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ESTUDO DE VARIÁVEIS VIRAIS, CLÍNICAS E IMUNOLÓGICAS ASSOCIADAS AO DENGUE VÍRUS TIPO 2 CIRCULANTE NO BRASIL

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Rio de Janeiro

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NIELI RODRIGUES DA COSTA FARIA

Estudo de variáveis virais, clínicas e imunológicas associadas ao Dengue vírus tipo 2 circulante no Brasil

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para a obtenção do título de Doutor em Medicina Tropical.

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À Niete minha irmã,

À Laryssa, minha sobrinha,

e ao meu bebê

Com amor.

“Faça do Senhor a sua grande alegria
e Ele dará a você os desejos do seu coração”
Salmo 37:4

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Siglas e Abreviaturas

3’NC	Região 3’ não-codificante
5’NC	Região 5’ não-codificante
aa	Aminoácido
ALT	Alanina aminotransferase/do inglês aminotransferase de alanine
AST	Aspartato aminotransferase/do inglês aspartate transaminase
APC	Células apresentadoras de antígeno/ do Inglês antigen-presenting cell
C	Proteína estrutural do capsídeo do vírus
CCL2/MCP-1	Ligante 2 de CC quimiocina/proteína quimiotática de monócitos-1
CCL3/MIP-1α	Ligante 3 de CC quimiocina/proteína 1α inflamatória de macrófagos
CCL5/RANTES	Ligante 5 de CC quimiocina/regulada sob ativação expressa e secretada por células T normais
CD 1a	Cluster de diferenciação 1a
CD 14	Cluster de diferenciação 14
CD 38	Cluster de diferenciação 38
CD 40	Cluster de diferenciação 40
CD 80	Cluster de diferenciação 80
CD 86	Cluster de diferenciação 86
CHIKV	Vírus Zika
DC	Dengue clássica
DCC	Dengue com complicações
DCs	Células dendríticas
DC-SIGN/CD 209	Molécula não integrina, captadora da molécula de adesão intracelular 3 específica de células dendríticas (do inglês DC-specific ICAM-grabbing nonintegrin)
DENCO	Controle da dengue/do Inglês dengue control
DENV	Vírus dengue
DENV-1	Sorotipo 1 do vírus dengue
DENV-2	Sorotipo 2 do vírus dengue

DENV-3	Sorotipo 3 do virus dengue
DENV-4	Sorotipo 4 do virus dengue
DSSA/DwoWS	Dengue sem sinais de alarme/Dengue without warning signals
DCSA/DwWS	Dengue com sinais de alarme/Dengue with warning signals
DG/SD	Dengue grave/Severe dengue
E	Proteína Estrutural do Envelope
ECP	Efeito citopático
E/NS1	Proteína estrutural do Envelope/Proteina não estrutural 1
ELISA	Ensaio imunoenzimático
Fem	Feminino
FD	Febre da dengue
FIOCRUZ:	Fundação Oswaldo Cruz
FHD	Febre hemorrágica da dengue
FUNASA	Fundação Nacional de Saúde
GRP78	Proteína regulada pela glicose-78 (do Inglês GRP78 – Glucose-regulated protein 78)
HI	Inibição de hemaglutinação/do Inglês hemagglutination inhibition
HSP	Proteoglicanos de heparano sulfato (*do inglês* HSP - *heparan sulphate proteoglycans*)
HSP 70/90	Proteína de choque térmico genes 70 e 90/do Inglês Heat Shock Proteins 70 and 90 genes
ICAM-3	do inglês Intercellular adhesion molecule 3
IFN-α	Interferon alfa
IFN-β	Interferon beta
IFN-γ	Interferon gama
IgG	Imunoglobulina G
IgM	Imunoglobulina M
IHA/ALF	Insuficiência hepática aguda/Acute liver failure
IL	Interleucina
IRF-7	Fator regulador de interferon 7/do Inglês Interferon regulatory factor 7
JAK/SAT	Janus quinase/ transdutores de sinal e ativadores de

	transcrição /do Inglês Janus kinase/signal transducers and activators of transcription
kb	Kilobase
MAC-ELISA	Ensaio imunoenzimático para detecção de anticorpos IgM
M	Proteína estrutural da Membrana
Masc	Masculino
MHC	complexo principal de histocompatibilidade /do Inglês major histocompatibility complex
MoDCs	Células dendríticas derivadas de monócitos
MV	Máxima verossimilhança *(do inglês* ML - *Maximum Likelehood)*
NS	Proteína Não estrutural do vírus
NS1	Proteína não estrutural 1
NS2A	Proteína não estrutural 2ª
NS2B	Proteína não estrutural 2B
NS3	Proteína não estrutural 3
NS4A	Proteína não estrutural 4ª
NS4B	Proteína não estrutural 4B
NS5	Proteína não estrutural 5
OMS	Organização Mundial de Saúde
ORF	do Inglês *Open reading frame* (Cadeia aberta para leitura)
OX 40L	
PAHO	Pan American Health Organization (Organização Panamericana da Saúde)
pb	Pares de bases
pH	Potencial hidrogeniônico
PCR	Reação em cadeia pela polimerase
PNCD	Programa Nacional de Controle da Dengue
prM/M	Proteínas estruturais Pré-membrana/Membrana
RE	Retículo endoplasmático
RNA	Ácido ribonucleico
RT-PCR	Transcrição reversa seguida da reação em cadeia pela polimerase
SCD	Síndrome de choque por dengue

SE	Semana epidemiológica
SINAN	Sistema de Informações de Agravos de Notificação
SVS	Secretária de Vigilância em Saúde
TNF	Fator de necrose tumoral/do Inglês Tumor necrosis factor
ZIKV	Vírus Zika

RESUMO

Atualmente a dengue é a arbovirose de maior importância mundial e um dos principais problemas de saúde pública no Brasil desde sua introdução na década de 1980. Dengue é uma doença febril aguda e geralmente de evolução benigna podendo evoluir para forma grave quando na presença de distúrbios hemodinâmicos, caracterizados por manifestações hemorrágicas graves, derrames cavitários e choque. Apesar do conhecimento crescente a cerca da doença e seu vetor, a evolução da dengue para quadros clínicos graves não está totalmente esclarecida. Os quatro sorotipos do vírus dengue (DENV) variam em termos de patogenicidade e virulência, embora essas diferenças ainda não sejam bem compreendidas. O entendimento dos mecanismos envolvidos na patogênese da doença e a identificação precoce de marcadores de gravidade constitui um dos principais desafios das pesquisas envolvendo dengue. Desta forma, o objetivo deste estudo foi de analisar os aspectos virais, epidemiológicos, clínicos e imunológicos de infecções causadas por DENV-2 circulantes no Brasil. Para isto, amostras de DENV-2 isoladas de pacientes com diferentes manifestações clínicas e representativas de seis estados brasileiros, de 1990 a 2010 foram sequenciadas. Avaliamos 260 casos confirmados de dengue atendidos nos centros de saúde dos estados do Rio de Janeiro (RJ) e Mato Grosso do Sul (MS) nos anos de 2010 e 2013. Os pacientes foram classificados de acordo com a nova classificação da OMS, 2009 nos grupos: 163 (62.7%) dengue sem sinais de alarme (DSSA), 75 (28,8%) dengue com sinais de alarme (DCSA) e 16 (6.2%) dengue grave (DG). Os resultados obtidos pelo sequenciamento parcial e / ou total do genoma caracterizaram as amostras de DENV-2 brasileiras como pertencentes ao genótipo do Sudeste Asiático e duas linhagens dentro deste genótipo foram identificadas. As cepas circulantes na introdução do DENV-2 1990-2003) pertencem à Linhagem I e cepas isoladas após a reintrodução do DENV-2 em 2007 pertencem à Linhagem II. A análise da carga viral das duas linhagens de DENV-2 demonstrou altos títulos em amostras originárias de pacientes graves e do período 2007-2010 (linhagem II) quando comparados com as amostras do período 1990-2003. Ainda, a análise comparativa de epidemias ocorridas no MS em dois períodos distintos (DENV1/2 em 2010 e DENV-4 em 2013) demonstrou que apesar da circulação dos 4 sorotipos de DENV no estado, a reintrodução do DENV-4 não resultou numa maior incidência de casos graves. Por outro lado, pacientes infectados pelo DENV1/2 (2010) apresentaram menores contagens de plaquetas, maiorfrequência de hospitalização e casos graves quando comparados com os pacientes infectados pelo DENV-4 (2013). A proteína não estrutural NS1 é frequentemente associada com casos graves de dengue e, de fato, altos níveis circulantes de NS1 foram encontrados em pacientes infectados pelos sorotipos DENV1/2 e apresentando formas mais graves da doença. Um caso fatal decorrente de hepatite fulminante, uma rara complicação da infecção pelo DENV, com diagnostico confirmado de infecção pelo DENV-2 (linhagem II) foi reportado em um paciente sem histórico prévio de doença hepática. Por fim, avaliamos a resposta imunológica inata *in vitro* induzida pelas duas linhagens de DENV-2 determinando as taxas de replicação viral e a indução de um perfil de citocinas após infecção de células dendríticas derivadas de monócitos humanos realizando análises comparativas. Os resultados demonstraram que ambas linhagens de DENV-2 foram capazes de infectar células dendriticas humanas e não foram observadas diferenças quanto a taxa de replicação viral ou produção de citocinas quando estas foram comparadas entre si. Interessantemente, sobrenadantes das células dendriticas infectadas com linhagem II apresentaram altos níveis da proteína NS1. Estes resultados em conjunto sugerem que a filogenia e caracterização molecular das cepas de DENV circulantes podem predizer o impacto de cepas virulentas na população. Além disso, a identificação de biomarcadores, relacionados tanto ao hospedeiro quanto ao vírus, poderá contribuir na prevenção das formas graves da doença.

ABSTRACT:

Currently dengue is the most important arbovirus of world and is the main public health problems in Brazil since its introduction in the 1980s. Dengue is an acute febrile illness usually benign and may progress to severe form in the presence of hemodynamic disturbances characterized by severe hemorrhagic manifestations, cavitary stroke and shock. Despite the increasing knowledge about the disease and its vector, the evolution of dengue to severe clinical forms is not fully understood. The four DENV serotypes vary in terms of pathogenicity and virulence, although these differences are poorly understood. Understanding mechanisms involved in the pathogenesis of disease as well as early markers of severity identification is one the main challenges of dengue research. In this context, the aim of this study was to analyze virus, epidemiological, clinical as well as immunological aspects of infections caused by DENV-2 serotypes circulating in Brazil. For this, DENV-2 strains isolated from patients with different clinical manifestations and representative of six Brazilian states, from 1990-2010 were sequenced. We evaluate 260 confimed dengue cases that were treated at health centers in Rio de Janeiro (RJ) and Mato Grosso do Sul (MS) states in 2010 and 2103. Patients were classified according to the new dengue classification WHO 2009 in the following groups: 163 (62.7%) dengue without warning signs, 75 (28,8%) dengue with warning signs, and 16 (6.2%) severe dengue. The results obtained by partial sequencing and / or total genome characterized samples of DENV-2 Brazilian as belonging to the genotype of Southeast Asia and two lineages within this genotype were identified. Strains circulating prior DENV-2 emergence (1990-2003) belong to Southeast Asian genotype, Lineage I and strains isolated after DENV-2 emergence in 2007 belong to Southeast Asian genotype, Lineage II. Analysis of viral loads of both DENV-2 lineages showed higher titers in samples originated from severe patients 2007-2010 (lineage II) when compared with from 1990-2003. Also, comparative analysis of epidemics occurred in MS during two different periods (DENV1 / 2 in 2010 and DENV-4 in 2013) showed that despite circulation of four serotypes in the state, the reintroduction of DENV-4 did not result in a higher incidence of severe cases. Moreover, DENV1/2 2010 infected patients showed lower platelets counts, higher frequency of hospitalization and severe cases as compared with patients infected with DENV-4 (2013). The nonstructural protein NS1 is often associated with severe cases of dengue and, indeed, high circulating levels of NS1 were found in DENV1/2 infected patients and those presenting more severe forms of disease. A fatal case evolving fulminant hepatitis associated with DENV-2 (lineage II), a rare complication of dengue infection, was reported in patient without a previous history of liver disease.. Finally, we evaluated the innate immune response induced by both DENV-2 lineages determining viral replication rates and the induction of a cytokine profile after infection of dendritic cells derived from human monocytes in vitro performing comparative analyses. Our results showed that both DENV-2 lineages were able to infect human dendritic cells and no difference was found in the viral replication ratio or in the cytokine production after comparative analysis. Interestingly, infected dendritic cells supernatants from lineage II exhibited high levels of NS1 protein. These results together suggest that the phylogeny and molecular characterization of circulating DENV strains can predict the impact of virulent strains in the population. Furthermore, the identification of biomarkers, related to both host and virus will contribute in the prevention of severe forms of disease.

1. INTRODUÇÃO

A dengue é uma doença viral aguda sistêmica que se estabeleceu globalmente através de transmissão endêmica e epidêmica. Endêmica em mais de 100 países nas Américas, Sudeste Asíatico, Oeste do Pacífico, África e as regiões do Mediterrâneo oriental, sua incidência aumentou 30 vezes nos últimos 50 anos (Guzman & Harris, 2015). Estima-se que ocorram 390 milhões de novas infecções por ano e que 96 milhões apresentem algum tipo de manifestação aparente (Bhatt *et al*., 2013). Os atuais esforços para conter a transmissão da dengue estão focados no combate ao vetor através da combinação de métodos químicos e biológicos (WHO/TDR, 2009).

O custo e as perdas econômicas com dengue têm crescido e estima-se que dois bilhões de dolares sejam gastos anulamente nas Américas e que cerca de 60% deste gasto correspondam aos custos indiretos, principalmente perda de produtividade. O Brasil é o país onde o custo da dengue é mais elevado nas Américas (42% da carga total). Com aproximadamente 1.35 bilhões de doláres anuais, gastos anualmente (Shepard *et al*., 2011).

A infecção pelos vírus dengue (DENV) em seres humanos na maioria das vezes apresenta–se de forma assintomática (Endy *et al*., 2011; Simmons *et al*., 2012), mas pode também apresentar uma ampla variedade de manifestações clínicas, desde uma febre branda até as formas graves potencialemte fatais conhecidas como Febre Hemorrágica do dengue/Síndrome de Choque por Dengue (FHD/SCD) (WHO/TDR, 2009). A imunidade vitalícia é desenvolvida após a infecção por um dos quatro sorotipos dos DENV que é específica para o sorotipo infectante (Simmons *et al*., 2012).

1.1. Histórico da dengue

Sintomas de uma doença febril compatível com a dengue foram descritos em uma Enciclopédia Chinesa de sintomas e remédios em 265 a 420 d.C. durante a Dinastia Chin e formalmente editados em 610 d.C. (Dinastia Tang). Posteriormente em 992 d.C. (Dinastia Norte Sung), esta doença que se apresentava com manifestações clínicas com febre, exantema, artralgia, mialgia e manifestações hemorrágicas, passou a ser reconhecida como "veneno da água" devido a associação dos insetos voadores com a água..

Porém, as primeiras epidemias bem documentadas ocorreram entre 1779 e 1780, nos continentes Asiático, Norte Americano e Africano (Gubler, 1997; 1998). O termo dengue foi introduzido na literatura médica inglêsa, entre 1827 e 1828, durante uma epidemia

ocorrida no Caribe onde as principais características clínicas observadas foram exantema e artralgia. Em 1869, foi estabelecido pelo “London Royal College of Physicians” a denominação desta enfermidade, até então referida na literatura como "febre articular", "febre quebra ossos", "dinga", "polka", entre outros (Halstead, 1974; Halstead, 1980).

No início da década de 1900, foi demonstrada que a transmissão dos DENV era realizada por mosquitos e estudos desenvolvidos por Bancroft em 1906 demonstraram que o vetor *Aedes aegypti* era capaz de transmitir os DENV (Gubler, 1997).

Durante a II Segunda Guerra Mundial pesquisadores japoneses (Hotta, 1952) e americanos (Sabin, 1952) isolaram as primeiras cepas dos DENV e, neste período, os sorotipos 1 e 2 foram identificados, seguidos pelos sorotipos 3 e 4 nas Filipinas e na Tailândia em 1954 (Hammon *et al*., 1960).

Após a II Guerra Mundial o DENV se dispersou mundialmente para áreas tropicas e subtropicais e a dengue passou a ser considerada uma das mais importantes doenças reemergentes (Yamada *et al.,* 2002). A distribuição global de todos os sorotipos dos DENV atingiu quase todos os continentes no fim da década de 1990, início do século 21. Este processo foi facilitado pela convergência gradual de diversos fatores, incluindo expansão das populações urbanas, o aumento da densidade vetorial por insustentáveis programas de controle e o aumento nas viagens aéreas comerciais (Vasilakis & Weaver, 2008).

Atualmente, todos os quatro sorotipos DENV circulam na África, Sul e Sudeste da Ásia, regiões do Pacífico Ocidental, na bacia do Caribe, e nas Américas Central e do Sul, com registro de frequentes introduções nos estados do sul da América do Norte embora, até momento, não existam relatos de surtos epidêmicos nos EUA (Rodriguez-Roche & Gould, 2013).

1.2 Epidemiologia da dengue

A dengue atualmente é a arbovirose de maior importância mundial, considerada um dos principais problemas de saúde pública. Nos últimos 50 anos a sua incidência aumentou 30 vezes. A dengue é endêmica em mais de 100 países nas Américas, Sudeste Asíatico, Oeste do Pacífico, África e as regiões do Mediterrêneo oriental (Guzman & Harris, 2015) (Figura 1.1). Estima-se que ocorram 390 milhões de novas infecções por ano e, destas, 96 milhões apresentam algum tipo de manifestação aparente (Bhatt *et al*., 2013).

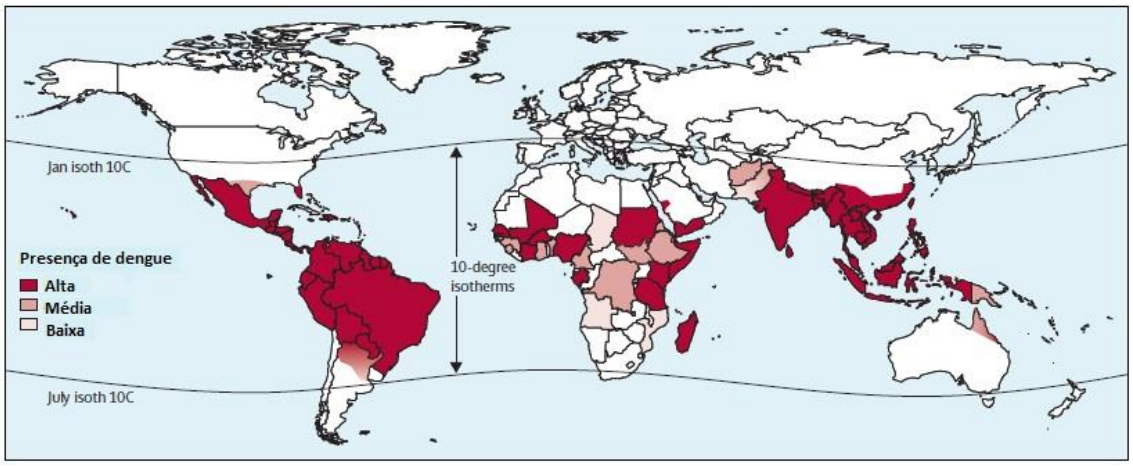

Figura 1.1: Distribuição global da dengue, 2014 (Adaptado de Guzmán and Harris, 2015).

1.3. Dengue nas Américas

Relatos de uma doença similar à dengue identificados em Martinica e Guadalupe e, posteriormente, no Panamá são considerados, oficialmente, os primeiros casos de dengue nas Américas no século XVII. No século XIX foram descritos inúmeros surtos, em intervalos irregulares, nas cidades portuárias do Caribe, Américas do Norte, Central e Sul (Brathwaite Dick *et al*., 2012).

Apesar de inúmeros surtos e epidemias de dengue descritos no final do século XIX e início do século XX, os sorotipos envolvidos só foram identificados em 1953, quando as primeiras amostras de DENV foram isoladas em Trinidad, o DENV-2 foi o primeiro sorotipo isolado nas Américas (Anderson *et al*, 1956). Dez anos depois, com o isolamento das primeiras amostras de DENV-3 em Porto Rico, as epidemias ocorridas no continente a Americano na década de 1960 passaram a ser causadas pelossorotipos 3 e 2 (Gubler,1992).

Nas décadas de 1940 e 1950 a Organização Pan-Americana de Saúde (OPAS) iniciou um programa de erradicação do mosquito vetor *A. aegypti* com o objetivo de prevenir epidemias urbanas de febre amarela o que resultou na diminuição significativa das epidemias de dengue nas Américas. No entanto, a descontinuidade do programa de erradicação no início dos anos 70, resultou na reinfestação deste vetor pelo continente (Schliesman & Calheiros, 1974; Pinheiro, 1989; Gubler, 1997). O DENV-1 foi introduzido em 1977 e foi responsável pelas epidemias ocorridas na Jamaica e Cuba e em 1978 pelas

epidemias em Porto Rico e Venezuela. Em 1981, o DENV-4 foi introduzido no leste das Ilhas do Caribe e se dispersou rapidamente para outras regiões, causando epidemias em regiões com recente epidemia pelo DENV-1 (Gubler, 1997; Guzman & Kouri, 2003). Neste mesmo ano, uma nova variante do DENV-2 de origem asiática foi introduzida no continente americano, causando em Cuba, a primeira epidemia de FHD/SCD das Américas (Kouri *et al.*, 1986; Pinheiro & Corber, 1997). Nesta epidemia foram notificados cerca de 344.000 casos, com aproximadamente 116.000 internações (Kouri *et al*., 1986).

Em 1994, o DENV-3 foi reintroduzido na Nicarágua e Panamá e, em 1995, no México. Esta variante de DENV-3 mostrou-se geneticamente distinta daquela que anteriormente circulava nas Américas (genótipo IV) e foi caracterizada como genótipo III. Este genótipo foi associado à ocorrência de epidemias de FHD/SCD no Sri Lanka e Índia e casos de FHD no México e nos países da América Central (Lanciotti *et al*, 1994; Gubler, 1997; Gubler & Meltzer, 1999, Brathwaite Dick *et al*., 2012). Nos anos seguintes, o DENV-3 foi detectado em outros países do continente, chegando à América do Sul (Pinheiro *et al*, 1997; Rigau-Perez *et al*, 2002).

Sucessivas epidemias com aumento no número de notificação de casos e da gravidade da doença começaram a ser registradas no final dos anos 90 e início dos anos 2000. Em 2002 um número recorde de 1.015,420 casos foi registrado, com 14.374 casos de FHD e 255 óbitos (Brathwaite Dick *et al*., 2012) com mais de 75% do número total de casos registrados no território brasileiro (Nogueira *et al*., 2002).

Em 2008 foram registrados nas Américas 908.926 casos de dengue, com 25.696 casos de FHD e 306 óbitos. No Brasil a reemergência do DENV-2 causou uma epidemia com mais de 700 mil casos suspeitos, destes, 9.957 casos de FHD e 212 óbitos, até a semana epidemiológica 35 (PAHO, 2009).

Em 2010 mais de 1,7 milhões de casos foram notificados, com 50.235 casos graves, 1.185 mortes e uma uma elevada incidência de 200 casos / 100 mil habitantes países. A taxa de letalidade de dengue nas Américas naquele ano foi de 2,6%. Vários países da região sofreram surtos de dengue excedendo o número total de casos já registrados, incluindo a introdução de dengue em áreas sem relato de casos, como em Key West, Florida (Brathwaite Dick *et al*., 2012).

Durante o ano de 2013, 2.386,836 casos de dengue foram registrados nas Américas, com um total de 37.903 casos de dengue grave e 1.318 casos fatais, em um período de

cocirculação dos quatro sorotipos em diversos países das Américas. (PAHO, 2013). Um ano depois, em 2014, foram notificados 1.176,529 casos de dengue com 16.238 casos graves e 798 óbitos (PAHO, 2014). Em 2015, até a 52ª semana epidemiológica, 2.326,829 casos de dengue foram notificados nas Américas, com 10.276 casos de dengue grave e 1.181 óbitos e mais uma vez o Brasil foi responsável pelo o maior número de casos notificados no continente americano (71%) (PAHO, 2015).

1.4. Dengue no Brasil

Os primeiros surtos de dengue foram descritos no município do Rio de Janeiro (RJ), em 1864, na cidade de Curitiba e no estado do Rio Grande do Sul (RS) e em 1917 e,posteriormente, nos anos de 1922 e 1923 novamente a cidade do Rio de Janeiro (Figueiredo, 1998; Figueiredo, 2000).

A primeira identificação dos sorotipos de dengue, responsáveis por epidemias ocorreu em 1981, na cidade de Boa Vista em Roraima (RR), quando 7.000 casos da doença foram notificados e as primeiras amostras de DENV-1 e DENV-4 foram isoladas (Osanai *et al*., 1983). Em 1986 foi registrada uma epidemia de DENV-1 no município de Nova Iguaçu, que se espalhou para vários municípios do estado do Rio de Janeiro (RJ). O intenso movimento de pessoas permitiu a rápida dispersão do vírus, avançando para os estados de Alagoas (AL) e Ceará (CE) e, no ano seguinte, para os estados de Pernambuco (PE), São Paulo (SP), Bahia (BA) e Minas Gerais (MG) (Schatzmayr *et al*., 1986; Miagostovich *et al*., 1993; Figueiredo, 1996).

A introdução do DENV-2 em 1990, na Região Metropolitana do Estado Rio de Janeiro, na cidade de Niterói, resultou em uma grande epidemia no período de 1990-91 com a cocirculação de DENV-1 e DENV-2 (Nogueira *et al*, 1990; Nogueira *et al*, 1993). Neste período a situação do DENV no país foi agravada pela introdução deste novo sorotipo, quando, então, foram notificados os primeiros casos de (FHD/SCD) no RJ com oito óbitos (Nogueira *et al.*, 1990; 1991; Teixeira *et al*., 1999).

