval corresponding to the ingestion of the infected blood meal until the vector becomes infectious is called the extrinsic incubation period (EIP). The ZIKV EIP has an average range of 3-14 days (13,14).

The permissiveness of a vector to infect and transmit a pathogen is called vector competence (VC). Multiple extrinsic and intrinsic factors can influence the VC. The extrinsic factors can be related to environmental conditions, the mosquito's geographic origin, breeding water quality, and pathogen virulence (14). The intrinsic factors are associated with the vector-pathogen interaction, including genetic variability, innate immunity-related pathways, the composition of the mosquito gut microbiota, and tissue barriers. The midgut infection and escape barrier (MIB and MEB) and salivary gland infection and escape barrier (SGIB and SGEB) are inside main challenges the virus should overcome to infect and disseminate inside the vector (15).

Despite the impact of Zika fever on the global health systems, the features that control vector competence (VC) in *Ae. aegypti* populations still need to be elucidated. Only a few studies have evaluated the ability of *Ae. aegypti* local populations to be infected and transmit ZIKV in the context of mosquito populational variability. This study assessed the VC and related parameters such as the infection rate (IR), disseminated infection rate (DIR), viral load (VL), and transmission efficiency (TE) of *Ae. aegypti* populations derived from five geographically distinct areas of Manaus, an endemic Brazilian city. Understanding the patterns of ZIKV infection and transmission response among mosquito populations may contribute to future vector-borne disease control programs.

2. Materials and Methods

2.1. Mosquito collection

1,200 *Ae. aegypti* eggs were collected using ovitraps distributed in five health districts of Manaus, State of Amazonas (latitude 3°6'26'' S, longitude 60°1'34'' W, and 39m above sea level). Manaus occupies an area of 11,401 km². The city is the capital of the State of Amazonas and has 2,255,903 inhabitants and a population density of 158 inhabitants/km². It is divided into five health administrative regions: North, South, East, West, and Central (16).

Eggs from each health administrative district were allowed to hatch and reared through 3 successive generations as a single local colony until enough specimens were used for the experimental infections. The *Ae. aegypti* mosquitoes were selected and kept at a controlled temperature of 28°C, 80% relative humidity, and 12 h/12 h light-dark photoperiod. They were separated and named according to their district of origin. The parental generation of mosquitoes derived from the field-collected eggs was checked for ZIKV natural infection using polymerase chain reaction (qPCR) before starting the mosquito colonization to obtain the next generations. A well-established Brazilian colony of *Ae. aegypti* (strain PP-Campos) was the positive control group in this study to validate the infection procedures.

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2.2. ZIKV Culture

Virus stocks of a ZIKV strain that currently circulates in Brazil (ZikaSPH2015) (17) were passaged in an *Aedes albopictus* cell line (C6/36), grown in Leibowitz L-15 medium supplemented with 2% inactivated fetal bovine serum (FBS), 20 µg/ml Gentamicin, 5 µg/ml Amphotericin B, and 200 U/ml Penicillin (18). Virus titration followed the 50% tissue culture infectious dose method (19).

2.3. ZIKV infection of Aedes aegypti

3 to 5-day-old *Ae. aegypti* female mosquitoes (n=150) from the five-health district were placed in separate cages and experimentally infected with ZIKV via a membrane feeding assay (11,20). A blood meal with a virus titer of 1×10^6 PFU/mL was mixed with fresh mouse blood (ratio 2:1) and offered to the mosquitoes as described elsewhere (19). Mosquitoes were allowed to feed for 1h on the ZIKV-infective blood meal. Fully engorged females were separated into new cages and maintained on 10% glucose solution *ad libitum* for up to 14 days post-infection (dpi) until the completion of the extrinsic infection period (EIP).

2.4. Mosquito dissection and viral RNA extraction

At 14 dpi, twenty-five mosquitoes from each health district were randomly chosen, anesthetized on ice, and dissected under a stereoscope. Their bodies and head/salivary glands (head/SG) (salivary glands attached to the heads) were individualized and transferred to separate microtubes. The viral RNA was extracted using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -80ºC. The quantification of ZIKV was carried out by the Real-Time Reverse Transcription qPCR with cDNA production using a random primer and M-MLV enzyme, and subsequent TaqMan-based qPCR assay (Thermos Fisher Scientific, Hampton, USA) (2,20).

