Molecular epidemiology of dengue viruses in Brazil

Epidemiologia molecular dos vírus dengue no Brasil

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Abstract *Dengue viruses (DEN) are found as four antigenically distinct serotypes designated DEN-1, 2, 3, and 4. Laboratory evidence that strain-intratypical variation occurs among DEN viruses has been demonstrated since the 1970s, although only with the advances in molecular technologies has it been possible to determine the genetic variability of each serotype. Genotypical identification has proven to be a useful tool for determining the origin and spread of epidemics and to correlate virulence of strains. In this report we present the results of molecular epidemiological studies with the DEN-1 and DEN-2 viruses that caused dengue epidemics in Brazil during the last decade.*

Key words *Dengue Viruses; Dengue; Molecular Epidemiology*

Resumo *Os vírus dengue (DEN) apresentam propriedades antigênicas distintas que caracterizam quatro sorotipos denominados DEN-1, 2, 3 e 4. Desde a década de 70, evidências laboratoriais têm demonstrado a ocorrência de variação intratípica entre os vírus DEN; entretanto, somente com o avanço das metodologias moleculares foi possível estabelecer variantes genéticas para cada sorotipo. A identificação genotípica tem sido uma importante abordagem para determinar a origem e a dispersão de epidemias e para tentar estabelecer correlação de virulência entre as variantes dos vírus DEN. Neste trabalho, apresentamos resultados obtidos através de estudos de epidemiologia molecular realizados com amostras de vírus DEN-1 e DEN-2, que causaram epidemias no Brasil, na última década.*

Palavras-chave *Vírus Dengue; Dengue; Epidemiologia Molecular*

The development of new techniques in molecular biology has meant a huge step in various fields of science and technology, including that of health. The possibility of precisely determining the composition of microorganisms' genomes has created new prospects for epidemiological studies, allowing for the molecular characterization of circulating viral samples and knowledge of their geographic distribution.

The molecular epidemiology of dengue viruses (DEN) has been used to determine the origin of the viruses that have caused outbreaks and epidemics, especially in the attempt to establish a correlation between the virulence of samples and the impact of these viruses on the population.

The DEN viruses belong to the *Flaviviridae* family (Westaway et al., 1985) and the *Flavivirus* genus, which includes 68 species in 8 serologicallly related groups (4 transmitted by mosquitoes, 2 by ticks, and 2 still without known vectors) and a group of viruses that is not classified within these serogroups by neutralization, including the yellow fever virus. They display different antigenic properties that characterize the 4 serotypes called the DEN-1, DEN-2, DEN-3, and DEN-4 viruses (Sabin, 1952; Hammon et al., 1960). They are spherical, enveloped viruses, with a diameter of approximately 40-50 nm and a genome consisting of single-strand RNA with a positive polarity of some 11 kb. The viral RNA is surrounded by a nucleocapsid with an icosahedral symmetry, made up of a single so-called C protein, surrounded by a double lipid layer associated with membrane (M) and envelope (E) proteins. Protein E is the principal structural protein and is directly related to immunity and probably the virulence of the samples. These viruses have seven other non-structural proteins that are related to viral replication (Brinton, 1986; Deubel et al., 1993).

Broad dispersal of the mosquito vector *Aedes aegypti* made dengue the most important human arbovirus disease in the world, with approximately 2.5 billion individuals exposed to risk of infection in some 100 countries with tropical and subtropical climates (Knudsen, 1996).

Infection with any of the serotypes leads to a febrile illness known as dengue fever (classic dengue). The severe form, characterized by the appearance of hemorrhage and/or hypovolemic shock, is termed hemorrhagic dengue (DHF) or dengue shock syndrome (DSS) and occurs in some 0.5% of cases (OMS, 1987). Worldwide estimates suggest 100 million cases of dengue and hundreds of thousands of DHF per year, depending on epidemic activity. From 1981 to 1997, 24 countries in the Americas reported laboratory-confirmed cases of DHF (Gubler, 1998). The case fatality rate for DHF/DSS in the Americas is 1.4%, although wide variation (1 to 11.9%) has been reported from one country to another (Pinheiro & Chuit, 1998).