Em dezembro de 2000, o DENV-3 foi detectado no município de Nova Iguaçu/RJ, quando foi sorotipo responsável pela maior e mais grave epidemia de dengue no país até o ano de 2002 (Nogueira *et al.*, 2000; 2001; PAHO, 2002). O DENV-3 predominou na maior parte dos estados do Brasil entre os anos de 2002 e 2006 (SVS, 2010).

A reemergência do DENV-2 em 2007 foi responsável pela mais grave epidemia ocorrida em 2008 quando foram notificados 806.036 casos, com 17.961 casos de dengue com complicaçãoes (DCC), 4.195 casos de FHD e 478 óbitos (SVS, 2009). A introdução de uma nova linhagem de DENV-2 (Oliveira *et al*, 2010; Faria *et al*., 2013) nesta epidemia apresentou um aumento significativo no número de FHD e de mortes em menores de 15 anos (Teixeira *et al*., 2009, Macedo *et al*., 2013).

O monitoramento de sorotipos circulantes ao longo de 2009 apontou para uma nova mudança no sorotipo predominante, com a reemergência do DENV-1 (dos Santos *et al*., 2011), fato que determinou um alerta para a possibilidade de uma nova epidemia tendo em vista a baixa circulação deste sorotipo desde o início da década. Naquele ano, foram notificados 528,883 casos suspeitos, 8,223 casos de graves e 298 óbitos (SVS, 2009; PAHO, 2009). De fato, a reemergência do DENV-1 em 2009 foi responsável pela alteração na epidemiologia com a ocorrência de óbitos em pacientes que apresentavam comorbidades, com o aumento das taxas de internações em maiores de 60 anos de idade (Siqueira Júnior, 2011).

Apesar de Figueiredo *et al* (2008) ter relatado a detecção de DENV-4 em pacientes residentes na cidade de Manaus, entre 2005 e 2007, estes dados não foram confirmados pelo Ministério da Saúde. Contudo, em julho de 2010, o DENV-4 foi detectado 30 anos após sua primeira identificação em uma unidade sentinela de monitoramento viral de Roraima (RR) (SVS, 2010). Os primeiros casos decorrentes da dispersão do DENV-4 só foram detectados em 2011, nas regiões Norte, Nordeste e Sudeste do Brasil, quando 775.060 casos foram notificados no país (Nogueira & Eppinghaus, 2011). Deste total, 764.032 foram referentes a casos de febre do dengue, 10.546 casos graves (7.744 por DCC e 2.802 por FHD) e 482 casos fatais (191 por FHD e 291 por DCC). No ano de 2012 foram notificados 594.343 casos da doença, com 589.591 casos de febre do dengue, 4.425 casos graves (3.429 por DCC e 996 por FHD) e 327 óbitos (121 por FHD e 206 por DCC) (SVS/MS, 2013).

Em 2013 o país viveu a maior epidemia de número de casos já registrada até o momento, com 1.468,873 casos de dengue, 6.969 casos graves e 545 mortes (PAHO, 2013), com predominância do DENV-4, que correspondeu a 60% dos casos. A região Sudeste, foi responsável por 63.4% de casos, seguida pela região Centro-Oeste (18.4%), Nordeste (10.1%), Sul (4.8%) e Norte (3.3%) (Rede Dengue, FIOCRUZ).

No ano de 2014, dos 591.080 casos notificados, 689 foram dengue grave com 410 óbitos (PAHO, 2014), em um período no qual circulavam os quatro sorotipos de DENV no

Brasil (SVS, 2014). Até a 52ª semana epidemiológica de 2015, foram registrados 1.649,008 casos prováveis de dengue no país, com 764 casos de dengue grave e 20.329 casos de dengue com sinais de alarme confirmados. Em relação aos óbitos por dengue em 2015, é pertinente registrar que, diante da confirmação de 863 óbitos por dengue, houve um aumento de 82.5% em comparação com o mesmo período de 2014. A região Sudeste concentrou 65.2% dos óbitos ocorridos no país, com o maior número de óbitos registrado no estado de SP. A cocirculação dos quatro sorotipos foi detectada nas regiões Norte, Nordeste e Sudeste, com a predominância dos DENV-1 (93.8%) dos casos confirmados (SVS, 2015).

1.5. Vetor e ciclo de transmissão

Os DENV são mantidos na natureza através de dois ciclos distintos: silvestre e humano. No ciclo silvestre a transmissão é realizada entre os *Aedes* spp. arborícolas e primatas não-humanos, parecendo este ser o único hospedeiro amplificador. Na África os principais vetores são *Ae.* (Stegomyia) *luteocephalus*, *Ae.* (Diceromyia) *furcifer* e *Ae.* (Diceromyia) *taylori.* O mosquito ancestral arbóreo, *Ae. aegypti formosus*, principal vetor doméstico de transmissão do DENV não se mostrou capaz de se infectar pelo DENV silvestre (Weaver & Vasilakis, 2009; Chen & Vasilakis, 2011). Os hospedeiros primatas na África incluem o macaco Patas (*Erythrocebus pernas*), macaco verde africano (*Chlorocebus sabaeus*), Babuínos Guiné (*Papio papio*) e espécies possivelmente relacionados como *Papio anubis, Papio ursinus* e *Papio cynocephalus.*

Na Ásia, os principais vetores incluem os mosquitos primatofílicos do complexo *Ae*. (Finlaya) *niveus* sl., um grupo que inclui *Ae. pseudoniveus*, *Ae. subniveus*, *Ae. vanus*, *Ae.albolateralis*, *Ae. niveoides* e *Ae. novoniveus*. Estas espécies são também conhecidas por descer das árvores até o solo para se alimentar em humanos. Os hospedeiros primatas asiáticos incluem os macacos cinomolgos (*Macaca fascicularis*), macacos de rabo de porco do sul (*Macaca nemestrina*) e macacos folhas prateadas (*Presbytis cristata*) e, possivelmente, macacos folha verde-mitrados (*Presbytis melaphos*). Embora até o momento apenas dois focos de transmissão do DENV silvestre tenham sido documentados, é provável que ocorra a transmissão do DENV silvestre, ainda não descrito, em outros locais de África tropical e da Ásia. Até o momento não há nenhuma evidência concreta da existência da transmissão do ciclo silvestre nas Américas (Chen & Vasilakis, 2011).

Atualmente, quase todas as infecções humanas são devidas à circulação do DENV exclusivamente em ambientes domésticos e peridomiciliares ao longo dos trópicos, onde o

homem serve como único reservatório de amplificação. Neste ciclo humano, *Ae. aegypti aegypti* é o principal vetor que transmite o DENV, enquanto outras espécies de *Aedes,* por exemplo, *Ae. albopictus*, *Ae. polynesiensis*, servem como vetores secundários. (Chen & Vasilakis, 2011).

A transmissão ocorre durante o repasto sanguíneo, quando as fêmeas adultas dos mosquitos do gênero *Aedes* transmitem o vírus da dengue ao hospedeiro humano. O ciclo de transmissão envolve a ingestão de sangue virêmico (sangue periférico contendo as partículas virais circulantes) pelo mosquito vetor e após um período de incubação extrínseco ocorre a transmissão do vírus ao hospedeiro vertebrado. O período de incubação no vetor dura de 8 a 12 dias e é requerido para que ocorra a replicação e disseminação viral na hemocele e em todos os tecidos do inseto, até finalmente infectar as glândulas salivares tornando o mosquito capaz de transmitir o vírus para um novo hospedeiro humano susceptível. O mosquito permanece infectado e assintomático durante toda sua vida e é capaz de transmitir o vírus da dengue para diversas pessoas, devido a sua capacidade de se alimentar em múltiplos indivíduos em sucessão. Outra forma importante de transmissão que ocorre entre os mosquitos do gênero *Aedes* é a transmissão transovariana (TTO), em que o vírus da dengue é transmitido diretamente para a prole, dispensando o homem no ciclo mantenedor, está transmissão tem sido sugerida como um mecanismo de manutenção dos DENV em ambos os ciclos de silvestre e humano.

O hospedeiro humano torna-se virêmico após um período de incubação de 3 a 14 dias, após a picada por um mosquito infectado, concomitante ao início do período febril (Vaughn, 1996; Gubler, 1998, Chen & Vasilakis, 2011). Outra forma associada à manutenção dos DENV, pode ser devido ao elevado número de casos assintomáticos de dengue tornando a transmissão silenciosa em humanos por um número reduzido de vetores. Este mecanismos é importante na transmissão do DENV em perídos interepidêmicos (Kyle & Harris, 2008).

1.6. Agente etiológico

1.6.1. Classificação e organização do genoma dos vírus dengue (DENV)

Os DENV pertencem à família *Flaviviridae* e ao gênero *Flavivirus.* São classificados como arbovírus que compreendem um grupo taxonômico de diversos vírus transmitidos por artrópodes (Calisher, 1989). Os DENV são mantidos na natureza por um ciclo de transmissão envolvendo hospedeiros vertebrados e mosquitos hematófagos do

gênero *Aedes*, o homem, é o único hospedeiro capaz de desenvolver as formas clínicas da infecção (Gubler, 2002).

Possuem propriedades antigênicas distintas, caracterizando quatro sorotipos específicos denominados vírus dengue tipo 1 (DENV-1), vírus dengue tipo 2 (DENV-2), vírus dengue tipo 3 (DENV-3) e vírus dengue tipo 4 (DENV-4) (Lindebach & Rice, 1999). Estudos recentes descrevem o isolamento do DENV-5 a partir de material coletado de um homem em 2007 na Malásia, porém até o momento não foi disponibilizada a sequência genômica deste novo vírus (Normile, 2013).

Os DENV são esféricos, envelopados e com cerca de 40 a 50 nanômetros de diâmetro. O RNA fita simples e polaridade positiva, com aproximadamente 11 kb, é envolto por um nucleocapsídeo de simetria icosaédrica, composto por uma única proteína, a proteína de capsídeo (C) circundada por uma bicamada lipídica associada às proteínas de membrana (M) e envelope (E) (Lindebach *et al*., 2007) (Figura 1.2).

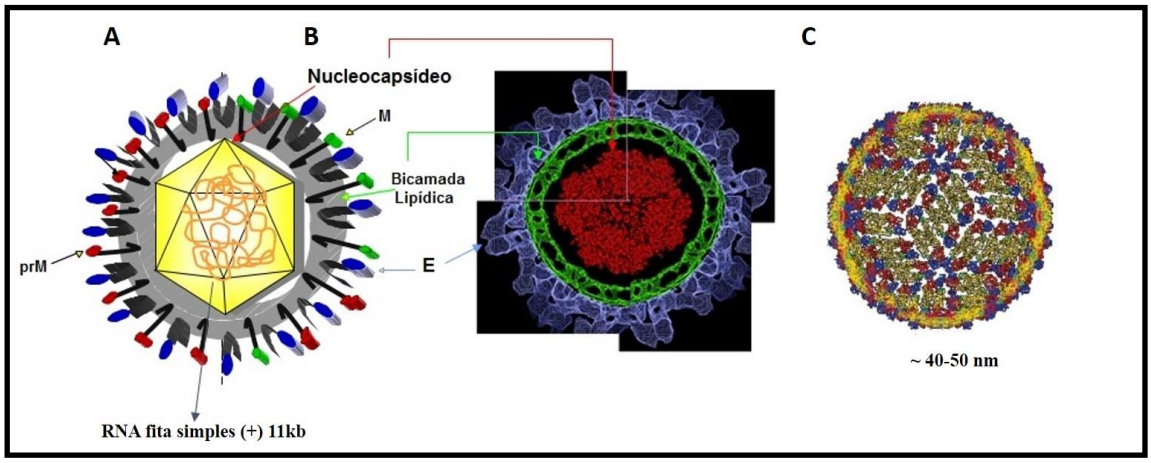

Figura 1.2: Estrutura do DENV. A: Diagrama esquemático dos DENV (adaptado de Heinz & Allison, 2001), B e C: Estrutura do DENV apresentando os dímeros da proteína E (Kuhn *et al*, 2002).

O RNA que é modificado em sua extremidade 5' pela adição da estrutura *cap*, mas é destituído de cauda poli-A na extremidade 3', apresenta uma única fase de leitura aberta que codifica uma poliproteína precursora das proteínas virais. Esta proteína precursora é clivada pelas proteases celulares do hospedeiro e pela protease viral, dando origem a dez proteínas, destas, três são proteínas estruturais C, pré-membrana/ membrana (prM/M) e E, e sete proteínas não estruturais NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5. As proteínas estruturais traduzidas são incorporadas às partículas virais durante a maturação, enquanto as proteínas não estruturais estão envolvidas na replicação e/ou montagem dos virions

(Figura 1.3). As regiões não-codificantes 3’ e 5’ são importantes na replicação viral (Mukhopadhyay *et al*., 2005; Qi *et al*., 2008).

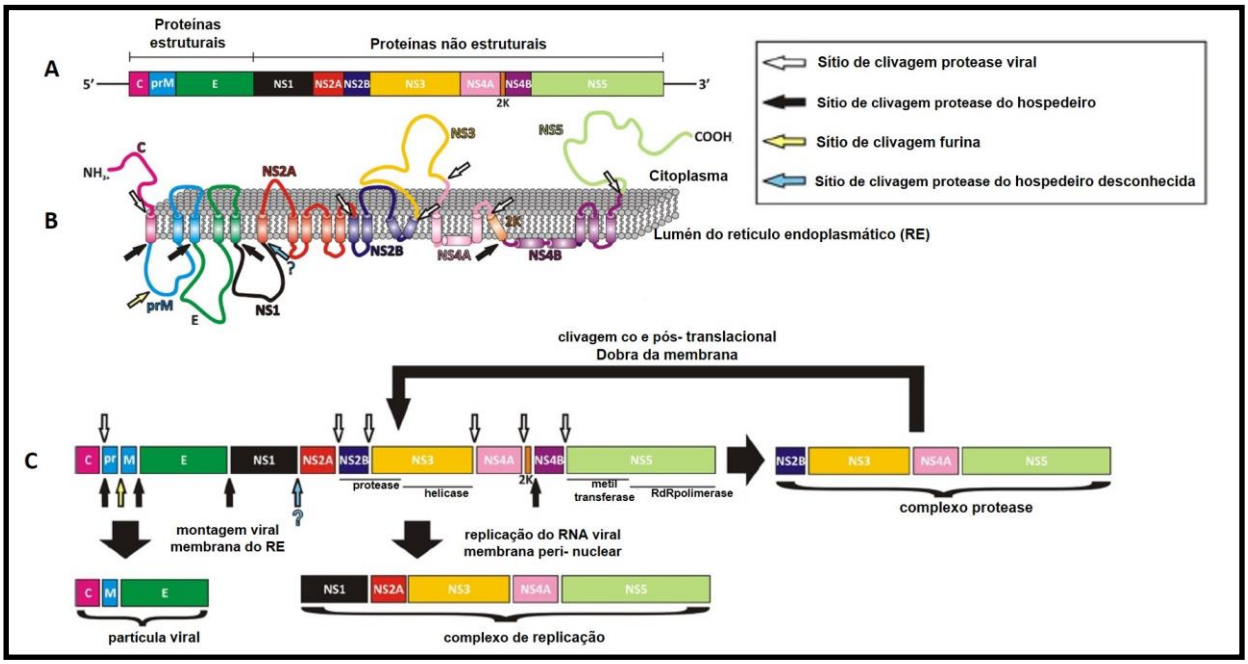

Figura 1.3: Diagrama esquemático da organização e processamento da poliproteína nos flavivírus. (A) Poliproteína viral – proteínas estruturais e não estruturais. (B) Topologia a membrana putativa da poliproteína prevista a partir de análises bioquímicas e celulares, as quais são processadas por proteases celulares e virais (indicadas pelas setas). (C) Diferentes complexos que surgemnos diferentes compartimentos durante e após o processamento da poliproteína. (Adaptado de Assenberg *et al*., 2009).

A proteína C (13-14 kDa) é altamente básica e que, associada ao RNA viral, forma o nucleocapsídeo. Possue resíduos básicos concentrados nas extremidades amino e carboxi terminais que são separados por uma região interna hidrofóbica que irá mediar a associação com a membrana da célula (Ma *et al*., 2004; Mukhopadhyay *et al*., 2005).

A PrM (~ 26 kDa) é gerada pela clivagem da extremidade N-terminal no retículo endoplasmático (RE) por uma peptidase sinal do hospedeiro. Durante a saída do virion, a proteína prM é clivada pela enzima furina residente no trans-Golgi originando a proteína M presente na partícula madura (Stiasny & Heinz, 2006; Lindebach & Rice., 1999).

A proteína M (22,0 kDa) é sintetizada sob a forma imatura, denominada prM. É uma proteína de transmembrana que interage com a proteína E (Muylaert *et al.*, 1997). A função

da prM é estabilizar a proteína E, impedindo a exposição prematura do peptídeo de fusão ao pH reduzido encontrado na via exocítica. A retenção da proteína prM pode afetar a conformação e antigenicidade da proteína E e reduzir a infectividade viral inibindo a fusão dependente de ácido (Stadler *et al.*, 1997, Heinz & Allison, 2001).

A glicoproteína E (51-60 kDa) é a principal e a maior proteína estrutural do vírus, é responsável por atividades biológicas do ciclo viral, tais como a montagem da partícula, a interação com receptores celulares e a fusão com a membrana celular do hospedeiro. Alguns de seus epítopos induzem resposta imune humoral capaz de impedir a entrada do vírus na célula alvo, promovendo a neutralização viral. O domínio III da proteína é responsável pela ligação do vírus à célula hospedeira e os anticorpos produzidos contra este domínio são capazes de obstruir o sítio de ligação viral ao receptor de membrana celular (Chambers *et al*.,1990).

A glicoproteína NS1 (~ 46 kDa) pode ser encontrada de três formas, (i) no interior das células infectadas pelo vírus, (ii) associada à membrana destas células (Chambers, 1990; Lindebach, Triel & Rice., 2007) e (iii) na forma solúvel, é secretada em grandes quantidades, o que possibilita a sua detecção no soro de pacientes na fase aguda da infecção (Young *et al*., 2000). A NS1 é translocada pela via do RE utilizando a extremidade C-terminal da proteína E . (Falgout *et al*., 1989; Lindebanch *et al* 2007). A proteína NS1 constitui uma subunidade do complexo de replicação do RNA viral (Mackenzie *et al*., 1996; Westaway *et al*., 1997). A proteína NS1 na forma secretada forma imunocomplexos podendo ativar a via do sistema complemento, contribuindo, assim, na patogênese (Avirutnan *et al*., 2006). Embora os efeitos da NS1 na patogênese da dengue ocorram devido à formação de imunocomplexos com anticorpos anti-NS1 (Avirutnan *et al*., 2006; Cheng *et al*., 2009), recentes estudos têm demostrado o papel patogênico da proteína NS1 por si só. (Beatty *et al*., 2015; Modhiram *et al*., 2015).

A NS2A, que é relativamente pequena (~ 22 kDa), é uma proteína hidrofóbica transmembrana. Esta proteína reconhece a porção 3'NC do RNA genômico e é uma subunidade do complexo de replicação (Khromykh *et al.,* 1999). A NS2B (~ 14 kDa) é uma proteína associada à membrana com dois domínios hidrofóbicos os quais rodeiam uma região conservada do genoma. Esta proteína forma um complexo com a NS3 e é um cofator necessário para a função serina protease de NS3 (Lindebach *et al* 2007).

A NS3, com aproximadamente 70 kDa, é a segunda maior proteína, que caracterizada como multifuncional, contém diversas atividades necessárias para o processamento da poliproteína e replicação do RNA. Estas características sugerem que a enzima NS3

desempenhe um papel importante durante os eventos da síntese de RNA viral juntamente com a RNA polimerase do vírus conhecida como NS5 (Chambers *et al*., 1990, Lindebach *et al*., 2007).

As proteínas NS4A e NS4B são pequenas e hidrofóbicas e estão associadas à membrana. A proteína NS4A (~ 17 kDa) promove a interação do complexo NS5-NS3-NS2A-RNA à proteína NS1, que se encontra no lúmen do RE, além de ser uma subunidade do complexo de replicação. A proteína NS4B (~ 24,0 kDa) encontra-se dispersa na membrana citoplasmática e, possivelmente, no núcleo. NS4A e NS4B podem bloquear a via de sinalização do interferon (IFN) tipo I (α e β) e escape viral (Lindenbach & Rice, 2007).

A proteína NS5 (~ 103 kDa), a maior proteína viral altamente conservada entre os *Flavivirus*, é uma proteína multifuncional com atividades de metiltransferase e de RNA polimerase dependente de RNA (RdRp) (Lindenbach & Rice, 2007). NS5 dos DENV-2 induz a transcrição e secreção de IL-8, podendo aumentar a propagação viral ou doença através do recrutamento de células inflamatórias para o local da infecção (Medin et al, 2005). Também bloqueia a resposta do interferon tipo 1 (α e β) (Kelley *et al*., 2011).

1.7. Variabilidade genética dos vírus dengue

Os DENV exibem um alto grau de variabilidade genética devido (i) à falta do mecanismo de correção da RNA polimerase viral durante a replicação, (ii) as rápidas taxas de replicação, (iii) ao grande tamanho populacional e pressão frente a reposta imunológica do hospedeiro (Twiddy *et al*., 2003). Esta alta variabilidade resulta na existência dos quatro sorotipos que apresentam homologias entre 62% e 67% ao nível de aminoácidos (aa), o que poderia levar os sorotipos de DENV a classificação como grupos virais separados (Westaway & Blok,1997). Historicamente, as variantes dentro de cada sorotipo de DENV foram classificadas de formas distintas, acompanhando o progresso tecnológico.

Na década de 1970, estudos apontaram a existência de variantes antigênicas dentro dos DENV-3,e que cepas de DENV-3 de Porto Rico e do Taiti eram antigênica e biológicamente distintas das cepas da Ásia (Russel & McCown, 1972). As primeiras evidências para as diferenças genéticas entre os sorotipos de dengue ocorreram na década de 1980, baseado na técnica de "fingerprinting" do RNA utilizando enzimas para digestão da fita do RNA, com o número e tamanho das fitas variando de acordo como tamanho da sequência do vírus, agrupando os vírus em topotipos (Rico-Hesse, 2003; Chen & Vasilakis, 2011). Nos anos 90, a utilização de métodos de sequenciamento do genoma viral e análise filogenética permitiram a classificação dos DENV em grupos geneticamente distintos ou

genótipos dentro de cada sorotipo (Rico-Hesse, 1990; Lewis *et al*, 1993; Lanciotti *et al*., 1994). Rico-Hesse (1990) definiu esses "genótipos" como grupos de vírus que apresentam uma divergência em sua sequência nucleotídica menor que 6% dentro de uma determinada região do genoma viral (junção E/NS1).

Um estudo detalhado de análises filogenéticas baseadas no sequenciamento do gene E determinaram cinco genótipos para os DENV-1: Genótipo I, Genótipo II, Genótipo III, Genótipo IV e Genótipo V (Quadro 1.1) (Weaver & Vasilakis, 2009).

Análises filogenéticas baseadas no sequenciamento do gene E determinaram seis genótipos distintos para os DENV-2: Genótipo Asiático I, Genótipo Asiático II, Genótipo Cosmopolita, Genótipo Americano, Genótipo Sudeste Asiático/Americano, Genótipo Selvagem (Quadro 1.1) (Weaver & Vasilakis, 2009).

O DENV-3 está classificado em cinco genótipos: Genótipo I, Genótipo II, Genótipo III, Genótipo IV, e Genótipo V. Estudos realizados por Araújo *et al* (2009) confirmaram a circulação do genótipo V que, embora considerado extinto, foi representado por cepas de FD/FHD isolados entre 2002 e 2004 da América do Sul (Weaver & Vasilakis, 2009) (Tabela 1).

Amostras selvagens de DENV-3 ainda não foram isoladas, mas é possível que existam na Malásia, uma vez que apresentaram macacos sentinelas apresentaram anticorpos contra esses vírus (Weaver & Vasilakis, 2009).

Os DENV-4 apresentam quatro genótipos distintos baseados na análise da sequência do gene E: Genótipo I, Genótipo II, Genótipo III e Genótipo IV (Weaver & Vasilakis 2009) (Quadro 1.1).

Recentemente foi anunciada a descoberta do DENV-5 em uma única amostra, a partir de material coletado de um homem em 2007 na Malásia, porém até o momento não foi disponibilizada a sequência genômica deste novo vírus e novos casos ainda não foram descritos (Normile, 2013). É importante ressaltar que a classificação dos DENV em genótipos dentro de cada sorotipo está constantemente sendo modificada, à medida que sequenciamento e análises evolutivas são aperfeiçoados e os bancos de dados de genomas dinamicamente se expandem.

Quadro 1.1: Classificação genotípica dos DENV baseada na análise filogenética do sequenciamento do gene que codifica para a proteína E, de acordo com Weaver & Vasilakis (2009) e Araújo *et al* (2009).

Sorotipo	*Genótipos*	Distribuição geográfica
DENV-1	I	Sudeste Asiático, China, Leste da África
	II	Tailândia (1950-1960)
	III	Malásia (cepas selvagens)
	IV	Ilhas do Oeste do Pacífico e Austrália
	V*	Américas, Oeste da África Africano, Ásia
DENV-2	Asiático I	Malásia e Tailândia
	Asiático II	Vietnã, da China, Taiwan, Sri Lanka e Filipinas
	Cosmopolita	Austrália, Leste e Oeste Africano, Ilhas dos oceanos Pacífico e Índico, Subcontinente Indiano e Oriente Médio
	Americano	América Latina, Caribe (1950-1960), Subcontinente Indiano e Ilhas do Pacífico
	Sudeste Asiático/Americano*	Tailândia, Vietnã, Américas (últimos 20 anos)
	Selvagem	Oeste Africano e Sudeste Asiático (cepas isoladas em humanos, mosquitos silvestres ou macacos sentinelas)
DENV-3	I	Indonésia, Malásia, Filipinas e Sul da Ilhas do Pacífico
	II	Tailândia, Vietnã e Bangladesh
	III*	Sri Lanka, India, África, Samoa, Tailândia (1962)
	IV	Porto Rico, Américas Latina e Central, Taiti (1965)
	V	Filipinas (1956), Japão (1973), China (1980) América do Sul (2002-2004)
DENV-4	I*	Tailândia, Filipinas, Sri Lanka e Japão (provenientes do Sudeste Asiático)
	II*	Indonésia, Malásia, Taiti, Caribe e Américas
	III	Tailândia (cepas recentes)
	IV	Malásia (cepas selvagens)

*Genótipos circulantes no Brasil.

