DENGUE-2 VIRUS ARTIFICIAL INFECTION OF BRAZILIAN COLONIZED *AEDES AEGYPTI*

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ABSTRACT

Dengue is a viral disease transmitted by mosquitoes and caused by four viral serotypes (DENV 1-4). This mosquito-borne disease is a major public health problem and a threat to more than 2.5 billion people, who live in endemic areas. *Aedes aegypti* is the primary vector of dengue in the Americas. The present study shows the susceptibility of a colony strain of *A. aegypti* to Dengue 2 virus (DENV-2). *A. aegypti* females from a laboratory colony were infected with DENV-2 using a membrane feeding technique. The engorged females were dissected on different days (3, 6, 9, 12 and 15). The midguts, ovaries, carcasses and heads were separated. The samples were fixed and immunolabeled with anti-DENV-2 antibody to be analyzed by laser confocal microscopy (LCM). Infected mosquitoes from the 15th day had their heads and bodies dissected, separated and analyzed by RT-PCR with specific primers. Immunolabeling of the midguts and ovaries showed the presence of DENV-2 in the mosquitoes dissected from the third day until the last day of the experiment. The RT-PCR reactions of the heads and the bodies confirmed the mosquito infection with DENV-2. These results showed that the methodology used for the artificial infection with dengue virus was successful and demonstrated that our colonized *A. aegypti* mosquitoes were susceptible to artificial dengue infection. Future studies are in progress to better understand the process of invasion by dengue virus in *Aedes* mosquito.

INTRODUCTION

Dengue viruses (genus *Flavivirus*, family Flaviviridae) are the most important arboviruses affecting humans. There are four distinct serotypes of dengue viruses (DENV 1-4) that co-circulate in many tropical regions of the world (Rigau-Perez et al.

1998). Dengue viruses are a threat to more than 2.5 billion people (WHO 2002). Recently, in Brazil, three of the four serotypes are present in the majority of the country and the vector, *Aedes (Stegomyia) aegypti* (Linnaeus, 1762), is spread throughout the country (SVS 2005, Schatzmayr 2000).

Arboviruses are naturally maintained in cycles by hematophagous arthropods that biologically transmit the virus between vertebrate hosts (Woodring et al. 1996). The most common vector of dengue fever is *A. aegypti* (Gubler 2002), a container-breeding mosquito that has adapted to cohabitation with humans and has a cosmopolitan distribution in tropical and subtropical areas (Bennett et al. 2002).

Vector competence is defined as the intrinsic permissiveness of a vector to infection, replication and transmission of a virus (Hardy 1988, Woodring et al. 1996). Studies have shown that *A. aegypti* exhibits continuous variation in its competence to transmit flaviviruses (Black et al. 2002, Severson et al. 2004, Gorrochotegui-Escalante et al. 2005). As the susceptibility varies among mosquito strains, it is important to evaluate the competence of these vectors in each epidemic and endemic area (Chen et al. 1993).

In this work the oral susceptibility of a colony strain of *A. aegypti* to infection with DENV-2 was experimentally tested. This study is the first step, in our laboratory, for establishing artificial infection of the mosquitoes with the dengue virus. This strain of *A. aegypti,* is going to act as a positive control when testing mosquitoes collected in the field.

MATERIAL AND METHODS

Mosquitoes.

A. aegypti mosquitoes (Campos dos Goytacases – Rio de Janeiro State strain) were obtained from a colony established in 1999, and kept in the Laboratory of Medical Entomology, Centro de Pesquisas René Rachou-Fiocruz in the state of Minas Gerais. The mosquitoes were kept in an acclimated insectary with an average temperature of 26-28ºC and relative air humidity around 70-80%, in a cycle of 12 hours in the dark and 12 hours in the light. The mosquitoes were provided with 10% glucose solution and water *ad libitum* until the time of the experiments.

Virus strain.

The virus strain used in the mosquito infection was DENV-2/SPH. This strain was isolated from a serum sample of a dengue hemorrhagic fever case in the city of Ribeirão Preto (São Paulo State) during 1991. To prepare stock viruses, the frozen DENV-2 sample was amplified in C6/36 cells. The titer was determined by plaque-forming units on LLC-MK_2 cells and the titer was 4×10^4.

Oral infection of mosquitoes.

Two hundred five-day-old *A. aegypti* females were deprived of sucrose solution 24 hours before the experiments. Then, they were allowed to feed through a chick-skin membrane in a glass feeder apparatus heated by circulating water at 37ºC during 1 hour (Rutledge et al. 1964, Billingsley & Rudin 1992). The infected blood meal was constituted of two-thirds of heat-inactivated heparinized mouse blood mixed with one-third of virus-infected C6/36 cells and adenosine triphosphate as a phagostimulant. The

engorged females were preserved in cages at 28ºC and provided with 10% glucose solution, for extrinsic incubation of the virus.

Mosquito dissection.