Infection with one serotype confers partial and temporary protection against the other serotypes, and secondary infection is possible after a relatively short period of time (OMS, 1987).

Prior infection with a given serotype is considered an important risk factor for the development of DHF/DSS, although the occurrence of primary DHF/DSS cases suggests that viral virulence may also be responsible for the variation in the disease's clinical expression (Halstead, 1970; Rosen, 1977; Thein et al., 1997). Individual, epidemiological, and viral risk factors are currently being explored in relation to the development of DHF/DSS (Kouri et al., 1987).

Antigenic and genetic diversity of dengue viruses

Intratypical variation in the dengue viruses was initially studied by means of serological techniques that demonstrated antigenic and biological differences between samples from the same serotype (McCloud et al., 1971; Russel & McCown, 1972).

Other methodologies, like antigenic analysis using a panel of monoclonal antibodies (Monath et al., 1986), cDNA-RNA hybridization (Block et al, 1984; Block, 1985), hybridization using synthetic peptides (Kerschener et al., 1986), and restriction endonuclease analysis of RT-PCR products (Vorndam et al., 1994), demonstrated the antigenic and genetic variability among the dengue viruses.

Establishing a "fingerprinting" pattern for each dengue serotype (Vezza et al., 1980) allowed for the molecular analysis of genetic variants within each serotype (Repik et al., 1983; Trent et al., 1983, 1989). In 1990, the term "topotype" was used to define genetic variants displaying homology in at least 70% of the larger oligonucleotides, representing samples from the same geographic region (Trent et al., 1990).

Subsequently, the viral genome sequencing technique replaced the so-called fingerprinting studies, since it allowed for greater discrimination in the analysis of genetic relations among samples. Sequencing of 240 nucleotides in the E/NS1 region, performed by Rico-Hesse

(1990), characterized five genomic groups for the DEN-1 viruses and five groups for the DEN-2 viruses. Complete sequencing of the gene coding for protein E in the DEN-2 virus (Lewis et al., 1993) established five subtypes corresponding essentially to those suggested by Rico-Hesse (1990). Partial sequencing of different samples of DEN-3 and DEN-4 virus demonstrated the existence of four and two genetic subtypes, respectively (Lanciotti et al., 1994; 1997).

Dengue viruses in Brazil

Reinfestation of Brazil by *Aedes aegypti* in 1977, the DEN-1 virus pandemic, and the introduction of the DEN-4 virus into the Americas marked the reintroduction of the DEN viruses into Brazil. In 1981, the first samples of DEN-1 and DEN-4 viruses were isolated in an outbreak in Boa Vista, Roraima (Osanai et al., 1983). However, it was only from 1986 onward that dengue became a nationwide public health problem, with the introduction of DEN-1 virus into the State of Rio de Janeiro and its subsequent spread to various other States of the country (Schatzmayr et al., 1986; Figueiredo, 1996).

The situation was aggravated in 1990 by the introduction of DEN-2 virus into the State of Rio de Janeiro (Nogueira et al., 1990). Difficulty in implementing an effective nationwide vector control program resulted in the rapid spread of the virus and consequently the occurrence of epidemics in various States. Currently, 22 of the 26 Brazilian States have reported dengue epidemics, totaling some 1,513,784 cases; from its introduction in 1986 until the 35th epidemiological week of 1999, simultaneous circulation of the DEN-1 and DEN-2 viruses was confirmed by laboratory analysis with viral isolation in 16 States. In 1998, Brazil accounted for some 85% of the dengue cases reported in the Americas.

