Vectorial Competence Potential of Culex Quinquefasciatus (Say, 1923) from the Amazon Region to Transmit West Nile Virus Isolated in Brazil

Lúcia Aline Moura Reis
State University of Pará
Eliana Vieira Pinto da Silva
Instituto Evandro Chagas
Maria Nazaré Oliveira Freitas
Instituto Evandro Chagas
Rossela Damasceno Caldeira
Federal University of Para
Pedro Arthur da Silva Araújo
Federal University of Para
Fábio Silva da Silva
Instituto Evandro Chagas
José Wilson Rosa Junior
Instituto Evandro Chagas
Roberto Carlos Feitosa Brandão
Instituto Evandro Chagas
Daniele Barbosa de Almeida Medeiros
Instituto Evandro Chagas
Lívia Caricio Martins
Instituto Evandro Chagas
Daniel Damous Dias
State University of Pará
Joaquim Pinto Nunes Neto (joaquimneto@iec.gov.br)
Instituto Evandro Chagas

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Abstract

The *West Nile virus* is characterized as a neurotropic pathogen that causes the West Nile fever. It is transmitted by mosquitoes, mainly of the *Culex* genus. In 2018, the Evandro Chagas Institute carried out the first isolation of WNV strain from a horse brain sample and in 2021 the same institute also isolated the WNV in a pool of mosquitoes of the *Culex spp*. from the Carajás region (Pará). Thus, this study aimed to determine the vectorial competence of *Culex quinquefasciatus* from the Amazon region of Brazil for transmission the WNV Brazilian strain. Oral infection of *Cx. quinquefasciatus* females belonging to F1 generation was performed with infected blood with WNV. Subsequently, analysis of infection, dissemination and transmission rates was performed, as well as verification of viral titers in the samples. The study demonstrated that *Cx. quinquefasciatus* can act as a potential vector of WNV in Brazil, since it was found that the Brazilian strain was able to overcome the host's anatomical barriers and spread to various regions, among them in saliva, in which, despite the low viral titers identified, it had a transmission rate of 100% on the 21st day after infection.

Introduction

Arboviruses (Arthropod-borne virus) are viruses that carry out part of their replicative cycle inside arthropods and are thus transmitted to vertebrate animals [1,2]. The transmission of arboviruses, such as *West Nile virus (WNV)*, occurs from the bite of a hematophagous arthropod, like *Culex (Cx.) pipiens*, in a viremic wild animal, including wild birds, followed by viral replication in the arthropod organism reaching the saliva by whereby it can be transmitted to other animals and humans [1,3,4].

The WNV is characterized as a neurotropic pathogen that causes West Nile fever and encephalitis. It is transmitted by mosquitoes, mainly of the *Culex* genus and can also be transmitted through contact with blood and tissues of infected animals and through organ transplants, blood transfusions, breastfeeding and transplacental pathway [5,6,7,8].

The WNV was first isolated in 1937 from a human case in the West Nile district of Uganda and was also detected in birds in the Nile Delta. In 1999 a strain found in Tunisia and Israel was introduced in the Western hemisphere, in New York City, and since then the virus has spread to other countries such as Canada, Mexico, Caribbean and Venezuela [9,10,11].

In Brazil, the first isolation was carried out by the Evandro Chagas Institute (IEC) in 2018, of brain samples from horses from Espírito Santo state, however, there are reports of samples of horses from Mato Grosso, Paraíba, Ceará and São Paulo and chicken from Mato Grosso, which showed WNV-seropositive [12,13, 14, 15].

According to a report by the Ministry of Health (MS) on the monitoring West Nile Fever, until July 2019, only two human cases were confirmed between the years 2014 and 2017, both in Piauí state. However, the MS affirms that after the confirmation of infections, there was an increase in the number of notifications of suspected cases [16].

Sequencing analyzes have shown that the first WNV strain isolated belongs to the *Japanese encephalitis virus* (JEV) serogroup and can be grouped into five lineages. In Brazil, sequencing analyzes demonstrated that the Brazilian strain belongs to the 1a lineage that circulates in the United States and Mexico [8,12,17].

The enzootic cycle of WNV consists of hematophagous arthropods, wild birds as amplification hosts and mammals (horses and humans) as accidental hosts [7,18,19]. However, other animal species have already been serologically identified with WNV, such as deer, mouflons, raccoons, skunks, bears and foxes [20].

Several birds are susceptible to WNV infections, among them *Corvus brachyrhynchos*, *Cyanocitta cristata* and *Circus aeruginosus* and several species of mosquitoes have also been identified carrying the virus, although, the *Culex* genus are accepted as the main vectors, as *Culex pipiens* and *Culex quinquefasciatus* [9,11,18,21,22].

The *Cx. quinquefasciatus* species is mainly found in tropical countries and is widely adapted to the urban environment, being easily found in human and animal dwellings [27,23,24,35]. In the urban environment, the females find several breeding sites conducive to their reproduction, since they lay their eggs, organized on rafts, in small collections of stagnant water containing a high content of organic matter, making such a species resistant to the effects of water pollution [23,26].

The species is characterized as the main vector of *Wuchereria bancrofti* and *Brugia malayi* that causes Lymphatic Filariasis and Dirofilariosis, respectively. However, this species is also identified as a vector or potential vector of several arboviruses, such as WNV, SLEV, JEV and *Oropouche orthobunyavirus* (OROV) [17,19,27,28,29].