At days 3, 6, 9, 12 and 15 after the artificial feeding, females were: 1) anesthetized on ice for 3 minutes; 2) dissected in phosphate buffer solution (PBS, pH 7,2); 3) midguts, ovaries, carcasses and heads were separated and stored at 4ºC until use.

Immunolabeling for Dengue Virus.

The samples dissected were fixed in 4% formaldehyde for 2 hours at 4ºC. The fixed samples were washed three times with PBS, incubated in RPMI (Roswell Park Memorial Institute) medium for 2 hours, then with PBS/bovine serum albumin (BSA)/Triton and finally with anti-DENV-2 antibody overnight. Afterwards, the samples were washed three times with PBS/BSA/Triton. Subsequently the samples were incubated with a FITC (fluorescein isothiocyanate) secondary antibody for 2 hours. After this procedure, samples were washed three times in PBS/BSA/Triton and then three times with PBS. The samples were mounted with anti-fading medium (Mowiol) and observed under Laser Confocal Microscopy (LCM).

Detection of the viral RNA using RT-PCR.

On the 15th day post infection, females had their bodies and heads separated and the viral RNA was extracted using a silica methodology (Boom et al. 1990). RT-PCR for detection of dengue virus was performed according to Lanciotti et al. (1992). Briefly, after reverse transcription, the cDNA was used in a first PCR and a subsequent

semi-nested PCR reaction was completed in order to confirm the dengue virus serotype. PCR products were visualized on 8% polyacrylamide gel electrophoresis (PAGE) with a positive control of DENV-2 and stained with silver nitrate.

RESULTS

The *A. aegypti* females fed successfully on a blood meal containing the virus and infected C6/36 cells (Figures 1 and 2). The presence of the virus in the samples was observed using RT-PCR and Laser Confocal Microscopy.

The samples analyzed under the Laser Confocal Microscopy showed infection after the infected blood meal. On the 3rd day post infection, midgut (Figures 3 and 4) and ovary (Figures 5 and 6) were positive for dengue virus and this observation was confirmed until the last day of the experiment. The infection of the salivary glands (Figures 7 and 8) was observed only in the samples collected on the 15th day post infection.

The infection rate was defined as the percentage of mosquitoes that had both heads and bodies infected among all surviving engorged females. This rate was achieved by the analysis of mosquitoes through RT-PCR on the 15th day post infection. Sixty orally infected mosquitoes were tested for the presence of dengue viruses. From these mosquitoes, forty-five were positive for the dengue virus, with an infection rate of 75%.

DISCUSSION

The analysis of the material by LCM showed that the infection of the dengue virus in the midgut was not homogeneous. It was observed that only certain epithelial cells showed specific fluorescence characteristic of viral infection. Similar results were reported with *A. aegypti* infected with DENV-1 (Chen et al. 1993), *Aedes albopictus* infected with DENV-2, *Culex tritaeniorhynchus, Culex pipiens* infected with Japanese encephalitis virus (Hardy et al. 1983) and *Culicoides variipennis* infected with blue tongue virus (Sieburth et al. 1991). Therefore, these infected cells may be subpopulations of cells acting as receptors for virus dissemination from the midgut to the hemocoel . In addition, the amount of infected cells in the midgut could be related to mosquito vector competence.

It is known that the virus can replicate in tissues within the hemocoel only after passing the midgut barrier (Kuberski 1979, Hardy et al. 1983, Schoepp et al. 1991, Chen et al. 1993) and when this happens, virions disperse in circulating hemolymph and replicate in many tissues and organs, similar to the Rift Valley fever virus in *Culex pipiens* (Faran et al. 1988). The analysis of the *A. aegypti* reproductive organs showed the same pattern of infection visualized in the midgut. Viruses were detected in only one part of the ovaries (oocytes), suggesting that the infection is located in the follicular epithelium. Although verification of vertical transmission did not occur in this study, the detection of the virus in the ovaries suggests that these mosquitoes are capable of transmitting the virus to its progeny (Cecílio et al. 2004).

In addition, dengue virus infection was detected in the fat body (not shown) and in the salivary glands, demonstrating that this mosquito strain was susceptible to this virus strain. After the infection of the salivary glands, the virus must escape into the

lumen of the gland, where it can be transmitted to vertebrates during a blood meal (Woodring et al. 1996). The presence of the virus in the salivary glands indicates that the virus is able to replicate within this organ. Even so, this fact does not allow us to affirm that the mosquitoes are capable of transmitting the virus, because the saliva of these samples were not tested.

In order to confirm the infection and estimate the infection rate, the methodology chosen was RT-PCR for its high sensitivity and specificity. The infection rate obtained in our experiment was 75%. Lourenço-de-Oliveira et al. (2004) showed variation in the infection rates with DENV-2 of mosquitoes collected in different parts of Brazil (21.6% to 99.0%). When we compare these results to ours (75%) it is possible to say that our colony has a similar behavior to other Brazilian mosquitoes towards DENV-2 infection. Although many authors suggest that the colonization process could affect the susceptibility of the mosquitoes to DENV infection (Leake 1984, Vazeille et al. 2003, Moncayo et al. 2004), this data is not yet confirmed.

