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Dengue virus in *Aedes aegypti* larvae and infestation dynamics in Roraima, Brazil

ABSTRACT

OBJECTIVE: To detect the presence of dengue virus in larval forms of *Aedes aegypti* and to associate vector presence with rainfall and incidence of disease.

METHODS: Eighteen households were randomly selected for egg collection in a neighborhood of the city of Boa Vista, Roraima, in Northern Brazil. Two oviposition traps were installed per home, and removed after one week. This was repeated on a monthly basis between November 2006 and May 2007. Trap positivity rate and egg density were calculated. Following the eclosion of 1,422 eggs, 44 pools of at least 30 larvae each were formed, which were evaluated for presence of dengue virus using RT-PCR and hemi-nested PCR. Dengue incidence rates in the period were correlated with rainfall rates. The association between these two variables and the number of eggs collected was determined using Pearson correlation.

RESULTS: None of the pools tested positive for presence of dengue virus, despite the high incidence of dengue in the neighborhood during the studied period. The density of *Ae. aegypti* increased with rainfall, but was not correlated with incidence of dengue.

CONCLUSIONS: The results suggest that transovarial transmission of dengue virus in mosquitoes occurs at a very low frequency, and therefore virus persistence in urban settings may not depend on such transmission. The mosquito population increased during the rainy season due to increased formation of breeding sites; the lack of correlation with incidence of dengue may be due to underestimation of incidence data during epidemics.

DESCRIPTORS: Dengue, transmission. *Aedes*. Insect Vectors. Entomology. Epidemiologic Surveillance.

INTRODUCTION

The first dengue epidemic in Brazil to be confirmed by laboratory diagnosis took place in Boa Vista, Northern Brazil, in 1981-82. During this epidemic, dengue virus serotypes 1 and 4 were isolated. Beginning in 1982, an intensive vector control campaign took place which eradicated the vector from the region, and no epidemics were reported in Boa Vista until 1999. From then onwards, incidence rates have been among the highest in the country, and have included serotypes 2, 1, and 3, which characterizes the state of Roraima as a hyperendemic area, a condition which is critical for the occurrence of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS).⁴ Roraima borders Guyana to the northeast and Venezuela to the northwest, and is therefore considered to be a potential route for entry of dengue in Brazil. According to data from

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the Pan-American Health Association, the four dengue serotypes are currently in circulation in Venezuela, and there is a possibility of reintroduction of serotype 4 through this border.

In the absence of animal reservoirs, an issue that remains unclear is how the dengue virus is able to persist in nature between epidemics and when vector density is low. Transovarial transmission of virus in mosquitoes is believed to contribute to viral persistence,¹⁶ since mosquito eggs are capable of survive in the environment under adverse conditions for over one year. Although transovarial transmission has been found to occur both in the laboratory and in the field, its role in viral persistence in nature has not been completely established.¹⁸

Detection and serotyping of dengue virus by reverse transcription followed by polymerase chain reaction (RT-PCR) using material from clinical or vector samples is a powerful tool for epidemiological surveillance.^{1,10,17} Presence of virus in mosquitoes collected in the field allows for the detection of epidemics from six to eight weeks in advance of their onset.^{1,17}

The aim of the present study was to identify the presence of dengue virus in *Aedes aegypti* larvae and to associate presence of vector with rainfall and number of cases of dengue in the period.

METHODS

Boa Vista, capital of the state of Roraima, is located in the Northern Hemisphere (02°49'11"N, 60°40'24"W). The Mecejana neighborhood was chosen because of its high incidence of dengue and high building infestation rate (percentage of buildings positive for *Ae. aegypti*) in the first semester of 2006, according to data from the Secretariats of Health of the state of Roraima and of the city of Boa Vista.^a

Ovitrap (oviposition traps) were installed on a monthly basis between November 2006 and May 2007, so as to span both the dry (December 2006 to March 2007) and rainy (November 2006 and April-May 2007) seasons. The flight range of *Ae. aegypti* in urban settings is of approximately 800 m around the breeding site where the mosquito was born.¹² Eighteen houses distributed throughout the neighborhood were randomly selected. Selected houses were located on average 360 m from each other, and no neighboring houses were located more than 600 m apart, thus covering the entire neighborhood.

In order to make inferences regarding vector infestation, we calculated two indicators of presence of eggs: the ovitrap positivity rate (OPR), defined as the percentage of positive ovitraps; and egg density index (EDI), de-

defined as the mean number of eggs per positive trap. OPR is a measure of the spatial distribution of infestation within a location, whereas EDI indicates the periods of greater or lesser reproduction for mosquito females.