1.8. Replicação e interação dos vírus dengue com células alvo

A infecção pelos DENV se inicia após a picada do mosquito *Aedes* que juntamente com a secreção salivar libera o vírus na corrente sanguínea. A partícula viral se liga a receptores específicos na membrana plasmática da célula alvo e é internalizada (Lindebanch & Rice, 1999; Mukhopadhyay *et al*., 2005). Os alvos primários da infecção pelo DENV são as células dendríticas (DC), monócitos e macrófagos (Clyde *et al*., 2006; Kou *et al*., 2008).

Os DENV entram na célula através de endocitose mediada por clatrina e a proteína Eassim como as glicoproteínas do DENV atuam como ligantes para diversos receptores celulares, tais como: (i) DC-SIGN/CD 209 (dendritic cell ICAM-3 grabbing nonintegrin) presente em subpopulações de DC; LC-SIGN (liver/lymph node-specific ICAM-3 grabbing nonintegrin), expresso em células endoteliais dos sinusóides do fígado, órgãos linfoides e capilares placentários; (ii) receptores de manose presentes em macrófagos e proteoglicanos heparan sulfato (HSP) expressos em células endoteliais; (iii) ICAM-3, CD14, HSP70/90, GRP78 e (iv) receptores de laminina (revisado por De La Guardia & Lieonard, 2014). O pH ácido induz a fusão do envelope do virion com a membrana celular e, em seguida, após o desnudamento, o RNA viral é liberado no citoplasma. Com a entrada e exposição do RNA no citoplasma, inicia-se a tradução da poliproteína viral precursora. A poliproteína viral é clivada pela combinação de proteases virais e do hospedeiro. As proteínas estruturais e não estruturais são codificadas na porção N-terminal da poliproteína.

A replicação inicia com síntese da fita negativa de RNA que serve como molde para a síntese de fitas positivas de RNA da progênie. A montagem das partículas virais ocorre em associação às membranas celulares internas, dentro do lúmen do reticulo endoplasmático (RE) das células hospedeiras. A formação das partículas virais ocorre através do revestimento do nucleocapsídeo viral com as proteínas do envelope, ancoradas na membrana do RE celular. Os vírus brotam para dentro do lúmen do RE, adquirindo uma membrana lipídica (envelope) e, assim, os vírus se mantêm solúveis nas cisternas do RE e as partículas virais são liberadas pela via exocítica através do sistema de Golgi (Lindenbach *et al*., 2007) (Figura 1.4).

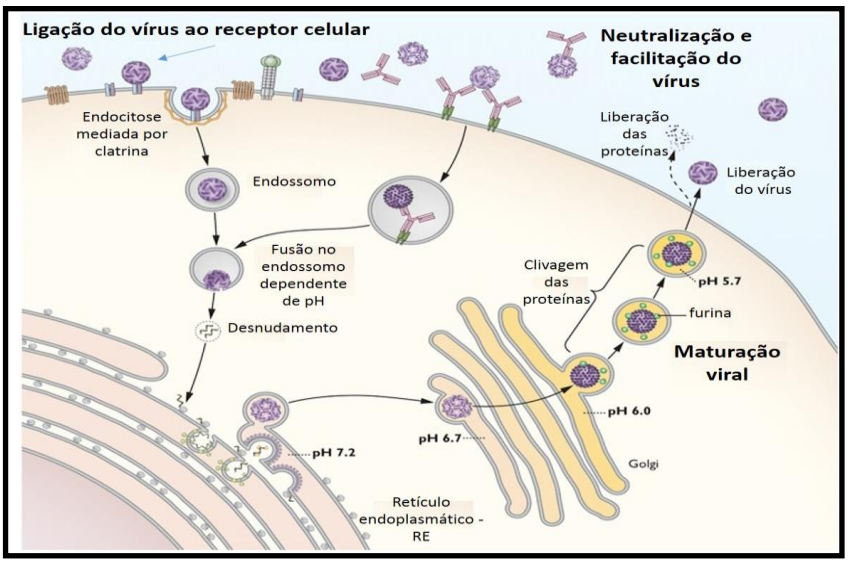

Figura 1.4: Ciclo de replicação dos flavivírus (Adaptado de Pierson & Diamond, 2012).

1.9. Manifestações clínicas e classificação dos casos de dengue

A dengue é uma doença febril aguda causada por qualquer um dos quatro sorotipos virais (Edelman & Hombach, 2008), que vão desde uma infecção inaparente, uma doença febril aguda indiferenciada, até formas mais graves (OMS, 2009). A doença sintomática segue tipicamente três fases: uma fase febril inicial com duração de 3 a 7 dias; uma fase crítica em torno da defervescência durante a qual complicações podem aparecer em alguns pacientes; e uma fase de recuperação espontânea (Simmons *et al*., 2012). Devido à dificuldade que existia na classificação das diferentes formas clínicas, uma categorização simplificada para a classificação da gravidade da dengue foi proposta pelo Programa Especial para Pesquisa e Treinamento em Doenças Tropicais da Organização Mundial da Saúde em 2009, intitulada *Dengue Control* [DENCO], cujo objetivo é orientar médicos na identificação precoce de casos graves (OMS, 2009). Observações preliminares do DENCO confirmaram que utilizando um conjunto de parâmetros clínicos e/ou laboratoriais, é possível a distinção entre pacientes com um quadro grave ou não da dengue (OMS, 2009). Os seguintes critérios de gravidade da doença foram estabelecidos (Figura 1.5):

1) dengue sem sinais de alarme (DSSA), caracterizada por náusea, vômito, erupções cutâneas, mialgia, cefaleia, artralgia, teste do torniquete positivo, sem sinais de hemorragia e leucopenia com confirmação laboratorial;

2) dengue com sinais de alarme (DCSA) que inclui dor ou sensibilidade abdominal, vômitos persistentes, acúmulo de líquido, letargia, agitação, hepatomegalia (aumento > 2 cm), elevação das transaminases hepáticas e diminuição na contagem de plaquetas;

3) dengue grave (DG) que é caracterizada por extravasamento plasmático grave, podendo levar ao choque (sindrome de choque da dengue - SCD) e acúmulo de fluído acompanhado de desconforto respiratório, além de hemorragias graves e comprometimento de órgãos como fígado (níveis de AST ou ALT> 1000), comprometimento do sistema nervoso central e/ou outros órgãos.

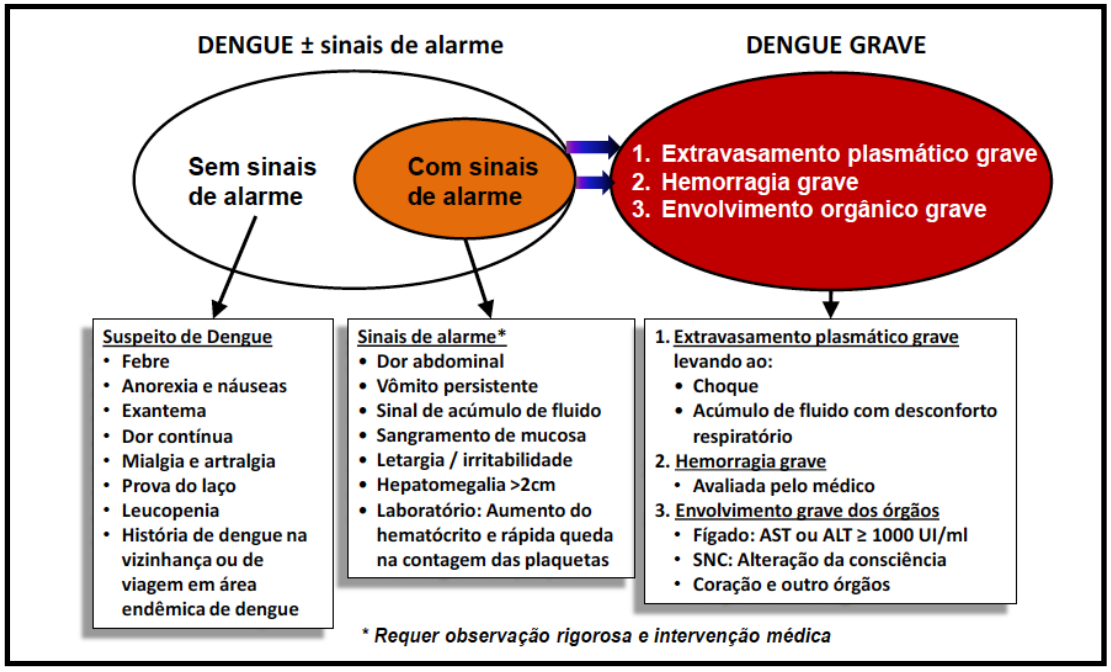

Figura 1.5: Classificação clínica das infecções por dengue segundo a OMS, 2009 (OMS 2009).

1.10. Principais mecanismos envolvidos na imunopatogênese das infecções pelos DENV

Diversas teorias são propostas para explicar o alto grau de variação das manifestações clínicas causadas pelos DENV. A falta de um modelo animal adequado que reproduza as formas graves da doença tem dificultado a compreensão da patogenia do dengue, levando a hipóteses que tentam associar diferentes fatores de risco à gravidade da

doença (Halstead, 1988; Thein *et al*., 1997; Srikiatkhachom and Green, 2010). Até o momento acredita-se que a infecção primária seja capaz de criar proteção efetiva e duradoura para toda vida contra uma possível infecção pelo mesmo sorotipo viral, porém uma proteção cruzada contra outros sorotipos por um curto período também foi descrita (Rothman, 2004). A neutralização realizada por anticorpos específicos pode ocorrer pela inibição da entrada do vírus, através de seus receptores específicos na célula-alvo (Nybakken *et al*., 2005) ou pela inibição da fusão viral no citoplasma celular (Vogt *et al*., 2009).

1.11. Teoria da virulência viral

Estudos anteriores sugeriram que a gravidade da doença poderia estar relacionada às variações genéticas e antigênicas em diferentes cepas virais infectantes (Rico-Hesse,1990, Leitmeyer *et al*., 1999; Cologna *et al*., 2005) e a altos níveis de carga viral (Vaugh *et al*., 2000; Libraty *et al*., 2002). Neste contexto, estudos preliminares observaram diferenças na patogenicidade e virulência entre os quatro sorotipos, com a evidência de que os DENV-2 e DENV-3 de origem asiática foram associados com casos graves e fatais, além de apresentarem um maior potencial de epidemia e virulência (Mackenzie *et al*., 2004; Guzmán *et al*., 2010). Estudos filogenéticos e epidemiológicos comprovam a importância da teoria de determinantes virais com estudos do sequenciamento nucleotídico dos genes dos DENV-2 do genótipo americano associado à FD e do genótipo Sudeste Asiático associado à FHD (Rico-Hesse, 2003).

1.12. Teoria da facilitação dependente de anticorpos ou ADE

A facilitação dependente de anticorpo ou ADE, "antibody dependent enhancement", preconiza uma associação entre infecções secundárias e o aparecimento das formas graves da doença. Essa teoria sugere que a formação de imunocomplexos entre o sorotipo viral infectante e anticorpos heterólogos da classe IgG existentes em níveis sub-neutralizantes de uma infecção anterior, facilitariam a infecção. Estes complexos, ao serem reconhecidos e internalizados por fagócitos mononucleares, resultariam no aumento da infecção e replicação viral. Essas células infectadas liberariam na corrente sanguínea, mediadores vasoativos capazes de aumentar a permeabilidade vascular, a ativação do sistema complemento e de coagulação (Halstead1988).

A ADE ocorre quando a endocitose do complexo antígeno- anticorpo (anti-DENV) é mais eficiente do que a entrada das partículas virais livres na célula. A ADE é mediada por anticorpos específicos contra a proteína E, que, em condições de baixa concentração ou

baixa avidez de anticorpos, não são capazes de neutralizar o vírus, por estarem abaixo do limite necessário para neutralização viral (Pierson *et al*., 2007). Assim sugere-se que o processo aumente potencialmente o risco de desenvolvimento das formas graves da dengue devido ao aumento de carga viral (Gúzman *et al*., 1990; Halstead 2003; Mathew & Rothman, 2008).

1.13. Teoria do pecado antigênico original

A infecção secundária por um sorotipo diferente do DENV resulta na ativação e expansão de células T CD4 e CD8 (células T de memória), com baixa avidez, e de reatividade cruzada para o sorotipo da infecção. (Mentor & Kurane, 1997; Remy, 2014). Esses clones de memória não exerceriam suas funções efetoras realizando o "clearance" viral, porém, teriam alta capacidade na produção de mediadores inflamatórios e poderiam, de fato, promover a imunopatogenia do dengue (Mongkolsapaya *et al.*,2003). Sugere-se que a patogênese da doença possa ser causada pela ativação e expansão dessas células, pois o sistema imune apresenta uma diminuição na eficácia em desenvolver uma resposta imune celular específica adequada ao DENV (Remy, 2014). A liberação de citocinas pró-inflamatórias por essas células, como IFN-γ, pode agir diretamente sobre o endotélio vascular e resultar no extravasamento de plasma, um evento característico das infecções graves por DENV (Pang *et al.*, 2007).

Estudos de polimorfismos genéticos têm possibilitado a correlação de diversos genes com o aumento da susceptibilidade a FHD (Lan et al., 2011). Assim, especula-se que uma mutação funcional na região promotora do receptor DC-SIGN1-336 poderia estar associada à proteção ou a uma maior susceptibilidade a FHD (Sakuntabhai *et al*., 2005; Wang *et al*., 2011).

Por fim, acredita-se que a patogênese da dengue seja multifatorial assim como o desenvolvimento de um quadro mais grave envolvem diversos fatores, entre eles, os fatores nutricionais e genéticos do hospedeiro, a idade, o sexo, o estado imunológico e as variações genéticas entre os sorotipos e genótipos virais infectantes (Gúzman & Kouri., 2002 Malavige *et al*., 2004).

1.14. Células dendríticas

As células dendríticas (DCs) foram reconhecidas pela primeira vez em 1973 por Steinman and Cohn como um "tipo celular originário de órgãos linfóides periféricos de ratos" (Steinman & Cohn, 1973). Estas células foram caracterizadas pela sua morfologia

dendrítica, baixa densidade, excepcional mobilidade e capacidade de apresentar antígenos para células T em repouso. Embora tenham sido identificadas pela primeira vez em órgãos linfóides, células com as mesmas características foram isoladas a partir de tecidos não linfóides em muitos roedores e no homem, incluindo a pele, pulmão, articulação sinovial e trato gastrointestinal, bem como de sangue periférico (Thomas & Lipsky, 1996).

Estão distribuídas por todos os tecidos do corpo, como células imunes parcialmente diferenciadas, localizadas preferencialmente em sítios estratégicos onde existe a possibilidade de entrada de um patógeno. As linhagens de DCs representam um sistema complexo, constituído por diversas subpopulações celulares em distintos estágios de maturação (Banchereau & Steinman, 1998). As DCs têm sido classificadas de acordo com a localização anatômica, expressão de moléculas de superfície e função no sistema imunológico. A importância dos vários grupos de DCs tem sido atribuída principalmente pelas diferenças na capacidade de ação na resposta imunológica envolvendo produção de citocinas, quimiocinas, receptores de fagocitose de antígenos e receptores de reconhecimento de padrões de microrganismos (PPR) (Lipscomb & Masten, 2002). Em humanos, três subpopulações de DCs podem ser identificadas: (i) células de Langerhans que estão localizadas apenas nos epitélios (como a epiderme), (ii) células dendríticas intersticiais que se infiltram em outros tecidos e (iii) células dendríticas plasmocitóides que circulam como precursores no sangue e órgãos linfóides (Banchereau *et al*., 2000).

As DCs estão presentes na maioria dos tecidos em uma fase de diferenciação chamada "imatura". As DCs imaturas (imDCs) são caracterizadas por intensa atividade endocítica e capturam vários tipos de antígenos como patógenos, células infectadas, células mortas e seus produtos atráves de fagocitose, micropinocitose e endocitose mediada por receptores (Steinman & Nussenzweig 2002).

Durante o processo de maturação das DCs, a capacidade de captura e processamento de antígenos se encontra reduzida, propiciando a restrição de apresentação de peptídeos endocitados na periferia. Ocorre um aumento de expressão de moléculas MHC classe I e classe II na superfície celular e também de moléculas co-estimulatórias como CD80, CD86, CD83, CD40, CD38 e OX40L (Quah & O'Neill 2005).

As DCs compreendem menos de 0.1% do número total de células brancas no sangue periférico (Steinman, 1991) e que, além de desempenharem um papel único na iniciação da resposta imune, são fundamentais na ligação entre a resposta imunológica inata e adaptativa. Devido à sua capacidade de apresentar antígenos aos linfócitos T naive, as DCS são denominadas também de células apresentadoras de antígenos (APC) (Gatti &

Pierre, 2003). Essas células são capazes de produzir citocinas e fatores solúveis quando infectadas pelos DENV (Chen *et al*.,2002).

O reconhecimento de patógenos intracelulares ativa as vias de produção pró-inflamatórias pelas DCs. As citocinas produzidas pelas DCs atuam de forma autócrina e parácrina, isto é, citocinas que atuam na própria célula ou em células vizinhas, respectivamente. Dentre algumas dessas citocinas e quimiocinas produzidas pelas DCs podemos destacar:

(i)TNF-α –citocina multifuncional, consideradao mediador inflamatório mais importante queexerce diversas funções tais como aumentar a habilidade fagocítica de macrofágos, induzir a maturação de células dendríticas, induzir a expressão de moléculas de adesão e o aumento da permeabilidade no endotélio, propiciando o aumento do número de células mononucleadas no local da inflamação (Vassalli, 1992; Tracey & Cerami,1993). A sua ação biológica propicia intensa lesão tecidual, quando liberado sistêmicamente em altas concentrações, podendo ser o responsável pelos quadros de hipotensão, supressão do miocárdio, extravasamento plasmático e estimulação de cascatas de coagulação em condições patológicas como na sepse bacteriana e na FD (Pinto *et al*., 1999; Pfeffer, 2003).

(ii)IFN-α. –citocina anti-viral produzida em grandes quantidades principalmente pelas DCs plasmacitóides mediante a infecção viral. A ligação com o seu receptor na célula, ativa as vias do JAK-STAT que, consequentemente, induzem a expressão dos genes ISGs (*Interferon- Stimulated Genes*) através da ativação de seu fator de transcrição, o IRF7. Os genes ISGs interferem na tradução e na edição do RNA mensageiro viral e ainda possuem ação enzimática direta sobre este RNA (Katze *et al*., 2002). O IFN-α é um diferenciador do desenvolvimento das repostas do tipo Th1 (do inglês *T helper*) e da imunidade celular, principalmente da ativação de células NK (Marshall *et al*., 2006).

(iii) IL-10 - citocina moduladora da resposta imunológica que diminui a expressão dos marcadores de maturacão das DCs assim como a secreção de citocinas pró-inflamatórias como IL-1β, TNF-α e IL-12, além da expressão de moléculas coestimulatórias (Wallet *et al*., 2005). As DCs produtoras de IL-10 podem induzir a diferenciação de linfócitos T virgens em células T regulatórias 1. Não se sabe ao certo se a IL-10 produzida seria um mecanismo compensatório ou imunopatológico, apesar da função modulatória na produção de TNF-α ser fundamental na patogênese do choque septico (Scumpia, 2005).

(iv) IL-6 – considerada uma citocina pleiotrópica com inúmeras atividades biológicas, é produzida por diversas células linfóides e não linfóides. A IL-6 auxilia na regulação da reatividade imunológica, na resposta de fase aguda, na inflamação, na oncogênese e na

hematopoiese. A produção de IL-6 é estimulada por TNF-α e IL-1β e persiste por mais tempo no plasma do que estas citocinas pró-inflamatórias. Níveis plasmáticos de IL-6 foram correlacionados com mortalidade no choque séptico (Song & Kellum, 2005).

(v) IL-1 e IL-1Ra – a família dessas citocinas está relacionada com a inflamação e com a defesa frente infecção. A IL-1 é uma citocina pró-inflamatória que pode ativar DCs (Blanco *et al*., 2008).

1.15. Papel das citocinas e mediadores inflamatórios na imunopatogenia da dengue

Altos níveis de mediadores inflamatórios (citocinas e quimiocinas) têm sido encontrados no soro de pacientes com FD. A maioria desses achados está presente em pacientes com FHD/SCD, sugerindo que as formas graves da doençase manifestam em um contexto de amplificação da produção de citocina, chamada de tempestade de citocinas (do inglês *cytokine storm),* que atinge o epitélio endotelial e causa um aumento da permeabilidade vascular, levando a manifestações hemorrágicas, hemoconcentração e, em alguns casos, o desenvolvimento do choque que pode levar à morte (Costa *et al.*, 2013). Estudos clínicos realizados em crianças e adultos infectados com DENV demonstram o aumento significativo de vários mediadores inflamatórios solúveis, alguns dos quais presentes nas formas mais graves da doença e outros nas formas brandas (Srikiatkhachorn & Green, 2010).

Estudos clínicos com dosagens de citocinas e de outros mediadores são descritos na literatura, mas os resultados entre eles diferem, com alguns demonstrando associação do IFN-γ com casos graves de dengue (Chakravarti et al., 2006; Bozza et al., 2008) enquanto que outros estudos relatando um papel protetor para o IFN- γ. Alguns autores afirmam que a produção de IFN-γ foi essencial na resposta antiviral em camundongos infectados com DENV-2. (Fagundes et al., 2011; Costa et al., 2012). Essas diferenças provavelmente estão associadas ao sorotipo do vírus ou ao polimorfismo genético individual envolvido, além da cinética de produção desses mediadores ao longo da evolução da doença.

Algumas citocinas pró-inflamatórias parecem exercer um papel patológico durante a infecção. Estudos experimentais com o modelo murino demostraram que o bloqueio do TNF-α resultou na redução da taxa de mortalidade induzida durante as infecções primárias e secundárias (Atrasheuskaya *et al.*, 2003; Zellweger *et al*., 2010). Linfócitos T isolados de pacientes com FHD produziram níveis elevados de TNF-α após estimulação com DENV *in vitro* (Mangada et al., 2002). Além disso, os altos níveis

circulantes dessa citocina têm sido associados com casos mais graves da doença (Costa el al., 2013).

Os níveis circulantes das interleucinas (IL) IL1β, 4, 6, 10 18, TGF-β também se encontram elevados em pacientes infectados com diferentes sorotipos e manifestações clínicas graves (Srikiatkhachorn & Green, 2010). As quimiocinas MCP1/CCL2, MIP1α/CCL3, IL8/CXCL-8 e IP-10/CXCL10 são importantes mediadores inflamatórios e também parecem estar associadas com a gravidade (Lee et al., 2006; Bozza et al., 2008).

A hipótese da tempestade de citocinas (do inglês cytokine storm) tem sido estudada por diversos grupos. Análises de soros de pacientes com FHD/SCD no Vietnã, Índia e Cuba demonstrou a presença de níveis elevados de IFN-γ, TNF-α e IL-10 (Perez et al., 2004; Nguyen et al., 2005, Chakravarti et al., 2006). Um estudo recente demonstrou um aumento significativo de MCP-2, IP-10 e TRAIL no soro de pacientes venezuelanos durante a fase febril (Becerra et al., 2009). Apesar da extensa investigação sobre o papel das citocinas na progressão da dengue grave (Chaturvedi et al., 2000; Mustafa et al., 2001; Chatuverdi et al., 2009; Priyadarshini et al., 2010), os perfis de citocinas, especialmente, na fase defervescence e a sinergia entre o que conduz o extravassamento plásmatico ainda não está ainda totalmente compreendido

As citocinas e mediadores inflamatórios exercem um importante papel na patogênese da dengue, podendo agir tanto de forma protetora como na exacerbação da resposta imune, o que leva a um descontrole das respostas que culminará na gravidade da doença. Ainda não está claro de que forma essa produção excessiva de citocinas é induzida e controlada, porém estudos recentes sugerem que casos graves da doença acionam uma descontrolada ativação de células do sistema imune, assim como a ativação da produção de mediadores pró-inflamatórios com uma consequente disfunção das células endoteliais provocadas pela infecção (Costa *et al*., 2013).

1.16. Diagnóstico laboratorial

O diagnóstico preciso e eficiente da dengue é primordial para o cuidado clínico adequado, detecção precoce de casos graves, diagnóstico diferencial com outras doenças infecciosas, atividades de vigilância, controle de epidemias, entendimento da patogênese, desenvolvimento de pesquisas acadêmicas, vacinas e ensaios clínicos (OMS, 2009).

Os métodos laboratoriais para a confirmação de infecção pelos DENV envolvem detecção do vírus, do ácido nucléico viral, captura de antígeno (NS1) e detecção de

anticorpos específicos (IgM/IgG), ou a combinação dessas técnicas (OMS, 2009). O período de doença determinará o método laboratorial ideal de investigação da infecção (Figura 1.6). O vírus pode ser detectado no soro, plasma, células sanguíneas e tecidos (OMS, 2009).Atualmente estudos utilizando amostras alternativas como saliva e urina têm possibilitado a detecção do vírus por qRT-PCR e detecção de anticorpos e, assim, mesmo não apresentando resultados similares às amostras de plasma, a utilização desses fluídos torna-se importante em situações nas quais a coleta de sangue não seja possível (Andries *et al*., 2015).