In Brazil, *Cx. quinquefasciatus* has a wide distribution, in an entomological study [30] carried out in the states of Pará, Mato Grosso, São Paulo and Rio de Janeiro between 1968 and 1976 years, the presence of the species has been demonstrated, including areas belonging to the Amazon Region. According to the Guidelines for Surveillance of *Culex quinquefasciatus* [31], the species is associated with high rates of Lymphatic Filariasis in cities such as Recife, Maceió and Belém.

Thus, the blood of vertebrate animals is rich in several nutrients for egg maturation. However, the blood supply is also a rich source of infection due to mosquitoes' exposure to various pathogens, such as bacteria, fungi and viruses, so mosquitoes have several physiological barriers to prevent infection [32, 33].

The insects' digestive tract consists of a layer of epithelial cells that extends from the mouth (opening in the anterior region) to the anus (posterior region) and it is subdivided into three regions, the anterior, middle and posterior intestines [32].

Thus, mosquitoes have several anatomical barriers of protection. The midgut infection barrier (MIB) works by preventing pathogens from infecting mesenteronal epithelial cells, since this tissue is rich in chitins and other proteins, in addition to having a strong immune response to the virus. The midgut escape barrier (MEB) works by preventing the pathogen from crossing the basal layer of the mesentery into the hemocele, a region that favors the multiplication of viral particles. The Salivary gland infection barriers (SGIBs) and the salivary gland escape barriers (SGEBs) has the function of preventing the entry of pathogens in the salivary gland and subsequent exit to the lumen of the salivary gland [9,33,34].

Therefore, vector competence is defined as the ability of a vector to become infected with a pathogen (susceptibility to infection), keep it multiplying through tissues (extrinsic incubation period - EIP) and transmit it from a high viremia in the salivary glands and saliva [9,19,35].

Thus, the present study aims to determine the vectorial competence of *Cx. quinquefasciatus* from the Amazon region of Brazil for transmission of the *West Nile virus* strain isolated in Brazil in 2018 (WNV-BE AN 854747).

Results

Infection, Dissemination and Transmission Rates

A total of 25 mosquitoes were tested. Infection rates remained similar to all post-infection days (pi), however dissemination and transmission rates varied widely according to the day of post-infection assessed (Fig. 1).

The infection rate at the 7th, 14th and 21st pi was 100%, demonstrating that all mosquitoes were infected by WNV, however due to the low quantity of body samples and there are no negative samples at any pi, it was not possible to obtain a p-Value for trend analysis.

In relation to the dissemination rate, in the 7th pi, 40% of positive samples were obtained, in the 14th pi a rate of 37.50% and in the 21st pi 100%. According to the trend analysis, it was found that, although there is an increasing trend in the number of positive head samples as pi increases ($A>0$ with $A = 3.000$), this growth is not yet statistically significant with $p>\alpha$.

Regarding the transmission rate, it was observed that only one saliva sample of the 7th pi was positive after a second passage in $C_{6/36}$ cells, demonstrating a 10% positive rate, however the viral titer of the sample could not be quantified by viral plaque titration test, suggesting a low viral load (Fig. 2 / Table 1). At 14th pi, no saliva sample was positive for viral isolation and at 21st pi all samples were positive for infection rate (100%). In relation to the trend analysis, the saliva samples showed a growth trend ($A> 0$ with $A = 4.3636$), presenting statistical significance with $p <\alpha$, that is, there is a tendency for a greater number of saliva samples infected with WNV as the post-infection days increase.

Viral Titration

All the samples were subjected to the viral plate titration test (Table 1).

When analyzing the viral titers of the body samples referring to the 7th, 14th and 21st pi by the KW test, there was statistical significance between the titers ($p = 0.0057$) and Dunn's test revealed that the difference between the mean scores of the 14th and 21st pi groups presented $p <\alpha$ (<0.05) with high significance, demonstrating that there is a significant increase in the viral titer in the body samples between 14th and 21st pi.

About head samples, the KW test also showed a variation in the values of the viral titers, with $p <\alpha$ ($p = 0.0172$), however the sample groups that showed greater variation in the titers by the Dunn's test referred to the 7th and 21st pi, with $p <\alpha$ (<0.05), showing a significant increase in the viral titer of the head region between the 7th and 21st pi.

Regarding saliva samples, the KW test also identified a statistical difference ($p = 0.0091$) between the sample groups, with variation in the values of viral titers between them. Dunn's test, on the other hand, showed a difference in the mean titer scores between the groups of 7ths and 21st pi and between 14th and 21st pi, both

with $p < \alpha$ (<0.05), demonstrating a significant increase in viral titer in saliva between the 7th and 21st pi and between the 14th and 21st pi.

Discussion

In Brazil, WNV circulation has been identified by serological methods in samples from various animals such as horses, domestic and human chickens since 2013 [12,13,15,16,36], however, it was only in 2018 that the first virus isolation was carried out on a sample of an adult horse from Espírito Santo state [12] and in 2021 the Evandro Chagas Institute (IEC) confirmed, through an Information Bulletin, the isolation of the virus in a pool of mosquitoes of the *Culex spp*. from the region of Carajás (Pará) (unpublished data).

Considering the isolation of the WNV carried out in Brazil, the present study evaluated the vectorial competence of the *Cx. quinquefasciatus* mosquito from the Amazon region, thus, considering that the analysis of the vectorial competence of the vectors is extremely important for the study of their participation in the transmission cycle of the arbovirus, mosquitoes belonging to the F1 generation were used because it is the closest generation to that collected in the field, resembling to mosquitoes present in a natural environment.