Minimal infection rate (MIR) was defined as the presence of virus in larvae pools, calculated as the number of positive tests divided by the total number of larvae and multiplied by 1,000.

Mosquito eggs were counted on the trap paddle, and then prepared for eclosion. Larvae were raised until the fourth stage and identified according to the dichotomous key of Consoli & Lourenço-de-Oliveira.² Larvae were then washed twice in deionized water, separated according to month and place of collection into pools of up to 30 individual larvae, and then frozen while still alive at -20°C. Of the 1,422 eggs collected, 767 (60.7%) reached the third and fourth larval stages, and were separated into 44 pools (1,172 larvae).

Pools were macerated using a micropestle and autoclaved sand in 1 ml L-15 media in a 1.5 ml microfuge tube. Macerated larvae were then centrifuged at 8,000 g for 10 min. Supernatants were collected and RNA was extracted for determining the presence of dengue virus by RT-PCR and hemi-nested RT-PCR. Using the same methods, we also extracted RNA from a positive control (the supernatant from a culture of dengue-infected cells). In order to ensure the absence of inhibition of molecular reactions by organic compounds present in larvae, ten stage-four *Ae. aegypti* larvae were macerated using a micropestle and autoclaved sand with the addition of 330 µl of supernatant from dengue-infected cells. RNA was extracted from this preparation using the same procedure as used for the experimental group. A 511 bp band was visible after PCR, and the 119 bp band characteristic of dengue 2 was visible following hemi-nested PCR in this sample. In addition, for each sample group, we also used a standard amplicon control from VDEN-2 diluted 1:100 in the PCR and hemi-nested PCR reactions. RNA extraction was performed using TRIzol® reagent according to the instructions provided by the manufacturer.

RT-PCR and hemi-nested PCR were performed using the primers described by Lanciotti et al.⁹ For reverse transcription we used primer D2, and for PCR, primers D1 and D2, which amplify a 511 bp fragment located in the confluence between gene C and prM. The hemi-nested PCR was carried out using primer D1 together with primers TS1, TS2, TS3, and D4, which amplify fragments of 482 bp, 119 bp, 290 bp, and 392 bp, characteristic of dengue virus serotypes 1, 2, 3, and 4, respectively.

Incidence of notified and of serologically confirmed cases of dengue (number of cases per 100,000 population) in the city were calculated according to data

^a Coordenação de Entomologia e Setor de Epidemiologia da Secretaria de Saúde do Estado de Roraima. Unpublished data.

provided by the State Secretariat of Health and the Brazilian Institute for Geography and Statistics (*Instituto Brasileiro de Geografia e Estatística* – IBGE). Monthly rainfall levels in the city were provided by the National Institute of Meteorology (*Instituto Nacional de Meteorologia* – INMET). The association between these variables and number of eggs collected was analyzed by calculating the Pearson coefficient (ρ), using this function in an electronic spreadsheet.

RESULTS

The 1,422 eggs collected were distributed unevenly across the study period. Table 1 presents the monthly values for OPR and EDI.

Correlation coefficients between tested variables are presented in Table 2. The strongest correlation obtained was between incidence of notified and of confirmed cases (+0.98). This was expected, given that these variables should show similar behavior because both deal with the same type of data. Number of eggs was positively correlated (+0.91) with rainfall, suggesting that increased rainfall contributes to the increase in *Ae. aegypti* population. Both number of eggs and rainfall showed a negative, albeit weak, correlation (around -0.57) with dengue incidence rates.

Figure 1 shows a scatter plot for number of eggs and rainfall, showing the positive correlation between these

Table 1. Ovitrap positivity rate and egg density index for *Ae. aegypti*. Boa Vista, Northern Brazil, November 2006 to May 2007.

Month	Nº of traps: positive/installed	OPR	Nº of eggs	EDI
Nov	14/18	77.78	276	19.7
Dec	3/18	16.67	56	18.7
Jan	0/18	0.00	0	0.0
Feb	3/18	16.67	57	19.0
Mar	4/18	22.22	283	70.8
Apr	5/18	27.78	189	37.8
May	10/18	55.56	561	56.1

OPR = ovitrap positivity index
EDI = egg density index

Table 2. Correlation coefficients between rainfall, number of *Ae. aegypti* eggs, and cases of dengue. Boa Vista, Northern Brazil, November 2006 to May 2007.

Variable	No. of eggs	IRN	IRC
Rainfall (mm)	+0.91	-0.55	-0.58
Number of eggs	-	-0.57	-0.57
IIN	-	-	+0.98

IIN= incidence rate for notified cases of dengue
IRC= incidence rate for confirmed cases of dengue

variables. Graphs in Figures 2 and 3 illustrate the correlation between number of eggs, rainfall, and dengue incidence by month.