According to our results, the methodology used for the artificial infection with dengue virus was successful. Moreover, laser confocal microscopy presented itself as a great tool for visualization of the presence of DENV in different organs of *A. aegypti*. Future studies are in process to better understand the invasion of the *Aedes* mosquito by dengue viruses.

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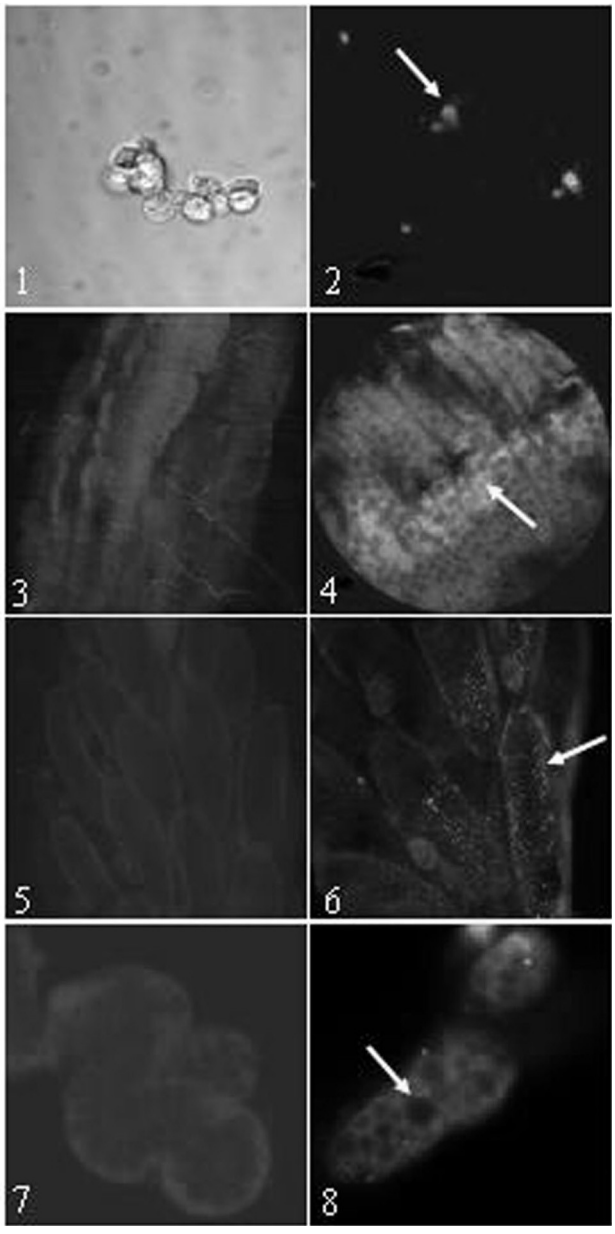
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Fig. 1. Interference contrast view of dengue virus infected C6/36 cells, showing the characteristic aspect of syncytia. Fig. 2. Immunolabeled dengue virus infected cells. Viral particles are grouped and located next to the cell nucleus (arrow). Fig. 3. Midgut, negative control (non-infected midgut). The mosquito midgut presents auto-fluorescence. Fig. 4. Immunofluorescence of an infected midgut at the third day post-infection. The bright points (arrow) are characteristic of viral infection. Fig. 5. Ovary, negative control (non-infected ovary). The mosquito ovary presents a soft auto-fluorescence. Fig. 6. Immunofluorescence of a dengue virus infected ovary at the sixth day post-infection. The bright points (arrow) show groups of viral particles. Fig. 7. Salivary gland, negative control (non-infected salivary gland). Fig. 8. Immunofluorescence of a dengue virus infected salivary gland. The bright points (arrow) are characteristic of viral infection.

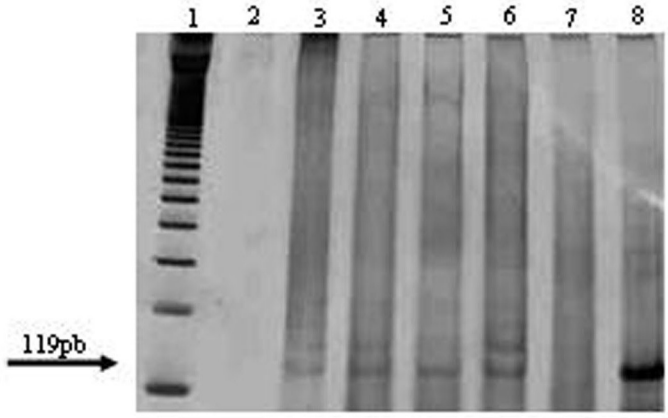

Fig. 2. Polyacrylamide gel electrophoresis (PAGE 8%) of PCR products of C/PrM junction. Lanes: 1- ladder (100bp); 2- negative control; 3 and 4- positive head sample; 5 and 6- positive body sample; 7- negative body sample; 8- positive DENV-2 control (119bp).