2.5. Infection Rate (IR), Disseminated Infection Rate (DIR), Transmission rate (TR), and transmission efficiency (TE)

The intensity of the infection was estimated by determining the number of viral cDNA copies, and viral load (VL), present in the sample. The IR, DIR, and VC were determined for the five *Ae. aegypti* populations. The well-established Brazilian colony of *Ae. aegypti* (strain PP-Campos) was used as a control group. The IR was calculated as the proportion of infected mosquito bodies related to the total number of tested mosquitoes (n = 25). The DIR was the proportion of infected mosquito heads/salivary glands related to the number of infected mosquito bodies (23). The TR and TE were assessed by evaluating the presence of ZIKV in forced-secreted saliva's of infected *Ae. aegypti*. At 14 dpi, 25 mosquitoes from each experimental group were cold-anesthetized and had their legs and wings removed. The proboscis of each one was inserted into a filter tip containing 6 µL of sucrose solution plus fetal bovine serum (1:1) for 30 min. The secreted saliva samples were individually cultivated in C6/36 cells for 5 days as described above, and supernatants were processed by qPCR for ZIKV cDNA copy quantification. The mosquitoes were analyzed for the transmission rate (TR), which corresponds to the proportion of mosquitoes with infected saliva among mosquitoes with disseminated infection; and for the transmission efficiency (TE), which corresponds to the proportion of mosquitoes with the virus in saliva among the total number of mosquitoes tested (21,22).

2.6. Ethics Approval

The Ethics approved this study in Animal Use Committee of Tropical Medicine Foundation Dr. Heitor Vieira Dourado, Manaus – AM, Brazil (protocol number 001979/2019.015/FMT-HVD/CEUA).

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2.7. Statistical Analyses

The body, head/SG and saliva viral loads were evaluated among all populations using Kruskal-Wallis one-way ANOVA tests. Mann-Whitney U tests were used to evaluate the significance among viral load medians in each infected mosquito population's bodies and head/SG. All statistical analyses were performed in GraphPad Prism Prism (version 8.0.2, La Jolla, CA, USA), and P values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Infection Rate (IR) and Disseminated Infection Rate (DIR) of the Ae. aegypti populations

The IRs and DIRs of the five *Ae. aegypti* populations were measured at 14 dpi, an incubation time enough to allow the viral establishment and spreading in the mosquito, making it infectious. The results are shown in Figure 1. To facilitate the comparisons of the infection-related rates among the mosquito populations, we assign arbitrary levels to measure their susceptibility to ZIKV related to infectivity (IR) and viral dissemination (DIR). Rates in the range of 0 - 40% were considered low levels; 40 - 70%, intermediate levels; and 70 – 100%, high levels. Considering that classification, the IRs and DIRs values were high in Northern, Eastern, Western, and Central health districts (ranging from 84 to 100%); and the Southern district had an intermediate IR (48%) and high DIR (92%). No mosquito population had IRs or DIRs rated below 40% (Table 1 and Fig. 1).

