Natural vertical transmission by *Stegomyia albopicta* as dengue vector in Brazil

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Abstract

The mosquito *Stegomyia albopicta* is among the most important arbovirus vectors in the world, particularly for *Dengue viruses*. Their natural history suggests that biologically these viruses are highly adapted to their mosquito hosts and they were most likely mosquito viruses prior to becoming adapted to lower primates and humans. As well as being maintained by transmission among susceptible humans, *Dengue viruses* may also be maintained by vertical transmission in mosquitoes during inter-epidemic periods. The larvae and mosquitoes of *Stegomyia albopicta* were used to identify the vertical transmission of the dengue virus in nature and to confirm the vectorial capacity concerning the *Dengue virus* type 2 infection. The minimum infection rate concerning *S. albopicta* infection with the *Dengue virus* was 1:36.45. In Brazil this was the first time that high minimum infection rates of vertical transmission of *S. albopicta* were detected in this species.

Keywords: Dengue virus, vertical transmission, *Stegomyia albopicta*, vectorial capacity.

Transmissão vertical natural de *Stegomyia albopicta* como vetor de dengue no Brasil

Resumo

O mosquito *Stegomyia albopicta* está dentre os mais importantes vetores de arbovírus do mundo, particularmente para o *Dengue virus*. A história natural sugere que biologicamente esses vírus são altamente adaptados aos seus mosquitos vetores e foram, provavelmente, os vírus que infectavam mosquitos antes de se tornarem adaptados a primatas não humanos e humanos. Além de serem mantidos entre os homens susceptíveis, os *Dengue viruses* podem também ser mantidos pela transmissão vertical em mosquitos durante os períodos interepidêmicos. As larvas e mosquitos da espécie *Stegomyia albopicta* foram utilizados para a identificação da transmissão vertical do vírus dengue na natureza e para confirmar a capacidade vetorial em relação à infecção pelo DENV-2 infection. A taxa mínima de infecção em relação à infecção do *S. albopicta* com o *Dengue virus* foi de 1:36,45. No Brasil, esta é a primeira vez que altas taxas de infecção mínima da transmissão vertical de *S. albopicta* foram detectadas nessa espécie.

Palavras-chave: Dengue vírus, transmissão vertical, *Stegomyia albopicta*, capacidade vetorial.

1. Introduction

The mosquito *Stegomyia albopicta* (Reinert and Harbach, 2005) is among the most important arbovirus vectors in the world, particularly for *Dengue viruses* (DENV) (Gratz, 2004). DENV are members of the genus *Flavivirus*. They are plus-sense ssRNA viruses that cause dengue in humans. Their natural history suggests that biologically these viruses are highly adapted to their mosquito hosts and they were most likely mosquito viruses prior to becoming adapted to lower primates and humans (Fontenille and Toto, 2001). *Dengue viruses* are comprised of four closely related but antigenically distinct serotypes, designated DENV-1, DENV-2, DENV-3 and DENV-4 (Joshi, 2002). The spectrum of illness ranges from unapparent, mild disease to a severe and occasionally fatal hemorrhagic clinical picture (Johnson et al., 2002). DENV are transmitted to humans by *Stegomyia* mosquitoes and the two most frequent vector species are the peri-domestic and competitive *S. aegypti* and *S. albopicta* (Guzman and Kouri, 2002).

The microhabitats of *S. albopicta* larvae are mainly tree holes and a wide variety of containers including natural and artificial containers, although this vector prefers common forested habitats in suburban and rural areas. The eggs can survive desiccation for several months. The adult biology of *S. albopictus* is similar to that of the urban population of *S. aegypti*, a dengue and yellow fever vector (Gratz, 2004). *S. albopicta* was recorded in North America as early as 1972. Its presence was re-

ported in Brazil in 1986, in the states of Espírito Santo (ES), Minas Gerais (MG), Rio de Janeiro (RJ) and São Paulo (SP) (Hawley et al., 1987). Recently *S. albopicta* has been found in 14 Brazilian states (Forattini, 1986).

