

RESEARCH NOTE

Molecular Epidemiology of Den-2 Virus in Brazil

MP Miagostovich/⁺, RMR Nogueira,
HG Schatzmayr, RS Lanciotti*

Laboratório de Flavivirus, Departamento de Virologia,
Instituto Oswaldo Cruz, Av. Brasil 4365, 21045-900
Rio de Janeiro, RJ, Brasil *Centers for Disease
Control and Prevention, CDC, Fort Collins, CO, USA

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Dengue (DEN) viruses belong to the family *Flaviviridae*, genus *Flavivirus*, and occur as four antigenically related, but distinct serotypes designated DEN-1, 2, 3 and 4 (EG Westaway et al. 1985 *Intervirology* 24: 183-192). The viruses are characterized by a single strand of RNA associated with a core protein, in a nucleocapsid surrounded by a lipid envelope. The genome consists of a single open reading frame coding for core protein (C), precursor of the membrane protein (prM) and envelope (E) structural proteins, followed by the non structural proteins NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (FZ Heinz & JT Roehring 1990 p. 289-305. In MHV Van Regenmorte, AR Neurath (eds), *Immunochemistry of Viruses, II. The Basis for Serodiagnosis and Vaccines*, Elsevier).

The genetic variation among DEN viruses has been demonstrated by numerous methods including oligonucleotides fingerprinting, restriction enzymes, primer extension sequencing and nucleotide sequences from different fragments of genome (R Rico-Hesse 1990 *Virology* 174: 479-493, JA Lewis et al. 1993 *Virology* 197: 216-224, V Deubel et al. 1993 *Arch Virol* 129: 197-210, V Vorndam et al. 1994 *Arch Virol* 136: 191-196, DW Trent et al. 1989 *Virology* 172: 523-535, RS Lanciotti et al. 1994 *J Gen Virol* 75: 65-75, E Chungue et al. 1995 *J Gen Virol* 76: 1877-1884, KZ Thant et al. 1995 *Microbiol Immunol* 39: 581-590).

Since intra-serotypic antigenic variations (genetic subtypes) could be associated with severe disease it is important to monitor the distribution and eventual introduction of new genotypes of

existing serotypes into areas where dengue activity are troublesome (Vorndam et al. 1994 *loc. cit.*).

In this report we sequenced the E fragment from geographically and temporally distinct DEN-2 viruses isolated in Brazil during 1990-1995, in order to investigate the genetic subtype distribution of this serotype virus in the country.

DEN-2 viruses analyzed in this study were obtained from the collection of the Laboratory of Flavivirus, Department of Virology, IOC, Fiocruz. These strains were isolated from sera by inoculation into *Aedes albopictus* clone C6/36 cell line (A Igarashi 1978 *J Gen Virol* 40: 531-544) and were identified by immunofluorescence using type-specific monoclonal antibodies. Virus seeds were amplified once by inoculation into C6/36 (DJ Gubler et al. 1984 *Am J Trop Med Hyg* 33: 158-165).

Viral RNA was extracted from infected C6/36 cells by using the acid-guanidin isothiocyanate procedure previously described (RS Lanciotti et al. 1992 *J Clin Microbiol* 30: 545-551). Oligonucleotide primers used in the amplification and sequencing protocols were designed with the aid of the oligo program (National Bioscience Inc., Plymouth, MN).

Nucleotides from positions 1685 to 2504 coding for the fragment of E gene were amplified using RT-PCR. The RT reaction was performed in 4 µl of 5X RT reaction buffer (BRL), 4 µl of water, 2 µl of 0.1M DTT, 5 µl of 25 µM dNTP's, 0.2 µl of Rnasin (40U/µl), 2µl of 10 mM downstream primer D2CP2504 (5' GGGGATTCTGGTTGGAAGCTT ATATTGTTCTGTCC 3'), 2 µl of RNA and 1 µl of 200 U Superscript RT (Gibco). The reaction was incubated at 50°C for 10 min then 50 min at 45°C. A PCR amplification was followed by adding 10 µl of RT reaction to 90 µl of PCR reaction mix (74 µl of water, 9 µl of 10X C buffer, 5 µl of 25 mM dNTP, 2µl of 10 mM upstream primer D2P1685 (5'CTAGGATCTCAAGAAGGAGC AATGCA 3') and 0.5 µl Taq. The DNA molecules were denatured at 94°C for 4 min and subjected to a 35 amplification cycles (94°C for 1 min, 55°C for 1 min, 72°C for 8 min) and to one of 72°C for 10 min.

After an eletrophoresis on a 1% agarose gel, the amplified DNA bands were excised and purified by using the Bio 101 Gene-Clean kit. Purified DNAs were then sequenced by using the following primers: D2P1685- 5'CTAGGATCTCAA GAAGGAGCAATGCA 3'; P760 - 5' GGATCA CAAGAAGGAGCCATGCA 3'; CP1171 - 5' ATGGAGCTTCCTTTCTTCTTGAACCA 3'; CP1234 - 5' CCAAAGTCCCAGGCTGTGTCTC CCAGAATGGCCAT 3'. The sequencing reaction was performed by using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystem, Inc., USA). Cycle sequencing parameters were exactly as described in the manufactures protocol.