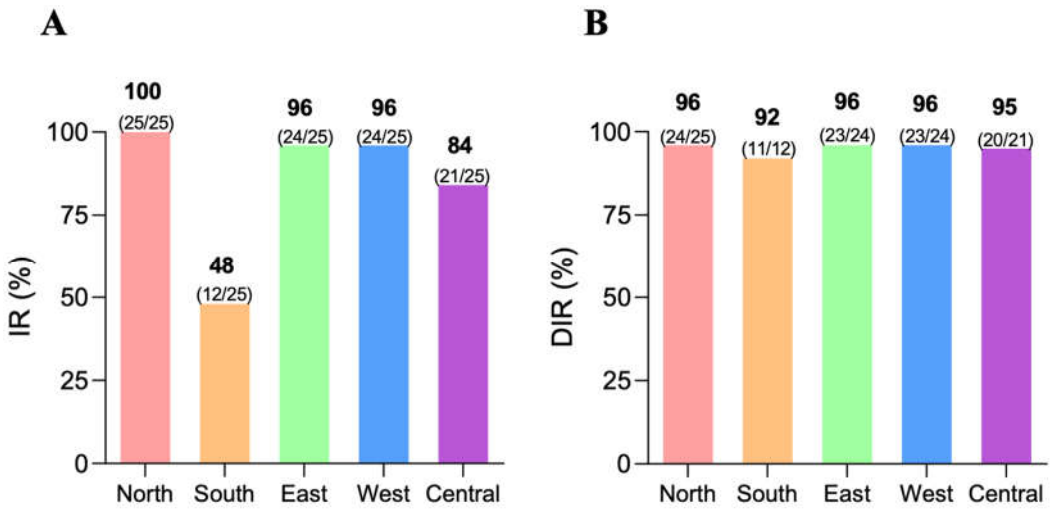

Figure 1. Infection Rates (**A**) and Disseminated Infection Rates (**B**) of *Aedes aegypti* mosquitoes from five districts of Manaus to transmit Zika virus, at 14 days post-infection.

3.2. Viral Load (VL) of the ZIKV by qPCR of Ae. aegypti populations

The VLs of ZIKV in the mosquito populations were determined by the median number of virus cDNA copies in the mosquitoes' bodies and head/SGs at 14 dpi. In ascending order, the median VL of ZIKV cDNA copies in the mosquito bodies were: Central district (4×10^6), Northern (2×10^6), Western and Eastern (1×10^6), and Southern district (2×10^1) (Figure 2A). The median ZIKV cDNA copies in the mosquito heads were: Central district (3×10^6), Northern (2×10^6), Western and Eastern (4×10^5), and Southern district (4×10^2) (Figure 2B). Comparatively, the ZIKV VLs in the mosquito body were similar among all tested populations ($p = 0.7252$). Differently, the head/SG VLs varied among populations ($p = 0.0074$), with the Southern population having a smaller value than the others (Fig. 2A-B).

3.3. Transmission Rate (TR) and Transmission Efficiency (TE)

The mosquito populations were tested for the TR corresponding to the proportion of mosquitoes with infected saliva among mosquitoes with disseminated infection, and for

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the TE, determined by the number of samples with infected saliva among the total samples tested. The highest TR value was from the population from the Central district (50%), while the lowest was from the Northern district (8%). The other values in ascending order were from Eastern (13%), Western (17%), and Southern (27%) districts (Fig. 2C).

For the TEs, the highest and lowest values were from the populations from the Central (40%) and the Northern (8%) districts. The TE for mosquitoes from the Western district was 16%, and for the Southern and Eastern districts, the values were 12 and 8%, respectively (Fig.2D).