The viral stock used in the present study had a viral titer of $1.4x10^8$ PFU/mL, however, no analysis was performed of the viral titer of the final infectious blood sample after it was mixed with defibrinated goose blood. However, the titer of the stock used is similar to other studies of vector competence, Goddard *et al*. [37] used a viral stock of WNV with a viral titer of $10^{4.9}$ to $10^{7.1}$ PFU/mL in conjunction with 1mL of rabbit blood, demonstrating in their study that the higher the viral load of the infectious blood meal, the higher the viral transmission rate. Richards, Anderson and Lord [38] used viral stock with titration of $10^{9.2}$ PFU/mL and Romo *et al*. [39] obtained, after mixing viral supernatant and calf blood, an infectious feed with 10^7 and 10^8 PFU/mL. Rochlin *et al*. [40] in their review, emphasized that *Culex* mosquitoes are capable of transmitting WNV when fed on infected blood containing a viral titer greater than 10^5 PFU / mL.

It was observed that the studied population has high susceptibility to the WNV strain used, since the virus was identified in all body samples of 7th, 14th and 21rst pi, as well as it was possible to quantify the viral titer in the same samples in the same period, corroborating with data presented by Bakhshi *et al.* [41] which demonstrates that *Cx. pipiens* species also showed high susceptibility to WNV strain belonging to 1a lineage isolated in Iran, with infection rates varying from 70% to 88% at 7th and 21rst pi.

Sudeep *et al.* [42] evaluated *Cx. quinquefasciatus* population from India for transmission of three different strains of WNV, demonstrating the susceptibility of the species to the virus and Jiang *et al.* [43] who studied the potential for vectorial competence of four different species of mosquitoes of *Culex* genus for WNV transmission (strain Eg101), noting that the *Cx. quinquefasciatus* species had an infection rate of 40.5%.

In a study carried out by Romo *et al.* [39] the *Cx. quinquefasciatus* when orally infected with different WNV strains belonging to lineage 2, presented an infection rate of 100%, as well as in research carried out by Micieli *et al.* [44] in which *Cx. quinquefasciatus* from the United States had an infection rate of 95.5% when fed with the NY99-3356 strain, data similar to that found in the present study.

Regarding the analysis of viral titers of the bodies samples evaluated, our data showed a high increase in viral titer when compared samples from the 14th and 21st pi, demonstrating that the *Cx. quinquefasciatus* population studied maintained WNV replication for an extended period and in high titles, favoring possible viral transmission. However, Richards, Anderson and Lord [38] highlight in their study that despite the high viral titers in bodies and legs in *Cx. quinquefasciatus* samples orally infected with different WNV isolates, the high infection rate is not necessarily related to a similar increase in the transmission rate. Bakhshi *et al.* [41] highlights that although the infection rates found in their study did not show statistical differences, the viral titer of the body samples also exhibited significant growth as the days post-infection increased.

Regarding the dissemination rate, our study showed a variation in the viral spread percentage, but did not show a continuity of growth, a fact that can be explained by the low number of samples from the 14th and 21st pi, indicating the necessity to carry out new tests of vector competence with a greater amount of samples, thus making it possible to establish with greater precision the viral kinetics of the WNV Brazilian strain. Despite the low sample number, when evaluating the viral titer of the head samples, there was a variation between the titers of the 7th and 21st pi samples, with a significant increase in the viral titer.

Brackney *et al.* [10] in their work observed that the dissemination viral of WNV in the *Cx. quinquefasciatus* body occurred in a similar way to our study, in which the viral dissemination rate at the 7th pi was 58%, followed by a decrease in this rate for only 36% at the 14th pi and for a new growth at the 21st pi (55%). In the study by Bakhshi *et al.* [41] with *Cx. pipiens*, there was a dissemination rate of 92.3% on the 21st pi, but no statistical differences in the analysis of viral titles.

In our study, we observed the detection of WNV, by viral isolation, in a saliva sample referring to the 7th pi, however it was not possible to determine its viral titer. This observation may indicate a rapid dissemination of the virus in the host organism, reaching the saliva region, but with a low viral load, and its transmission through hematophagy is not possible. In a vector competence survey, Brackney *et al.* [10] verified the presence of WNV in *Cx. quinquefasciatus* saliva from the 7th pi, with a transmission rate of 25%. In our study, 100% of saliva samples were positive at 21st pi, while the authors obtained 35% positivity at 21st pi.

According to Sudeep *et al.* [42] work, the WNV can be identified in Culex quinquefasciatus saliva samples already in 5th pi with a viral titer ranging from 1.4 to 1.17 PFU/mL. The authors also point out that the highest viral titer detected was from a sample referring to the 11th pi with a viral load above 2.4 PFU/mL, a value similar to the viral load identified in the present study in samples of 21rst pi.

Richards *et al.* [45] point out that several environmental aspects may be involved in the extrinsic incubation process of arboviruses, including temperature, in which, in environments with high temperatures, arboviruses tend to have a shorter extrinsic incubation period, that is, they have a faster viral dissemination. Lima-Camara [46] and Lopes, Nozawa and Linhares [1] also point out that tropical countries, such as Brazil, have extremely favorable environmental conditions for the occurrence of the transmission cycle of arboviruses, including WNV, since the climate reduces the incubation period of arboviruses, as well as reduces the time of larval development of various species, increasing the population density of mosquitoes favoring the transmission of arboviruses.

In our study, saliva samples from the 14th pi were not positive in any of the tests performed, being possible to identify and quantify the viral load only in the samples of 21rst pi, with titers ranging from $2x10^2$ to $4x10^2$ PFU/mL, values that are high when analyzing saliva in isolation, but when evaluating the saliva samples together with the head and body samples belonging to the same pi, a reduction in the viral load in the saliva is observed. These data are similar to those identified by Bakhshi *et al.* [41] in research with *Cx. pipiens*, where it was observed that despite the presence of WNV in saliva after 21 days after infection, the viral titer is lower than that observed in body and head samples belonging to the same group.