Molecular epidemiology of dengue viruses in Brazil

The growing activity of DEN viruses in Brazil in the 1980s led to the establishment of a National Dengue Diagnosis Network (Schatzmayr et al., 1996), with the implementation of primary diagnostic methods, including the specific antibody capture immunoenzymatic test (Mac-Elisa), routine serology, and the use of *Aedes albopictus* clone C6/36 cell line and monoclonal antibodies for viral isolation (Igarashi, 1978; Gubler et al., 1984).

Widespread application of these methodologies, using sera from patients, produced hundreds of samples of the DEN-1 and DEN-2 viruses, the only ones circulating in the country in the last 12 years (Miagostovich et al., 1993; Nogueira et al., 1993; 1999). During this period, the Flavivirus Laboratory of the Oswaldo Cruz Institute, through a molecular epidemiology program, established different methodologies for genetic analysis of these samples.

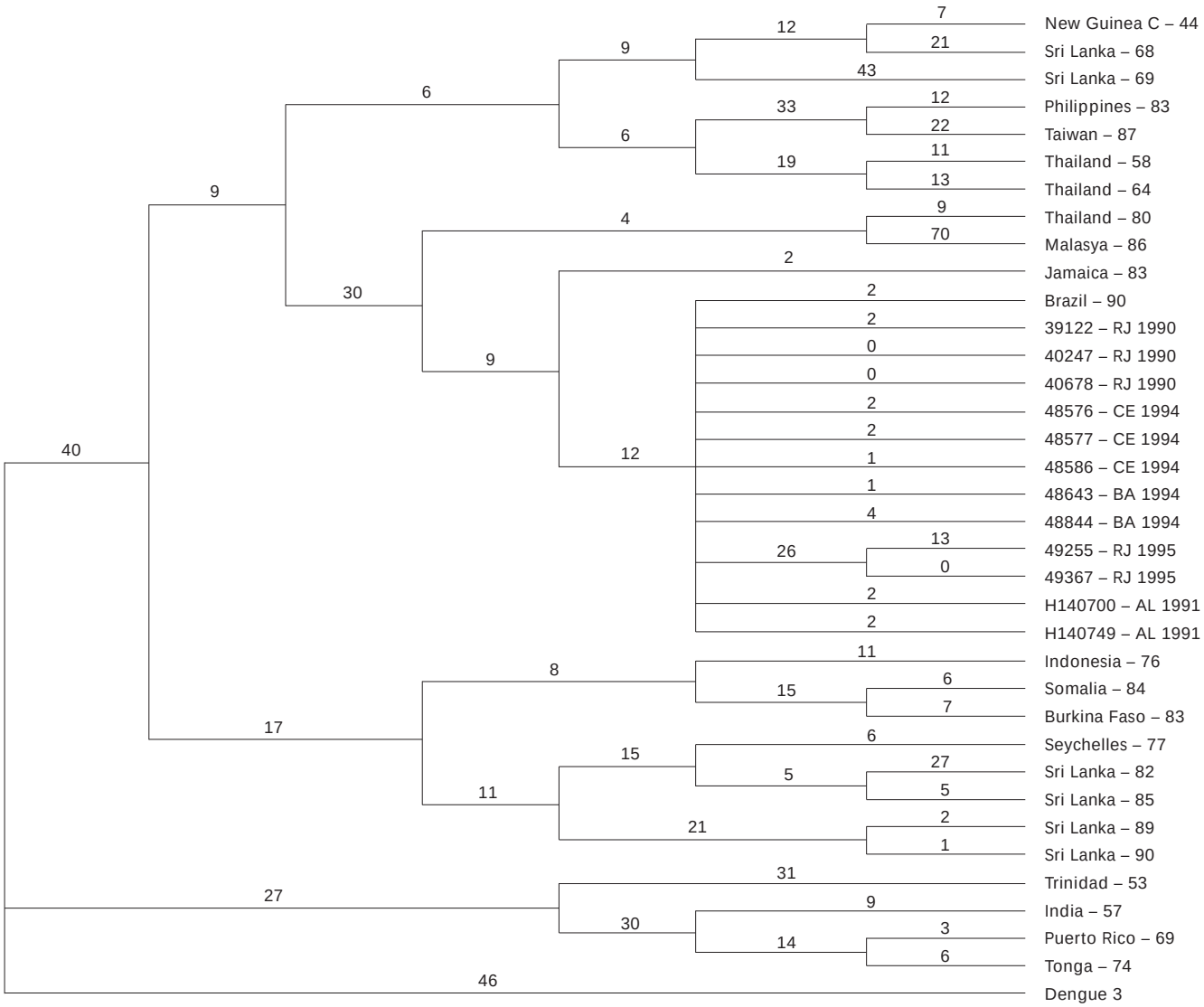
Analysis was initially performed on genome fragments from DEN-1 and DEN-2 viruses isolated in the State of Rio de Janeiro, using restriction endonuclease on the Hae III enzyme. This investigation was directly applicable to the determination of circulating viruses, identifying the Caribbean and Jamaica genotypes for the DEN-1 and DEN-2 viruses, respectively (Vorndam et al., 1994). These results were confirmed by the partial sequencing of a fragment of the gene coding for the envelope (E) protein, between nucleotides (nts) 85 and 282, after extension and amplification by reverse transcription followed by the polymerase chain reaction (RT-PCR) (Deubel et al., 1993; Chungue et al., 1995). Comparison of the DEN-2 viruses isolated in Rio de Janeiro (two samples obtained from classic dengue cases and one sample isolated from a fatal case) showed the same sequence of nucleotides, hence with no identification of markers for virulence in the region studied (Deubel et al., 1993).