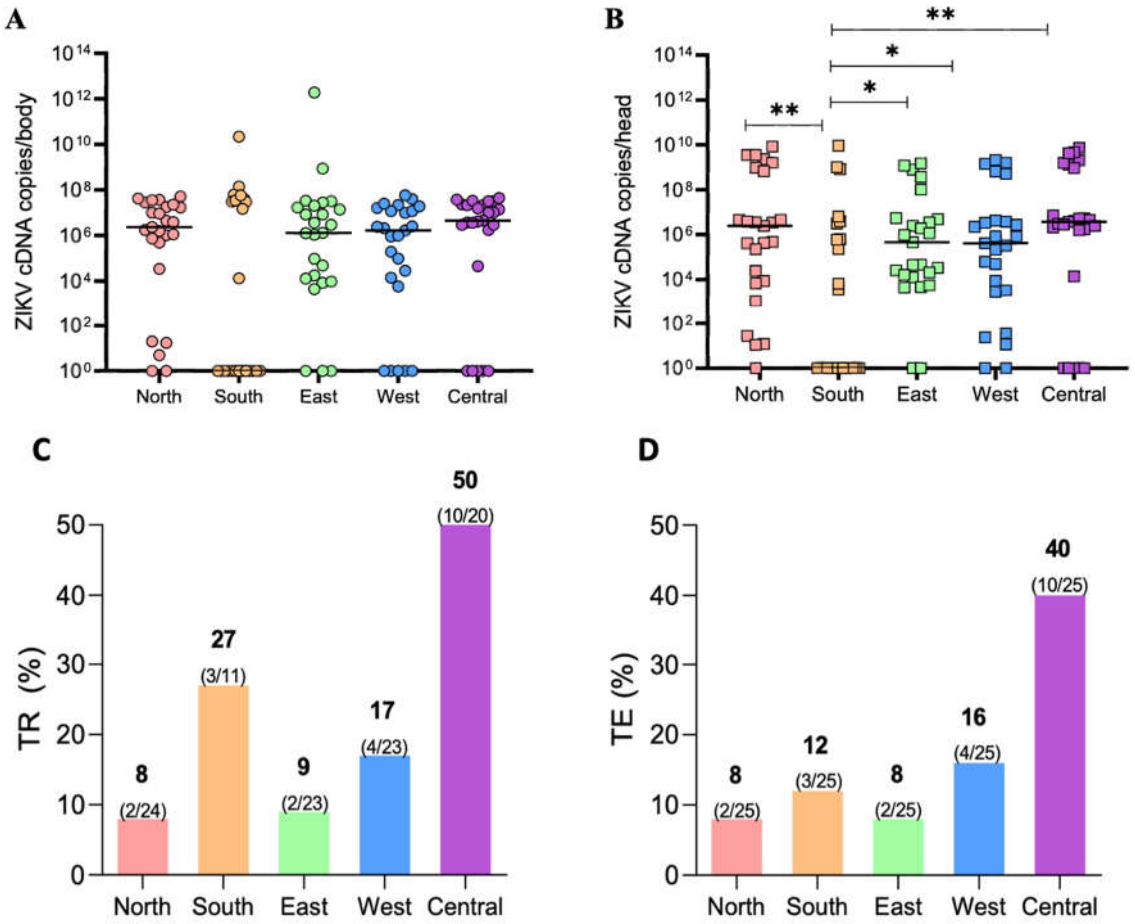

Figure 2. ZIKV viral loads (VLs) per body (**A**) and head/salivary gland **(B**) in *Ae. aegypti* populations from the five districts of Manaus. The VLs were measured as cDNA copies by qPCR at 14 days post-infection. Transmission Rate (**C**) and Transmission Efficiency (**D**) of the five *Ae. aegypti* populations from Manaus to ZIKV at 14 days post-infection. The *p* values 0.05 and 0.01 are summarized with one (*) and two (**) asterisks, respectively.

4. Discussion

Assessing the patterns of infectivity and dissemination of the arboviruses inside their natural vectors is essential to understanding the vector susceptibility to these pathogens and the transmission to the vertebrate hosts. This study evaluated two types of infection parameters: (***i***) the susceptibility of *Ae. aegypti* to the ZIKV infection, an analysis based on the IR (viral ability to infect the mosquito body) and DIR (viral ability to disseminate the infection to secondary organs, such as the salivary glands); and (***ii***) the TR and the efficiency of ZIKV transmission by mosquitoes through secreted saliva analysis. It was already demonstrated in previous studies that the infection parameters of *Ae. aegypti* after arbovirus challenge can vary in mosquitoes from distinct geographic areas (24), including that belonging to the same city (7,10), corroborating the results presented here for the field-derived populations of *Ae. aegypti* from Manaus. The primary factors that may

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explain these variabilities in susceptibility among mosquito populations related to their geographical area of origin include their intrinsic genetic differences and differences in the composition and abundance of midgut microbiota. The genetic differences may reflect in distinct antiviral efficiency of the anatomical barriers and other antiviral responses; and the microbiota in the mosquito midgut can improve or prevent virus infection, replication, and dissemination in the tissues, depending on the bacterial species present there and its quantity.