None of the larva pools tested was positive for dengue virus. Therefore, we were unable to detect transovarial transmission of *Ae. aegypti*.

DISCUSSION

Transovarial transmission of dengue virus in mosquitoes has been reported both in the laboratory and in nature. Rosen et al¹⁵ found a higher rate of vertical transmission in *Ae. albopictus* when compared to *Ae. aegypti*, and were able to detect transmission of serotype 1 by the latter specie. In other works, transovarial transmission of serotypes 2, 3, and 4 was also detected in *Ae. aegypti*.⁵⁻⁷ According to Joshi et al,⁶ transovarial transmission allows dengue virus to persist in successive generations of mosquitoes, at rates of 5% to 26% in the lab, although rates in nature are likely not to exceed 20%.

In the present study, in spite of the choice of a neighborhood with high incidence of dengue and high infestation rates for *Ae. aegypti*, we were unable to obtain positive results for dengue virus in any of the pools of larvae tested. These results are in agreement with those of other field studies, such as the one carried out by Chow et al,¹ which failed to detect dengue virus in 53 pools of larvae (of 1 to 10 individuals each) collected during household visits. Pinheiro et al,¹¹ in a study carried out in the city of Manaus, failed to detect dengue virus in 1,142 larvae collected during household visits, although these authors obtained an MIR of 16 % among adult mosquitoes. Fouque et al³ found an MIR of 0.36% (1 infected mosquito in 2,755 tested) in mosquitoes raised from eggs collected in from ovitraps; and Khin & Than⁷ found an MIR of 0.48% (1:2,067) in larvae collected in the field. Kow et al⁸ found a rate of 0.133% when isolating dengue virus from adult *Ae. aegypti*

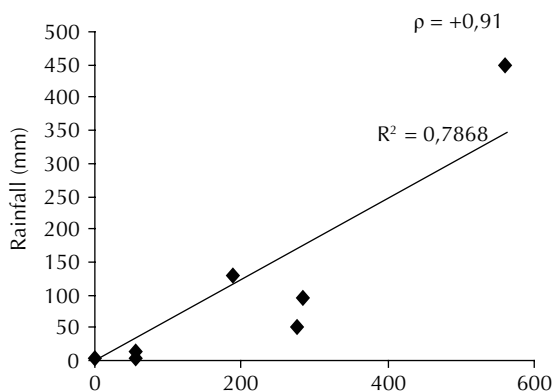


Figure 1. Positive correlation (ρ) between number of *Ae. aegypti* eggs and rainfall. Boa Vista, Northern Brazil, November 2006 to May 2007.

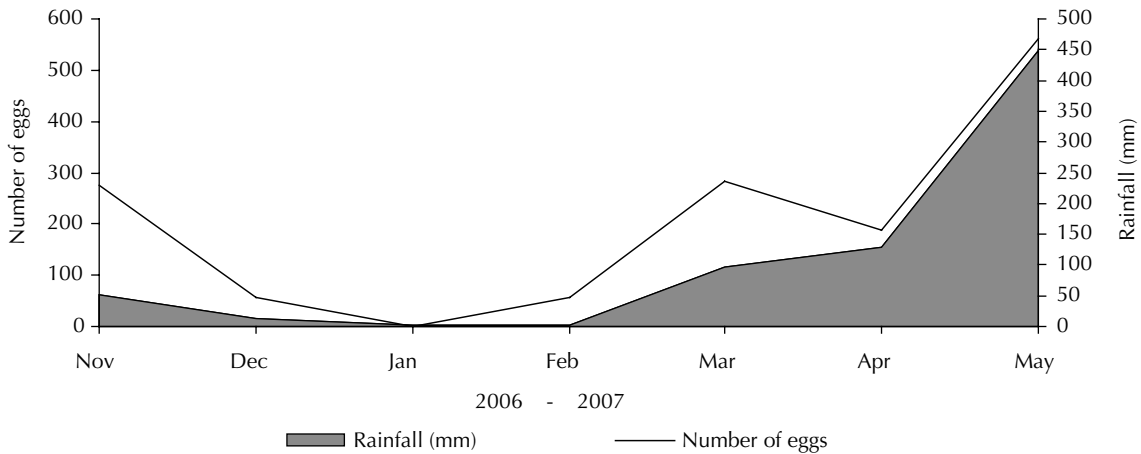


Figure 2. Number of *Ae. aegypti* eggs and rainfall. Boa Vista, Northern Brazil, November 2006 to May 2007.

