

SIMULTANEOUS INFECTION WITH DENGUE 1 AND 2 IN A BRAZILIAN PATIENT

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SUMMARY

Dengue outbreaks have occurred in several Brazilian States since 1986 involving serotypes 1 (DEN-1) and 2 (DEN-2). In view of the few cases of double infection documented in the literature, we report here a case of simultaneous infection with DEN-1 and DEN-2 in a patient residing in the municipality of Miranda, State of Mato Grosso do Sul, Western region of Brazil. DEN-1 was introduced in this State in 1989 and DEN-2 in 1996, both of them circulating in some municipalities. This double infection was identified by virus isolation and by indirect immunofluorescence using monoclonal antibodies and confirmed by the polymerase chain reaction (PCR). This is the first documented case of simultaneous infection with serotypes DEN-1 and DEN-2 in Brazil.

KEYWORDS: Dengue; Simultaneous infection; Virus isolation; PCR.

INTRODUCTION

The incidence of disease induced by the Dengue viruses have drastically increased over the last few years, affecting millions of persons all over the world. Because of its morbidity and mortality, dengue, with four distinct serotypes (DEN-1 to 4) is currently considered to be the most important viral disease transmitted by arthropods (HALSTEAD⁶). Dengue hemorrhagic fever and Dengue shock syndrome (DHF/DSS) has become a serious public health problem in various regions of the world. In the Americas, starting in the 1980's the problem of dengue has worsened, with the disease spreading throughout the continent and reaching most American countries (PAHO⁸). Up to the 1980's, many epidemics were caused by one or occasionally two serotypes, but today the various serotypes are endemic in many countries (GUBLER³). Although the different serotypes may be maintained in the same population for a long period of time, few cases of double infection have been reported in the international literature (GUBLER⁵, LAILLE et al.¹², MANEEKARN et al.¹⁵, KANESA-THASAN et al.¹⁰, SISOUK et al.¹⁹). In a review of the Brazilian literature we did not find any report on the simultaneous occurrence of infection with two different serotypes.

Dengue epidemics have been occurring in various states of Brazil since 1986, involving DEN-1, DEN-2 or both (FIGUEIREDO², NOGUEIRA et al.¹⁷).

In the present report we describe a case of simultaneous infection with DEN-1 and 2 during the 1996 epidemics in the State of Mato

Grosso do Sul, Western region of Brazil. DEN-1 was introduced in this state in 1989 and DEN-2 in 1996, with both serotypes circulating in some municipalities.

MATERIAL AND METHODS

Case report

The patient, a 30-year old female teacher, lives in Miranda, State of Mato Grosso do Sul, a town of approximately 22,000 inhabitants located 194 km from the capital city of Campo Grande. The municipality of Miranda borders others where transmission by DEN-1 or DEN-1 and 2 occurs. During the first semester of 1996, 80 cases of dengue were notified in the town, 23 of which were confirmed in the laboratory by MAC-ELISA (KUNO et al.¹¹). Two days before the onset of symptoms the patient had been in Campo Grande where serotypes 1 and 2 circulate.

The symptoms were fever, headache, sudoresis, photophobia and prostration for three days, followed by a mild course with full recovery and with no hemorrhagic manifestation or other more severe alterations.

Virus isolation and identification

The viruses were isolated by inoculating a 30- μ l serum aliquot obtained during the acute phase of the disease into cell

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cultures from the mosquito *Aedes albopictus*, clone C6/36 (IGARASHI⁹) grown in Leibovitz medium (L-15) supplemented with 1% nonessential amino acids, 10% tryptose phosphate and 10% fetal calf serum. The tubes were maintained for 10 days at 28°C in a bacteriologic incubator and the inoculated cells were then submitted to the indirect immunofluorescence test using mouse anti-dengue ascitic fluid and mouse anti-total immunoglobulin conjugate (SIGMA). The isolated viruses were then typed by another indirect immunofluorescence test using monoclonal antibodies for each of the 4 serotypes (HENCHAL et al.⁸, GUBLER et al.⁴), provided by Center for Disease Control, Fort Collins, CO.

RNA extraction

Viral RNA obtained after inoculation into C6/36 was first extracted and purified with a mixture of 150 µl trizol (GIBCO) and 150 µl cell fluid supplemented with 50 µl chloroform (-20°C) and centrifuged at 13,000 rpm for 5 min (Spin I). The supernatant (200 µl) was transferred to a microtube containing 400 µl isopropanol and centrifuged again at 13,000 rpm for 5 min. The supernatant was discarded and the sediment was air dried under a laminar flow.