Concerning the IR and DIR in mosquitoes from Northern, Eastern, Western, and Central populations, ZIKV establish in a high proportion of infected individuals and disseminate the infection in the tissues, maintaining high viral loads upon arrival in the salivary glands. It indicates that these mosquitoes are highly permissive to ZIKV establishment. Differently, mosquitoes from the Southern population are less permissive to infection than the other populations, with lower rates of IR and DIR. The high IR and DIR observed in all mosquito populations except the Southern one show that ZIKV could transpose MIB and MEB in several individuals, disseminating through the hemocoel and infecting secondary organs, such as the salivary glands. For the Southern population, less than half of mosquitoes challenged with ZIKV were permissive to infection. Variations in IR and DIR among geographically diverse mosquito populations of the same species are observed in the literature for different arboviruses in *Aedes* spp. (7,10,24-26). These results are in line with that made with *Ae. aegypti* populations from the city of Belo Horizonte in Brazil (7), with high values of IR and DIR for ZIKV infections. A similar vector competence study was conducted in Manaus with 4 serotypes of DENV and demonstrated that the *Ae. aegypti* populations have variabilities in the IR and DIR, indicating different efficiencies to deal with the virus considering both infection and dissemination (10).

Concerning the VLs of the five mosquito populations, the similarity in the body values indicates homogeneity in the infection and virus replication in the Northern, Eastern, and Central populations. Also, it demonstrates that the individuals with positive infections from the Southern population had high enough VLs to compensate for the impact of the mosquitoes with a negative one. Additionally, for the VLs in the head/SG, the lowest value for the Southern population compared with the others may be explained by its low IR. These VL, IR, and DIR results demonstrate no homogeneity in the virus spreading inside the *Ae. aegypti* body concerning the five mosquito populations.

After forced salivation, we could detect the mosquitoes with viruses in the saliva and calculate the TR and TE. All *Ae. aegypti* populations from Manaus had individuals presenting ZIKV-positive saliva, demonstrating that the SG barriers have been overcome and that mosquitoes can effectively transmit ZIKV. SG infection is the last step in the mosquito infection cycle, and to reaching the salivary glands, arboviruses must overcome natural barriers, infect various tissues, and counteract innate and cellular defenses (28). In the SG, the viruses can be inside the saliva secretory cells after overcoming the SGIB, but to be transmitted to the vertebrate host, they need to cross the SGEB and disseminate to the SG ducts to be present in the mosquito saliva. The TE results showed that even though the Southern population has the lowest IR compared to the others, their TR of 27 %, the second higher considering all five populations, allowed it to reach pattern TE rates close to that of the Northern, Eastern, and Western ones. Despite high IR and DIR, the low TE highlights that even if the virus can establish infection and spread in the mosquito body, SG barriers may still prevent virus transmission. It shows the importance of assessing all the steps of the viral invasion, concerning infection, dissemination, and the reach of the SG. The Central population presented high IR, DIR, and TR, thus having the most competent mosquitoes compared to the other ones and demonstrating that in this population, the ZIKV had success in all these three steps of viral invasion.

It is known that genetic factors are responsible for most of the characteristics that contribute to the success of the insect vectors, such as susceptibility, vector competence, and insecticide resistance (31). However, a previous study of population genetics performed with *Ae. aegypti* in Manaus showed no genetic difference between mosquito populations of distinct geographical areas (32). It is possible that a potential divergence in the

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midgut microbiota of the mosquitoes could explain the differences seen in IR rates, VLs, and TEs. Further studies with *Ae. aegypti* from distinct geographical areas of Manaus are necessary to assess if the microbiota composition differs and contributes to their different susceptibilities to ZIKV.

5. Conclusion

In this study, we saw that all *Ae. aegypti* populations from Manaus have individuals competent to transmit ZIKV. The Southern population stood out for being the least susceptible to ZIKV, which supported the least virus quantity at all, and even presented equivalent results to the other populations of high infectivity because there was a high proportion of individuals with infected saliva. The Central population was the most competent population due to the high proportion of individuals susceptible to ZIKV infection, and permissive to the virus spreading and crossing the SG cells to reach saliva. Our results show that determining whether an infected mosquito can transmit an arbovirus is essential to understanding the transmission dynamics and emphasizes the importance of analyzing vector-arbovirus interactions for mosquito populations in urban environments. The understanding of the susceptibilities of *Ae. aegypti* mosquitoes from distinct locations to ZIKV, especially in endemic cities to Zika fever, can cooperate with transmission control studies and mitigation of arboviral diseases. Future studies aimed at elucidating factors besides genetics capable of interfering with *Ae. aegypti* susceptibility/resistance to ZIKV in geographically close mosquito populations is necessary to trace the diversity of antiviral behaviors they can develop against arboviral infections.

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