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⁺Corresponding author. Fax: + 55-21-270.6397. E-mail: marizepm@gene.dbbm.fiocruz.br

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The overlapping nucleic acid sequences obtained from individual sequencing reactions were combined for analysis and edited using the DNASTAR program (Madison, WI). The DEN-2 virus nucleic acid sequences were then aligned with each other, and with DEN-2 envelope sequences obtained from GENEbank, using the multiple sequence alignment algorithm CLUSTAL (D Higgins, Heidelberg, Germany). Phylogenetic trees were reconstructed from the aligned nucleic acid sequences using algorithms based upon parsimony (program PAUP, D Swofford, Champaign, IL).

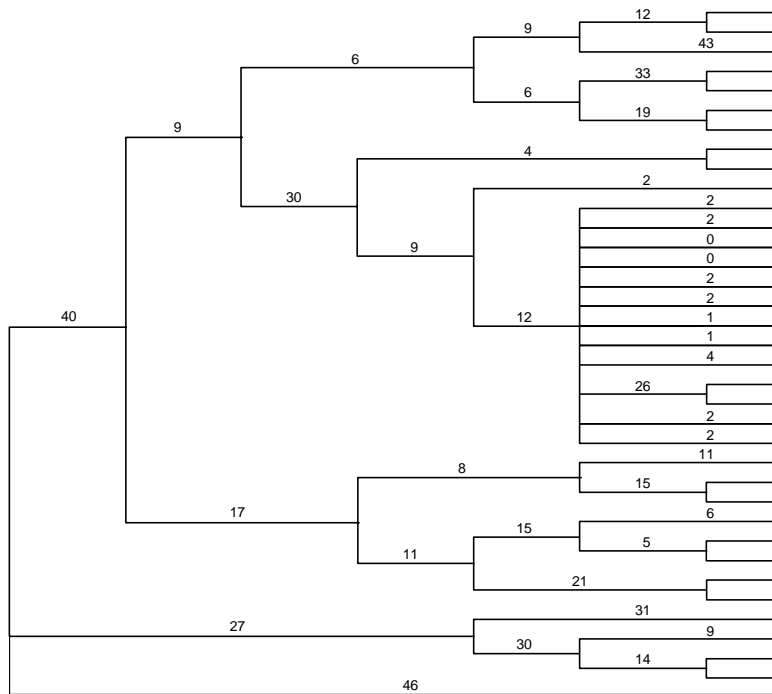
The comparison of our results with the phylogram generated by the sequencing of the entire E gene (Lewis et al. 1993 *loc. cit.*) showed that all the isolates belong to subtype III (Figure). The results confirmed the asiatic origin of DEN-2 strains isolated in the State of Rio de Janeiro previously demonstrated by Rico-Hesse (1990 *loc. cit.*) and Lewis et al. (1993 *loc. cit.*). The circulation of the same genotype in all areas studied demonstrated the dispersion of DEN-2 virus from Rio de Janeiro to the other states of the country. The subtype III has been referred to have a greater potential to cause severe disease causing concern in those areas in which high rates of antibody to DEN-1 and DEN-4 viruses predispose populations to severe disease (Vorndam et al. 1994 *loc. cit.*).

In Brazil, the increasing incidence of DHF/

DSS was associated with the introduction of the DEN-2 viruses in the states of Rio de Janeiro, Ceará and recently in the State of Rio Grande do Norte (RMR Nogueira et al. 1993 *Epidemiol Infect* 111: 163-170, RV Souza et al. 1995 *Mem Inst Oswaldo Cruz* 90: 345-346, PFC Vasconcelos et al. 1995 *Rev Inst Med Trop São Paulo* 37: 253-255, SMO Zagne et al. 1994 *Trans R Soc Trop Med Hyg* 88: 677-679) after a period of high DEN-1 virus activity. In the states of Bahia and Espírito Santo, where DEN-2 virus was responsible initially for primary infections, signs and symptoms of classic dengue fever were observed (RMR Nogueira et al. 1995 *Rev Inst Med Trop São Paulo* 37: 507-510). In those states a higher percentage of exanthema and pruritus were observed when compared with signs and symptoms due to DEN-1 primary infection during 1986 in Rio de Janeiro.

Recently, R Rico-Hesse et al. (1997 *Virology* 230: 1-8) demonstrated the direct association between the introduction of southeast Asian DEN-2 viruses severe disease in America and showed a circulation of a new subtype responsible for DHF epidemics in Mexico and Venezuela, in 1995. This data point out the need to continue molecular epidemiological studies in dengue endemic areas in order to monitor the introduction of a new subtype and the impact of it over the population.

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Phylogram generated by parsimony analysis of nucleic acid sequences from the prM/M and E genes of 12 DEN-2 Brazilian strains and 24 DEN-2 viruses obtained from Genebank.