Conclusions

This study demonstrated that Culex quinquefasciatus presents itself as an important potential vector of WNV in Brazil, as it is a widespread species throughout the country and its reproductive cycle is adapted to urban environments.

Stands out that although arthropods have several anatomical barriers that act by hindering viral dissemination, it was found that the Brazilian strain of WNV was able to overcome these barriers by spreading to different regions of the mosquito's body, including saliva, by which can be transmitted. However, it is noteworthy that although viral dissemination occurs, the present study showed that the viral titers present in saliva were much lower than those found in the body and head regions of the mosquitoes evaluated at 21st pi, a fact that may indicate that the anatomical barriers despite of not being able to inhibit viral spread, they act by minimizing it.

Another aspect that should be considered in future studies of vectorial competence analysis is the action of the mosquito's innate immune system, an essential aspect to determine the vectorial competence of an arthropod in the natural environment, since innate immune response pathways, such as the RNAi and the JAK-STAT pathway, act to inhibit viral replication, thus reducing the spread and transmission of arboviruses.

Thus, it is noteworthy that the low number of samples in this study demonstrates the necessity for further research to analyze the vector competence of *Culex quinquefasciatus* belonging to the Amazon region for transmission the WNV Brazilian strain, using more sensitive analysis methods such as Molecular Biology, given that although the virus has been isolated from a sample from the southeast region of Brazil, several human cases have been reported in Piauí state (northeast region) showing the large circulation of the virus and the risk of new outbreaks of human and animal cases of West Nile Fever throughout the Brazilian territory.

Materials And Methods

Study Population

The colonies of *Cx. quinquefasciatus* (Cidade Nova-Ananindeua/Pará) were maintained in conditioned insectary, with temperature of 28ºC ± 1ºC, humidity of 80% ± 10% and light/dark cycles of 12/12 hours, according to Vogels *et al.* [19] protocol.

The egg rafts were created in basins containing 700 mL of distilled water and supplemented with crushed and sterilized fish food, with 12/12 light and dark photoperiod, average temperature of 28ºC ± 1ºC. As the pupae evolved, they were transferred to a transparent polypropylene container (Firstlab®) containing 50 mL of distilled

water, then they were placed in entomological cages with 30 cm^3 of dimensions. The winged forms were maintained with cotton wool soaked in 10% sugar solution *ad libitum*, according to the protocol established by SILVA *et al.* [47].

Viral Strain

The BEAN854747 strain of WNV was originally isolated from a sample of the Central Nervous System (CNS) of an adult male horse from the city of Pedra Grande, state of Espírito Santo, Brazil. The isolation of the strain was carried out by the Evandro Chagas Institute (IEC), Ananindeua/Pará. The virus was isolated using the Viral Isolation technique on $C_{6/36}$ cells and the Indirect Immunofluorescence technique, which demonstrated approximately 75% of positive cells for Flavivirus and WNV antibodies. Already in horse brain sample, the strain was identified by RT-qPCR [12].

Viral Stock Preparation

The viral stocks were prepared in Vero cells from African green monkey kidney (*Chlorocebus aethiops*) (ATCC® CCL-81 ™), in which 150µL of virus was inoculated in a polystyrene cell culture flask (Sarstedt®) with a capacity of 21 mL to 55 mL, then the bottle was incubated at a 37ºC in an incubator oven at 5% CO_2 for one hour for adsorption, shaking the flask every 15 minutes. After adsorption, 25mL of maintenance culture medium 199 (GIBCO) containing 2% fetal bovine serum, penicillin (100 UI/mL) and streptomycin (100 µg/mL) are added. Infected cells are maintained in an incubator oven and monitored daily with an inverted microscope (ZEISS) to assess the occurrence of cytopathic effects for a period of five to six days. After the identification of the cytopathic effects in 90% of the monolayer, the flask is shaken to release the monolayer from the wall, 10% of fetal bovine serum is added and 2mL of the culture supernatant was aliquoted in tubes for cryogenic freezing KMA (Mylabor®), keeping them in a freezer -70ºC until the moment of use [48,49].

The viral stock of WNV was titrated, obtaining $1.4x10^8$ PFU/mL of titer.

Mosquitoes Infection

After the emergence of adulthood, approximately 106 females with five to seven days of life were separated and deprived of sugar for 24 hours before feeding an infected blood meal. The oral infection was carried out with the assist of an artificial glass feeder, in which the blood supply area for mosquitoes is surrounded by a bovine liver peritoneum membrane acquired from a fridge. The artificial feeder was filled with 2 mL of defibrinated goose blood acquired from the IEC vivarium and 2 mL of the WNV stocks. The blood was constantly kept at 37ºC in a water bath (Digital Bath NI 1255-25L) (Fig. 3A). The females were exposed to the infected blood meal for 60 minutes. The mosquito control group was composed of uninfected females belonging to the same generation used in the infection, according to the protocols defined by Salazar *et al.* [50], Serrão [51], Goenaga *et al.* [52] and Vogels *et al.* [19].

After the period of exposure to the infectious blood meal, a total of 46 engorged females were obtained, which were separated and transferred to an entomological cage. Forty-eight hours after the infective blood feeding, a transparent polypropylene container (Firstlab®) containing 15 mL of distilled water and organic matter was deposited inside the cages for oviposition, remaining inside the cage for a period of up to seven days after infective feeding, as proposed by Pesko & Mores [34].