O isolamento viral é considerado "padrão ouro" através do qual é possível a identificação direta do vírus. É uma técnica demorada e requer pessoal técnico especializado na executação. A inoculação da amostra suspeita pode ser realizada em mosquitos, cultura de células ou em camundongos recém-nascidos, considerando, no entanto, que a viremia coincide com a fase febril e que o isolamento é significaticamente maior nas amostras coletadas até o 6º dia de doença, (Kao *et al*., 2005). O isolamento viral em células de mosquitos clone C6/36 (Igarashi, 1978) tem sido o mais utilizado, pois demostrou alta sensibilidade aos DENV e por sua fácil manutenção (Miagostovich *et al*., 1993). A presença do vírus pode ser detectada pelo efeito citopático (ECP) na monocamada celular ou pela detecção do antígeno viral através da técnica de imunofluorência indireta que utiliza anticorpos monoclonais específicos para os quatro sorotipos (DENV-1 a 4) (Gubler *et al.*, 1984).

Para a detecção do ácido nucléico viral e do sorotipo infectante diversos protocolos de amplificação genômica utilizando transcrição reversa seguida da reação em cadeia pela polimerase (RT-PCR), têm sido utilizados no diagnóstico rápido das infecções por dengue (Henchal *et al*., 1991, Lanciotti *et al.*, 1992; Figueiredo *et al*., 1997; De Paula *et al*., 2002).

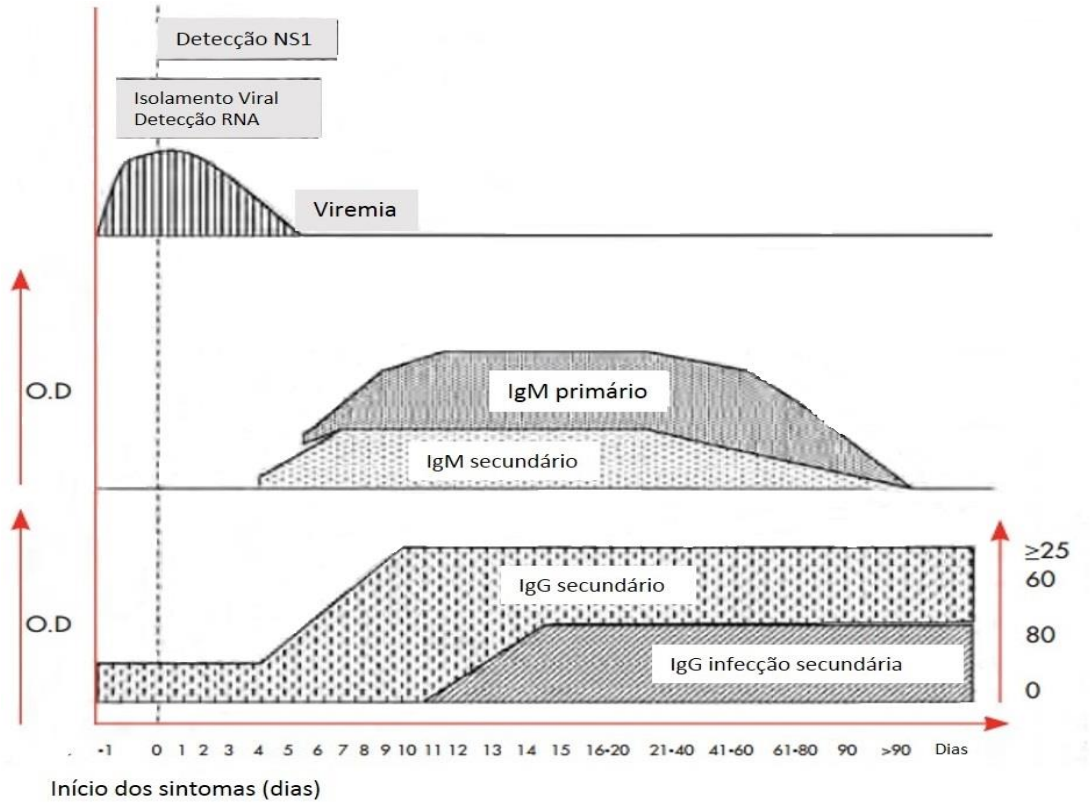

Figura 1.6: Linha do tempo aproximada das infecções primária e secundária pelos vírus dengue e métodos de diagnóstico de acordo com o dia de doença (Adaptado de OMS 2009).

As técnicas sorológicas utilizadas para a detecção de anticorpos específicos, possibilita a identificação de infecções atuais ou recentes, através da captura das imunoglobulinas de classe M (IgM) por ELISA (MAC ELISA) (Kuno *et al*., 1987). Teste para a detecção de anticorpos da classe IgG (IgG-ELISA), que foi desenvolvido por Miagostovich *et al*.(1999) em substituição ao teste de HI, é utilizado para classificar o tipo de infecção para a dengue (primária ou secundária) de acordo com os títulos observados no teste.

A detecção de antígeno NS1 pode ser realizada através de método imunoenzimático (ELISA) ou por testes imunocromatográficos rápidos, disponíveis comercialmente, que permitem a detecção de antígenos NS1 virais específicos, precocemente. Estudo recente que avaliou estes kits comerciais para a detecção do antígeno NS1, confirmou que, além de serem utéis para o diagnóstico laboratorial da dengue primária e secundária na fase aguda, devem ser usados em combinação com o MAC-ELISA para a detecção de casos e como triagem para complementar o isolamento viral (Lima *et al*., 2010).

A imunohistoquímica, também utilizada para o diagnóstico das infecções por DENV, é realizada em cortes de tecidos fixados em formalina e emblocados em parafina, permitindo a detecção de antígenos virais. Os cortes são corados pela fosfatase alcalina ou peroxidase marcados com anticorpos específicos. Essa técnica é bastante sensível e específica, levando em conta que se trata de um exame confirmatório que deve ser utilizado após diagnóstico histopatológico presuntivo (SVS/MS, 2010).

2. JUSTIFICATIVA

Há exatos 30 anos, o Brasil vem sofrendo o impacto de epidemias causadas pelos DENV, desde a introdução do DENV-1 em 1986. O aumento da frequência e da intensidade das epidemias de dengue ocorridas nos últimos anos deve-se à intensificação do processo de urbanização, à falta ou irregularidade do abastecimento de água potável e à deficiência na coleta de lixo, fatores que acometem com mais frequência as comunidades pobres. Neste sentido, o manejo dos determinantes socioambientais que resultam na exposição aos DENV representa a medida mais efetiva de controle da doença.

A identificação precoce de marcadores associados à gravidade e à letalidade por dengue pode ser capaz de predizer o impacto que uma epidemia pode exercer sobre determinada comunidade.

O avanço nas pesquisas de evolução viral através de estudos filogenéticos, combinados a dados epidemiológicos, possibilitou a identificação de genótipos dentro de cada sorotipo associados com uma maior ou menor gravidade da doença (Rico-Hesse, 1990; 2007; Messer *et al*., 2002; 2003). Dentre os sorotipos de DENV, o DENV-2 tem sido o mais estudado devido a sua associação com grandes epidemias e com manifestações clínicas mais graves (Rico-Hesse, 2003), estando, portanto, frequentemente associado a casos de FHD/SCD (Balmaseda *et al*., 2006). Porém, ainda não estão bem definidos quais são os fatores virais e do hospedeiro que determinam porque certos indivíduos apresentam FD e outros desenvolvem formas graves da doença. Em regiões onde o dengue é hiperendêmico, estudos têm demonstrado a circulação de linhagens virais com diferentes características fenotípicas, incluindo virulência e transmissibilidade distintas dentro dos genótipos circulantes. Atualmente, não há um consenso na utilização das diferentes classificações descritas para os genótipos de DENV. Portanto, durante a apresentação dos resultados deste estudo, a classificação adotada para a genotipagem dos DENV brasileiros foi de acordo com a descrita por Weaver & Vasilakis (2009).

O DENV-2 foi introduzido no estado do Rio de Janeiro em 1990 e, após um curto período, sua presença já era detectada em 24 dos 26 estados da federação (Nogueira *et al*. 1990). Neste período, foram registrados os primeiros casos de FHD/SCD e um aumento no número de hospitalizações (Nogueira *et al*. 1990, Zagne *et al*. 1994). Após sete anos sem atividade, este sorotipo reemergiu em 2007 e causou a epidemia mais grave de dengue no país até então, caracterizada por um maior número de hospitalizações e óbitos em crianças em 2008 (Teixeira *et al*., 2009; SVS, 2009; Macedo *et al*., et al 2013).

A falta de modelos animais que reproduzam a forma grave da doença levou ao desenvolvimento de modelos *in vitro* utilizando linhagens humanas primárias, como monócitos e células dendríticas diferenciadas (MoDC) e ainda linhagens endoteliais, capazes de produzir citocinas e fatores solúveis quando infectadas pelo DENV. Embora vários estudos *in vitro* já tenham sido realizados utilizando MoDC, a maioria destes utiliza cepas asiáticas, com escassos estudos utilizando cepas brasileiras.

Diante do exposto, visamos neste estudo, analisar os aspectos filogenéticos, epidemiológicos, laboratoriais, clínicos e imunológicos de cepas de DENV-2 circulantes no Brasil. A filogenia e caracterização molecular dos vírus circulantes podem predizer o impacto de cepas virulentas na população enquanto que a identificação de marcadores, relacionados tanto ao hospedeiro quanto ao vírus, poderá contribuir na predição de risco aumentado para o desenvolvimento de formas graves da doença, contribuindo, consequentemente, para as decisões terapêuticas mais adequadas e precoces.

3. OBJETIVO GERAL

Analisar e caracterizar aspectos virais, epidemiológicos, laboratoriais, clínicos e imunológicos de infecções causadas por DENV-2 circulantes no Brasil.

3.1. Objetivos específicos:

3.1.1 Análise de aspectos virais através de estudos filogenéticos:

- Realizar a caracterização molecular e a filogenia das cepas de DENV-2 circulantes no Brasil durante o período de 20 anos (1990-2010);

3.1.2. Análise dos aspectos epidemiológicos, laboratoriais, clínicos e/ou imunológicos de casos de dengue:

- Analisar os casos de DENV-2 associados à gravidade da doença durante epidemia ocorrida no Rio de Janeiro em 2008;
- Analisar as epidemias causadas pela co-circulação de DENV-1 e DENV-2 em 2010 e por DENV-4 em 2013, em Campo Grande, Mato Grosso do Sul.
- Correlacionar infecção por DENV-2 à hepatite fulminante durante epidemia ocorrida em Campo Grande, Mato Grosso do Sul em 2010;
- Analisar casos de DENV-2 associados à gravidade da doença e comparando aos casos de DENV-4 em epidemias ocorridas em Campos dos Goytacazes, Rio de Janeiro em 2010 e 2013.

3.1.3. Análise dos aspectos virais através de estudos de infecção in vitro de células dendríticas:

- Padronizar a infecção das duas linhagens de DENV-2 circulantes no Brasil em MoDCs humanas e determinar as taxas de infecção viral, a expressão de moléculas fenotípicas de DC, de citotoxidade, de ativação e produção de citocinas.

4. RESULTADOS

Os resultados serão apresentados sob a forma de artigos publicados, aceitos e submetidos à publicação em revistas indexadas:

4.1. Análise dos aspectos virais através de estudos filogenéticos:

Artigo 1: Twenty years of DENV- 2 activity in Brazil: molecular characterization and phylogeny of strains isolated from 1990 to 2010. (Publicado na PLoS Neglected Tropical Disease. 7(3): e2095, 2013. doi: 10.1371/journal.pntd.0002095).

4.2. Análise dos aspectos epidemiológicos, laboratoriais, clínicos e/ou imunológicos de casos de dengue:

Artigo 2: Dengue Severity Associated with Age and a New Lineage of Dengue Virus Type 2 during an Outbreak in Rio de Janeiro, Brazil. (Publicado na Journal of Medical Virology).

Artigo 3: Dengue epidemics in two distinct periods reveal distinct epidemiological, laboratorial and clinical aspects in a same scenario: analysis of the 2010 and 2013 epidemics in Mato Grosso do Sul, Brazil (Publicado na Transactions of the Royal Society of Tropical Medicine and Hygiene).

Artigo 4: Fulminant hepatitis associated with Dengue Virus type 2- a case report (Submetido à Journal of Medical Virology).

Artigo 5: Analysis of clinical and laboratorial alterations related to dengue cases severity: a comparative study between serotypes 2 and 4 in Brazil. (Aceito pela American Journal of Tropical Medicine and Hygiene).

4.3. Análise dos aspectos virais através de estudos de infecção in vitro de células dendríticas:

Artigo 6: Analysis of the infection human dendritic cell “in vitro” by different lineages of the DENV-2 genotype Southeast Asian circulation in Brazil. (Em fase de preparação).

Artigo 1: Vinte anos de atividade do DENV-2 no Brasil: caracterização molecular e filogenia de cepas isoladas entre 1990 e 2010.

- **Objetivo específico:** Realizar a caracterização molecular e filogenia de cepas de DENV-2 circulantes no Brasil durante 20 anos (1990-2010);

Situação do manuscrito: Artigo publicado na revista PLoS Neglected Tropical Disease.

Classificação QUALIS : Área de Medicina II – A1

Referência: **Faria NRC**, Nogueira RM, de Filippis AM, Simões JB, Nogueira Fde B, da Rocha Queiroz Lima M, dos Santos FB.

Resumo: No Brasil, a dengue tem sido um grande problema de saúde pública desde a sua introdução na década de 1980. Estudos filogenéticos constituem uma ferramenta valiosa para monitorar a introdução e a propagação de vírus, bem como para prever as possíveis consequências epidemiológicas desses eventos. Com o objetivo de realizar a caracterização molecular e análise filogenética do DENV-2 durante vinte anos de atividade viral no país, cepas virais isoladas de pacientes que apresentam diferentes manifestações da doença (n = 34), representando seis estados do país, de 1990 a 2010, foram sequenciadas. O sequenciamento parcial do genoma (genes C / prM / M / E) foi realizado em 25 cepas de DENV 2 (região codificante) e o sequencimento do genoma completo foi realizado em 9 cepas. A análise da similaridade entre os DENV-2 com cepas de referência identificou a formação de dois grupos epidemiologicamente distintos: um formado por cepas isoladas entre os anos de 1990 a 2003 e outro por cepas entre 2007 e 2010. Não foram observadas diferenças consistentes entre as seqüências do gene E das cepas dos diferentes casos clínicos de dengue, sugerindo que a gravidade da doença tem uma origem genética e que, não se deve apenas as diferenças observadas no gene E viral. Os resultados obtidos pelo sequenciamento completo do genoma do DENV-2 não apontou diferenças consistentes relacionadas com uma doença mais grave. A análise com base no sequenciamento parcial e/ou total do genoma caracterizou as amostras de DENV-2 brasileiras como pertencente ao genótipo do Sudeste Asiático, no entanto, uma distinção de duas linhagens dentro deste genótipo foi identificada. Ficou caracterizado que as cepas do período pré-emergência (1990-2003) pertencem ao genótipo do Sudeste Asiático, Linhagem I e as cepas isoladas

após a re-emergência deste sorotipo em 2007, pertencem ao genótipo Sudeste Asiático, Linhagem II. Além disso, todas as cepas analisadas neste estudo possuem uma asparagina (N) em E_{390}, previamente caracterizada como um provável determinante genético desencadeador de FHD detectado em cepas de origem asiática. O percentual de similaridade destas cepas com a cepa isolada na República Dominicana em 2001 combinado ao percentual de divergência com as cepas introduzidas no país na década de 90 sugere que estes vírus não sofreram uma evolução local, e sim uma introdução no país de uma linhagem viral distinta provavelmente importada do Caribe.

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Twenty Years of DENV-2 Activity in Brazil: Molecular Characterization and Phylogeny of Strains Isolated from 1990 to 2010

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Abstract

In Brazil, dengue has been a major public health problem since its introduction in the 1980s. Phylogenetic studies constitute a valuable tool to monitor the introduction and spread of viruses as well as to predict the potential epidemiological consequences of such events. Aiming to perform the molecular characterization and phylogenetic analysis of DENV-2 during twenty years of viral activity in the country, viral strains isolated from patients presenting different disease manifestations ($n = 34$), representing six states of the country, from 1990 to 2010, were sequenced. Partial genome sequencing (genes C/prM/M/E) was performed in 25 DENV-2 strains and full-length genome sequencing (coding region) was performed in 9 strains. The percentage of similarity among the DENV-2 strains in this study and reference strains available in Genbank identified two groups epidemiologically distinct: one represented by strains isolated from 1990 to 2003 and one from strains isolated from 2007 to 2010. No consistent differences were observed on the E gene from strains isolated from cases with different clinical manifestations analyzed, suggesting that if the disease severity has a genetic origin, it is not only due to the differences observed on the E gene. The results obtained by the DENV-2 full-length genome sequencing did not point out consistent differences related to a more severe disease either. The analysis based on the partial and/or complete genome sequencing has characterized the Brazilian DENV-2 strains as belonging to the Southeast Asian genotype, however a distinction of two Lineages within this genotype has been identified. It was established that strains circulating prior DENV-2 emergence (1990–2003) belong to Southeast Asian genotype, Lineage I and strains isolated after DENV-2 emergence in 2007 belong to Southeast Asian genotype, Lineage II. Furthermore, all DENV-2 strains analyzed presented an asparagine (N) in E_{390}, previously identified as a probable genetic marker of virulence observed in DHF strains from Asian origin. The percentage of identity of the latter with the Dominican Republic strain isolated in 2001 combined to the percentage of divergence with the strains first introduced in the country in the 1990s suggests that those viruses did not evolve locally but were due to a new viral Lineage introduction in the country from the Caribbean.

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Introduction

Dengue viruses (DENV) are the most important human arboviruses worldwide, transmitted by mosquitoes of the genus *Aedes*, *Aedes aegypti* is the main vector. Explosive epidemics have become a public health problem, economic impact, socially and politically significant [1,2].

Currently it is estimated that 70 to 500 millions dengue infections occur annually in 124 endemic countries. Nearly 3.6 billion people (55% of world population) are at risk of contracting the disease (DVI). The rapid global spread of DENV in the last 50 years resulted in the dispersal of genotypes associated with increased severity [3].

The four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) are closely related yet antigenically distinct and contain a positive-sense RNA genome that is translated as a single polyprotein and post-translationally cleaved into three structural proteins, capsid (C), premembrane (prM) and envelope (E), and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The RNA genome is packaged in an icosahedral capsid, and the nucleocapsid is surrounded by a lipid bilayer containing the E and M proteins [4,5].

DENV infection causes a spectrum of clinical disease ranging from an acute debilitating, self-limited febrile illness - dengue fever (DF) - to a life-threatening syndrome - dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [6]. Despite the similar disease manifestations, the DENV are genetically diverse with approximately 40% of amino acid sequence divergence. Distinct DENV genotypes can be characterized when the genetic divergence are higher than to 6% [7].

A recent analysis of 1,827 complete E gene sequences supported the existence of six genotypes for DENV-2: Asian genotype I,

Author Summary

In Brazil, the first dengue haemorrhagic cases were reported after the DENV-2 introduction in Rio de Janeiro, which spread to other states in the country. Aiming to perform the molecular characterization and phylogenetic analysis of DENV-2 during twenty years of viral activity in the country, strains isolated from patients presenting different disease manifestations were sequenced. Phylogeny characterized the DENV-2 as belonging to the Southeast Asian genotype, however a distinction of two Lineages within this genotype has been identified. Furthermore, all strains presented an asparagine in E_{390}, previously identified as a probable genetic marker of virulence. The results show a temporal circulation of genetically different viruses in Brazil, probably due to the introduction of a new viral lineage from the Caribbean, which lead to the re-emergence of this serotype after 2007, causing the most severe epidemic already described in the country.

Asian genotype II, Southeast Asian/American genotype, Cosmopolitan genotype, American genotype and the Sylvatic genotype, the most genetically distinct genotype. Furthermore, the Southeast Asian/American genotype's topologies suggested a spatial division of this genotype into two major subclades [8].

In the Americas, the first DHF epidemics in the 80's were due to the introduction of the Southeast Asian/American genotype which replaced the American genotype and more severe cases with higher viremia were reported [9–11].

In Brazil, the disease has become a public health problem with explosive epidemics after the introduction of DENV-1 in 1986 in Rio de Janeiro [12]. However, the first DHF/DSS cases were only reported after the DENV-2 introduction in 1990 in the country [13,14]. From 1990 until the 26th epidemiological week of 2010, a total of 5,481,921 cases, including 17,203 cases of dengue hemorrhagic fever (DHF) and 1954 deaths were reported in the country [15].

Aiming to perform the phylogeny of the DENV-2 and its impact in the disease severity during 20 years of viral activity in Brazil, strains isolated from DF, DHF/DSS and fatal cases occurred since its introduction in 1990 until 2010, were analyzed. In this scenario, the partial sequencing (C/prM/M/E genes) of 25 DENV-2 strains was performed. To determine whether the evolutionary relationships observed for the C/prM/M/E genes were applicable to the complete genome, we further fully sequenced the coding regions of nine DENV-2 strains. In order to avoid mutations introduced by *in vitro* passages of the virus in cell cultures we used DENV-2 strains extracted directly from serum or originally isolated from cell cultures.

Materials and Methods

Ethical statement

The strains analyzed in this study belong to a previously-gathered collection from the Laboratory of Flavivirus, IOC/FIOCRUZ, Rio de Janeiro, Brazil, obtained from human serum through the passive surveillance system performed by the Laboratory from an ongoing Project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health-Brazil. Samples were chosen anonymously, based on the laboratorial results and clinical manifestations input on the Laboratory database.

Viral strains

Viral strains consisted of DENV- 2 ($n = 34$) isolated during epidemics occurred from 1990 to 2010 in six states in Brazil (Table 1). Each sample was accompanied by identification form containing clinical and epidemiological data. All strains were determined as DENV-2 serotype by reverse transcriptase polymerase chain reaction (RT-PCR) and or/virus isolation from DF ($n = 19$), DHF ($n = 3$), DSS ($n = 1$) and fatal cases ($n = 4$; 1 from DF, 2 from DHF and 1 with no classification available). Seven cases were not classified due to data unavailability.

RNA extraction

Viral RNA was extracted from infected cell culture supernatant or directly from the patients' serum using QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's instructions and stored at −70°C for DENV typing and sequencing.

RT –PCR (Reverse transcriptase- polymerase chain reaction)

RT—PCR for detecting and typing DENV was performed as described previously [16]. Briefly, consensus primers were used to anneal to any of the four DENV types and amplify a 511-bp product in a reverse transcriptase-polymerase reaction. A cDNA copy of a portion of the viral genome was produced in a reverse transcriptase reaction. After a second round of amplification (nested PCR) with type-specific primers, DNA products of unique size for DENV-2 (119 bp) were generated.

Dengue virus isolation

Virus isolation was performed by inoculation into C6/36 *Aedes albopictus* cell line [17] and isolates were identified by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies [18]. Briefly, patients' sera were inoculated into C6/36 *Aedes albopictus* cell monolayers in L-15 Medium (Leibovitz, Sigma) supplemented with 2% fetal calf serum (FCS, Invitrogen) and 0.2 mM of nonessential amino acids (Invitrogen). Cells were incubated at 28°C for 5 to 7 days and observed for cytopathic effects. Infected supernatant was clarified by centrifugation and virus stocks stored in 1-mL aliquots at −70°C until use.

Sequencing

Reverse transcription (RT) was performed using 5 µL of extracted RNA in 25 µL of AccessQuick RT-PCR System (Promega Corporation) and specific oligonucleotides primers (Table 1). To amplify the C/prM/M/E region of 2,325 bp, specific primers (1 to 4) were used to produce 4 overlapping amplicons of approximately 900 bp and to amplify the complete coding region (10,173 bp), 15 overlapping amplicons of approximately 900 bp (1 to 15). Thermocycling conditions consisted of a single step of 42°C for 60 minutes and 40 cycles of denaturation at 94°C (30 seconds), annealing at 56°or 63°C (60 seconds) depending on the set of primers, extension at 72°C (2 minutes) and a final extension at 72°C (10 minutes). Amplification was conducted using a Model 9700 thermal cycler (Applied Biosystems). PCR products were purified from 1.0% agarose gels using QIAquick Gel extraction Kit or QIAquick PCR purification Kit (Qiagen) and used as template for cycle sequencing. Sequencing reactions were performed as recommended in the BigDye Dideoxy Terminator sequencing kit (Applied Biosystems) and the products were analyzed using an automated 3130 DNA Sequencer (Applied Biosystems). Partial sequences (C/prM/M/E) and complete coding sequences for the unprocessed polyprotein (5′ and 3′ noncoding regions excluded) were deposited in GenBank (Table 2).

Table 1. Primers used for amplification of the partial and complete genes (coding region) from Brazilian DENV-2.

Primers Designation	Sense A (5'→3')	Anti-sense B (5'→3')	Position in the genome (according to AF489932)	Amplicon (pb)	Tm (°C) A/B
1	CGT GGA CCG ACA AAG ACA GA	GGA GCG ACG GCT GTC AGT AA	14–906	892	62/64
2	GAT CAG TGG CAC TCG TTC CA	CTC CGG GTAGCCATGGTAAC	708–1580	878	62/62
3	ATG GCA CTG TCA CGA TGG AG	CAC TAT CAG CCTGCACCATAGCT	1467–2405	938	62/63
4	GGA TCC CTG GGA GGA GTG TT	TCC ATT GCT CCA GAG GGT GT	2202–3106	904	63/63
5	GAC TCA AAA CTC ATG TCA GCG G	GTG CTT TGG GAA AGG AGT GC	2958–3800	842	62/62
6	GGG CGT TAC CAT GAC GGA T	GCC CAT GAT GGT TCA ATC CTT	3656–4709	1053	63/63
7	AAT TAC GGC AGC AGC ATG GT	GGA GGA GTG GCT GTC ATG AAA	4475–5456	981	63/63
8	CAG CCA TCA GAA CCG AGC A	CCA CCT TCT GTC TGC GTA GTT G	5254–6185	931	64/62
9	ACA CAC CTG AAG GAA TCA TTCCTA G	TGA CAA ATG TTG TAG CCA CGG	6016–6948	932	62/62
10	AGC CAT CCT CAC AGT GGT GG	TCT CAG TTT TGC TGA GCC TCG	6791–7737	946	64/63
11	CTA TTT GGC CGG AGC TGG A	TTT CAA TTC CAA TGT TGC GG	7508–8354	846	63/62
12	ATG GAG GAG CTT TAG TGA GGA ATC	CGT GCT CCA AGC CAC ATG TA	8170–8994	824	61/63
13	GAA ATC GGC TCG TGA GGC T	TCA TCT TGG TTT CTG CAT GGG	8825–9746	921	63/63
14	GAC AGT CAC AGA AGA AAT CGC TGT	CTA TGG CTT GAT CCG ACC TGA	9473–10304	831	62/62
15	CGG CTC ATT GAT TGG GCT AA	TTC TGT GCC TGG AAT GAT GCT	10109–10662	553	63/63

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Sequences and phylogenetic analysis

The analysis of similarities, percentage of identity and divergence among the strains analyzed were performed using Megalin Program (DNAstar, www.dnastar.com). The multiple alignment was performed using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and the phylogenetic analysis by MEGA 4 software (www.megasoftware.net), using the Maximum Likelihood method (ML), according to the Tamura-Nei model, with a bootstrap of 1,000 replications. Strains representative from the five genotypes available in Genbank (www.ncbi.nlm.nih.gov) were used for the comparison, DENV-1 (GenBank accession number GU370049), DENV-3 (accession number EF629369), and DENV-4 (accession number AF289029) strains were used as outgroup to root the trees (Table 3).

