

CO-INFECTION OF DENGUE VIRUS BY SEROTYPES 3 AND 4 IN PATIENTS FROM AMAZONAS, BRAZIL

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SUMMARY

The natural co-infection with dengue virus can occur in highly endemic areas where different serotypes have been observed for many years. We report here four cases of DENV-3/DENV-4 co-infection detected by serological and molecular tests among 674 patients with acute undifferentiated fever from the tropical medicine reference center of Manaus City, Brazil, between 2005 and 2010. Analysis of the sequences obtained indicated the presence of genotype 3 and 1 for DENV-3 and DENV-4 respectively.

KEYWORDS: Brazil; Dengue; Co-infection; Flavivirus.

Dengue Fever is the most important arboviral disease worldwide. Dengue viruses (DENVs) belong to the genus *Flavivirus*, family *Flaviviridae*. These are single-stranded positive-sense RNA viruses grouped into four antigenically related, but distinct, serotypes named DENV-1, 2, 3 and 4⁵. Since the first laboratory-confirmed DENV cases at Roraima 1981-1982, more than four million cases of dengue have been reported in Brazil¹¹. In Manaus, the capital of the Amazonas State in Brazil, all four dengue serotypes have already been reported^{3,4}. Dengue virus co-infection cases are poorly documented in literature, although our results demonstrate that such cases might be more common than expected, mostly in hyperendemic areas.

From January 2005 to December 2010, 674 patients with acute undifferentiated fever were treated at a reference center of Tropical Medicine (Fundação de Medicina Tropical Doutor Heitor Vieira Dourado - FMT-HVD, Manaus, Brazil). These patients were tested for malaria by thick blood analyses, and all patients with negative results were asked to participate in this study. The participants signed an informed consent form that was approved by the FMTAM Ethical Committee Board (272/2005). Two blood samples were collected from each patient, one in the acute phase of the disease and the other in the convalescent form. Sera from the convalescent phase were used for detection of anti-dengue immunoglobulin M (IgM) specific antibodies by MAC-ELISA, as previously described⁹.

Acute-phase samples were used in two tests: one for viral isolation in C6/36 *Aedes albopictus* cell line, followed by viral identification using an indirect immunofluorescent test with dengue type-specific monoclonal antibodies⁷, kindly provided by Centers for Disease Control

and Prevention (CDC), Atlanta, Georgia USA; and the other for specific DENV nucleic acid amplification. RNA was extracted directly from serum samples with the QIAamp Viral RNA Mini-Kit (Qiagen, USA), following manufacturer's instructions and submitted to RT-PCR to be followed by semi-nested multiplex PCR as previously described for DENV detection and typing¹⁰. When samples were positive for any serotype of DENV, a second semi-nested PCR, this time in a singleplex format, with a type-specific primer (DENV-1 to DENV-4) was performed for confirmation. Amplicons from the C/PrM region were purified and sequenced in both directions by using the BigDye Terminator Cycle Sequence Kit (Applied Biosystems, USA). The genotypes were detected by a search tool <http://www.denguedb.org/submitGenotypeRequest.aspx?type=Dengue>.

One hundred and thirty-five samples were positive for DENV: 2 DENV-1, 25 DENV-2, 71 DENV-3 and 37 for DENV-4. Four individuals, three women and one man, ages 40-70 years, had co-infections of DENV-3 and 4 detected by immunofluorescence assay, RT-PCR and nucleotide sequencing (Table 1). Co-infections including distinct dengue serotypes are, probably, more common in tropical regions of the world where dengue is hyperendemic, with circulation of the four serotypes⁸. Risk factors for dengue infections and co-infections include the virulence of the virus and the density of *Aedes*⁶. Larval Index Rapid Assay (LIRA) varied from 2.6 to 2.8% between 2005 and 2007 (Table 1). In 2008, DENV-4 was reported in Brazil after 25 years of absence⁴. Although the paper was published in 2008, the DENV-4 positive samples were, in fact, obtained in 2005 and 2006, kept at -80 °C in FMT-HVD fever serum collection and analyzed in the second half of the year 2007 (Figueiredo, R.M. personal communication), which indicates that DENV-4 might have