A second phase included the sequencing of the region encompassed between nts 1685 and 2504 in the protein E gene from samples of DEN-2 virus isolated in the States of Rio de Janeiro (RJ), Ceará (CE), Bahia (BA), and Alagoas (AL), in order to investigate the genotypes circulating in Brazil in 1990-1995. Analysis of these results, shown in Figure 1, confirmed the origin of the DEN-2 viruses circulating in Brazil and identified the presence of only one genotype (Jamaica) as of 1995, demonstrating the spread of this virus from Rio de Janeiro to other States. There was also great uniformity among our samples, which underwent few modifications over the course of the years in which they circulated in the country. The samples isolated in Rio de Janeiro in 1995 were the only ones to present a larger number of alterations in the nucleic acid bases, reflecting the evolution of the DEN-2 viruses since their introduction into the State (Miagostovich et al., 1998).

The Jamaica genotype consisted of a set of samples known by this name, since the group includes a sample isolated in that country. This variant, of Asian origin, was isolated in the Americas during an extensive outbreak affect-

Figure 1

Phylogram generated by partial analysis of the nucleic acid sequence from a fragment of gene E from 12 Brazilian samples of DEN-2 virus and 24 obtained from Genebank.



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ing various areas of the Caribbean in 1981. This confirms that the DEN-1 and DEN-2 samples circulating in Brazil originated in the Caribbean, reaching the country through infected persons or vectors (Rico-Hesse, 1990).

The Jamaica genotype represents samples with a high potential for causing severe disease, especially in areas where the DEN-1 and DEN-4 viruses circulated previously (Vorndam et al., 1994). Its introduction into the Americas resulted in both an increase in the severe forms of the disease and various epidemics (Venezuela, 1989; Brazil, 1990-91) in which there was a significant number of DHF/DSS cases. Until the 1980s, cases of DHF/DSS in the Americas were sporadic and associated with the Puerto Rico genotype, isolated for the first time in 1953 in Trinidad and responsible

for extensive epidemics in the 1960s and 70s (Gubler, 1992).

The genomic analysis recently performed by Rico-Hesse et al. (1997) proved the existence of a new variant of the DEN-2 virus in the Americas, related to samples circulating in Asia and highly virulent when involved in secondary infections. Circulation of this variant in 1994, 1995, and 1996, especially in Venezuela, resulted in a large number of DHF/DSS cases and high case fatality.