Mosquito processing

The segmentation of mosquitoes and the saliva extraction were performed after the 7th, 14th and 21st day of post-infection (pi) with legs and wings, body and saliva being separated individually. The segmentation of the body, legs and wings was performed according to the technique described by Consoli & Oliveira [53] in which the infected females were cooled to inactivity in an ice bath, then deposited from the belly upwards on a sterilized microscope slide (Kolplast ®) being removed first, with the aid of entomological forceps, the wings and legs. Then, the saliva extraction procedure was performed according to the adapted technique described by Castro *et al.* [54] and Nuñez *et al.* [55], in which the female's proboscis was inserted into a 10µL polypropylene micropipette tip (Axygen®) containing 5µL of fetal bovine serum (GIBCO) and waiting for 30 minutes for salivation (Fig. 3B). After, the medium containing the saliva was transferred to *epperdorf* tubes (Sarstedt®) containing 45µL of Leibowitz L-15 medium (GIBCO), then the saliva was immediately stored in a freezer at -70ºC.

After the saliva extraction, the body, legs and wings was transferred to *epperdorf* tubes (Sarstedt®) and 1000µL of Dulbecco's Phosphate-Buffered Saline (DPBS) (Life technologies, Carlsbad, CA, USA) containing 2% penicillin and streptomycin, 1% of fungizone and 5% of fetal bovine serum was added, as well as a 3mm stainless steel bead to perform the maceration procedure in TissueLyser II (Qiagen, Hilden, Germany), at a speed of 25 turns (Hz) for one minute, followed by the storage of the samples in a freezer -70ºC for a minimum period of 24 hours, according to Vazeille *et al.* [56] protocol.

Viral Isolation

The attempts of viral isolation in cell culture were carried out according to the Gubler *et al.* [57] protocol. After the process of maceration and incubation of the samples in a freezer -70ºC, the *eppendorfs* were centrifuged (Hettich® model Mikro 220R) at a speed of 10.000 rotations per minute (rpm) for 10 minutes at a temperature of 4ºC, then 100µL of the macerate supernatant was collected and inoculated into the monolayer of *Aedes albopictus* $C_{6/36}$ cell culture (ATCC: CRL-1660) grown in tubes 16 x 125mm for cell culture (TPP®), after inoculation the tubes were maintained in an incubator at a 28◦C for one hour for adsorption and homogenized every 15 minutes. Then, based on Beaty *et al.* [49] protocol, 1.5 mL of Leibowitz L-15 maintenance medium (GIBCO) prepared with 2.95% tryptose phosphate, non-essential amino acids, penicillin, streptomycin and 2% fetal bovine serum was added to monolayer.

The inoculated $C_{6/36}$ cells were stored in an oven (NAPCO) with 5% CO_2 at 28ºC (± 2ºC) and evaluated daily under an inverted optical microscope (OLYMPUS model CK-2) for seven days to check the occurrence of a cytopathic effect. Positive and negative controls were also used to provide greater reliability to the test.

Indirect Immunofluorescence Test (IFI)

The Indirect Immunofluorescence Test (IF) was performed according to the Gubler *et al.* [57] protocol. 25µL of the inoculated sample was added in $C_{6/36}$ cell culture in each hole of the immunofluorescence assay slide, waiting 10 minutes, then, remove the sample excess from the slide and fixed with acetone for 10 minutes at -20ºC, followed by acetone (propanone) PA for 10 minutes at -20ºC. After that, the slides were immersed in bovine albumin solution (3% and 1% concentrations) and in PBS (pH 7.4) for 10 minutes at 25ºC and 25µL of the polyclonal antibody, with West Nile hyperimmune ascitic fluid (*in house*), was added and the slides were packaged in a humid chamber in an oven (NAPCO) for 30 minutes at 37ºC. Subsequently, the sample was washed in phosphate-buffered saline solution (PBS) pH 7.4 for 10 minutes, then the slides were briefly washed with distilled water and 50 µL of the conjugated anti-mouse antibody (Cappel) diluted was added at 1:900 to each slide orifice, the conjugated anti-mouse was marked with fluorochrome allowing the visualization of positive samples and Evans Blues (0.5%) was used as a dye. Then, the slides were conditioned again in a humid chamber in the oven for 30 minutes at 37ºC and 5% CO_2, repeating the washing in PBS for 10 minutes, finishing the preparation of the samples with 50µL of buffered glycerin (pH 8.2) in each slide orifice and fixed the coverslip for observation under a fluorescence microscope (Olympus BX51, UPlanFL N 20X / 0.5 lens and WB and U-25nd filters).

Cells inoculated with samples of head, body and saliva from uninfected mosquitoes were used as negative controls.

The images of the samples were acquired in 200X magnification in the fluorescence microscope with Canon PowerShot G6 camera (Canon, Tokyo, Japan).

Viral Titration

Viral titration was performed on the saliva, body, legs and wings samples, as well as the viral stocks used in the infection process.

Viral titration was carried out according to Dulbecco & Vogt [58] protocol, in which a 96-well cell culture plate (Kasvi®) divided in half to allow a 10-fold serial dilution of the samples (10^{-1} to 10^{-6}) in PBS diluent with 0.75% bovine, penicillin (100 IU/mL), streptomycin (100 µg/mL) and 2 % fetal bovine serum. 180µL of maintenance culture medium 199 (GIBCO) was added to the plate, then 20µL of the “-1” dilution was added to the orifice identified as “-1” and the mixture was homogenized and 20µL was aspirated from "-1" and transfers to the next hole identified by "-2", repeating the procedure until the last dilution of "-6".