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Table 1
Results of different methods used for identification of dengue virus serotypes co-infecting 4 patients

Sample	Accession numbers	RT-PCR and virus isolation		Genotyping	MAC-ELISA	LIRAa
		Onset of symptoms	Collection date		Collection date	
AM707	JF923865 DENV-3 JF923866 DENV-4	N.I.	02/25/2005	(DENV-3 / GIII*) (DENV-4 / GI)	ND	2.6
AM726	JF923879 DENV-3 JF923880 DENV-4	N.I.	02/28/2005	(DENV-3 / GIII*) (DENV-4 / GI)	ND	2.6
AM750	EU127898 DENV-3 EU127899 DENV-4	04/28/2005	05/06/2005	(DENV-3 / GIII*) (DENV-4 / GI)	Negative 05/06/2005	2.6
+AM2220	JF923873, DENV-4	11/27/2007	11/28/2007	(DENV-4 / GI)	Positive 12/05/2007	2.8

N.I. = not informed; ND = Test not done; + The sequence of DENV-3 was of poor quality; * The genotypes presented were of highest probability despite the small size of the PCR fragment sequenced; The genotypes were those most likely, although the reliability is not 100% due to the small size analyzed.

been circulating in a silent cryptic way in northern Brazil in the last few years. In 2010, the LIRA was 1.5%, showing an alarming situation in Manaus which led to the dengue fever outbreak in the first three months of 2011 (unpublished data).

According to the period of the disease, (Table 1) it was possible to observe during convalescence the presence of the DENV by the RT-PCR¹⁰ method, irrespective of the presence or absence of antibodies.

Nucleotide sequence analysis shows genotype 3 for DENV-3, although the reliability is not 100% due to the small size analyzed (Table 1), whereas DENV-4 samples were typed as genotype 1, in accordance with previous analyses². Regarding the circulation of DENV-4 of genotype I, causing cases of human infection in Manaus, it is possible that DENV-4 of genotype I has been introduced by international visitors or by imported mosquitoes from Asia². The isolates reported here were from patients with no travel history, which indicates autochthonous infection.

In Brazil, one case of co-infection by DENV-1 and DENV-2 was reported in a patient with classic dengue fever (DF) from the southeast region, in 2001¹². Another case of co-infection by DENV-2 and DENV-3 was reported in 2005 in a patient DF from the northeast region, who recovered without a relapse¹. During an outbreak of dengue in São José do Rio Preto, in the state of São Paulo, 365 samples showed positive to DENV-3, five samples were positive to DENV-2, and 8 to St. Louis encephalitis flavivirus (SLEV). Among the positive samples, one co-infection was detected between DENV-2 and DENV-3¹³. Hence, co-infection with distinct DENV serotypes during outbreaks may be expected.

The co-infected patients presented benign clinical examinations and recovered without sequel, corroborating with previous reports¹². Here, we report cases of co-infection by dengue in the state of Amazonas, concurrent with the first reported cases of DENV-4 in Brazil after Roraima 1981-1982. Viral evolution of DENV-4 cases from Manaus should be studied in detail since it is an Asian genotype that has been associated with DHF in the Asian continent.

RESUMO

Co-infecção pelo vírus dengue 3 e 4 em pacientes da Amazônia brasileira

A co-infecção natural com os vírus dengue pode ocorrer em áreas altamente endêmicas onde diferentes sorotipos têm sido transmitidos por muitos anos. Relatamos aqui quatro casos de co-infecção com DENV-3/ DENV-4 detectados por testes sorológicos e moleculares entre 674 pacientes com febre indiferenciada aguda, atendidos em um centro de medicina tropical de referência da cidade de Manaus, Brasil, entre 2005 e 2010. As análises das sequências obtidas indicaram a presença dos genótipos 3 e 1 para DENV-3 e DENV-4 respectivamente.

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