Results

In this study, the strains BR64022/98 isolated in the 90's and Jamaica 1983 were considered as reference strains for comparison purposes. The percentage of similarity among the 25 DENV-2 strains ranged from 80.3 to 99.9% when those compared to each other and to strains representative of the different genotypes available on GenBank. The partial genome sequencing analysis characterized the Brazilian DENV-2 strains from this study as belonging to the Southeast Asian genotype, however a distinction of two Lineages within this genotype has been identified. It was observed that strains circulating prior DENV-2 emergence (1990–2003) belong to Southeast Asian genotype, Lineage I and strains isolated after DENV-2 emergence in 2007 belong to Southeast Asian genotype, Lineage II (Figures 1 and 2). Furthermore, the latter were more closely related to strains from the Dominican Republic (DR59/01), representative from the Southeast Asian genotype, Lineage II.

When the 25 DENV-2 strains were compared to the strain BR64022/98, amino acid substitutions leading to change in the biochemical properties were observed on the C and prM genes. On the E gene, a total of twelve substitutions were observed, with nine resulting in a change on the amino acid change of biochemical property (Supplementary material 1). No consistent differences were observed on the E gene from strains isolated from cases with different clinical manifestations analyzed, suggesting that if the disease's severity has a genetic origin, it is not only due to the differences observed on the E gene.

To determine whether possible amino acids differences on other genes were related to disease severity, we fully analyzed (coding region) DENV-2 strains ($n = 9$), representative of DF cases isolated from 1990 to 1999 and strains isolated from fatal cases occurred after the DENV-2 re-emergence after 2007 until 2010. The strain 0450/2008, representative of the DENV-2 re-emergence isolated from a DF secondary case who evolved to death was fully sequenced and its comparison to the strain from the Dominican Republic (DR59/2001), representative of the DENV-2 re-emergence, showed 22 amino acid substitutions. Likewise, the strain 0690/2008 isolated from a DHF case occurred also during the re-emergence of DENV-2 had nine had amino acid substitutions when compared to the strain DR59/2001, with seven of those leading to amino acid biochemical property change (Table S1).

The DENV-2 strain 0337/2008 isolated from a newborn presenting a high anti-DENV IgG titer who evolved to death, infected probably due transplacental transmission as his mother was diagnosed with acute DENV infection, showed substitutions on NS2A, NS4A and NS5, which were shared with the other two strains isolated from fatal cases (Table S2). The results obtained by the DENV-2 full-length genome sequencing did not point out consistent differences related to a more severe disease.

A substitution on E_{390} (N→D) was reported as resulting in a reduction in viral replication in macrophages and dendritic cells [19] whereas E_{390} (D→N) resulted in enhanced replication, maturation and activation of macrophages, enhancement of the immune response with an increased production of cytokines, increased vascular permeability and consequently a greater chance of developing DHF [20].All DENV-2 strains analyzed presented an asparagine (N) in E_{390}, previously identified as a probable genetic marker of virulence observed in DHF strains from Asian origin.

The percentage of identity of the re-emergent DENV-2 with the Dominican Republic strain isolated in 2001 combined to the

Table 2. DENV-2 used in this study for partial ($n=25$) and complete coding region ($n=9$) sequencing.

Strain #	Year of isolation	State of origin	Clinical manifestation	Imunne response	Age	Gender	Sequence region	Acess number GenBank
44298	1991	BA	DF	S	NA	Fem	C/prM/M/E	HQ012508
48578	1994	CE	NA	ND	NA	Male	C/prM/M/E	HQ012509
51222	1995	RJ	NA	ND	NA	Fem	C/prM/M/E	HQ012510
52477	1995	RJ	NA	ND	NA	Fem	C/prM/M/E	HQ012511
55769	1996	RS*	DF	ND	10	Male	C/prM/M/E	HQ012512
55803	1996	BA	NA	S	NA	Fem	C/prM/M/E	HQ012513
58448	1997	RN	DF	ND	NA	Male	C/prM/M/E	HQ012514
59382	1997	RN	DHF/Fatal	ND	NA	Male	C/prM/M/E	HQ012515
63291	1998	RJ	DF	ND	16	Male	C/prM/M/E	HQ012516
64625	1999	RJ	DF	ND	34	Male	C/prM/M/E	HQ012517
66985	2000	RJ	DF	ND	39	Male	C/prM/M/E	HQ012518
67955	2000	RJ	DHF	ND	27	Male	C/prM/M/E	HQ012519
69221	2001	RJ	DF	ND	28	Male	C/prM/M/E	HQ012520
72308	2001	RJ	DF	ND	62	Fem	C/prM/M/E	HQ012521
75103	2002	RJ	DF	ND	61	Masc	C/prM/M/E	HQ012522
76012	2002	ES	NA	ND	41	Fem	C/prM/M/E	HQ012523
77395	2003	ES	NA	ND	50	Male	C/prM/M/E	HQ012524
86977	2007	RJ	DHF	ND	7	Male	C/prM/M/E	HQ012525
88034	2007	RJ	DF	ND	12	Male	C/prM/M/E	HQ012526
0030	2008	RJ	DF	S	13	Male	C/prM/M/E	HQ012527
0832	2008	RJ	DHF	S	8	Fem	C/prM/M/E	HQ012528
066	2009	BA	DF	ND	1 month	Male	C/prM/M/E	HQ012529
0145	2009	ES	DF	ND	16	Male	C/prM/M/E	HQ012530
023	2010	RJ	DF	ND	73	Male	C/prM/M/E	HQ012531
0199	2010	RJ	DSS	S	50	Fem	C/prM/M/E	HQ012532
39143	1990	RJ	DF	ND	41	Fem	Complete CR	HQ012538
41768	1990	RJ	DF	ND	10	Male	Complete CR	HQ012533
42727	1991	RJ	DF	P	NI	Fem	Complete CR	HQ012534
48622	1994	CE	NA	ND	NI	Fem	Complete CR	HQ012535
61310	1998	RJ	DF	ND	47	Fem	Complete CR	HQ012536
64903	1999	RJ	DF	ND	52	Fem	Complete CR	HQ012537
0337	2008	RJ	Fatal	S	5 days	NA	Complete CR	NA
0430	2008	RJ	DF/Fatal	S	46	Male	Complete CR	NA
0690	2008	RJ	DHF/Fatal	S	32	Male	Complete CR	HQ026763

BA: Bahia, CE: Ceará, RJ: Rio de Janeiro, RS: Rio Grande do Sul, RN: Rio Grande do Norte, ES: Espirito Santo; DF: Dengue Fever; DHF: Dengue Hemorrhagic Fever; DSS: Dengue Shock Syndrome; Fem: Female; Male; C/prM/M/E: Capsid/pré-membrane/Membrane/Envelope; Complete CR: Complete coding region;
*Imported case; NA: Not available; ND: Not done; P: primary infection; S: secondary infection.
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percentage of divergence with the strains first introduced in the country in the 90's suggests that those viruses did not evolved locally but were due to a new viral Lineage introduction in the country from the Caribbean.

Discussion

In the Americas, the first DENV-2 was isolated in 1953 in Trinidad [21] and the first DHF epidemic caused by this serotype occurred in Cuba in 1981 after the introduction of DENV-2 genotype originated in Southeast Asia [10,22]. Epidemics studies showed that the DENV-2 introduced in Brazil, Colombia, Venezuela and Mexico had a common ancestor with isolates from Southeast Asia, suggesting the direct transmission from that region to the Americas [23].

In Brazil, the first DHF/DSS cases were reported after the DENV-2 introduction in Rio de Janeiro [13,24,25], which spread to other states in the country. Phylogenetic analysis of DENV-2 strains circulating at that time confirmed the genotype circulating in Southeast Asia [26,27]. This observation was further corroborated in an extensive analysis of viruses from the states of Rio de Janeiro (1990 and 1995), Ceará (1994), Bahia (1994 and 1999), Maranhão (1996 and 1998), Mato Grosso (1997), Pará (1998), Rio Grande do Norte (1998), Paraiba (1999) Sergipe (1999), Espirito Santo (1995 and 2000) and forty strains isolated in Pernambuco (1995–2002) [28,29].

Table 3. Strains representative of the different DENV-2 genotypes and strains used as outgroup for comparison purposes.

Strain #	Year of isolation	Country	Genotype	GenBank Accession #
BR64022	1998	Brazil	Southeast Asia (Lineage I)	AF489932
BID-V3496	1990	Venezuela	Southeast Asia (Lineage I)	GQ868540
NL1409	1983	Jamaica	Southeast Asia (Lineage I)	M20558
BID-V2683	1999	Nicaragua	Southeast Asia (Lineage II)	GQ199895
BID-V2996	2007	Nicaragua	Southeast Asia (Lineage II)	GQ199868
BID-V595	2006	Puerto Rico	Southeast Asia (Lineage II)	EU482726
BID-V1439	2005	Puerto Rico	Southeast Asia (Lineage II)	EU687216
DR23/01	2001	Dominican Republic	Southeast Asia (Lineage II)	AB122020
DR59/01	2001	Dominican Republic	Southeast Asia (Lineage II)	AB122022
BID-V3653	2008	Brazil	Southeast Asia (Lineage III)	GU131885
China-04	1985	China	Asian II	AF119661
New Guinea C	1944	New Guinea	Asian II	AF038403
Strain 44	1989	China	Asian II	AF204177
T81GI	2004	Indonesia	Asian I	AY858036
98900666 DSS DV-2	1998	Indonesia	Asian I	AB189124
IQT1797	1995	Peru	American	AF100467
strain 131	1992	Mexico	American	AF100469
isolate 1328	1977	Puerto Rico	American	EU056812
Dak Ar D75505	1991	Senegal	Sylvatic	EF457904
DENV-1-SGEHI(D1)1494Y08	2008	Singapore	-	GU370049
BRDEN3 290-02	2002	Brazil	-	EF629369
DENV-4-Guangzhou B5	2000	China	-	AF289029

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After seven years without activity in Brazil, DENV-2 re-emerged in April of 2007 in the state of Rio de Janeiro causing the more severe dengue epidemic in the country in 2008 [30,31]. Phylogenetic analysis of DENV-2 circulating in 90's and after its re-emergence identified two distinct lineages within the Southeast Asian genotype [32].

In the present study, the analysis based on the sequencing of the C/prM/M/E genes (2,325 bp) from 25 DENV-2 Brazilian isolates divided those strains in two distinct groups, one formed by DENV-2 isolated from 1991 to 2003 and another with strains isolated from 2007 to 2010 following the re-emergence of this serotype in the country. Corroborating previous phylogeny [26–29] strains isolated from 1991to 2003 were classified as Southeast Asian genotype, Lineage I and presenting similarities with the Brazilian strain BR64022/98 and the strain Jamaica/83. However, the strains isolated between 2007 and 2010, showed higher similarity with the strain DR59/01, from the Dominican Republic, representing the Southeast Asian genotype, Lineage II, corroborating the analysis by Oliveira *et al* [32]. A study by Aquino *et al* [33] demonstrated that DENV-2 strains from Paraguay could also be grouped into two distinct lineages within the Southeast Asian genotype and suggested the introduction of a new lineage possibly associated a serotype shift from DENV-3 to DENV-2, as observed in Brazil in 2007 and 2008 [31].

The absence of DENV-2 circulation in the years prior to its re-emergence and the high similarity observed between those viruses and the strain isolated in the Dominican Republic in 2001, suggests the introduction of a new lineage of DENV-2 causing the 2008 epidemic in Brazil. Romano *et al* [34] also demonstrated that DENV-2 strains isolated in Sao Paulo State in 2010 were in a monophyletic group with the strains circulating in Rio de Janeiro in 2007 and 2008 and that those were closely related to strains isolated in Cuba and Dominican Republic, with a small genetic distance, suggesting that this new lineage of DENV-2 re-emerged in of Brazil may have been imported the Caribbean. Although genetic variants of DENV have been implicated in disease severity in the past [35,36], it was with the advance of evolutionary studies based on phylogenetic analysis combined to epidemiological data that genotypes within the distinct serotypes were associated with a greater or lesser disease severity [11,37–40].

The strain isolated from a DHF case in 2000 (strain RJ/67922/2000) presented an exclusive substitution on prM_{143} (T→I) when compared to the other strains analyzed in this study. However, substitutions related to DHF/DSS cases were identified on prM_{16} and prM_{81} [41].

Substitutions were found on the residues E_{129} (V→I) and E_{131} (L→Q), and these are related to the division of the Southeast Asian genotype in two distinct clades, corroborating the observations that amino acids on E_{129} and E_{131} are in critical markers for genetic classification of DENV [33,42].

All 34 strains analyzed in this study presented an asparagine (N) on E_{390}, previously characterized as a probable trigger for DHF detected in strains of Asian origin [43]. Mutations on the flaviviruses domain III of E protein can induce virulence or attenuation of the virus to escape from the immune system [44,45] and in this study, changes were observed throughout this domain (aa 297 to 394). The DHF case, which culminated in death (59382/1997) showed amino acid differences only in

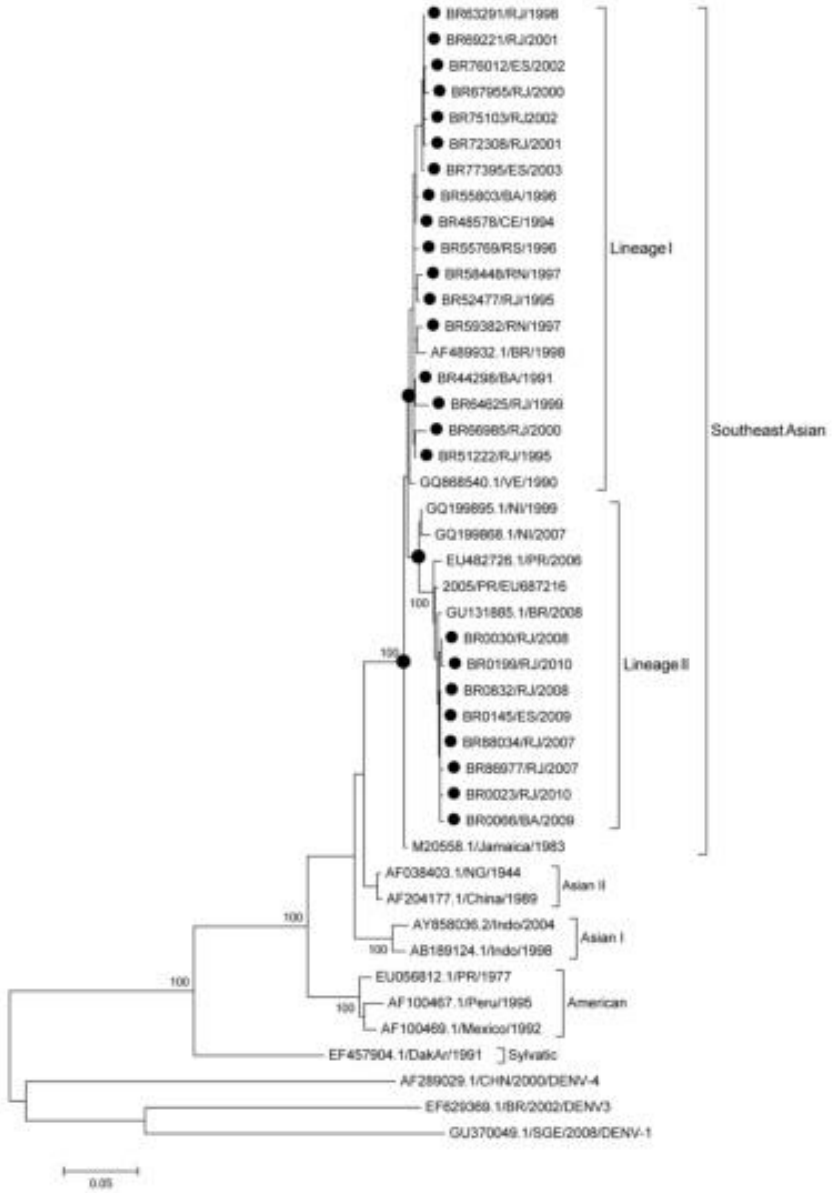

Figure 1. Maximum likelihood phylogeny based on the C/prM/M/E genes of 25 Brazilian DENV-2, 1991–2010. Black circles represent DENV-2 sequences generated in this study. Strains representative from the four genotypes available in Genbank (www.ncbi.nlm.nih.gov) were used for the comparison, DENV-1, DENV-3 and DENV-4 strains were used as outgroup to root the trees. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. DENV strains used were named as follows: Country/strain number/state/year. RJ: Rio de Janeiro, ES: Espírito Santo, CE: Ceará, BA: Bahia, RS: Rio Grande do Sul, RN: Rio Grande do Norte.
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the E gene, but those differences were shared with other DF cases strains, when they were compared to the strain BR64022/98.

In this study, a substitution on prM_{30} was observed on the strain 0690/2008 isolated from a DHF case with a fatal outcome, on the strain 55769/1996 from a DF case and on the strain 0199/2010.. Catteau *et al* [46] demonstrated that the intracellular production of M ectodomain of all four DENV serotypes of DENV induce apoptosis in host cells. The carboxy terminus of prM protein with nine amino acids (aa 32–40) of some flaviviruses was designated as Apopto M [46] and appears to play an important role in inducing apoptosis and cytopathic effects [46–48].

Several changes were observed along the NS protein genes. Studies conducted by Yábar, [49] show that mutations in NS1 are related to the development of DHF/DSS cases when they were compared to patients with DF.

Despite the functional importance of mutations in NS genes remains unknown, future studies can elucidate their role in the

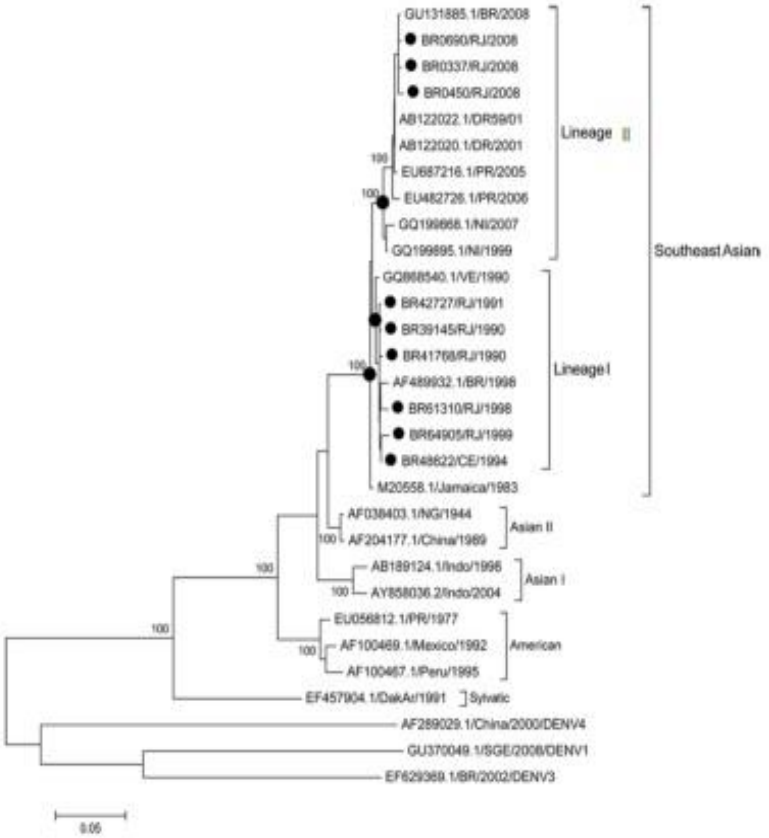

Figure 2. Maximum likelihood phylogeny based on the complete coding region sequencing of 9 Brazilian DENV-2, 1990–2008. Black circles represent DENV-2 sequences generated in this study. Strains representative from the four genotypes available in Genbank (www.ncbi.nlm.nih.gov) were used for the comparison, DENV-1, DENV-3 and DENV-4 strains were used as outgroup to root the trees. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. DENV strains used were named as follows: Country/strain number/state/year. RJ: Rio de Janeiro and CE: Ceará.
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emergence of strains and/or pathogenesis of the disease. It was not possible to correlate the role of Lineage II emergence with an increased severity of cases observed in the period between the years 2007–2010. Furthermore, the occurrence of secondary infection may have been the risk factor for the development of more severe cases.

In conclusion, this result shows a temporal circulation of genetically different viruses in Brazil probably due to the introduction of a new viral lineage from the Caribbean which lead to the re-emergence of this serotype after 2007. In 2007–2008, DENV-2 was responsible for most severe epidemic already described in the country, with 787,726 cases reported and 491 deaths [31]. Moreover, the Caribbean has been suggested as an important region for the circulation of DENV-2, importation and exportation of strains from and to Central America and South America [42,50,51].

In the past 20 years, DENV-2 activity in Brazil has contributed significantly to changes in the disease morbidity and sudden age shift [30]. In dengue endemic countries, displacement of DENV serotypes, genotypes and lineages have been reported previously and have been associated with changes in the disease severity [40,52–55]. This emphasizes the need of straightening virological surveillance to monitor the emergence or re-emergence of DENV strains with pathogenic potential to cause epidemics.

Supporting Information

Table S1 Molecular characterization of DENV-2 strains isolated in Brazil based on the partial genes analysis.
(DOCX)

Table S2 Molecular characterization of DENV-2 isolated in Brazil based on the complete coding region analysis.
(DOCX)

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Author Contributions

Conceived and designed the experiments: FBdS RMRN AMBdF. Performed the experiments: NRdCF JBSS FdBN MdRQL. Analyzed the

data: NRdCF FdBN. Contributed reagents/materials/analysis tools: RMRN. Wrote the paper: NRdCF FBdS.

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Artigo 2: Dengue grave associada à idade e à nova linhagem do DENV-2 no Rio de Janeiro Brasil.

Objetivo específico: Analisar os aspectos epidemiológicos, laboratoriais e clínicos de casos de DENV-2 associados à gravidade da doença durante epidemia ocorrida no Rio de Janeiro em 2008;

Situação do manuscrito: Artigo publicado na Journal of Medical Virology

Classificação QUALIS: Aréa de Medicina II – B1

Referência: Nunes, PCG, Sampaio SA, **Faria NRC,** Lima de Mendonça M, Lima MRQ, Aráujo ESM, dos Santos FB, Simões JB, Gonçalves BG, Nogueira RMR, Filippis AMB.

Resumo: Vírus dengue tipo 2 (DENV-2) causou três epidemias, nos anos de 1990, 1998 e 2008, no Rio de Janeiro, Brasil. A epidmeia de 2008 foi a mais grave em número de casos relatados, hospitalizações e mortes. Para investigar fatores virológicos e epidemiológicos que podem ter contribuído para o perfil patogênico da epidemia de 2008, foram obtidos 102 soros durante as três epidemias e em períodos interepidêmicos estes foram analisados por qRT-PCR para estimar os níveis de viremia e sua correlação com as características clínicas, imunológicas, e demográficas de cada paciente . Os DENV-2 isolados durante as epidemias foram sequenciados. Duas linhagens (I e II) de DENV-2 do genótipo americano/asiático foram confirmados, um exclusivo para 1990-2002 e outro para os anos de 2007-2011, respectivamente. O nível de viremia significativo nas amostras 2008 era duas ordens de grandeza maior do que a das amostras 1990-2002. Os casos de dengue grave aumentaram de 31% em 1990-2002 para 69% em 2007-2011; em pacientes com idade ≤15 anos, de 3% em 1990-2002 para 37% em 2007-2011. A Linhagem II do DENV-2 e idade mais jovem contribuiram significativamente para o perfil patogênico da epidemia de 2008 no Rio de Janeiro.

Dengue Severity Associated With Age and a New Lineage of Dengue Virus-Type 2 During an Outbreak in Rio De Janeiro, Brazil

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Dengue virus-type 2 (DENV-2) caused three outbreaks, in the years 1990, 1998, and 2008, in Rio de Janeiro, Brazil. The 2008 outbreak was the most severe in reported cases, hospitalizations, and deaths. To investigate virological and epidemiological factors that may have contributed to the pathogenic profile of 2008 epidemic, 102 patients sera obtained during the epidemic and inter-epidemic periods of three outbreaks were analysed by qRT-PCR to estimate viremia levels and their correlation with the clinical, immunological, and demographic patient characteristics. DENV-2 isolates from the outbreaks were sequenced. Two DENV-2 lineages (I and II) of the American/Asian genotype were confirmed, each exclusive for 1990–2002 and 2007–2011, respectively. The mean viremia level in the 2008 samples was two orders of magnitude higher than that of the 1990–2002 samples. Severe dengue cases increased from 31% in 1990–2002 to 69% in 2007–2011; in patients aged ≤15 years, from 3% in 1990–2002 to 37% in 2007–2011. The DENV-2 lineage II and younger age significantly contributed to the pathogenic profile of 2008 epidemic in Rio de Janeiro. ***J. Med. Virol.*** © 2016 Wiley Periodicals, Inc.