S. albopicta shows aggressive anthropophilic behavior and a great adaptability to different habitats (Gomes et al., 1999; Miller and Ballinger, 1988). In addition to being maintained by transmission among susceptible humans, DENV may also be maintained by transovarial transmission in mosquitoes during interepidemic periods (Rodhain, 1995). These species have the possibility of naturally transmitting the serotypes 2 and 3 of DENV vertically (Hull et al., 1984). Serotype 1 of DENV was isolated from naturally infected larvae of *S. albopicta* in Campos Altos, Minas Gerais (Serufo et al., 1993). This mechanism could explain the survival of DENV during dry seasons. During the outbreak of 2000, a successful attempt was made to detect DENV infection in *Stegomyia aegypti* larvae collected from nature in Pompeu city, Minas Gerais state, Brazil. In Brazil, this was the first time that vertical transmission of DENV has been shown to occur in nature, using molecular detection to confirm this mechanism (Ibanez-Bernal et al., 1997). *S. albopicta* has been identified as a dengue vector in Asia, Japan, Indonesia, the Seychelles, Thailand, Malaysia (Lourenço-de-Oliveira et al., 2003).

In this study we could detect vertical transmission of DENV-2 in *S. albopicta* larvae and identify the importance of *S. albopicta* as a dengue vector in Brazil detecting the virus in mosquitoes.

2. Material and methods

2.1. Larval and mosquitoes collections

78 oviposition traps were placed weekly near the house of patients suspected or confirmed to be infected with *Dengue virus* in the Pampulha region of Belo Horizonte, Minas Gerais state in May, 2003. The oviposition trap consisted of a black plastic recipient filled with water and containing a carton paddle. Paddles were collected, and *Stegomyia* eggs were hatched in the insectary. Fourth stage *Stegomyia* larvae were identified up to the species level based on the morphology of scales at the base of the siphon and thorn in the thorax (Consoli and Oliveira, 1994). Pools of 50 larvae were stored in vials at –80 °C until required.

20 ovitraps were placed in the Pampulha region in the same position where DENV infections were detected in larvae in this study. Paddles were collected, and *Stegomyia* eggs were hatched in the insectary. Fourth stage *Stegomyia* larvae were identified as described above. The field-collected *S. albopicta* larvae were reared to adults in double cages as a biosafe measure. Emerged adults were fed on 10% sugar solution, and 5 days after emergence, the adults were identified to species for confirmation, separated into females and males and frozen at –80 °C.

2.2. RNA extraction

From the larvae collected, pools with approximately 50 larvae were ground using sterile sand. The supernatant of the ground larval pool was used to extract RNA. From larvae reared to adults, 18 mosquitoes were used to extract viral RNA. The head of each mosquito was separated from the thorax-abdomen using a sharp razor. The head was ground using sterile sand and the supernatant was used to extract RNA. RNAs were extracted by a modified silica method (Boom et al., 1990). Briefly, the supernatant was treated with a lysis buffer containing guanidine isothiocyanate, Tris 0.1 M, EDTA 0.2 M, Triton X-100 mixed with sterilized silica. After centrifugation the sample was washed with a washing buffer containing guanidine isothiocyanate and Tris 0.1 M, followed by several washes with 70% ethanol and acetone. The material was resuspended in TE (TRIS 10 mM, EDTA 1 mM, pH 8.0) treated with RNAsin and maintained at –80 °C until required.

2.3. cDNA synthesis

The cDNA synthesis was conducted in a 20 µL reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each of the four dNTPs, 50 pmol of the anti-sense primer, 250 ng of viral RNA in 5 µL volume and 200 U of reverse transcriptase enzyme (SUPERSCRIPT II RT, Invitrogen) at 42 °C for 50 minutes.