In Brazil, infections by DEN-2 viruses presented different clinical patterns, principally in relation to the severity of the disease. In regions where the DEN-2 virus accounted for primary type infections, as in the States of Bahia and Espírito Santo (Nogueira et al., 1995), the clinical picture was typical of classic dengue, with frequent exanthem, pruritis, and few severe cases. However, in other States, where the DEN-2 virus circulated after extensive epidemics caused by DEN-1, as in Rio de Janeiro, Ceará, Pernambuco, and Rio Grande do Norte, an increase in the number of severe cases was observed. Beginning with the introduction of DEN-2 virus in 1990, a total of 754 DHF/DSS cases were reported, with 34 deaths, as of the 39th epidemiological week, and it is believed that there may have more cases that were not officially confirmed (Nogueira et al., 1990; Zagne et al., 1994; Souza et al., 1995; Vasconcelos et al., 1995).

The above-mentioned data demonstrate the importance of on-going monitoring of samples of dengue viruses circulating in the country in order to identify the entry of a new serotype or genotype and a potential association between genotype and virulence, in addition to defining the pattern of endemicity in a given area. The increase in air travel has fostered a spatial shifting of individuals during the viremic phase, facilitating the introduction of new viruses and/or genotypes. Molecular characterization can identify the simultaneous circulation of antigenically similar (but genetically distinct) samples and follow their spread separately.

Knowledge of samples circulating in a region also has implications for the potential introduction of vaccines, making it possible to evaluate the genomic relations between the vaccinal virus and these samples. The same is true for the development of serological diagnostic tests, which should include viral samples identical or close to those circulating in the region.

Through genomic analysis, it is possible to reconstitute the probable evolution of the virus. For the DEN viruses, variants were estimated for the subtypes that emerged in the last 200 years and were related to population growth and conditions of ecological imbalance (Zanotto et al., 1996).

Given the limited options for the prevention and control of dengue epidemics, due not only to the lack of a tetravalent vaccine for large-scale application but also the difficulties in eradicating the mosquito vector, molecular epidemiology has become an indispensable tool for identifying more virulent genetic variants which can potentially cause more severe forms of the disease.

Acknowledgments

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Oswaldo Cruz (FIOCRUZ), Coordenação do Sistema Nacional de Laboratórios de Saúde Pública do Ministério da Saúde, International Development Research Centre/Canada, and Fundação Banco do Brasil for their financial support and the Centers for Disease Control and Prevention (CDC) in Puerto Rico and Fort Collins, USA, and the Pasteur Institute in Paris for their research collaboration in the molecular characterization of samples.

References

BLOCK, J., 1985. Genetic relationship of the dengue virus serotypes. *Journal of General Virology*, 66: 1323-1325.

BLOCK, J.; HENCHAL, E. A. & GORMAN, B. M., 1984. Comparison of dengue viruses and some other flaviviruses by cDNA/RNA hybridization analysis and detection of a close relationship between dengue virus serotype 2 and Edge Hill viruses. *Journal of General Virology*, 65:2173-2181.

BRINTON, M. A., 1986. Replication of flavivirus. In: *The Togaviridae and Flaviviridae* (S. Schlesinger & M. Schlesinger, eds.), pp. 327-365, New York: Plenum Press.

CHUNGUE, E.; CASSAR, O.; DROUET, M. T.; GUZMAN, M. G.; LAILLE, M.; ROSEN, L. & DEUBEL, V., 1995. Molecular epidemiology of dengue-1 and dengue-4 viruses. *Journal of General Virology*, 76:1877-1884.

DEUBEL, V.; NOGUEIRA, R. M. R.; DROUET, M. T.; ZELLER, M.; REYNES, J. & MA, D. Q., 1993. Direct sequencing of genomic cDNA fragment amplified by the polymerase chain reaction for molecular epidemiology of dengue 2 viruses. *Archives of Virology*, 129:197-210.

FIGUEIREDO, L. T. M., 1996. Dengue in Brazil I: History, epidemiology and research. *Virus Review and Research*, 1:9-16.