After the sample dilution process, in a six-well cell culture plate (Kasvi®) containing monolayer of cultured Vero cells (*Cercopithecus aethiops*) (ATCC® CCL-81 ™), each hole was identified from “-1” to “- 6” according to the dilution performed. Then, the culture medium 199 (GIBCO) used to grow VERO cells was replaced and 100µL of the diluted viral sample was added to each well according to the identification. Then, the plate was incubated in an oven (NAPCO) at 37ºC and 5% CO_2 for one hour, in which, every 15 minutes the plate was homogenized to increase viral adsorption.

After viral adsorption 3 mL of carboxymethylcellulose (CMC, 3% in 199 medium) supplemented with 5% fetal bovine serum, penicillin (100UI/mL) and streptomycin (100µg/mL) were added to each orifice, followed by incubation of the plate in an oven at 37ºC for five days. After that period, the cells were fixed with 3 mL of 10% formaldehyde in water and the plate was incubated in the oven for four to seven hours or overnight. Subsequently, the plate was washed until the product is completely removed and 3 mL of 0.1% Violet Crystal dye were added, waiting at room temperature for a period of 24 hours.

Viral titration was determined by multiplying the number of plaques obtained from a given dilution, by the dilution factor (Eq. 1), with the result being expressed in Plaque Forming Units per Milliliter (PFU/mL), according to the mathematical calculation established by Guerra [59].

$$\text{Viral Titer} = \text{No. of PFU x dilution factor x dilution} \qquad (1)$$

Calculation of Infection, Dissemination and Transmission Rates

Viral infection in mosquitoes starts from the mesentery, spreading through the hemolymph to other regions of the animal's body and ends with its multiplication in the salivary glands and ovaries [60]. Thus, the studies of vector competence use the calculations of infection, dissemination and transmission rates as parameters of the different phases of the viral multiplication in the mosquito's organism [19].

Thereby, the infection rate (Eq. 2) is calculated from the number of mosquitoes with the infected body among the engorged females, whereas the dissemination rate (Eq. 3) is calculated based on the number of mosquitoes with the infected head, wings and legs among mosquitoes previously identified with infected body and the transmission rate (Eq. 4) is calculated according to the number of mosquitoes with the infected saliva among those positive for infection and dissemination [61].

$$\text{Infection Rate: } \frac{\text{No. of females with infected body}}{\text{Total engorged females}} \qquad (2)$$

$$\text{Dissemination Rate: } \frac{\text{No. of females with infected head, wings and legs}}{\text{Total females with infected body}} \qquad (3)$$

$$\text{Transmission Rate: } \frac{\text{No. of females with infected saliva}}{\text{Total females with infected body, head, wings and legs}} \qquad (4)$$

Statistical Analysis

The analysis of the infection, dissemination and transmission rates were expressed in percentages and analyzed by the Chi-square test of Trend (α = 0.05) aiming to evaluate the growth tendency or decrease of the rates. The viral titers of bodies, heads and saliva were analyzed using the Kruskal-Wallis Test (KW) and Dunn's Test for multiple comparisons (α=0.05), aiming to determine the differences in the titers according to the day of post infection. Statistical tests were applied using the BioEstat 5.3 program (Instituto Mamirauá / Pará).

Declarations

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Author contributions:

L.A.M.R. wrote the main manuscript text, conducted the analyses. L.A.M.R., R.D.C., P.A.S.A., F.S.S., J.W.R.J., R.C.F.B., D.D.D. conducted the experiment(s). E.V.P.S., M.N.O.F. processed the experimental data. D.B.A.M., L.C.M., E.V.P.S. analyzed the results. J.P.N.N. conducted the analyses, analyzed the results. J.P.N.N. conceived the experiment(s). All authors reviewed the manuscript.

Competing interests:

The authors declare no competing interests.

Data Availability:

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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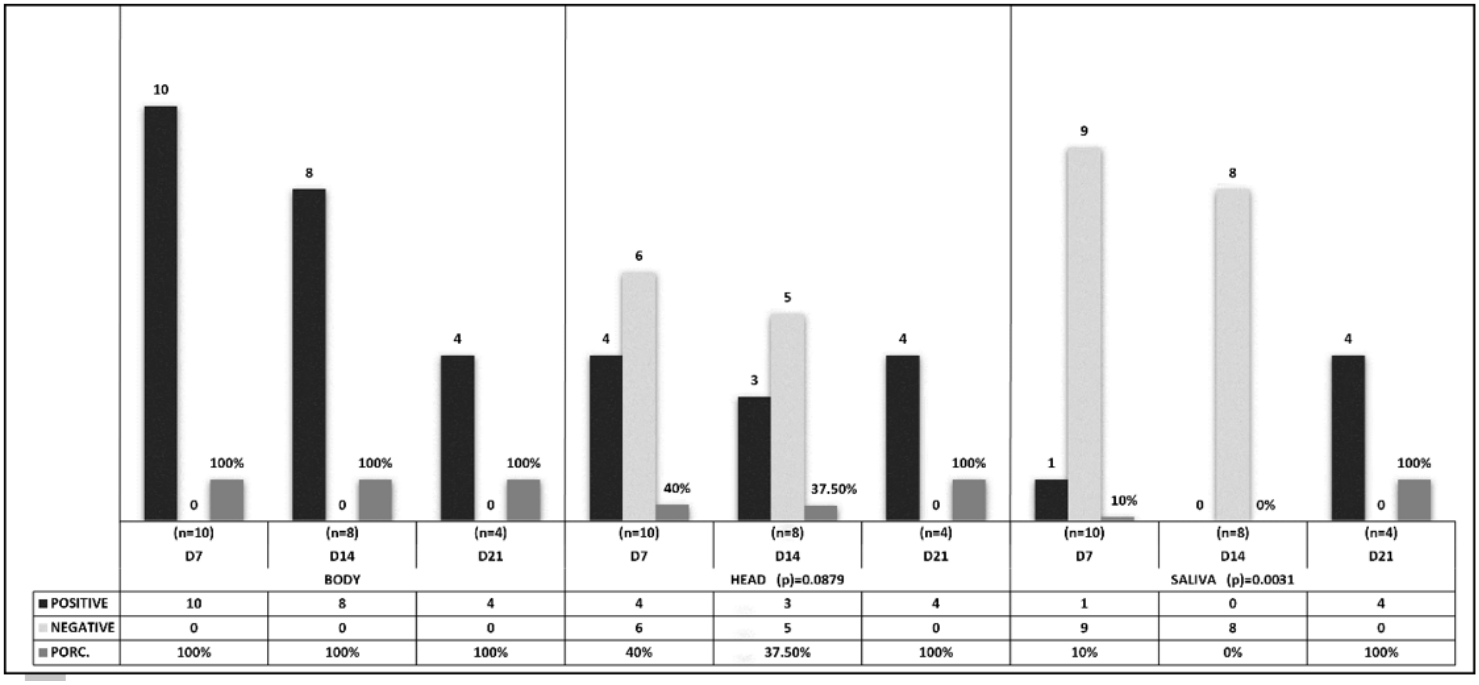
Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures



	BODY			HEAD (p)=0.0879			SALIVA (p)=0.0031		
	(n=10) D7	(n=8) D14	(n=4) D21	(n=10) D7	(n=8) D14	(n=4) D21	(n=10) D7	(n=8) D14	(n=4) D21
POSITIVE	10	8	4	4	3	4	1	0	4
NEGATIVE	0	0	0	6	5	0	9	8	0
PORC.	100%	100%	100%	40%	37.50%	100%	10%	0%	100%

Figure 1

Infection, dissemination and transmission rates according to the post-infection day of Culex quinquefasciatus mosquitoes orally exposed from the WNV.