RT-PCR

Amplimers and sequencing primers were designed based on published dengue sequence (LANCIOTTI et al.¹⁴). Extracted RNA was resuspended in a 25 µl volume that contained the following components: 5.0 µl 5X First Strand Buffer, 2.0 µl 0.1M dithiothreitol (DTT), 3.0 µl 25 mM MgCl₂, 2 µl 10 mM deoxynucleotide triphosphates (dNTP), 2.0 µl 200 U/µl reverse transcriptase (Kit SuperScript II Rnase H⁻ Reverse Transcriptase - GIBCO), 1.0 µl 10 U RNasin, 0.5 µl 20 uM primer DEN-2 (LANCIOTTI et al.¹⁴), and

9.0 µl DEPC water. The mixture was incubated at 42 °C for 60 min and at 92 °C for 10 min. A typical reaction mix contained in a 50 µl final volume: 10 µl of the cDNA, 3.5 µl 25 mM MgCl₂, 0.5 µl 20 mM each primer DEN-1, DEN-2 and TS 1 to 4 (LANCIOTTI et al.¹⁴), 2.0 µl 10 mM dNTP and 2.5 µl of 10X PCR buffer (GIBCO), 0.2 µl 5U/µl Taq (GIBCO) and 29.3 µl DEPC water. The PCR reactions were carried out in a Gene Amp PCR System 9600, PERKIN ELMER, thermal cycler, the process comprising an initial step of heating to 94 °C for 5 min to denature the cDNA, following by 35 cycles of 50 °C for 40 sec, 72 °C for 40 sec and 94 °C for 50 sec, followed by a final extension step of 72 °C for 7 min.

Identification of the PCR products

An aliquot of 10 µl of the RT-PCR products was analyzed on 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV-light. The size of the RT-PCR products with resulted from the amplification of DEN-1, DEN-2, DEN-3 and DEN-4 was 482 bp, 119 bp, 290 pb and 392 bp, respectively (LANCIOTTI et al.¹⁴).

RESULTS

Microscopic examination of the cell cultures inoculated with serum from the patient showed a clearly visible cytopathic effect with changes in the monolayer such as formation of vacuoles and of syncytial cells (Fig. 1). The immunofluorescence test was positive for dengue and typing with monoclonal antibodies showed positive reaction to DEN-1 and DEN-2. The reaction to DEN-1 was more marked than the reaction to DEN-2, and the reaction to serotypes 3 and 4 were negative. The results obtained after reisolation were identical to the previous ones.

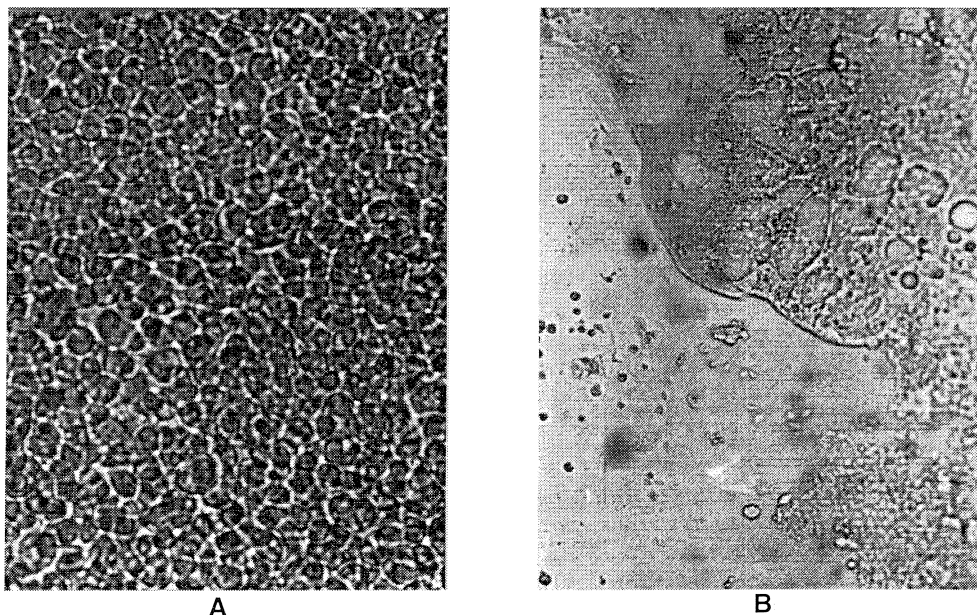


Fig. 1 - C6/36 *Aedes albopictus* cells line grown and maintained in L-15 medium. A: Negative control; B: Cytopathic effect observed in C6/36 inoculated with patient serum. (Amplified 400X)

