TRANSOVARIAL TRANSMISSION OF DENGUE VIRUS 1 AND 2 AS SHOWN BY DETECTION IN Aedes aegypti LARVAE

ABSTRACT

Dengue viruses are Flavivirus presenting a single-stranded RNA genome of approximately 11 Kb. Dengue fever, associated with dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) is an important public health problem in Minas Gerais. Outbreaks of dengue have been occurring periodically since 1996. In addition to being maintained by transmission among susceptible humans dengue viruses may also be maintained by transovarial transmission in mosquitoes especially during inter-epidemics periods. During the last outbreak attempts were made to detect the transovarial transmission of dengue viruses in Aedes aegypti larvae collected in Pompeu, MG. RT-PCR was used to amplify the genome and DNA was visualized on poliacrylamide gel stained with silver nitrate. From 31 pools with approximately 50 larvae each, dengue virus serotype 1 was detected in 11 and dengue virus serotype 2 in 7 pools. The detection of the two viruses occurred in 3 pools of larvae. The results indicate the co-circulating of two serotypes in Pompeu and could suggest different transovarial transmission rates of dengue virus serotypes 1 and 2.

INTRODUCTION

Dengue virus infections are among the most common arthropod-borne infections in tropical and subtropical areas. The four serotypes, dengue virus 1 (DENV-1), dengue virus 2 (DENV-2), dengue virus 3 (DENV-3) and dengue virus 4 (DENV-4), are transmitted by several mosquito species including Aedes (Stegomyia) aegypti (Linnaeus, 1762) and Aedes (Stegomyia) albopictus (Linnaeus, 1762) (Groen et al. 2000). Dengue viruses are members of Flaviviridae family with positive single-stranded RNA genome of approximately 11 kb (Chow et al. 1997). The four serologic types are recognized on the basis of plaque reduction neutralization tests and constitute a distinct antigenic complex (Monath & Heinz, 1996). In recent years, increases in human population movements and vector (Aedes aegypti) density have resulted in a greatly
increased frequency and severity of epidemics due to dengue viruses (Lee et al. 2000). Dengue virus outbreaks occurred in all Brazilian territory since the mid-1980s. After a number of outbreaks, DENV-1 and DENV-2 are endemic in many areas including Minas Gerais state. These viruses are transmitted to humans by *Aedes* mosquitoes and may cause simple dengue fever (DF), or the more severe illness, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Puri et al. 2000). Dengue viruses are most commonly in Brazil transmitted by *Aedes aegypti* and these viruses cause explosive outbreaks throughout the Brazilian territory. *Aedes aegypti* is a container-breeding mosquito that has an oviposition preference for artificial containers (e.g. tires and discarded jars) associated with human habitats (Bennet et al. 2002). *Aedes aegypti* has reinfested most of the neotropics since its partial eradication earlier this century, resulting in re-emergence of neotropical dengue (Wang et al. 2000). Viremic humans in the acute phase of their infection constitute the principal, and perhaps the only, source of infection for this mosquito. Once infected, mosquitoes remain capable of transmission by bite for life (Rosen et al. 1983). 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. These viruses are maintained in a “human-mosquito-human” cycle (Joshi et al. 2002). In addition to being maintained by transmission among susceptible humans, dengue viruses may also be maintained by transovarial transmission in mosquitoes during interepidemics periods (Hull et al. 1984). This mechanism could explain the survival of the virus during dry seasons. The role of viral maintenance during inter-epidemic periods in species other than its primary vectors has also been suggested (Joshi et al. 2002). During the outbreak of 2000, successful attempt was made to detect dengue virus from larvae collected in Pompeu city, Minas Gerais state. In this study, the dengue virus could be detected in larvae of *Aedes aegypti*. In Brazil, this was the first time that transovarial transmission of dengue viruses has been shown to occur in nature using molecular detection. The results indicate the co-circulation of DENV-1 and DENV-2 in Pompeu and could suggest different transovarial transmission rates of these viruses.

**MATERIALS AND METHODS**

**Mosquito larval collections.**

134 oviposition traps were placed weekly near the home of patients suspected or confirmed to be infected with dengue virus in Pompeu city, Minas Gerais state, in May/2000. Paddles were collected and eggs were hatched in the insectary. Fourth stage *Aedes aegypti* larvae were identified to species, pooled and washed in PBS pH 7.2 containing 50mg/mL of fungizone, 200mg/mL of gentamicin, 2000U/mL of penicillin and 2500U/mL of amikacin. Then pools of 50 larvae were stored in vials at −80°C until required.

**Extraction of RNA.**

Each pool with approximately 50 larvae was ground using sterile sand. The supernatant of the grounded larval pool was used to extract RNA. RNAs were extracted by a modified silica method according to Boom et al. (1990). Briefly, the supernatant was treated with a lysis buffer containing guanidine isothiocyanate, Tris 0.1M, EDTA 0.2M, Triton X-100 mixed with sterilized silica. After centrifugation the silica was washed first with a washing buffer containing guanidine isothiocyanate and Tris 0.1M, followed by several washes with 70% ethanol and acetone. The material was resuspended in TE (TRIS 10mM, EDTA 1mM, pH 8.0) treated with RNAsin and after centrifugation the RNA was collected in the upper phase.