KEY WORDS: viremia; qRT-PCR; genotype; dengue outbreaks

INTRODUCTION

Dengue virus (DENV) infections have unpredictable clinical outcomes ranging from asymptomatic or a mild febrile illness to severe and fatal disease. Globally it estimated that 3.6 billion people live in dengue risk areas [WHO, 2009; Bhatt et al., 2013].

Since the introduction of DENV in Brazil in 1981, about 12 million cases have been reported. In the last 3 decades, Brazil has accounted for 70% of all dengue cases in the Americas, with the case fatality rate varying from 1.45% (1995) to 11.25% (2007) [Teixeira et al., 2009; San Martín et al., 2010; SVS, 2012]. In particular, the state of Rio de Janeiro (southeast region of Brazil) has been marked with extensive dengue epidemics due to the introduction or re-emergence of different dengue serotypes during the last 28 years. After the introduction of DENV-2 in 1990, two additional DENV-2 outbreaks occurred in Rio de Janeiro in 1998 and 2008. The 2008 epidemic was considered to be of a greater magnitude with 806,036 cases reported across the country. During this epidemic, approximately 322,000 dengue cases were reported in Rio de Janeiro with 252 fatal cases [Nogueira et al., 2007; SVS, 2008; Gibson et al., 2013; Macedo et al., 2013]. Coincidently, a change in the epidemiological disease profile was observed during the 2008 epidemic with an increase in severity and the number of affected children ≤15 years of age [Teixeira et al., 2009].

According to the phylogenetic analysis of the DENV-2 strains isolated during the epidemics of 1990, 1998, and 2008 in Rio de Janeiro, the virus isolated in the 2008 epidemic was genetically different from the other epidemics despite belonging to the

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Conflict of Interest: None

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same genotype (American/Asian). Therefore, the 1990 and 1998 viruses were classified as lineage I and those of 2008 were considered as lineage II [Oliveira et al., 2010; Faria et al., 2013]. Mechanisms that determine the variations in the clinical manifestations of dengue infections are not clearly understood, but are frequently associated with types of infection, viral serotype, genotype, lineages, and host genetic factors [Simmons et al., 2007]. The two most accepted hypotheses for the pathogenesis of dengue hemorrhagic fever (DHF) are based on antibody-dependent-enhancement or virulence of the viral strain [Halstead et al., 1970; Rosen, 1986; Rico-Hesse et al., 1997; Silva et al., 2010]. According to the second hypothesis, disease severity depends on the degree of virulence of the DENV variants. Therefore, strains that replicate more efficiently and reach higher serum titres have a greater epidemic potential and higher incidence of DHF and dengue shock syndrome [Rico-Hesse et al., 1997; Vaughn et al., 2000; Rico-Hesse, 2003; Silva et al., 2010].

The circulation of different DENV-2 lineages in Brazil has been described in phylogenetic studies [Oliveira et al., 2010; Drumond et al., 2013; Faria et al., 2013; Romano et al., 2010]; however, the impact of these lineages in the Brazilian population has not been previously evaluated. In the present study, we analyzed the epidemiological profile of DENV-2 strains (lineages I and II) circulating in Rio de Janeiro through the correlation of viremia levels, disease severity and patients characteristics, in order to determine the factors that may have contributed to the pathogenic profile of the 2008 epidemic.

MATERIALS AND METHODS

Specimens

We obtained 102 serum from patients in Rio de Janeiro representative of periods of lineages circulation (American/Asian genotype), 1990–2002 (lineage I) and 2007–2011 (lineage II), respectively, which encompass the three DENV-2 epidemics (1990, 1998, 2008). Acute serum (days 1–4) were selected from patients previously confirmed as DENV-2 [Lanciotti et al., 1992] and clinically classified as dengue or severe dengue [WHO, 2009]. The major symptoms used for clinical classification of dengue cases were patients presenting fever with two or more of the following symptoms: headache, pain behind the eyes, muscle and joint pains, nausea, vomiting or rash. The severe dengue cases presented symptoms of respiratory insufficiency, severe bleeding, organ failure or death. Cases were further classified according to the immune status of the patient (i.e., primary or secondary dengue infection) [Miagostovich et al., 1999].

Quantification of Viral RNA

Viral RNA was extracted from 140 μl of human serum specimens with the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) in accordance with the manufacturer's suggested protocol. Viremia levels were measured by serotype-specific qRT-PCR assay using a previously reported protocol [de Oliveira Poersch et al., 2005]. The number of copies of viral RNA detected was calculated by generating a standard curve using five points by 10 times dilution of RNA extracted from a DENV-2 prototype, strain 40247, with a title of $8{,}7 \times 107$ PFU/ml, which was determined by a plaque assay [Azevedo et al., 2011].

Sequencing and Phylogenetic Analysis

Partial genome sequencing of the envelope (E) gene of DENV-2 isolates from 1990 to 2002 (n = 19) and 2007 to 2011 (n = 25) were performed on PCR amplified products as previously described [Faria et al., 2013]. A multiple alignment of the sequences was performed using CLUSTAL W (http://www.ebi.ac.uk/clustalw/); phylogenetic analysis was conducted using the MEGA 5 software (www.megasoftware.net) and the neighbor—joining method according to the [Tamura et al., 2011].

Statistical Analyses

SPSS software for Windows 21.0 (SPSS Inc., Chicago, IL) was used for all parametric tests (*t*-test and/or Fisher's test). Differences were considered significant for *P*-values <0.05.

Ethical Approval

Study approved by the Oswaldo Cruz Foundation Ethical Committee in Research (Resolution number: CSN196/96) and the Ministry of Health, Brazil.

RESULTS

Patient Serum Samples and Inter-Relationships Between Variables

A total of 102 serum samples were analyzed from patients affected with DENV-2 lineages I and II during 1990–2002 (n = 52; 51%) and 2007–2011 (n = 50; 49%), respectively. From 1990 to 2002, 77% (40/52) of samples were from 1990 (n = 20) and 1998 (n = 20) epidemics, respectively and approximately 80% (40/50) from the period 2007 to 2011 were from the 2008 epidemic; therefore, about two random samples were selected from each year for genetic characterization to represent the other years in both periods. Cases from the interim period (i.e., 2003–2006) and after 2011 were not included in this study because there was no DENV-2 activity in Rio de Janeiro during these periods.

The demographic characteristics of the patients included a male:female ratio of 1:1.04 (50 males and 52 females) and an age range of 1–88 years. Approximately 21% (18/85) of patients were aged ≤15 years and 79% (67/85) of patients were aged ≥16 years; age data were missing for 17% (17/102) of patients.

Regarding types of infection, primary and secondary infections were observed in 29% (30/102) and 71% (72/102) of patients, respectively. According to the clinical classification, 70% (67/102) and 34% (35/102) of patients were dengue and severe dengue, respectively. According to the correlation of types of infection versus clinical classification by age group, 72% (13/18) of the patients aged ≤15 years had secondary infection, which was classified as severe dengue 54% (7/13) or dengue 46% (6/13). Primary infection accounted for 28% (5/18) of patients aged ≤15 years and 80% (4/5) of those cases involved severe dengue. In the subgroup of patient aged ≥16 years, 27% (18/67) and 73% (49/67) of cases were primary and secondary infections, respectively. Out of the secondary infection cases, 33% (16/49) and 67% (33/49) of cases progressed to severe dengue and dengue, respectively. In contrast to the subgroup of patients aged ≤15 years, a smaller fraction of the population in the older subgroup had primary rather than secondary infection and 22% (4/18) of those progressed to severe dengue.

Correlation Between DENV-2 Infection Periods and Patient Variables

The correlations between the infection periods and patient characteristics (i.e., age, gender, disease severity, and types of infection) were analyzed. During 1990–2002, due to the epidemiological pattern of dengue in Brazil, the great majority of the cases involved patients aged ≥16 years (97%). In this period although we have received samples from 10 patients ≤15, only one fulfilled the inclusion criteria to have DENV-2 confirmed, IgM confirmed the remaining cases as dengue. Whereas, during 2007–2011, the number of patients age ≤15 years increased to 37%, furthermore, the number of severe dengue cases increased from 21% in 1990–2002 to 56% in 2007–2011. However, gender and types of infection were not significantly correlated with disease severity in the two periods.

The correlations between clinical classification (i.e., dengue and severe dengue) and key variables (i.e., age, gender, types of infection, and viral lineage) (Table I) were analyzed using Fisher's test. No correlations were observed between the clinical classification and types of infection ($P = 0.650$) or gender ($P = 0.060$), but more men than women presented with severe dengue (63% vs. 37%, respectively). However, age and lineage appeared to be correlated with the clinical classification. The relative frequency of severe dengue cases was significantly higher in patients aged ≤15 years than older patients ($P = 0.025$). There were significantly more cases of severe dengue than dengue cases with lineage II than lineage I ($P = 0.006$). Thus, after the emergence of lineage II, 69% of the cases were classified as severe dengue, when compared to the lineage I only 31% of the cases were serious. Furthermore, coincidently or not the younger population were more affected during lineage II circulation.

TABLE I. Comparisons of Different Categorical Variables in Relation to Dengue Disease Severity

Categorical variables	Severe dengue (%)	Dengue (%)	*P*-value
Age			
≤15 years	11/31 (35)	7/54 (13)	**0.025**
≥16 years	20/31 (65)	47/54 (87)	
Gender			
Female	13/35 (37)	39/67 (58)	0.060
Male	22/35 (63)	28/67 (42)	
Lineage			
I	11/35 (31)	41/67 (61)	**0.006**
II	24/35 (69)	26/67 (39)	
Types of infection			
Primary	09/35 (26)	21/67 (31)	0.650
Secondary	26/35 (74)	46/67 (69)	

Correlation Between RNA Viremia Level of DENV-2 Lineages I and II and Patient Characteristics

The analysis of the viremia level for studied subgroups is shown in Table II. In general, the viremia level was two orders of magnitude higher in sera from 2007 to 2011 (lineage II) than from 1990 to 2002 (lineage I). Notably, the mean serum titres of DENV-2 in patients, clinically classified as severe dengue cases from either period, were higher than those classified as dengue cases, but only severe cases of lineage II were statistically significant ($P = 0.006$). Age and lineage II were statistically significant ($P = 0.009$). There were no significant differences between the age subgroups when analyzed cumulatively for both periods (i.e., 1990–2011; $P = 0.680$). No significant relationship was observed between the viremia level and gender (lineage I; $P = 0.308$ and

TABLE II. Correlation of Viremia Level in Dengue Virus-Type 2 Lineages With Clinical Classification, Types of Infection, Gender, and Age

	1990–2002 (Lineage I)		2007–2011 (Lineage II)	
Categorical variables	Viral load (RNA/mL)	*P*-Value	Viral load (RNA/mL)	*P*-Value
Age				
≤15 years	$1{,}74 \times 10^5$	0.072	$9{,}68 \times 10^7$	**0.009**
≥16 years	$1{,}62 \times 10^4$		$1{,}17 \times 10^6$	
Gender				
Female	$7{,}13 \times 10^4$	0.308	$1{,}04 \times 10^6$	0.765
Male	$1{,}44 \times 10^4$		$1{,}23 \times 10^7$	
Clinical classification				
Severe dengue	$5{,}27 \times 10^4$	0.893	$1{,}42 \times 10^7$	**0.006**
Dengue	$4{,}44 \times 10^3$		$5{,}21 \times 10^5$	
Types of infection				
Primary	$1{,}61 \times 10^3$	0.060	$9{,}36 \times 10^5$	0.218
Secondary	$6{,}78 \times 10^4$		$9{,}27 \times 10^6$	

lineage II; $P = 0.765$) and types of infection (lineage I; $P = 0.060$ and lineage II; $P = 0.218$) using sera from both periods.

Phylogenetic Analysis

In order to confirm no co-circulation of the two lineages during the periods studied, 44 DENV-2 isolates from period encompassing the three DENV-2 epidemics (early, mid, and late) were randomly selected for sequencing the E gene. According to the sequencing results, there was no concomitant circulation of the two lineages. Lineage I alone was identified in the 1990–2002 samples, whereas samples from 2007 to 2011 had lineage II exclusively (Fig. 1). Samples from 2003 to 2006 and after 2011 were not included because there was no DENV-2 activity in Rio de Janeiro during these periods.

DISCUSSION

After 7 years without detectable activity, DENV-2 re-emerged in April 2007 in Rio de Janeiro causing one of the most severe dengue epidemics already reported in the State in 2008 [SVS, 2008; Macedo et al., 2013]. According to the phylogenetic analysis of the DENV-2 strains from the entire study period (i.e., 1990–2011), which encompass the introduction and the two re-emergences, the DENV-2 isolates in Rio de Janeiro from 2007 onwards were genetically distinct from those found earlier despite belonging to the same genotype (American/Asian); these viruses were grouped as lineage II.

Several investigators have sought to identify clinical-epidemiological and laboratory predictor patterns for dengue severity by examining correlations with viremia levels [Vaughn et al., 2000; Thomas et al., 2008]. An increase in dengue severity has been associated with DENV-2 and DENV-3 serotypes [Rico-Hesse, 2003]. In this study, results suggest that lineage was an important factor for disease severity because higher viremia levels and severe dengue were more frequent observed in patients infected by DENV-2 lineage II than by lineage I, both belong to American/Asian genotype.

The observed disappearance of lineage I viruses and temporal replacement by lineage II viruses after 2007, which was associated with a higher RNA viremia level and a greater disease severity during the 2008 epidemic, supports the hypotheses that viruses replicating more efficiently can out-compete and displace those that have lower epidemiological impact [Rico-Hesse et al., 1997; Rico-Hesse, 2003; Vu et al., 2010; OhAinle et al., 2011].

The association of viremia levels with the immune status and their relationship with disease progression remain contradictory. A secondary infection with DENV-2, which was characterized by a greater disease severity and high viremia level, was reported in patients who were previously infected with DENV-1 or DENV-3 [Alvarez et al., 2006]. The sequence of introduction and re-emergences of dengue viruses in Rio de Janeiro was as follows: DENV-1 in 1986, DENV-2 in 1990, DENV-3 in 2001, DENV-2 in 2007, DENV-1 in 2010, and DENV-4 in 2011. Severe cases and deaths began to be reported during the first epidemic of DENV-2 in 1990, this number increased during DENV-3 epidemic in 2002; however, during DENV-2 epidemic in 2008 there was an increase of twofold higher in the case fatality rate for dengue in the state, coincidently during the circulation of lineage II. According to our results, independent of the period of lineage circulation, secondary infection was not correlated with disease severity. There were no significant association between higher viremia levels and disease due to immune status in patients previously infected with DENV-1 and/or DENV-3, which circulated widely in Rio de Janeiro in 1986–1997 and 2001–2006, respectively, before the introduction and re-emergence of DENV-2 in 1990 and 2008 [Nogueira et al., 2007; Teixeira et al., 2009; SVS, 2012; Macedo et al., 2013].

In contrast with what is observed in other endemic regions of the world, in Brazil since its introduction, dengue was predominant among individuals >15 years; from 2007 to 2009, there was an age shift in which the disease affected mainly children aged ≤15 years. During 2008 epidemic, the state of Rio de Janeiro was responsible for 37% of reported dengue cases in Brazil [Ramos and Machado, 2014], from these, 50% of dengue cases and 86% of deaths occurred in individuals ≤15 years [Teixeira et al., 2009]. Furthermore, there was also a sevenfold increase in the number of hospitalizations of children under 1 year of age, shifting from 8% of the total number of cases per 100,000 inhabitants in 2002 during DENV-3 epidemic to 57% in 2008. This epidemiological profile changed in 2010 during the huge DENV-1 epidemic (more than one million cases) with a shift of the disease to age group >60 years. During DENV-1 epidemic the state of Rio de Janeiro, epicentre of 2002 (DENV-3) and 2008 (DENV-2) epidemics responded for only 2.9% of the dengue cases in the country.

In our casuistic besides the significant association with disease severity, higher RNA viremia levels was observed in ≤15 years group during lineage II circulation suggesting that age was an important factor for the peak of viremia. The association of an emergent lineage of DENV-2 with higher viremia was also described in paediatrics inpatients from Viet Nam [Vu et al., 2010].

Physiological, immunological, and behavioral conditions can contribute to a substantially higher rate of mortality. Furthermore, gender is one of the risk factors that have been associated with severe dengue and dengue deaths. In Brazil, the distribution of dengue cases by sex is proportional but with a slight increase for women, in a study conducted from 2002 to 2010, women represented 55% of dengue cases in the

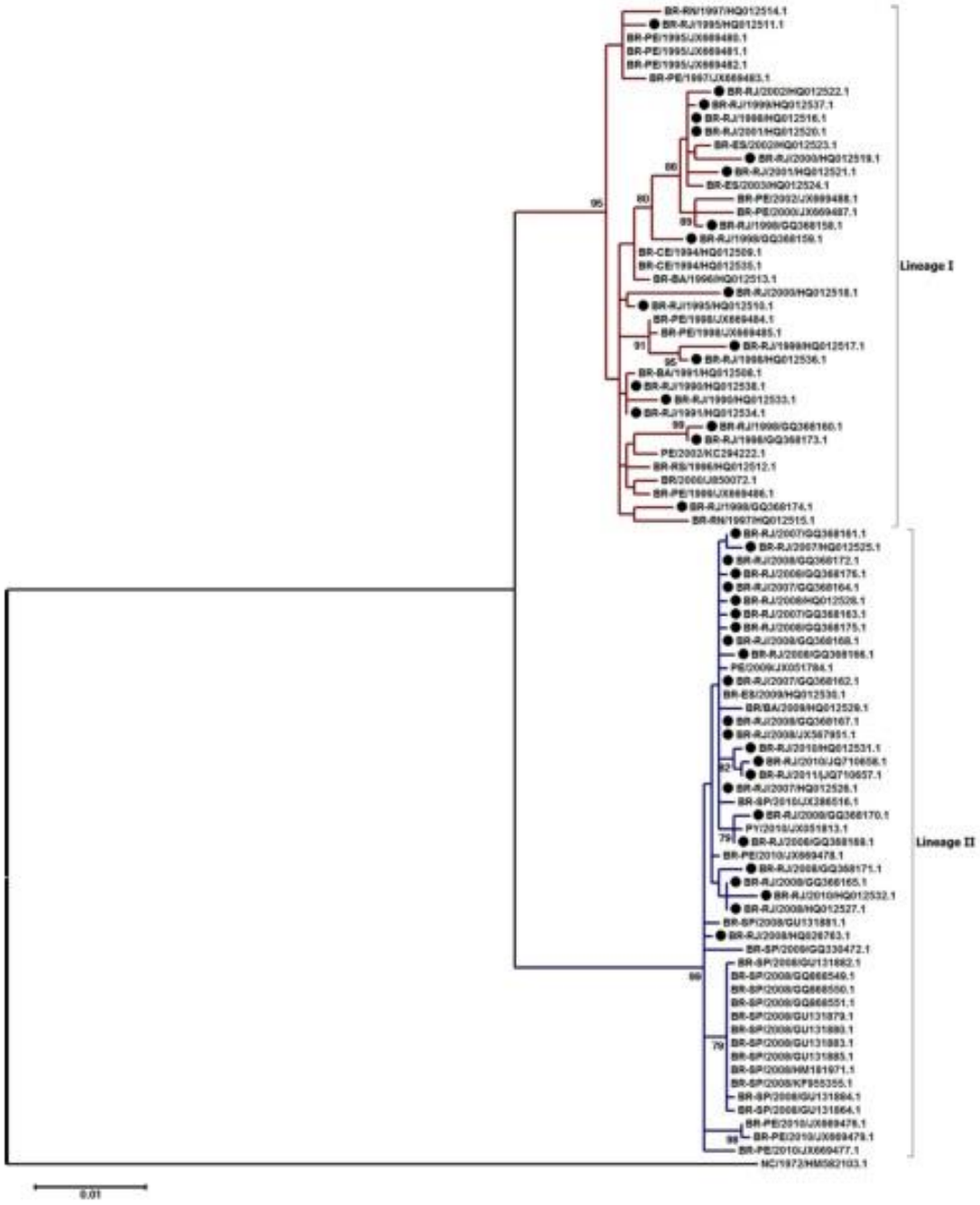

Fig. 1. Phylogenetic relation of DENV-2 isolates spanning the three DENV-2 epidemics and the two lineages 1990–2002 (Lineage I) and 2007–2011 (Lineage II). The sequenced strains representing the periods of our study are marked with black dots.

country [Siqueira et al., 2010]. In our study, the population was homogeneous for gender (52% women and 50% men), but 37% of women and 63% of men were classified as having severe dengue. Although the rate of severe dengue in men has been higher than that in women, disease severity was not significantly associated with gender in this study, which corroborates with previous observations [Wang et al., 2003].

There were no observed associations between disease severity and immune status, age, or gender during the lineage I period and the occurrence of higher viremia levels during the lineage II period reinforces the hypothesis that strains with higher virulence are an important factor for disease severity [Simmons et al., 2007; Romano et al., 2010], as observed during the 2008 epidemic.

In Brazil, the circulation and evolution of DENV-2 lineages were previously reported [Halstead et al., 1970; Drumond et al., 2013]; however, this was the first report in which the epidemiological impact of these lineages are described in the Brazilian population. According to our data, the ability of a lineage II to replicate at higher titres and the high susceptibility to DENV-2 in the individuals $\leq$15 years may have been key factors for the disease dynamics in the 2008 epidemic.

The hyperendemicity of dengue with co-circulation of multiple DENV serotypes, emergence or re-emergence of new serotypes, virulent genotypes or lineages, constant replenishment of susceptible individuals due to birth cohort, and high density of *Aedes aegypti* are a constellation of factors contributing to the serious dengue scenario observed in the last 10 years in Brazil. Until a vaccine is available, the only strategy to prevent the worsening situation is to improve measures of vector control and medical assistance to the population.

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Artigo 3: Epidemias de dengue em dois períodos distintos revelam aspectos epidemiológicos, laboratoriais e clínicos em um mesmo cenário: análise das epidemias ocorridas em Mato Grosso do Sul e, 2010 e 2013.

Objetivo específico: Analisar os aspectos epidemiológicos, clínicos e laboratoriais de epidemias causadas pela co-circulação de DENV-1 e DENV-2 em 2010 e por DENV-4 em 2013, em Campo Grande, Mato Grosso do Sul.

Situação do manuscrito: Artigo publicado na Transactions of the Royal Society of Tropical Medicine and Hygiene

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Resumo: Este artigo faz referência aos casos de dengue estudados durante duas epidemias de dengue ocorridas no estado do Mato Grosso do Sul, no anos de 2010 e 2013, a dengue foi confirmada em 78.7% (48/61) e 75.6% (118/156) dos casos estudados em 2010 e 2013, respectivamente. O DENV-1 e DENV-2 foram os sorotipos detectados na epidemia de 2010 e 2013 e o DENV-4 foi detectado somente na epidemia de 2013. A maioria dos casos foram classificados como dengue sem sinais de alarme, no entanto a dengue grave foi observada em 18,7% (9/48) dos casos em 2010 e com menor frequencia nos casos de DENV-4 em 2013. Os níveis de NS1 foram maiores nos pacientes dengue com sinais de alarme e dengue grave pertencentes a epidemia de 2010. O níveis circulantes de Aspartato aminotransferase (AST) e alanina transferase (ALT) foram alterados em todos os grupos, independentemente do sorotipo infectante ou epidemia. Pacientes com DENV-1 e DENV-2 apresentaram significativamente menores contagens de monócitos quando comparados a pacientes com DENV-4. Uma correlação inversa foi encontrada entre a contagem de plaquetas, leucócitos, monócitos e níveis NS1. Podemos concluir que as epidemias

causadas pela prevalência de distintos sorotipos de DENV possuem diferentes impactos e características clínicas em um mesmo cenário e, apesar da ocorrência de infecções secundárias, o surgimento do DENV-4 não foi associado a casos graves.

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ORIGINAL ARTICLE

Dengue epidemics in two distinct periods reveal distinct epidemiological, laboratorial and clinical aspects in a same scenario: analysis of the 2010 and 2013 epidemics in Mato Grosso do Sul, Brazil

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Background: Dengue is a major problem in Brazil. Epidemiological and clinical aspects were characterized in patients from two epidemics which occurred in Mato Grosso do Sul, Brazil.

Methods: Dengue cases were classified according to the 2009 WHO criteria, tested by serological and molecular biology tests and analysed for nonstructural protein 1 (NS1) antigenemia.

Results: Dengue was confirmed in 78.7% (48/61) and 75.6% (118/156) of the cases studied in 2010 and 2013, respectively. DENV-1 and DENV-2 were the serotypes involved in the 2010 epidemic and DENV-4 in the 2013 one. Most of the cases were classified as dengue without warning, however severe dengue was observed in 18.7% (9/48) of the cases in 2010 and less observed in DENV-4 cases. NS1 levels were higher in patients with dengue with warning signs and severe dengue in 2010. Circulating aspartate aminotransferase (AST) and alanine transferase (ALT) were altered in all groups, independently of the infecting serotype or epidemic. Patients with DENV-1 and DENV-2 presented significant lower monocyte counts when compared to patients with DENV-4. An inverse correlation was found between platelet count, leucocytes, monocytes and NS1 levels.

Conclusions: Epidemics caused by the prevalence of distinct DENV serotypes had different impacts and clinical characteristics in a same scenario and, despite the occurrence of secondary infections, the DENV-4 emergence was not associated with severe cases.