2.4. PCR assay

- PCR Control – Each reaction set was checked for contamination using negative control (all reagents included except template DNA). In addition to this negative control, for reactions using pools of larvae RT-PCR of a negative pool of larvae was included, and for reactions using isolated material, RT-PCR of cells not infected was included, and for reactions using mosquito heads, mosquitoes from a breeding colony DENV free was used. Each reaction set included a DENV-2 sample previously sequenced as a positive control either from isolated material or from larvae.
- Primers – One pair of primers was used in a first PCR assay followed by a semi-nested PCR assay using serotype specific primers according to Lanciotti et al. 1992.
- PCR assay – The PCR using 5 µL of the cDNA was conducted in reaction of 50 µL containing 10 mM Tris-HCl (pH 8.8), 80 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 80 mM of each of the four dNTPs, 50 pmol of each primers and 2 units of *Taq* DNA polymerase (Invitrogen). The thermal cycle used was that recommended by Lanciotti et al. 1992 using an annealing temperature of 58 °C.
- Semi-nested PCR – The semi-nested PCR was assayed using the same conditions of the first PCR assay. About 1 µL of the first PCR was used as a template for the semi-nested PCR. The thermal

cycle used was that recommended by Lanciotti et al., 1992 using an annealing temperature of 60 °C.

- Detection of amplified DNA – 10 µL of the semi-nested PCR amplified DNA underwent electrophoresis on a silver stained 8% poliacrylamide gel electrophoresis (PAGE).

3. Results

A total of 12.491 eggs were collected from 78 ovitraps from the Pampulha region in Belo Horizonte city in May, 2003. The number and percentage of hatching of *S. aegypti* and *S. albopicta* are given in Table 1.

After hatching, a total of 163 pools of larvae of *Stegomyia* sp. were analyzed. 76 pools were positive for DENV showing a minimum infection rate of 1: 45,81 for *Stegomyia* sp. From a total of 72 pools of *S. albopicta* larvae processed as fourth stage larvae, 33 pools were positive for DENV-2, 2 were positive for DENV-1 and DENV-2 and 37 were negative for DENV. The minimum infection rate concerning *S. albopicta* infection with DENV was 1:35,45 (Table 2).

The adult mosquitoes were obtained by rearing in the laboratory larvae collected from 20 ovitraps placed in areas with confirmation of transovarial transmission of DENV in *S. albopicta* larvae (results not shown). The mosquitoes were then tested for DENV infection. 18 *Stegomyia albopicta* mosquitoes were selected in order to be tested for DENV infection. From this group, 7 mosquitoes were females and 11 were males, with 4 females and 4 males positive for DENV-2 infection.

4. Discussion

In the year of 2003, there were 324,512 reported cases of dengue in Brazil. 618 reported cases were of dengue hemorrhagic fever (DHF) with 33 deaths among these patients (Pan American Health Organization, 2003). In the same year there were 22,781 reported cases of dengue in Minas Gerais state. Belo Horizonte city notified a total of 1,557 reported cases (Prefeitura de Belo Horizonte, 2005). The Pampulha region was responsible for 239 (15%) of the total number of reported dengue cases from the 9 regional districts (Barreiro, Center-south, East, Northeast, Northwest, North, West, Pampulha and Venda Nova). Pampulha represents the second region in terms of number of dengue cases, being behind only the northeast region (667 cases) of Belo Horizonte city. A percentage of 35,6% of *S. albopicta* hatching was registered in the Pampulha region, which represents a significant population of this species in an urban area.

Among public health authorities in the infested countries, there has been much concern that *S. albopicta* would lead to serious outbreaks of arbovirus diseases (*S. albopicta* is a competent vector for at least 22 arboviruses), notably dengue (all four serotypes) more commonly transmitted by *S. aegypti*. Results of many laboratory studies have shown that many arboviruses are readily transmitted by *S. albopicta* to laboratory animals and birds, and have frequently been isolated from wild-caught mosquitoes of this species, particularly in the Americas (Reinert and Harbach, 2005).

In Brazil, dengue outbreaks have been associated with *S. aegypti*, and the role of *S. albopicta* in virus transmission in nature remains to be confirmed (Schatzmayr, 2000). The results of Lourenço-de-Oliveira et al., 2003 showed that Brazilian *S. albopicta* were as efficient as the North American population in being infected with dengue and yellow fever viruses in the laboratory.