**RT-PCR**

(i) **Primers** – Two pairs of primers were used in a semi-nested PCR assay according to Lanciotti et al. 1992. (ii) **cDNA synthesis** – The cDNA synthesis was conducted in a 20ml reaction containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 2.5mM MgCl2, 10mM dithiothreitol, 1mM each of the four dNTPs, 50pmol of the anti-sense primer, 250ng of viral RNA in 5ml volume and 200U of reverse transcriptase enzyme (SUPERSCRIPT II RT, Invitrogen) at 42°C for 50 minutes. The enzyme was inactivated at 70°C for 15 minutes. (iii) **PCR assay** – The PCR using 5ml of the cDNA was conducted in reaction of 50ml containing 10mM Tris-HCl (pH 8.8), 80mM KCl, 2mM MgCl2, 0.01% gelatin, 80mM of each of the four dNTPs, 50pmol of each primers and 2 units of *Taq* DNA polymerase (Invitrogen). The termal cycle used was the recommended by Lanciotti et al. (1992) with modifications. The termal cycle used was
94°C-5min and then 94°C-1min, 58°C-1min, 72°C-1min for 30 times followed by a final extension of 72°C-10min. (iv) Semi-nested PCR – The semi-nested PCR was assayed using the same conditions of the first PCR assay. About 1ml of the first PCR was used as a template for the semi-nested PCR. The thermal cycle used was 94°C-5min and then 94°C-1min, 60°C-1min, 72°C-1min for 30 times followed by a final extension of 72°C-10min. (v) Detection of amplified DNA – Amplified DNA was detected by electrophoresis of a 10ml of the semi-nested PCR on a silver stained 8% polyacrylamide gel electrophoresis (PAGE).

RESULTS

For the mosquito larval collections, a total of 134 ovitraps were placed in areas suspected of occurrence of dengue cases being 99 ovitraps positive for *Aedes aegypti*. The eggs were hatched and after identification a total of 1518 larvae were obtained. The larvae were processed as fourth stage larvae in 31 pools. From 31 pools of approximately 50 larvae each, DENV-1 was detected in 11 and DENV-2 in 7 pools using RT-PCR and PCR semi-nested as a detection tool. The two serotypes were detected in the same group of larvae in 3 pools only. The *Aedes aegypti* larvae were used to detect the serotype of dengue virus circulating in the region of study. The larvae were used as well to identify the occurrence of transovarial transmission of dengue virus in larvae of *Aedes aegypti* in nature. Table 1 summarizes the minimum field infection rate obtained. The minimum field infection rate of DENV-1 observed was 1:138 and for DENV-2 was 1:217.

Table 1 - Minimum field infection rates of transovarial transmission in *Aedes aegypti* larvae collected in the field.

<table>
<thead>
<tr>
<th>Larvae examined</th>
<th>Nr. positive pools/ Nr. pools tested</th>
<th>Total larvae tested</th>
<th>Minimum field infection rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dengue virus 1</td>
<td>11/31</td>
<td>1518</td>
<td>1:138</td>
</tr>
<tr>
<td>Dengue virus 2</td>
<td>7/31</td>
<td>1518</td>
<td>1:217</td>
</tr>
<tr>
<td>Dengue virus 1 and 2</td>
<td>18/31</td>
<td>1518</td>
<td>1:84</td>
</tr>
</tbody>
</table>

* eggs were collected in the field, reared in laboratory to larvae, identified and tested for dengue virus infection.

DISCUSSION

We initiated our studies on transovarial transmission of dengue viruses in order to monitor the circulating serotypes of a region after noticing that the clinical serum samples most of the time are collected in a wrong phase of the infection in patients and are not conserved properly till they arrive at laboratory.

The epidemiology of dengue is of continuing health importance, as the incidence of DF and DHF/ DSS is tending to increase as outbreaks occur each year. It remains to be determined whether there is a direct association between specific serotypes or genotypes and severity of disease.

The occurrence of transovarial transmission of dengue virus 2 by *Aedes aegypti* in nature was demonstrated from the isolation of dengue virus from naturally infected mosquito larvae (Khin & Than 1983). Natural transovarial transmission of dengue DENV-4 by *Aedes aegypti* was demonstrated from adult mosquitoes reared in the laboratory from eggs collected in Trinidad (Hull et al. 1984).

Our findings suggest the occurrence of transovarial transmission of DENV-1 and DENV-2 by *Aedes aegypti* in nature although it has been stated before that transovarial transmission in nature seems not to occur in Brazil concerning this vector (Dégalier et al. 1996). The transovarial transmission of dengue viruses may be one mechanism of establishing endemicity and may be also a safety mechanism, which insures biological survival of the virus during dry seasons. The study indicate the co-circulation of two serotypes in Pompeo and could suggest different transovarial transmission rates of DENV-1 and DENV-2.
Laboratory experiments with *Aedes aegypti* showed very inefficient vertical transmission, with a minimum infection rate of 1:1543 for DENV-1 (Bosio et al. 1992). However, we found a minimum infection rate for DENV-1 of 1:138 which could indicate that transovarial transmission of dengue virus by *Aedes aegypti* may assist in the maintenance of the virus in nature during interepidemic periods and in the transmission of the virus to man.

This is the first report of transovarial transmission of dengue virus from field collected mosquito larvae in Brazil detecting using molecular tools.

The virus isolates from the outbreaks need to be studied in more detail in order to understand the true interplay and evolution of the dengue serotypes circulating in Minas Gerais state, Brazil.

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**REFERENCES**