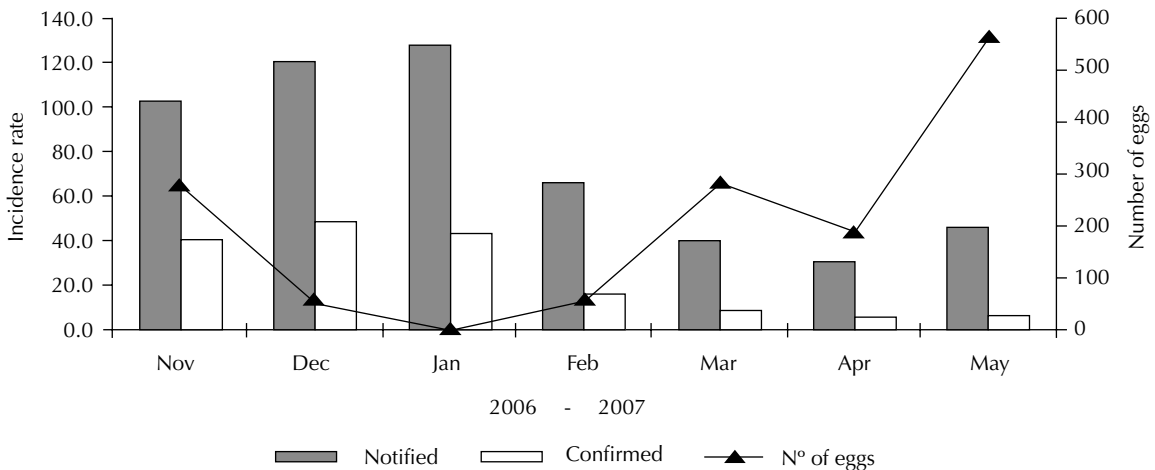


Figure 3. Number of *Ae. aegypti* eggs and incidence of notified and confirmed cases of dengue. Boa Vista, Northern Brazil, November 2006 to May 2007.

males collected in the field. These results suggest that transovarial transmission of dengue virus in *Ae. aegypti* seems to occur at a very low frequency in nature, and its role in viral persistence in urban settings may not be very extensive. Other sources of reintroduction of the virus, such as inter-state and international flow of people, are likely to contribute more to its persistence in this environment.

In contrast, studies using adult females collected in the field have obtained high MIRs, such as the 8.52% detected by Lourenço-de-Oliveira et al,¹⁰ the 16% detected by Urdaneta et al,¹⁷ who collected adults from the homes of persons infected with dengue and neighboring households, and the 57.6 % detected by Chow et al.¹ Therefore, screening for presence of virus in *Ae. aegypti* larvae is not the most adequate method for predicting epidemics, given its low rate of infection when compared to the screening of adult females. This can be related to the fact that females, who require blood

meals, are much more likely to become infected with dengue virus, positivity therefore not being limited to transovarial transmission in this population.

Egg collection in ovitraps is a practical method for monitoring vector population, showing high positivity rates even in areas of low population density.¹³ Transovarial transmission of dengue virus in *Ae. aegypti* was not detected in the Mecejana, neighborhood, even though this is a neighborhood with high incidence of dengue and vector infestation, suggesting that such transmission must occur at a very low rate. A greater number of eggs must be collected in order for the minimum infection rate to be calculated. Moreover, for this same reason, screening for presence of virus in larvae is not as efficient a surveillance tool as the screening of adults, which is proven to be efficient in predicting epidemics. However, use of ovitraps was confirmed as an efficient tool for monitoring vector population.

Rosa-Freitas et al.¹⁴ in a study of the temporal and spatial distribution of dengue notifications in Boa Vista between 1999 and 2001 failed to detect a correlation between number of notified cases and meteorological variables, which showed different distribution patterns in each year. Despite what occurs in the rest of the country, where the majority of cases occur during the rainy season, in Boa Vista, incidence peaks can occur both in the rainy and in the dry seasons. The results of the present study confirm this contradictory behavior, showing a negative correlation between rainfall and dengue incidence. It is possible that correlation may be due to inadequate case notification during epidemics. We suggest that further studies be based on cases of dengue sampled from health care facilities rather than on government notification data.

In conclusion, the *Ae. aegypti* population increased in the rainy season, probably due to increased accumulation of water in natural and/or artificial reservoirs, leading to a greater number of breeding sites for egg eclosion. We did not detect a correlation between vector

population and dengue incidence, a finding previously reported in earlier studies carried out in Boa Vista. This may be the result of reduced notification of dengue cases by health care professionals during epidemics. Transovarial transmission of virus in mosquitoes occurred at a very low frequency, and is therefore unlikely to be a decisive factor in the persistence of dengue in the urban environment, given that presence of the infected host in our settings occurs at a much greater frequency.

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