GUBLER, D. J., 1992. Dengue/dengue hemorrhagic fever in the Americas: Prospects for the year 2000. In: *Dengue, a Worldwide Problem, a Common Strategy. Proceedings of the International Conference on Dengue and* Aedes aegypti *Community-based Control* (S. B. Hasltead & H. Gomez-Dantes, eds.), pp. 19-27, Mexico City: Mexican Ministry of Health.

GUBLER, D. J., 1998. Dengue and dengue hemorrhagic fever. *Clinical Microbiology Reviews*, 11:480-496.

GUBLER, D. J.; KUNO, G.; SATHER, G. E.; VELEZ, M. & OLIVER, A., 1984. Use of mosquito cell cultures and specific monoclonal antibodies in surveillance for dengue viruses. *American Journal of Tropical Medicine and Hygiene*, 33:153-165.

HALSTEAD, S. B., 1970. Observations related to pathogenesis of dengue hemorrhagic fever. VI. Hypotheses and discussion. *Yale Journal of Biology and Medicine*, 42: 350-362.

HAMMON, W. McD.; RUDNICK, A. & SATHER, G. E., 1960. Viruses associated with epidemic hemorrhagic fever of the Philippines and Thailand. *Science*, 31:1102-1103.

IGARASHI, A., 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. *Journal of General Virology*, 40: 531-544.

KERSCHNER, J. H.; VORNDAM, V.; MONATH, T. P. & TRENT, D. W., 1986. Genetic and epidemiological studies of dengue type 2 viruses by hybridization using synthetic deoxyoligonucleotides as probes. *Journal of General Virology*, 69:2645-2661.

KNUDSEN, A. B., 1996. Global strategy for the prevention and control of dengue and dengue hemorrhagic fever. In: International Seminar on Dengue. 1st Dengue Rio, *Abstracts*, p. 27, Rio de Janeiro: Fundação Oswaldo Cruz.

KOURI, G.; GUZMAN, M. G. & BRAVO, J., 1987. Why dengue hemorrhagic fever in Cuba? II. An integral analysis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 81:821-824.

LANCIOTTI, R. S.; GUBLER, D. J. & TRENT, D. W., 1997. Molecular evolution and phylogeny of dengue-4 viruses. *Journal of General Virology*, 78: 2279-2286.

LANCIOTTI, R. S.; LEWIS, J. G.; GUBLER, D. J. & TRENT, D. W., 1994. Molecular evolution and epidemiology of dengue-3 viruses. *Journal of General Virology*, 75:65-75.

LEWIS, J. A.; CHANG, G. J.; LANCIOTTI, R. S.; KINNEY, R. M.; MAYER, L. W. & TRENT, D. T., 1993. Phylogenetic relationship of dengue – 2 viruses. *Virology*, 197:216-224.

McCLOUD, T. G.; CARDIFF, R. D.; BRANDT, W. E.; CHEWSILP, D. & RUSSEL, P. K., 1971. Separation of dengue strains on the basis of a nonstructural antigen. *American Journal of Tropical Medicine and Hygiene*, 20:964-968.

MIAGOSTOVICH, M. P.; NOGUEIRA, R. M. R.; SCHATZMAYR, H. G. & LANCIOTTI, R. S., 1998. Molecular epidemiology of Den-2 virus in Brazil. *Memórias do Instituto Oswaldo Cruz*, 93:625-626.

MIAGOSTOVICH, M. P.; NOGUEIRA, R. M. R.; CAVALCANTE, S. M. B.; MARZOCHI, K. B. F. & SCHATZMAYR, H. G., 1993. Dengue epidemic in the State of Rio de Janeiro, Brazil: Virological and epidemiological aspects. *Revista do Instituto de Medicina Tropical de São Paulo* 35:149-154.