Keywords: Brazil, Clinical aspects, Dengue virus, Diagnosis, Epidemics, NS1

Introduction

Dengue viruses (DENVs) are the most important human arboviruses worldwide, and are transmitted by mosquitoes of the genus *Aedes*, in the form of four distinct serotypes of DENV, 1 to 4. DENVs have become a major public health problem with relevant social and economical effects due to the increased geographic extension, number and severity of cases.[1] New estimates indicate that 3.6 billion people live at risk of contracting dengue with up to 390 million infections expected to occur annually.[2]

Since dengue was introduced in Brazil in 1982, more than 8 million cases have been reported, with the years 2002, 2008, 2010 and 2013 being the most critical for the country.[3] Currently, Brazil accounts for approximately 76.0% of reported cases of dengue in the Americas.[4] From 2010 to 2013, a total of 3 817 660 dengue cases were reported in Brazil, mainly due to epidemics that occurred in the Southeast and Midwest regions. In that same period, the state of Mato Grosso do Sul located in the Midwest region, reported a total of 160 189 cases, 4% of the total reported in Brazil and the seventh state in the number of notifications.[3] The simultaneous co-circulation of all four DENV serotypes characterizes clear evidence of dengue hyperendemicity.[5] In this scenario, the surveillance of DENV has been accepted as one of the most important tools for predicting epidemics.

Dengue has become a major public health problem in Brazil due to many factors such as the human host susceptibility,

virus emergences and re-emergences, as well as serotype shifts, vector abundance and environmental factors.[6] Since its establishment in Brazil, laboratory diagnosis of dengue has proven imperative for disease surveillance, and plays a role as an early warning tool.[7]

The dengue severity varies with an individual's immune status, DENV serotype/genotype, age, and population susceptibility.[8] Furthermore, pre-existing comorbidities are also likely to lead to more severe dengue infections.[9] Therefore, early diagnosis is critical, since some patients may progress from mild to severe disease in a short period of time. Repeated monitoring of the platelet count and hematocrit is recommended, as an abrupt decrease in platelet counts is an alarm sign and a significant hematocrit increase is an indirect sign of plasma leakage.[10]

Recently, a study referring the analysis of multiple epidemiological parameters associated with DENV cases in Brazil, including data from MS, identified highly heterogeneous outbreak characteristics in terms of frequency, duration and case burden, and stressed the need for a more thorough investigation.[11] Therefore, here we aim to analyze the epidemiological and clinical aspects of dengue cases associated with two distinct DENV epidemics that took place in 2010 and 2013 in Campo Grande, Mato Grosso do Sul, Midwest region of Brazil.

Material and methods

Enrolment, data and specimen collection

The serum and plasma specimens analyzed in this study were collected during the ongoing epidemics that occurred in 2010 and 2013, in the city of Campo Grande, Mato Grosso do Sul, Brazil, in the Midwest region of the country, as shown in Figure 1. Patients were enrolled at Federal University of Mato Grosso do Sul, Esterina Corsini Day Hospital or Reference Center for Infectious and Parasitic Diseases (CEDIP) and Guanandy Health Center.

An infectious disease physician collected data on demographic characteristics, symptoms and physical signs using a questionnaire. Dengue suspected cases (n=217) were obtained during an active surveillance performed by the Laboratory of Viral Immunology, IOC/FIOCRUZ. Laboratory diagnosis was performed by the Laboratory of Flavivirus, Regional Reference Laboratory for the Brazilian Ministry of Health, located in Rio de Janeiro. Acute serum samples (up to the 7th day after disease onset) were submitted to reverse transcriptase (RT)-PCR and nonstructural protein 1 (NS1) antigen capture ELISA. Convalescent samples (later than the 7th day after the onset of symptoms) were tested by IgM antibody capture (MAC)-ELISA and IgG-ELISA. Both acute and convalescent serum samples were stored at -70°C. Laboratory parameters from all patients were obtained in the hospitals in which they were treated. Each sample was accompanied by an identification form containing clinical and epidemiological data. The data include demographic information, vital signs, physical examination, complete blood count, liver enzymes (alanine transferase (ALT), alanine transaminase, aspartate aminotransferase (AST), aspartate transaminase), ultrasounds and X-ray findings, history of the previous diseases and medication use. All patients enrolled signed a written consent form.

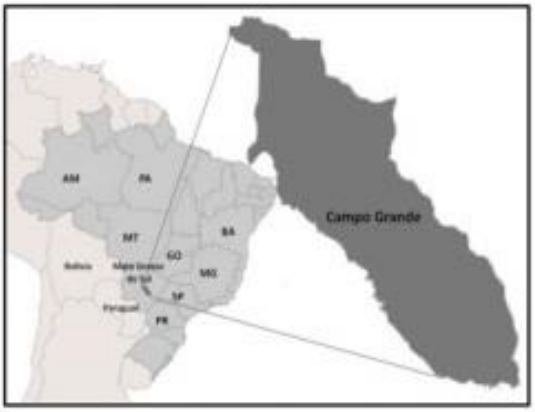

Figure 1. Map of Brazil, highlighting the studied area in Mato Grosso do Sul, Brazil in 2010 and 2013. AM: Amazonas; BA: Bahia; GO: Goiás; MG: Minas Gerais; MT: Mato Grosso; PA: Pará; PR: Paraná; SP: São Paulo.

Inclusion criteria for WHO dengue case definition

Dengue confirmed cases were classified according to the 2009 WHO[10] classification and grouped as follows: dengue without warning signs (DwoWS): patients living in and/or traveling to dengue endemic areas, presenting fever and two of the following symptoms: nausea, vomiting, rash, aches, pain, positive tourniquet test and leukopenia; dengue with warning signs (DwWS): dengue patients with any of the following warning signs: abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy or restlessness, liver enlargement >2 cm, and an increase in hematocrit concurrent with rapid decrease in platelet count; severe dengue (SD): dengue patients presenting at least one of the following: severe plasma leakage (leading to shock and fluid accumulation with respiratory distress), severe bleeding evaluated by clinicians, severe involvement of liver by AST or ALT>1000 U, central nervous system with impaired consciousness, and severe involvement of the heart and other organs and Group IV: dengue confirmed cases with insufficient information for classification according to 2009 WHO criteria. Non-dengue cases, excluded after a negative result by laboratory diagnosis, were considered in this study as other febrile diseases (OFD).

Laboratory diagnosis of dengue infection

Laboratory-positive dengue infection was defined as patients experiencing a febrile illness consistent with dengue according to WHO criteria and infection confirmed based on the results obtained by the laboratory diagnostic assays: RT-PCR, MAC-ELISA, NS1 ELISA and IgG-ELISA.

Serological diagnostics

Anti-DENV immunoglobulins

The Panbio Dengue IgM Capture ELISA (Panbio, Brisbane, Australia) was used for the qualitative detection of anti-DENV

IgM antibodies in serum for case confirmation according to the manufacturer's instructions.

The IgG-ELISA previously described by Miagostovich et al.[12] was performed for the characterization of dengue immune response as primary or secondary infections in dengue cases previously confirmed by virus isolation, RT-PCR and/or MAC-ELISA. Alternatively, the IgG Select Dx kit (Focus Diagnostics, Carlsbad, CA, USA) was used for characterization of dengue immune response as primary and secondary. Briefly, the acute samples presenting an NS1 and/or RT-PCR positive result with a negative IgG detection were considered as primary infections, and acute samples NS1 and/or RT-PCR positive with a positive IgG detection were classified as secondary ones.

NS1 antigen capture ELISA and NS1 antigenemia

For the NS1 antigen capture, the Platelia™ Dengue NS1 Ag-ELISA kit (Biorad Laboratories, Marnes-La-Coquette, France) was used according to the manufacturer's protocol. All NS1 negative samples were submitted to a NS1 immune complex dissociation protocol previously described.[7] Quantification of circulating DENV NS1 was performed as described previously,[13] with slight modifications, only on samples where the serotype was identified. All samples were tested 1–9 days after disease onset.

Molecular methods

The viral RNA was extracted using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and stored at -70°C for DENV detection and typing. The RT-PCR for detecting and typing DENV was performed as described previously.[14] All negative samples by laboratory diagnosis (OFD) were also subjected to a RT-PCR for detection of other flaviviruses and alphaviruses according to a previously published protocol.[15] Aiming to further exclude DENV infection in all negative cases (OFD), samples were tested by using the Simplexa™ Dengue Real Time RT-PCR (Focus Diagnostics, Cypress, CA, USA) according to the manufacturer's protocol, for viral qualitative detection and typing of DENV.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 6.0 (GraphPad Software Inc., San Diego, CA, USA). The Kruskal-Wallis non-parametric test and Dunn's multiple comparison test were used to compare the differences between study groups (OFD, DwoWS and DwWS/SD). Fisher test was used to compare clinical and laboratory characteristics between patients groups. Correlation was estimated by Spearman regression analysis. Values of $p<0.05$ were taken as being significant for all statistical analysis.

Results

Differences in clinical presentation and laboratorial parameters between the 2010 and 2013 epidemics

In 2010, the population of the city of Campo Grande was estimated by the Mato Grosso do Sul State Secretariat of Health at 786 797. A total of 82 597 dengue suspected cases were reported in that year, and of those, 41 293 (50.0%) cases and 47 deaths were confirmed in the capital, Campo Grande. In this study, dengue suspected cases (n=61), day 1–11 were analyzed and 79% (48/61) were confirmed as dengue by using any of the diagnostic laboratory tests performed. In 21% (13/61) of the cases, dengue infection was not confirmed after testing by laboratory diagnosis and therefore were considered as OFD. Females (29) were more affected than males (19); the mean age was 42 years.

In 2013, a total of 102 026 dengue suspected cases were reported in Mato Grosso do Sul. Only in the city of Campo Grande, 46 448 cases (45.5%) and 12 deaths were notified. Representing that epidemic, data on dengue suspected cases (n=156) were collected and analyzed. Males and females were similarly affected and the mean age was 36 years old. Dengue infections were confirmed in 75.6% (118/156) of the cases and 24.4% (38/156) were defined as OFD. All 51 negative samples from both epidemics were further evaluated using an RT-PCR for flavivirus and alphavirus[15] and using a commercial real-time RT-PCR kit to detect DENV.

In 2010, MAC-ELISA confirmed 85% (41/48) of the cases analyzed, the NS1 ELISA confirmed 29% (14/48) and RT-PCR, 25% (12/48). DENV-1 (6/12; 50%) and DENV-2 (6/12; 50%) were the infecting serotypes detected by RT-PCR and involved in the epidemic. The MAC-ELISA sensitivities according to the patient's number of days of illness were 41.5% (1–3 days), 43.9% (4–7 days) and 14.6% (>7days). The RT-PCR and the NS1 ELISA sensitivities on those same days were 58.4%, 33.3% and 8.3% and, 50.0%, 28.6% and 7.1%, respectively.

In 2013, MAC-ELISA confirmed 46.6% (55/118) of the cases, by using the NS1 ELISA, 71.1% (84/118) of the cases tested positive and the RT-PCR confirmed 30.5% (36/118) of the cases, identifying DENV-4 as the prevalent serotype, detected in 28.0% (33/118) of the cases. Moreover, this serotype accounted for 91.6% (33/36) of the confirmed cases by RT-PCR, as DENV-1 and DENV-2 were identified, in 2 and 1 case, respectively (Table 1). The MAC-ELISA sensitivities according to the patient's number of days of illness were 10.9% (1–3 days), 52.7% (4–7 days) and

Table 1. Laboratory confirmation of dengue suspected cases analyzed from epidemics in 2010 and 2013, in Campo Grande, Mato Grosso do Sul, Brazil

Dengue laboratorial diagnostic methods	Year	
	2010 (n=48) Positive/Tested (%)	2013 (n=118) Positive/Tested (%)
MAC-ELISA	41/48 (85.4)	55/118 (46.6)*
NS1 ELISA	14/48 (29.2)	84/118 (71.1)*
RT-PCR	12/48 (25.0)	36/118 (30.5)
[serotypes]	[DENV-1; 6/12]	[DENV-1; 2/36]
	[DENV-2; 6/12]	[DENV-2; 1/36]
		[DENV-4; 33/36]

DENV: dengue virus; MAC: IgM antibody capture; RT: reverse transcriptase.
*($p<0.001$). Statistical significance was assessed using Fisher test.

Table 2. Characteristics of dengue suspected cases from epidemics occurred in 2010 and 2013, in Campo Grande, Mato Grosso do Sul, Brazil

Cases characteristics	Epidemic year (Total of cases analyzed)	
	2010 (n=61) Positive/Tested (%)	2013 (n=156) Positive/Tested (%)
Dengue	48/61 (78.7)	118/156 (75.6)
Mean age (in years)	42	36
Gender (male:female)	19:29	58:60
Cases classification	(n=48) Positive/Tested (%)	(n=118) Positive/Tested (%)
DwoWS	22/48 (45.8)	84/118 (71.2)**
DwWS	14/48 (29.2)	32/118 (27.2)
SD	9/48 (18.7)	1/118 (0.8)***
Infecting serotype	(n=48) Positive/Tested (%)	(n= 118) Positive/Tested (%)
DENV-1	6/48 (12.5)	2/118 (1.7)
DENV-2	6/48 (12.5)	1/118 (0.8)
DENV-4	(0/48)	33/118 (28.0)
Total	12/48 (25.0)	36/118 (30.5)
Type of infection**	(n=45) Positive/Tested (%)	(n=114) Positive/Tested (%)
Primary	4/45(8.9)	16/114 (14.0)
Secondary	41/45 (91.1)	98/114 (86.0)
Factors associated to a more severe disease	(n=48) Positive/Tested (%)	(n=118) Positive/Tested (%)
Hospitalization	23/48 (47.9)	23/118 (19.5)***
Abdominal pain	19/48 (38.5)	19/118 (16.1)**
Plasma leakage[a]	2/48 (4.2)	0/118 (0)
Haemorrhage manifestation[b]	13/48 (27.1)	14/118 (11.8)**
Severe bleeding[c]	10/13 (76.9)	2/11 (18.1)
Hepatomegaly	7/48 (14.5)	2/118 (1.7)**
Comorbidity	17/48 (35.4)	27/118 (22.8)
Arterial hypertension	8/48 (16.7)	17/118 (14.4)
Diabetes mellitus	3/48 (6.2)	7/118 (5.9)
Other comorbidities	6/48 (12.5)	3/118 (2.5)

DENV: dengue virus; DwWS: dengue with warning signs; DwoWS: dengue without warning signs; SD: severe dengue.
[a] signs of vascular leakage (pleural or pericardial effusion, ascites); [b] includes skin haemorrhages, epistaxis, gingival bleeding, positive tourniquet test; [c] gastrointestinal bleeding, urinary tract haemorrhage.
*(p<0.05); **(p<0.01); ***(p<0.001). Statistical significance was assessed using Fisher test.

36.4% (>7days).). The RT-PCR and the NS1 ELISA sensitivities on those same days were 58.3%, 36.1% and 5.6%, and 33.3%, 53.6% and 13.0%, respectively.

According to the 2009 WHO classification, in 2010 46% (22/48) of the cases were classified as DwoWS, 29% (14) as DwWS, 19% (9) as SD and 3 (6%) were excluded due to lack of information (Table 2). The signs and symptoms more frequently observed were fever (89.5%), prostration (89.5%), myalgia (81.5%), arthralgia (60.4%), retro-orbital pain (52.1%), nausea (47.9%), petechia (47.9%), rash (35.4%), vomiting (35.4%), anorexia (31.2%), itch (29.2%), diarrhea (22.9%) and a positive tourniquet (22.9%). The main warning signs of groups DwWS were hemorrhagic manifestation (48.0%), persistent abdominal pain (33.0%), increased hematocrit with rapid decrease of platelets (14.0%) and fluid accumulation (10.0%).

In 2013, 71.2% (84/118) of the cases were classified in DwoWS, 27.2% (32) in DwWS, 0.8% (1) in SD; due to lack of information, 0.8% were not classified, Table 2. The main signs and symptoms observed were headache (82.0%), fever (81.0%), myalgia (78.0%), retro-orbital pain (72.0%), arthralgia (69.0%), anorexia (64.0%), nausea (56.0%), back pain (45.0%), itch (33.0%), vomiting (22.0%), petechia (10.0%), diarrhea (10.0%) and rash (7.0%). The most common warning signs observed in patients from DwWS were persistent abdominal pain (58.0%), increased hematocrit with rapid decrease of platelets (39.0%), mucosal bleeding (33.0%) and fluid accumulation (9.0%).

In 2010, 91% (41/45) of the cases were due to secondary infections. Hospitalization occurred in 48% (23/48) of the cases and comorbidities were described in 35% (14/48) of those. As observed in the 2010 epidemic, most of the cases were due to secondary infections in the 2013 epidemic (86.0%, 98/114). However, different from the 2010 epidemic, despite the high incidence of secondary infections, hospitalization was observed in 19.5% (23/118), Table 2. Comorbidities were described in 22.8% (27/118) of the cases analyzed in 2013.

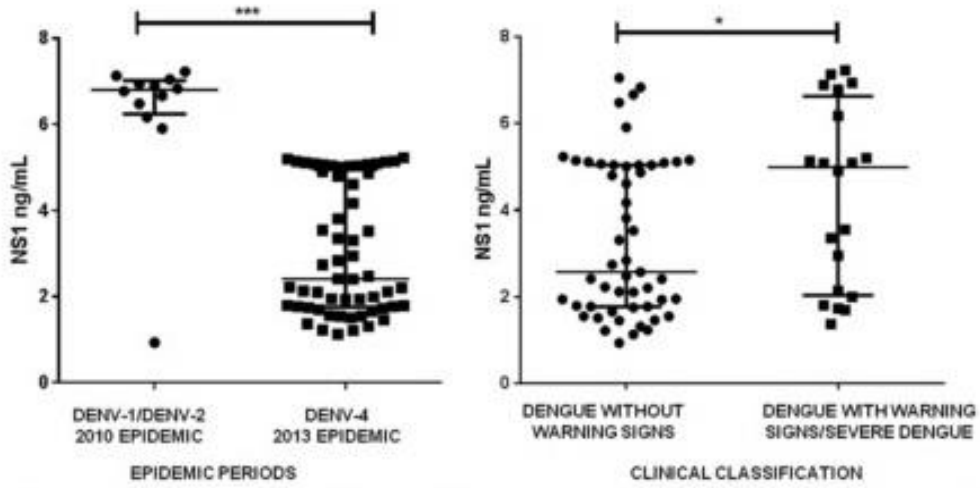

Figure 2. Circulating levels of nonstructural protein 1 (NS1) in patients with dengue according to (A) the disease severity and (B) DENV infecting serotype. The Mann–Whitney non-parametric test was used and *p<0.05 and ***p<0.001 were taken as significant for all statistical analysis.

Considering the manifestations associated with a risk of an unfavorable outcome, the patients from the 2010 epidemic cause by DENV-1 and DENV-2, presented more abdominal pain, bleeding hepatomegaly and hospitalizations. According to the new dengue classification, WHO 2009, the diagnosis of severe cases was less frequent in patients from DENV-4, in the 2013 epidemic (Table 2).

The NS1 protein was quantified and serum levels ranging from 1.98 to 7.22 ng/m in patients from the 2010 epidemic and from 1.12 to 5.23 ng/mL in those from the 2013 outbreak. Circulating levels of NS1 were highest in patients with DwWS/SD than in DwoWS and high levels were detected in patients from DENV1/2 2010 outbreak compared with those from DENV-4 2013 outbreak (Figure 2).

Hematological and biochemical analysis

The hemograms from patients were analyzed since leukopenia and thrombocytopenia have been associated with dengue severity. Hemoconcentration is detected by changes in hematocrit.[16] Similarly, alterations in liver functionally are commonly observed during dengue infection and elevation on transaminases enzymes is frequently found during infection and have been reported to be more pronounced in severe cases.[17] In relation to liver transaminases, circulating levels of AST and ALT were altered in 64.4% and 75.9% of dengue patients in 2010 and 2013, respectively. Elevated levels of ALT and AST were found in both DwoWS and DwWS/SD groups, independently of infecting serotype or epidemic when compared to the OFD group. Moreover, patients with DwWS/SD from the 2013 epidemic, showed high levels of AST and ALT (208.5 IU/L [170.7–412]; 184.5 IU/L [121.3–332.6], respectively) when compared to the DwoWS group (41 IU/L [46.7–72.6] and 59 IU/L [64.9–94.5], respectively), Table 3.

As demonstrated (Table 3), all dengue patients had low leukocytes counts compared to the OFD group, but the DwWS/SD group from the DENV-4 epidemic in 2013 showed low leukocyte count (3300 mm^3 [2953–3903]), compared to the DwoWS group (3950 mm^3 [4289–5480]). As expected, we observed that the platelets counts were lower in all dengue patients, when compared to the OFD group. In the 2010 epidemic caused by DENV-1 and DENV-2, the laboratory findings indicated low platelet count (68.000/mm^3 [66.2–108.7]) compared to OFD. Moreover, hemorrhagic manifestations and/or plasma extravasation were found in those patients (13/48 [27%] and 2/48, [19%], respectively). In comparison, patients from the DENV-4 2013 epidemic presented a higher platelet count (137.000/mm^3 [124.8–151.5]) with less frequent bleeding (11/118 [9.3%]) and no plasma leakage. Comparative analyses also indicated that patients from the 2010 epidemic presented significant lower monocyte count (291 mm^3 [217–378.3]) when compared to those from the 2013 epidemic (391 mm^3 [404–521.9]), Table 3.

Relationship between platelets, leukocytes and monocytes with circulating levels of NS1

We analyzed the relationship between circulating levels of NS1 with the clinical and laboratorial parameters. Interestingly, an inverse correlation was found between platelets counts and NS1 serum levels ($r=-0.377$, $p=0.0018$, $n=66$). Similarly, we also found an inverse correlation between leucocytes and monocytes counts with NS1 levels ($r=-0.366$, $p=0.0029$, $n=64$; $r=-0.333$, $p=0.016$, $n=51$ respectively), Figure 3.

Discussion

During the present study, we evaluated two epidemic periods corresponding to the years 2010 and 2013 in the state of Mato Grosso do Sul, Brazil, where the number of reported cases and the severity of the disease are rising.[19,20]

In the epidemics analyzed here, there was co-circulation of two or more serotypes. In the 2010 epidemic, DENV-1 and DENV-2 circulated in the same proportion. According to

Table 3. Hematological and biochemical parameters in patients with dengue infection and other febrile disease during the 2010 and 2013 epidemics in Campo Grande, Mato Grosso do Sul, Brazil

Characteristics	Clinical presentation/epidemic year							
	OFD (95% CI)	2010			2013			2010 vs 2013 epidemics p value
		DwoWS (95% CI)	DwWS/SD (95% CI)	Total (95% CI)	DwoWS (95% CI)	DwWS/SD (95% CI)	Total (95% CI)	
AST, IU/L[a]	23.5 (20.9–48.4) (n=22)	83 (43.7–132.2) + (n=11)	97 (78.2–240.4) ++++ (n=22)	95 (80.1–191) ++++ (n=33)	41 (46.7–72.6) +++, §§ (n=50)	208.5 (170.7–412) +++ (n=26)	53.5 (91.186.5) +++ (n=76)	NS
ALT, IU/L[b]	43 (36.2–61.2) (n=22)	70 (34.3–148.5) (n=11)	100.5 (56.1–239.6) ++ (n=22)	88 (66.5–191.6) ++ (n=33)	59.5 (64.9–94.5) +, §§§ (n=50)	184.5 (121.3–332.6) +++ (n=26)	77.6 (90.8–169.4) +++ (n=76)	NS
Platelets, 10^3 /mm^3 counts	184 (153.2–190) (n=40)	70 (65.5–126.8) +++, §§§ (n=19)	53 (48.9–111.5) +++ (n=23)	68 (66.2–108.7) +++ (n=42)	152 (143.9–172.7) §§§ (n=79)	60.5 (60.7–111.2) +++ (n=32)	137 (124.8–151.5) ++ (n=111)	***
Leukocytes, mm^3 counts	6150 (5290–7094) (n=38)	3300 (2927–5018) ++ (n=14)	3500 (3074–5758) ++ (n=19)	3400 (3378–5077) ++ (n=33)	3950 (4289–5480) ++, §§ (n=78)	3300 (2953–3903) +++ (n=32)	3800 (4004–4918) +++ (n=110)	NS
Monocytes, mm^3 counts %	470 (426.4–626.2) (n=33)	320 (206.6–522.4) (n=14)	255 (163.3–328.1) +++ (n=18)	291 (217.1–378.3) +++ (n=32)	434 (436.3–581) §§§ (n=78)	286.5 (252.3–419.9) ++ (n=28)	391 (404.3–521.9) (n=106)	**

[a] Transaminases, reference values for aspartate aminotransferase (AST): Female 31 IU/L, Male 37 IU/L; [b] Alanine aminotransferase (ALT): female 31 IU/L, male 41 IU/L.
$^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ and $^{++++}p<0.0001$ represent statistical difference. OFD vs clinical classification. Mann-Whitney nonparametric test was applied.
$^{§}p<0.05$, $^{§§}p<0.01$, $^{§§§}p<0.001$ represent statistical difference. DwoWS vs DwWS/SD (same outbreak). Mann-Whitney nonparametric test was applied.
$^{*}p<0.05$, $^{**}p<0.01$ $^{***}p<0.001$, represent statistical difference. 2010 outbreak vs 2013 outbreak. Mann-Whitney nonparametric test was applied.
DwWS/SD: dengue with warning signs/severe dengue; DwoWS: dengue without warning signs; NS: not significant; ODF: other febrile disease.