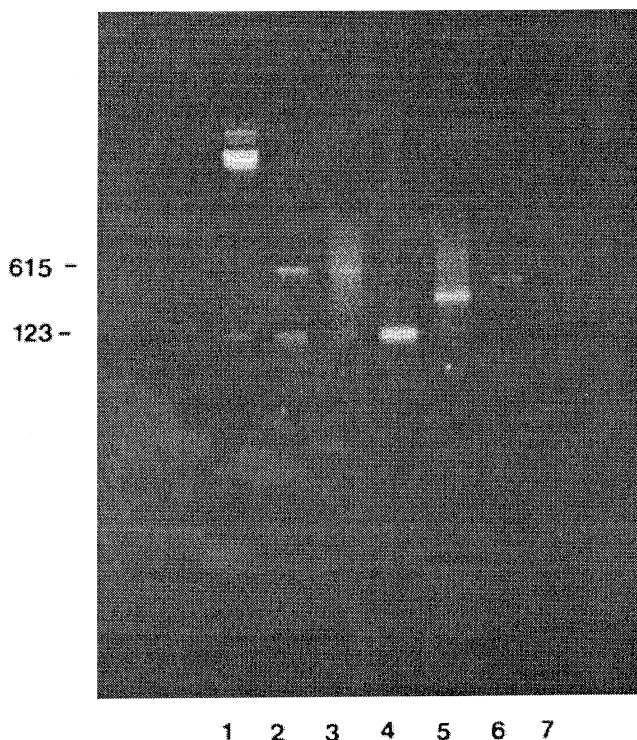


Fig. 2 - Agarose gel analysis of the DNA product from dengue viruses. Lanes: 1: molecular weight markers; 2: positive cells fluid for DEN-1 and 2; Controls: 3: DEN-1; 4: DEN-2; 5: DEN-3; 6: DEN-4; 7: cell negative control.

To confirm these results, we analyzed the supernatants of infected and non-infected cells by multiplex RT-PCR. The primers used recognized part of the genome of the structural proteins of DEN-1 and 2. The DNA fragments corresponded to 482 and 119 base pairs of DEN-1 and 2, respectively. The sequence size of amplified DNA was as expected for the target region of the dengue virus genome (Fig. 2).

DISCUSSION

Simultaneous infections with more than one virus have been studied in vertebrates and invertebrates. Experimental laboratory data have demonstrated that the mosquito *Aedes aegypti* may be infected with double combinations of different arboviruses and that it is also able to transmit the two viruses simultaneously. Viremia was also observed in mice inoculated with a suspension of macerated preparations of these mosquitoes (LAM & MARSHALL¹³). Similar results have been obtained with *Culex tarsalis* infected with Eastern and Western equine encephalitis (CHAMBERLAIN & SUDIA¹). Thus it is possible to assume that mosquitoes infected with the two dengue serotypes may transmit them both in areas where two or more serotypes of the virus exist, together with a high prevalence of the vector.

Although it is not known if transmission occurs through two different mosquitoes or through one double-infected mosquito, cases of simultaneous human infection with viruses Chikungunya and

Dengue 2 (MEYERS & CAREY¹⁶), with Dengue 1 and 4 (GUBLER et al.⁵), Dengue 1 and 3 (LAILLE et al.¹²), Dengue 1 and 2 (MANEEKARN et al.¹⁵, SISOUK et al.¹⁹) and Dengue 2 and 3 (KANESA-THASAN et al.¹⁰), have been reported.

Since the number of cases of infection with the Dengue virus has increased and the disease represents a serious public health problem in tropical zones, rapid and reliable methods are very important to confirm infection with the virus, to identify the serotype(s) and to carry out epidemiological studies.

In Brazil, the circulation of different serotypes occurs in various regions (FIGUEIREDO², NOGUEIRA et al.¹⁷). However, this is the first documented case of simultaneous infection with two dengue serotypes. The small number of these concomitant infections reported in the literature is still insufficient to formulate any assumption but, in contrast to previous suggestions (HAMMON⁷), double dengue infections do not appear to be related to situations of DHF/DSS in most of the reported cases^{5, 10, 12, 19}. Only in one report two patients with DEN-1 and 2 infection had a clinical diagnosis of dengue hemorrhagic fever¹⁵. In the present study, the clinical picture of the patient was not serious.

In the present case, double infection was identified by immunofluorescence and confirmed by PCR. Although the diagnosis by immunofluorescence was positive for the two dengue serotypes, the reaction was more marked for DEN-1 than for DEN-2. Analysis of the infected cell fluid submitted to RT-PCR permitted the visualization of two sharp bands of similar intensity, confirming once again the sensitivity of PCR. However, the specificity of the two techniques seems to be comparable since both showed positivity for the two serotypes.

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RESUMO

Infecção simultânea com Dengue 1 e 2 em uma paciente brasileira

Epidemias de dengue vem ocorrendo em vários Estados brasileiros desde 1986 envolvendo os sorotipos 1 (DEN-1) e 2 (DEN-2). Em vista dos poucos casos de dupla infecção documentados na literatura, relatamos aqui um caso de infecção simultânea com DEN-1 e DEN-2 em uma paciente residente no município de Miranda, Estado de Mato Grosso do Sul, Região Centro-Oeste do Brasil. O DEN-1 foi introduzido nesse Estado em 1989 e o DEN-2 em 1996, com a circulação de ambos em alguns municípios. Esta dupla infecção foi identificada por isolamento de vírus e imunofluorescência indireta usando anticorpos monoclonais e confirmada pela reação em cadeia da polimerase (PCR). Este é o primeiro caso de infecção simultânea com os sorotipos 1 e 2 documentado no Brasil.

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