MONATH, T. P.; WANDS, J. R.; HILL, L. J.; BROWN, N. V.; MARCINIAR, R. A.; WONG, M. A.; GENTRY, M. K.; BURKE, D. S.; GRANT, J. A. & TRENT, D.W., 1986. Geographic classification of dengue 2 virus strains by antigen signature analysis. *Virology*, 154:313-324.

NOGUEIRA, R. M. R.; MIAGOSTOVICH, M. P.; LAMPE, E. & SCHATZMAYR, H. G., 1990. Isolation of dengue virus type 2 in Rio de Janeiro. *Memórias do Instituto Oswaldo Cruz*, 85:253.

NOGUEIRA, R. M. R.; MIAGOSTOVICH, M. P.; LAMPE, E.; SOUZA, R. W.; ZAGNE, S. M. O. & SCHATZMAYR, H. G., 1993. Dengue epidemic in the state of Rio de Janeiro, Brazil, 1990-1991: Co-circulation of dengue 1 and dengue 2. *Epidemiology and Infection*, 111:163-170.

NOGUEIRA, R. M. R.; MIAGOSTOVICH, M. P.; SCHATZMAYR, H. G.; MORAES, G. C.; CARDOSO, F. M. A.; FERREIRA, J.; CERQUEIRA, V. & PEREIRA, M., 1995. Dengue type 2 outbreak in the south of the state of Bahia, Brazil: Laboratorial and epidemiological studies. *Revista do Instituto de Medicina Tropical de São Paulo*, 37:507-510.

NOGUEIRA, R. M. R.; MIAGOSTOVICH, M. P.; SCHATZMAYR, H. G.; ARAÚJO, E. S. M.; SANTOS, F. B.; FILIPPIS, A. M. B.; SOUZA, R. W.; ZAGNE, S. M. O.; NICOLAI, C.; BARAN, M. & TEIXEIRA FILHO, G., 1999. Dengue in the State of Rio de Janeiro, Brazil, 1986-1998. *Memórias do Instituto Oswaldo Cruz*, 94: 297-304.

OMS (Organização Mundial da Saúde), 1987. *Dengue Hemorrágico. Diagnóstico, Tratamento e Controle*. Genebra: OMS.

OSANAI, C. H.; TRAVASSOS DA ROSA, A. P. A. & TANG, A. T., 1983. Surto de dengue em Boa Vista em Roraima. *Revista do Instituto de Medicina Tropical de São Paulo*, 25:53-54.

PINHEIRO, F. P. & CHUIT, R.,1998. Emergence of dengue hemorrhagic fever in the Americas. *Infections in Medicine*, 15:244-251.

REPIK, P. M.; DALRYMPLE, J. M.; BRANDT, W. E.; McCOWN, J. M. & RUSSEL, P. K., 1983. RNA fingerprinting as a method for distinguishing dengue virus 1 strains. *American Journal of Tropical Medicine and Hygiene*, 32:577-589.

RICO-HESSE, R., 1990. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology*, 174:479-493.

RICO-HESSE, R.; HARRISON, L. M.; SALAS, R. A.; TOVAR, D.; NISALAK, A.; RAMOS, C.; BOSHELL, J.; MESA, M. T. R.; NOGUEIRA, R. M. R. & TRAVASSOS DA ROSA, A., 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. *Virology*, 230:652-658.

ROSEN, L., 1977. The emperor's new clothes revisited or reflections on the pathogenesis of dengue fever. *American Journal of Tropical Medicine and Hygiene*, 26: 337-343.

RUSSEL, P. K. & McCOWN, J. M., 1972. Comparison of dengue 2 and dengue 3 virus strains by neutralization tests and identification of a serotype of dengue 3. *American Journal of Tropical Medicine and Hygiene*, 21:97-99.

SABIN, A. B., 1952. Research on dengue during World War II. *American Journal of Tropical Medicine and Hygiene*, 1:30-50.

SCHATZMAYR, H. G.; NOGUEIRA, R. M. R. & MIAGOSTOVICH, M. P., 1996. Dengue in Brazil: Laboratory aspects and perspectives. *Virus Reviews and Research*, 1:17-21.