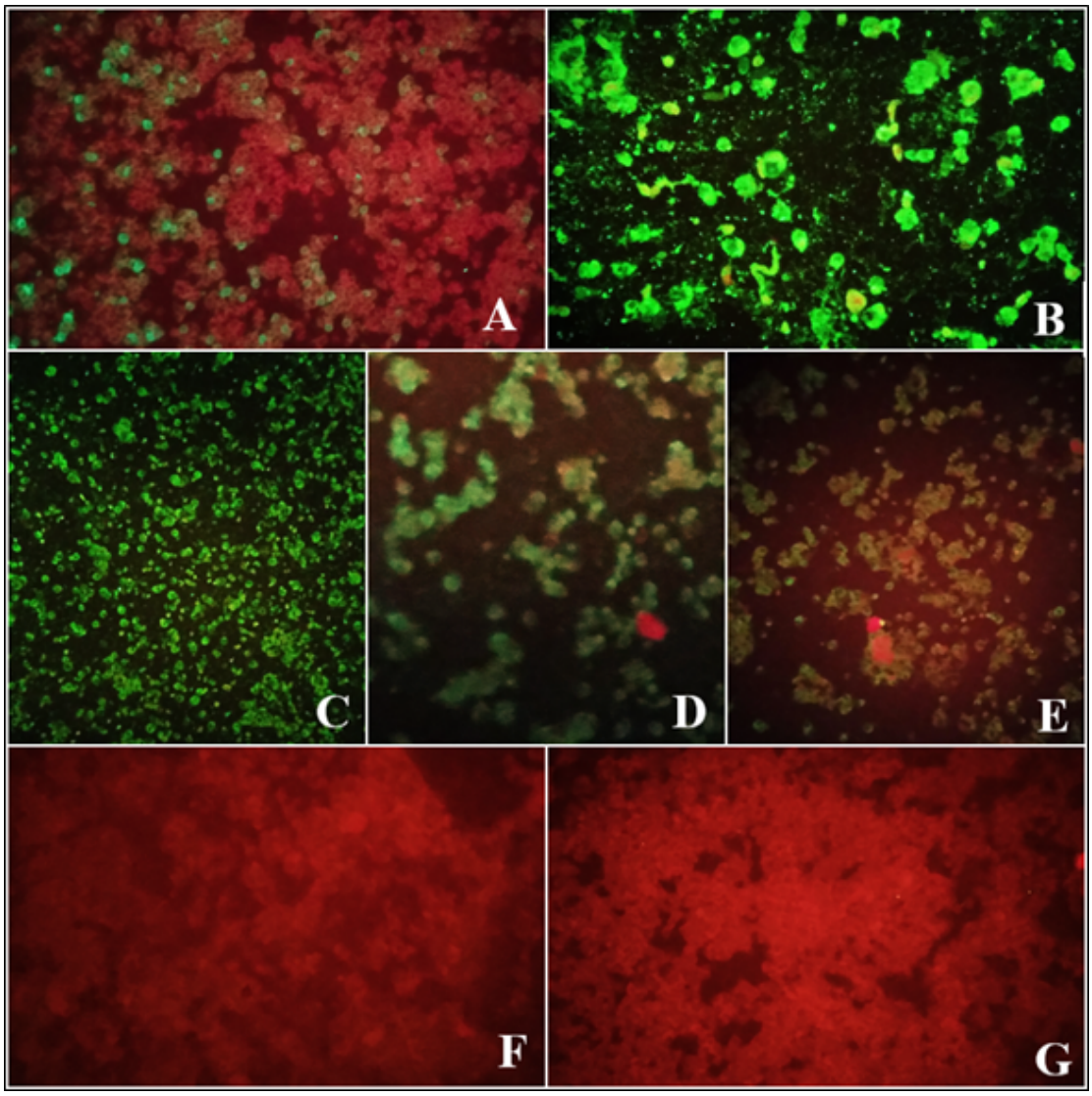


Figure 2

Indirect Immunofluorescence Test using West Nile virus polyclonal antibodies (in house) to identify WNV in C6/36 cells inoculated with body, head and saliva samples. Evans Blue was used for IFI staining. a. positive saliva sample of the 7th pi. b. positive head sample from 7th pi. c. positive body sample of the 7th pi. d. positive sample of saliva from the 21st pi. e. positive head sample of the 21st pi. f. negative saliva control. g. negative control of C6/36 cells. Images at 200x magnification.

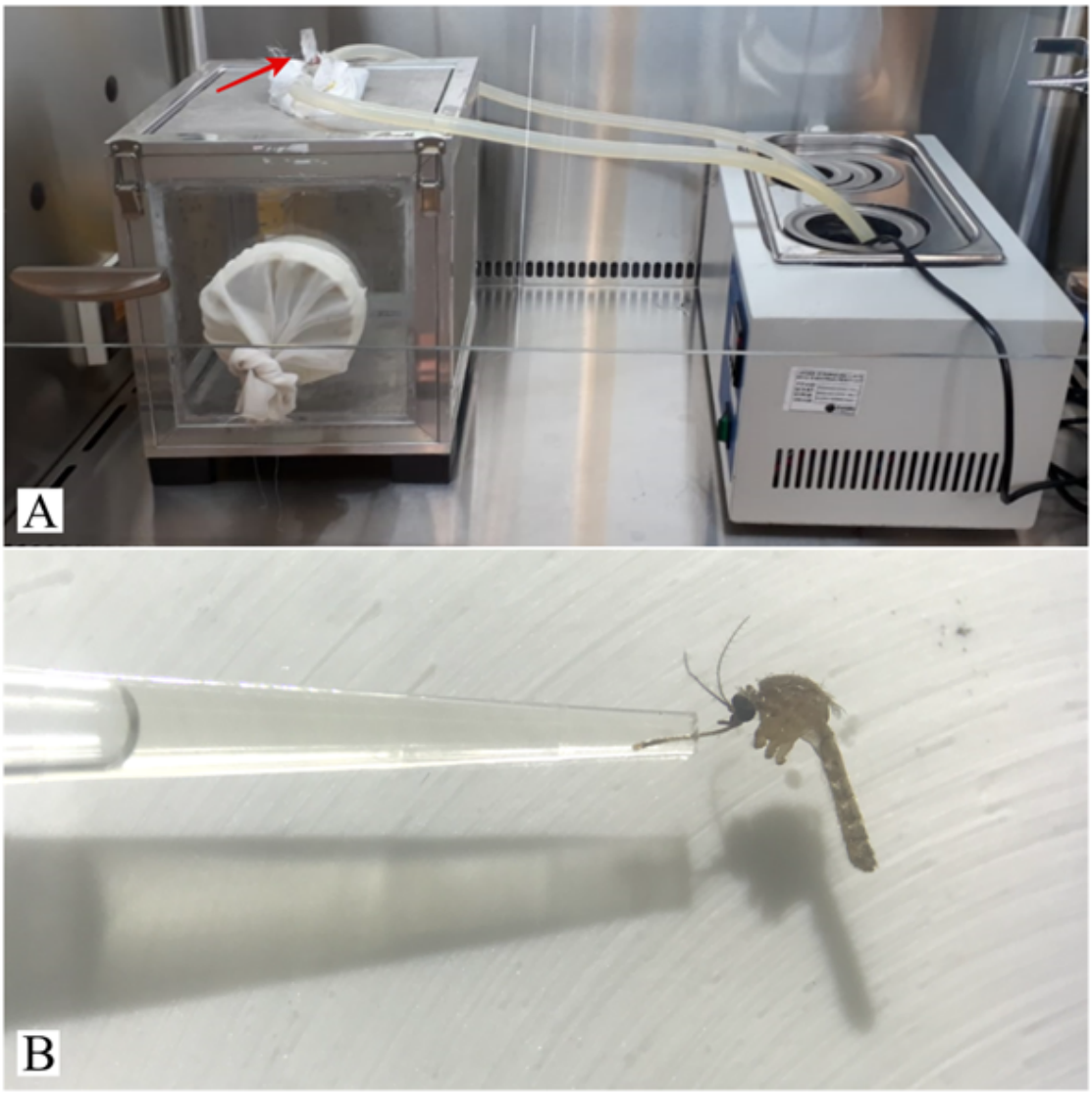


Figure 3

Representation of the oral infection process and saliva extraction. a. Biological safety cabinet containing entomological cage (30cm2) with Cx. Quinquefasciatus females, artificial glass feeder (red arrow) and a water bath used to heat the infected blood meal at 37 °C. b. Representation of the saliva extraction protocol, with the female proboscis introduced in a 10µL polypropylene micropipette tip (Axygen®), containing 5µL of fetal bovine serum.

Supplementary Files

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- Table1.docx