In this study we developed a rapid, specific diagnostic tool, RT-PCR, for the detection and typing of DENV in larvae and adult mosquitoes in populations collected from nature. Our RT-PCR is a sensitive method capable of detecting the viral RNA in a single mosquito sample and typing DENV. Therefore it could be used to monitor the infection rate of infection with higher precision. The method could be applied to an active epidemiological surveillance program in order to identify the circulation of DENV in a region. The technique was used to detect DENV-2 in *S. albopicta* salivary glands as we used smashed heads to molecularly identify the presence of the virus in these glands. If the salivary glands of *S. albopicta* are integral to the transmission of the dengue virus, it might be hypothesized that they are highly efficient producers of infectious virions and capable of producing high titers of virus in the presence of less demonstrable antigen than other infected tissues of the

Table 1. Total number of collected eggs and percentage of hatching of *Stegomyia aegypti* e *Stegomyia albopicta*.

Number of eggs collected	12,491	Percentage of hatching
Number of larvae identified	3,482	27.9
Number of *Stegomyia aegypti* larvae	2,241	64.4
Number of *Stegomyia albopicta* larvae	1,241	35.6

Table 2. Minimum infection rate.

Stage examined	Positive pools/tested pools	Total number of larvae	Minimum infection rate
Larvae	76/163	3,482	1:45.81 (*Stegomya* sp.)
Larvae	35/72	1,241	1:35.45 (*S. albopicta*)

mosquito. These data confirm the vectorial competence of *S. albopicta* in transmitting DENV-2 in the field remembering that genetic differences between *S. albopicta* populations through the country might exist (Consoli and Oliveira, 1994).

Thus, it seems that *S. aegypti* is the species involved in propagating the disease to epidemic proportion. This difference between the two species in vectorial capacity and competence could be explained by their oral susceptibility to *Dengue viruses* and feeding behavior (Chung and Pang, 2002). In the Americas, the introduction of *S. albopicta* has been associated with a decrease in the abundance of *S. aegypti* (Barrera, 1996; Khin and Than, 1983). Typically, *S. albopicta* prefers suburban and rural areas where it breeds in natural containers.

The occurrence of transovarial transmission of DENV-2 by *Stegomyia aegypti* in nature was demonstrated from the isolation of dengue virus from naturally infected mosquito larvae showing a minimum infection rate of 1:2,067 for DENV-2 (Sabin, 1952). Natural transovarial transmission of DENV-4 by *S. aegypti* was demonstrated from adult mosquitoes reared in the laboratory from eggs collected in Trinidad reporting a minimum infection rate of 1:1,855 for DENV-2 (Hull et al., 1984). The results of laboratory experiments described above with *S. aegypti* showed a very inefficient minimum infection rate. However we found a minimum infection rate for DENV-2 of 1:35,45 which indicates that transovarial transmission of dengue DENV by *S. albopicta* could provide a mechanism of maintenance of DENV when continuous mosquito breeding is interrupted. The transovarial transmission was confirmed by the detection of DENV-2 in male mosquitoes hatched from eggs which were collected in nature and reared in controlled conditions.

Transovarial transmission of DENV by *Stegomyia* could play an important role in the survival of these viruses in nature. Even low minimum infection rate might be sufficient to maintain the virus during dry seasons or during periods when insufficient numbers of mosquitoes are present to maintain the circulation of DENV in susceptible hosts. The minimum infection rate demonstrated in this study would appear more than sufficient to assist in the maintenance of DENV in nature.

While *S. albopicta* is less important than *S. aegypti* as a vector of DENV to man on a worldwide basis, it has been blamed as the principal vector in major outbreaks such as those in Japan during World War II (Metselaar et al., 1980) and Seychelles (Rosen et al., 1983). *S. albopicta* is predominantly rural in distribution but is also prevalent in urban areas especially in the absence of *S. aegypti*. *S. albopicta* is frequently associated with *S. aegypti*, as observed in different parts of the world. Some observations from regions where *S. albopicta* was recently introduced suggest it tends to supplant *S. aegypti* (Hobbs et al., 1991).

This is the first report of transovarial transmission and vectorial capacity of *S. albopicta* in Brazil concerning DENV-2 infection with molecular confirmation. Since *S. albopicta* shows susceptibly to DENV-2, vectorial capacity and occurrence of transovarial transmission of DENV-2, we can presume that the present expansion of this species is a threat to dengue control in Brazil specially using the same strategies as used for *S. aegypti* control.

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