SCHATZMAYR, H. G.; NOGUEIRA, R. M. R.; TRAVASSOS DA ROSA, A. P. A., 1986. An outbreak of dengue virus at Rio de Janeiro – 1986. *Memórias do Instituto Oswaldo Cruz*, 81: 245-246.

SOUZA, R. V.; CUNHA, R. V.; MIAGOSTOVICH, M. P.; TIMBÓ, M. J.; MONTENEGRO, F.; PESSOA, E. T. F. P.; NOGUEIRA, R. M. R. & SCHATZMAYR, H. G., 1995. An outbreak of dengue virus in the State of Ceará, Brazil. *Memórias do Instituto Oswaldo Cruz*, 90:345-346.

THEIN, S.; AUNG, M. M.; SHWE, T. N.; AYE, M.; ZAW, A; AYE, K.; AYE, K. M. & AASKOV, J.,1997. Risk factors in dengue shock syndrome. *American Journal of Tropical Medicine and Hygiene*, 56:666-672.

TRENT, D. W.; GRANT, J. A.; MONATH, T. P.; MANSKE, C. L.; CORINA, M. & FOX, G. E., 1989. Genetic variation and microevolution of dengue 2 virus in Southeast Asia. *Virology*, 172:523-535.

TRENT, D. W.; GRANT, J. A.; ROSEN, L. & MONATH, T. P., 1983. Genetic variation among dengue 2 viruses of different geographic origin. *Virology*, 128: 271-284.

TRENT, D. W.; MANSKE, C. L.; FOX, G. E.; CHU, M. C.; KLIKS, S. C. & MONATH, T. P., 1990. The molecular epidemiology of dengue viruses: Genetic variation and microevolution. In: *Applied Virology Research, vol 2. Virus Variation and Epidemiology* (E. Kurstak, R. G. Marusky & M. H. V. Regenmortel, eds.), pp. 293-315, New York: Plenum Press.

VASCONCELOS, P. F. C.; MENEZES, D. B.; MELLO, L. P.; PESSOA, E. T. F. P.; RODRIGUES, S. G.; TRAVASSOS DA ROSA, J. F. J.; ANDRADE, F. M. & TRAVASSOS DA ROSA, A. P. A., 1995. A large epidemic of dengue fever with dengue hemorrhagic case in Ceará state, Brazil. *Revista do Instituto de Medicina Tropical de São Paulo*, 37:253-255.

VEZZA, A.; ROSEN, L.; REPIK, P.; DALRYMPLE, J. M. & BISHOP, D. H. L., 1980. Characterization of the viral RNA species of prototype dengue viruses. *American Journal of Tropical Medicine and Hygiene*, 29: 643-652.

VORNDAM, V.; NOGUEIRA, R. M. R. & TRENT, D. W., 1994. Restriction enzyme analysis of American region dengue viruses. *Archives of Virology*, 136: 191-196.

WESTAWAY, E. G.; BRINTON, M. A.; GAIDAMOVICH, S. Y.; HORZINEK, M. C.; IGARASHI, A.; KAARIAINEN, L.; LVOV, D. K.; PORTERFIELD, J. E.; RUSSELL, P. K. & TRENT, D. W., 1985. Flaviviridae. *Intervirology*, 24:183-192.

ZAGNE, S. M. O.; ALVES, V. G. F.; NOGUEIRA, R. M. R.; MIAGOSTOVICH, M. P.; LAMPE, E. & TAVARES, W., 1994. Dengue hemorrhagic fever in the state of Rio de Janeiro, Brazil: A study of 56 confirmed cases. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88:677-679.

ZANOTTO, P. M. A.; GOULD, E. A.; GAO, G. F.; HARVEY, P. H. & HOLMES, E. C., 1996. Population dynamics of flavivirus revealed by molecular phylogenies. *Proceedings of the National Academy of Sciences*, 93:548-553.