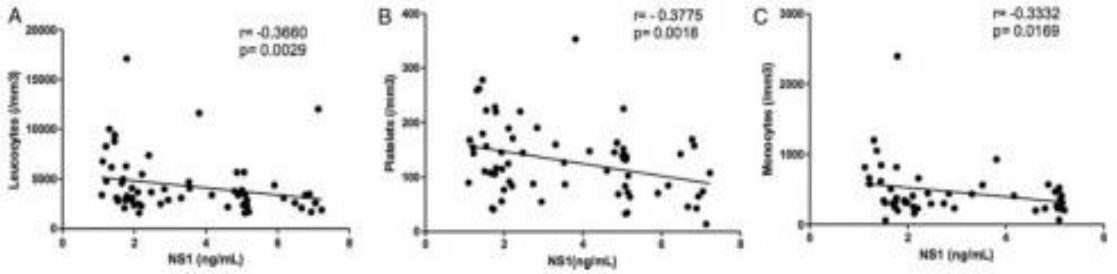

Figure 3. Correlation of levels nonstructural protein 1 (NS1) and (A) platelets, (B) leucocytes and (C) monocytes. A Spearman's rank correlation was calculated and r and p values are represented.

data from the Brazilian Ministry of Health, in that year 1815 severe cases with 47 deaths were confirmed in the state of Mato Grosso do Sul. In the 2013 epidemic, DENV-1, DENV-2 and DENV-4 co-circulated, and 695 severe cases with 30 deaths were confirmed, and a lower severity was observed. In Southeast Asia the co-circulation of two or more serotypes during the same epidemic has been a major cause of severe disease.[18] Although the number of cases in the 2010 epidemic was lower than in the 2013 epidemic, the number of severe cases and deaths were relatively higher. Furthermore, a higher number of hospitalizations was observed in 2010 (46.0%) when compared to 2013 (21.0%).[19,20]

According to the new clinical classification proposed by WHO in 2009,[10] the appropriate management of patients depends on early recognition of the warning signs. These signs are important because they occur before the onset of severe dengue.[21] Brazil officially adopted the new classification in 2014.[22] This study showed the prevalence of DwoWS (64.0%) in both epidemics studied. In the 2010 epidemic, 48% of the cases were of DwWS/SD, and DENV-1 and DENV-2 were the infecting serotypes detected. In the 2013 epidemic, 71.2% of the cases were of DwoWS and this epidemic was mainly caused by DENV-4 (90.4%), corroborating the data from the Brazilian Ministry of Health Persistent abdominal pain (16.1%) and mucosal bleeding (8.4%) were the most common warning signs in patients with DwWS, similar to other studies with new classification in Asia[23] and Brazil.[24]

The infections caused by DENV-4 are typically characterized by a milder clinical presentation compared to other serotypes.[25] However, several studies have reported cases of severe dengue and deaths related to infection by DENV-4, mainly in secondary infections.[26]

High detection rates of anti-DENV IgG in the studied population, characterizes a previous exposure to DENV infection. In this study, 84.0% of the cases were due to secondary infections, however, more severe cases were not observed in the DENV-4 cases which occurred in the 2013 epidemic. In the 2010 epidemic, on the other hand, a higher number of cases with warning signs and severe dengue were reported. In fact, disease severity is also caused by increased virulence and higher viral loads were found in patients with dengue hemorrhagic fever.[27] A recent study also in Rio de Janeiro showed that patients infected by DENV-3 and by DENV-2, as well as secondary cases had a significantly higher risk of developing a more severe disease.[6] In the present study we observed that patients infected during the 2010 epidemic caused by DENV-1 and DENV-2 presented a more severe disease than those infected during the 2013 epidemic mainly caused by DENV-4. Furthermore, the number of hospitalizations in 2010 was about 2 times higher than in 2013.

Several studies have evaluated biomarkers such as viremia, cytokines, laboratory parameters, genes expression associated with antigen presentation, MHC class II receptor and NK and T cell activities in order to identify potential predictors of severity.[28] Since we observed that DENV-4 was associated with less severity, the laboratory parameters in both groups of study were evaluated. In our study, 51.7% of patients had leukopenia and 27.7% thrombocytopenia corroborating previous studies carried out in Brazil.[16] Importantly, patients from the 2010 epidemic with prevalence of DENV-1 and DENV-2 had low platelet count, high frequency of hemorrhagic manifestations and plasma leakage. In comparison, patients from the DENV-4 epidemic in 2013 showed high platelet count with less frequent bleeding. Thrombocytopenia and coagulation disorders have been shown in severe cases of dengue infection. Circulating markers of coagulopathy are increased during the acute phase of the disease such as tissue factor, protein necessary for initiation of coagulation cascade, and have been associated with dengue pathogenesis and thrombocytopenia.[16] Interestingly, we observed that circulating levels of tissue factor were lower in patients infected with DENV-4 compared to those infected with DENV-2 (unpublished data).

The involvement of NS1 protein in dengue severity was suggested by several studies.[29] Aiming to confirm and expand those previous results, we analyzed NS1 levels in the Brazilian patients from those two epidemics in the same city. As demonstrated in Figure 2, patients with DwWS/SD presented high levels of NS1 compared to the DwoWS group, regardless of the infecting serotype. In addition, patients from the 2010 epidemic caused by DENV-1 and DENV-2 presented high levels of NS1 compared to those from the 2013 epidemic mainly caused by DENV-4. Previous results found that circulating levels of NS1 in DENV-1 cases were much higher than levels detected in DENV-2.[30]

Since most patients presented secondary infections in both epidemics analyzed here, soluble NS1 protein could be in the form of immune complexes with IgG antibodies. Despite all samples being heat dissociated according to Lima et al.,[7] circulating levels of NS1 were lower in samples from the 2010 epidemic when DENV-1 and DENV-2 co-circulated as compared to those from 2013, when DENV-4 was prevalent. Studies reporting the role of DENV NS1 as a biomarker are currently available, but insights on NS1 antigenemia on DENV-4 cases are scarce.

Interestingly, we found an inverse correlation between circulating levels of NS1 and platelets counts. Previous studies reported

associations between higher viral load and thrombocytopenia.[27] Importantly, increased platelet destruction was associated with immune complexes containing DENV antigen on the surface of platelets. Antibodies to NS1 antigen cross-react with platelets leading to platelet activation and opsonization in vitro.[16] NS1 activates the complement system leading generation of anaphylatoxins and terminal SC5b9 complement complex, contributing for pathogenesis of vascular leakage.[31] An inverse correlation between NS1 levels with leukocytes is in agreement with the study by Paranavitane.[30] Additionally, we also observed that monocyte counts were inversely correlated with NS1 levels. Monocytes are the main target cell for DENV replication and are the source of inflammatory cytokines that are involved in dengue pathogenesis. Patients from the 2010 epidemic presented the lowest monocyte count and increased levels of NS1. Circulating monocytes are activated during the acute phase of dengue infection[32] and this phenomenon could in part justify the low monocyte count observed in these patients. The reduced number of these cells may be also caused by apoptosis of these cells due DENV replication. In fact, CD14+ monocytes infected with DENV-2 expressed higher levels of death receptor Fas and may be susceptible to apoptosis.[33]

We also observed the rise in transaminase levels in most patients with dengue, regardless of infecting serotype or epidemic period. In the 2010 epidemic caused by DENV-1 and DENV-2, no differences were observed in AST and ALT levels according to the clinical classification, while, in the 2013 epidemic caused by DENV-4, the DwWS/SD group showed a significant increase in AST and ALT, compared to the DwoWS group. Liver involvement is one of the characteristics of DENV infection, and liver disorders, including hepatomegaly and transaminase elevations have been described in both mild and severe cases and is cause of death.[17] In fact, one patient infected by DENV-2 presented elevated levels of ALT and AST (5598 and 20618 IU/L, respectively) and died with fulminant hepatitis (data not included in the analyses).

A large number of samples collected in both periods were negative for DENV by using all laboratory methods available and were, thus, classified as OFD. Non-specific febrile illness, having fever can be caused by a number of other infectious diseases that may commonly be mistaken for dengue. Overall, the limitations of the study may include sampling from younger age groups and a more representative number of severe cases.

Conclusions

Even though patients with DENV-2 infection were more likely to develop severe forms of dengue, the number of patients studied here was small, and no significant association between severity and DENV serotype was found. DENV-4 infected patients presented symptoms of mild dengue and it has been shown that the epidemics caused by the prevalence of distinct DENV serotypes had different impacts and clinical characteristics in a same scenario.

Authors' contributions: FBS, ELA and RVC designed the study. NRCF, VEFS, JBSS, PCGN, FBN, MRQL and TCC implemented the study. NRCF, ELA, FBS, LMOP, RMRN and CFK analyzed the data and FBS, ELA and NRCF wrote the paper. All authors read and approved the final version of the paper. FBS and ELA are the guarantors of the paper.

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Competing interests: None declared.

Ethical approval: The samples were collected as part of an ongoing Project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05 and CEP100/00) and by the Ethics Committee of Plataforma Brasil, FIOCRUZ (CAAE 13318113.7.0000.5248). All participating subjects gave written informed consent.

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Artigo 4: Hepatite fulminante associada ao vírus dengue tipo 2: um relato de caso

Objetivo específico: Correlacionar infecção por DENV-2 à hepatite fulminante durante epidemia ocorrida em Campo Grande, Mato Grosso do Sul em 2010.

Situação do manuscrito: Submetido à Journal of Medical Virology

Classificação QUALIS: Área de Medicina II – B1

Referência: **Nieli Rodrigues da Costa Faria**, Elzinandes Leal de Azeredo, Rita Maria Ribeiro Nogueira, Fernanda de Bruycker-Nogueira, Thiara Manuelle Alves de Souza, Maurício Pompilio, Sandra Maria L de Oliveira, Flavia Barreto dos Santos, Rivaldo Venâncio da Cunha.

Resumo: Infecções por dengue podem ter um amplo espectro de manifestações clínicas e manifestações atípicas já foram descritas. O envolvimento hepático na dengue pode variar de uma forma assintomática com elevação das transaminases hepáticas à insuficiência hepática aguda fatal (IHA). IHA é uma condição na qual uma rápida deterioração da função hepática é observada , resultando em encefalopatia e/ou coagulopatia hepática. Este trabalho apresenta a descrição de um caso de paciente sem co-morbidades infectado pelo DENV-2 durante a epidemia de 2010 que evolui para obíto apresentando IHA. A identificação precoce de IHA em pacientes com dengue é importante na redução da morbidade e mortalidade.

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Fulminant hepatitis associated with Dengue Virus type 2 in Brazil: A case report

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Keywords:	Dengue virus < Virus classification. Flavivirus < Virus classification. Immune responses

Fulminant hepatitis associated with Dengue Virus type 2 in Brazil: A case report

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Abstract

Dengue infections can have a wide range of clinical manifestations and unusual manifestations have been described. The liver involvement in dengue can range from an asymptomatic hepatic transaminase elevation to fatal acute liver failure (ALF). ALF is a condition in which rapid deterioration of liver function, resulting in hepatic encephalopathy and/or coagulopathy. Early identification of ALF in dengue patients is important in reducing morbidity and mortality.

Key Words: Dengue virus – hepatitis – Brazil

Financial support: FIOCRUZ, CNPq, CAPES, FAPERJ

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INTRODUCTION

Dengue is actually main arboviruses in tropical and subtropical areas around the world. The incidence of dengue has grown dramatically worldwide in recent decades. Dengue virus (DENV) belongs to the *Flaviviridae* family, genus *Flavivirus* with four distinct serotypes known as DENV 1, 2, 3 and 4. The four serotypes circulate in Brazil and in 2015 were notified 1,649,008 suspected dengue cases in the country, according to the Ministry of Health [SVS, 2016]. DENV infections can range from asymptomatic to an acute febrile illness, dengue fever (DF). Some patients can progress to a severe form of the disease, characterized by increased plasma leakage, thrombocytopenia and hemorrhagic manifestations, which may be fatal. After mosquito feeding, DENV replicates in Langerhans dendritic cells (LDCs) and then infect circulating monocytes, the main target cells of infection. The virus also infects hepatocytes and liver is a target organ. Although DENV affect the liver, hepatic involvement is usually asymptomatic and severe liver disease is less frequent [Srikiatkhachorn and Green, 2010]. Here, we report a case of a Brazilian woman that develops severe dengue with complication of acute liver failure (ALF).

MATERIAL AND METHODS

Case Report

A 33-year Brazilian female, resident in Campo Grande, Mato Grosso do Sul, Brazil, was admitted in a health center near her residence, and then was referred to Professora Hesterina Corsini-NHU Dia hospital, Campo Grande, MS. She presented with 5-day history of fever, headache, retro-orbital pain, myalgia, arthralgia, prostration, anorexia, rash, vomiting, nausea, diarrhea, abdominal pain, paresthesia and dyspnea. There was a progressive laboratory worsening. Blood analysis on presentation

demonstrated thrombocypenia with a platelet count of 14000 mm^3 and leukocyte counts of 12000 mm^3. The liver enzymes were elevated, with AST more elevated than ALT (23618 and 5598 U/L respectively). Biochemical and hematological parameters, as well as vital signs are shown on table I. This study was approved by the Ethics Committees from the Instituto de Pesquisas Clinicas Evandro Chagas, Fiocruz (CAAE 3723.0.000.009-08) and Plataforma Brasil, Fiocruz (CAAE 13318113.7.0000.5248). The patient's husband gave written informed consent.

The infecting serotype was determined as DENV-2 by reverse transcriptase polymerase chain reaction (RT-PCR) [Lanciotti et al., 1992] and virus isolation [Igarashi, 1978] at the Reference Laboratory for Dengue and Yellow Fever, Oswaldo Cruz Institute, FIOCRUZ. Viral isolation was only possible through the lysis of the peripheral blood mononuclear cells (PBMC) followed by inoculation of the lysate into C6/36 *Aedes albopictus* cell line. A positive dengue non-structural protein 1 antigen (NS1) and a positive dengue IgM antibody ELISA, on day 5 confirmed the diagnosis of dengue infection. A secondary infection was confirmed by a positive IgG antibody detection according to previously established criteria (Focus Diagnosis, Cypress, CA, USA) [Limonta et al., 2014]. Hepatitis B sufarce antigen, hepatitis C, A and E antibodies were all negative. In addition, screening for Yellow fever virus, hantaviruses and arenaviruses were all negative. The level dengue viral RNA was investigated using quantitative RT-PCR [Johnson et al., 2005] and the viral load in the plasma was 4,46 x 10^5 copies/ml. Phylogenic analysis identified the Southeast Asian genotype, lineage II through partial genome sequencing (C/prM/M/E genes) according to Faria et al. [2013].

At the time of admission, medical examination revealed nervous system alteration, signs of meningeal irritation, irritability, drowsiness, hypothermia, fainting, oliguria, cyanosis, intense and continuous abdominal pain, massive hepatosplenomegaly

and petechiae on the lower limbs. The patient was hospitalized with liver failure, acute respiratory failure, severe metabolic acidosis, hepatomegaly voluminous and ascites. After hospitalization, the patient evolved with normalization of blood pressure, but with tachycardia frame, tachypnea and low level of consciousness. Due to the deterioration of respiratory symptoms associated with wheezing auscultation, the patient was intubated and subjected to mechanical ventilation. Despite full supportive care on intensive unit the patient died on the same day of admission.

DISCUSSION AND CONCLUSIONS

The spectrum of hepatic manifestations during dengue infections ranges from asymptomatic transaminases elevation to occurrence of severe ALF. Indeed, dengue patients present liver dysfunction and, elevated transaminases levels have been reported in children [Pires Neto et al., 2013] and adults [Ling et al., 2007; Souza et al., 2008]. Although hepatic involvement is frequently asymptomatic, dengue infection is an important cause of acute liver failure (ALF) in endemic countries [Samanta and Sharma, 2015].

The pathogenesis of dengue associated to liver injury is not well understood. It is believed to be due a direct viral infection of cells in the liver or by deregulated host immune response. Liver is the commonest target organ affected by DENV [de-Oliveira-Pinto et al., 2012; Povoa et al., 2014; Ferreira et al., 2015] and hepatocytes as well as Kupffer cells are important target during infection [Jessie et al., 2004]. Hepatic histological changes have been demonstrated during dengue infection such as hepatocyte necrosis, hyperplasia and destruction of Kupffer cells, Councilman Bodies and mononuclear cell infiltrates [Burke, 1968]. In fact, DENV antigens were detected in hepatocytes surrounding the necrotic foci and were associated with apoptosis in a fatal

case [Couvelard et al., 1999]. Cytokine storm is another factor that could be associated with liver injury. In this way, inflammatory cytokines are found in severe cases of dengue [Srikiatkhachorn and Green, 2010] and are frequently associated with hepatic dysfunction [Ferreira et al., 2015]. Additionally, the infecting serotype could also have a role in hepatic pathology as demonstrated with DENV-2 and DENV-3 serotypes [Kalayanarooj and Nimmannitya, 2003; Thomas et al., 2008]. In fact, the introduction of lineage II the Southeast Asian DENV-2 genotype was responsible for the most severe epidemic in 2008 in Brazil [Faria et al., 2013]. In the present study, high levels of viremia were found in plasma and PBMC in agreement with previous reports that observed an association between high viral loads with disease severity and hepatic dysfunction [Couvelard et al., 1999; Wang et al., 2003]. According to the WHO 2009 classification, the patient had clinical features of severe dengue and died with ALF caused by DENV-2 serotype. The majority of dengue related ALF described in the literature is mainly in children and there are few cases reported in adults [Souza et al., 2008; Pires Neto et al., 2013]. ALF is an unusual but serious complication of severe dengue and should be considered as a differential diagnosis of hepatitis. Further studies for investigation of the effect of the DENV infection and the biomarkers on involved in pathogenesis might contribute to establish the target tissues and cells involved in the pathogenesis of the disease, as well as the mechanisms behind it.

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CONFLICT OF INTEREST STATEMENT

We declare no conflict of interest.

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Table 01. Clinical and laboratorial parameters recorded on admission in fatal case with confirmed secondary DENV-2 infection, Mato Grosso do Sul, Brazil.

Patient's clinical and laboratorial parameters	
Hematocrit (%) (35-47)*	35
Platelets (x 10^3/L) (150-450)	14.000
AST (IU/L) (0-40 U/L)	23618
ALT (IU/L) (0-41 U/L)	5598
Blood pressure (120 x 80 mm of Hg)	95/80
Heart rate (60 – 100 beats/minute)	116
Viral load in plasma and PBMC (copies/mL)	2.33 x 10^7/4.46 x 10^6
Gama GT (8 – 61 U/L)	417
Alcaline phosphatase (40 - 129 U/L)	205

* Reference normal range is shown

AST, aspartate aminotransferase; ALT, alanine aminotransferase; Gama GT, gammaglutamyltranspeptidase

Artigo 5: Análise de alterações clínicas e laboratoriais relacionadas com a gravidade de casos de dengue: um estudo comparativo entre sorotipos 2 e 4 no Brasil

Objetivo específico: Análise dos aspectos laboratoriais, clínicos e imunológicos de casos de DENV-2 associados à gravidade da doença e comparados à casos de DENV-4 em epidemias ocorridas em Campos dos Goytacazes, Rio de Janeiro em 2010 e 2013.

Situação do manuscrito: Submetido à American Journal of Tropical Medicine and Hygiene

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Referência: **Nieli Rodrigues da Costa Faria**, Victor Edgar Fiestas Solorzano, Rita Maria Ribeiro Nogueira, Fernanda de Bruycker-Nogueira, Thaís Chouin-Carneiro, Jaqueline Bastos Santos Simões, Monique da Rocha Queiroz Lima, Luzia Maria de Oliveira Pinto, Claire Fernandes Kubelka, Luiz José de Souza, Elzinandes Leal de Azeredo, Flavia Barreto dos Santos.

Resumo: O aumento das formas graves de dengue tem causado grande impacto na saúde pública e tem preocupado as autoridades dos países onde a doença é endémica e as epidemias atingem altas proporções. O reconhecimento dos sinais de progressão para a forma grave da doença durante a fase febril pode ser difícil, uma vez que os sintomas são muitas vezes indistinguíveis de outras doenças febris. O objetivo deste estudo foi avaliar as manifestações clínicas e os parâmetros laboratoriais de pacientes provenientes de duas epidemias de dengue e sua associação com manifestações clínicas. O estudo foi realizado em pacientes (n = 153) com sinais e sintomas compatíveis com a dengue ocorridos durante duas epidemias distintas, 2010 e 2013, na cidade de Campos dos Goytacazes, Rio de Janeiro, Brasil. De acordo com os critérios da OMS de 2009, os pacientes foram classificados como dengue sem sinais de alarme ([DSSA], 60,6%; 57/94), dengue com sinais de alarme ([DCSA], 30,9%; 29/94) e dengue grave ([DG], 6.4%; 6/94). Independente da epidemia, pacientes com DCSA/DG apresentaram menores contagens de plaquetas e leucócitos e elevados níveis de transaminases quando comparados com os pacientes DSSA. Curiosamente, os pacientes da epidemia de 2010 causada pelo DENV-2 apresentaram menores contagens de plaquetas do que os pacientes da epidemia de 2013

causada pelo DENV-4. Embora estudos anteriores tenham amplamente relatado a vasta gama de aspectos clínicos da dengue, a caracterização de epidemias causadas pelo DENV-4 é muito importante, considerando as diferentes formas clínicas apresentadas durante as epidemias, este fato torna-se especialmente indispensável para as unidades de saúde e hospitais que executam a gestão do paciente.

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Analysis of clinical and laboratorial alterations related to dengue cases severity: a comparative study between serotypes 2 and 4 in Brazil

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Key Words:	Dengue, Epidemiology, Diagnosis

Analysis of clinical and laboratorial alterations related to dengue cases severity: a comparative study between serotypes 2 and 4 in Brazil

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Running title: Comparison of hematological and biochemical parameters on Dengue 2 and 4 cases

ABSTRACT

The increase on severe forms of dengue has caused great impact on public health and has concerned the authorities of countries where the disease is endemic and epidemics reach high proportions. The recognition of signs of progression to the severe form of the disease during the initial febrile phase can be difficult, since the symptoms are often indistinguishable from other febrile diseases. The aim of this study was to evaluate the clinical manifestations and laboratorial parameters in patients from two dengue outbreaks and their association with clinical manifestations. The study was conducted in patients (n=153) with signs and symptoms compatible with dengue occurred during two distinct epidemics, 2010 and 2013, in the city of Campos dos Goytacazes, Rio de Janeiro, Brazil. According to the 2009 WHO criteria, patients were classified as dengue without warning signs ([DwoWS], 60.6%; 57/94), dengue with warning signs ([DwWS], 30.9%; 29/94) and severe dengue ([SD], 6.4%; 6/94). Independent of the epidemic, patients with DwWS/SD presented lower platelets and leukocytes counts and higher transaminases levels when compared to the DwoWS ones. Interestingly, patients from the epidemic of 2010 caused by DENV-2 had lower platelet counts than patients of the 2013 epidemic caused by DENV-4. Although previous studies may have extensively reported the wide range of the clinical aspects of dengue, the characterization of DENV-4 is desirable considering the burden of the disease during epidemics, especially for the health units and hospitals performing patient's management.

Introduction

Dengue is currently considered globally as the most important mosquito-borne viral disease and the incidence has grown dramatically around the world in recent decades. It is estimated that 3.9 billion people in 128 countries are at risk of dengue infection.[1] One recent estimate indicates 390 million dengue infections per year, of which 96 million manifests clinically with any severity of disease.[2] Demographic changes, urbanization and international travel contribute to the expansion of geographical areas where transmission occurs and all four DENV serotypes are now circulating in Asia, Africa, and the Americas.[3] Not only is the number of cases increasing as the disease spreads to new areas, but explosive outbreaks are occurring. Currently, Brazil accounts for approximately 70.0% of reported cases of dengue in the Americas.[4]

In Brazil, the first dengue epidemic with laboratory confirmation occurred in 1981 in Boa Vista, Roraima in the North region, where DENV-1 and 4 were isolated.[5] However, it was only after DENV-1 was introduced in Rio de Janeiro (RJ) in 1986, that the disease became a nationwide public health problem.[6] In 2007–2008, the country experienced the most severe epidemic in terms of morbidity and mortality and severe cases in children due to the DENV-2 re-emergence.[7] DENV-4 was reintroduced in Brazil in 2010 and in RJ, the first cases were detected in Niteroi in 2011[8] and, despite the epidemic caused by DENV-1, DENV-4 could be isolated during the disease surveillance supported by the laboratorial diagnosis performed.[9]

In 2010 and 2013, Brazil reported a total 1.011,548 and 1.452, 489 dengue cases, respectively, and RJ was responsible for 2.9% (29.824) and 14.7% (213.058) of those case.[10]

Early diagnosis of dengue is critical, since some patients may progress from a mild to a severe disease in a short period of time.[11] Although dengue pathogenesis is not completely clear, it is believed that multiple circulating serotypes, host factors such as secondary infection, comorbidities, genetic polymorphism and factors related to the virus serotype or genotype would determine the evolution of the clinical forms of dengue.[12]

The white blood count (WBC) can reveal a leucopenia and neutropenia, lymphocytosis with lymphocytic atypia and monocytosis. Signs and symptoms such as severe abdominal pain and continuous, persistent vomiting, postural hypotension, hepatomegaly, mucosal bleeding, increased hematocrit or abrupt drop of platelets, are considered as a potential worsening of the disease.[13] Repeated monitoring of the platelet count and hematocrit is recommended, as an abrupt decrease in platelet counts is an alarm sign and a significant hematocrit increase is an indirect sign of plasma leakage.[14]

In this study, we aimed to analyze the laboratorial parameters and clinical findings during two distinct epidemics occurred in 2010 and 2013, caused by DENV-2 and DENV-4, respectively, in Campos dos Goytacazes, Rio de Janeiro, Brazil.

Material and Methods

Ethics Statement

The samples were collected as part of an ongoing Project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05 and CEP100/00) and by the Ethics Committee of Plataforma Brasil,

FIOCRUZ (CAAE 13318113.7.0000.5248). All participating subjects provided a written consent.

Enrolment, Data And Specimen Collection

The specimens (serum and plasma) analyzed in this study were collected during ongoing epidemics that occurred in 2010 and 2013, in the city of Campos dos Goytacazes, RJ, Brazil. Patients were assisted at the Hospital Plantadores de Cana where an infectious disease physician collected data on demographic characteristics, symptoms and physical signs using a structured questionnaire. Dengue suspected cases (n=153 [n=49 in 2010 and n=104 in 2013]) were obtained during an active surveillance performed by the Laboratory of Viral Immunology, IOC/FIOCRUZ. Laboratorial diagnosis was performed by the Laboratory of Flavivirus, Regional Reference Laboratory for the Brazilian Ministry of Health, located in Rio de Janeiro. Acute serum samples (up to the 7th day after the disease onset) were submitted to RT-PCR and NS1 antigen capture ELISA. Convalescent samples (>the 7th day after the onset of the symptoms) were tested by MAC-ELISA and IgG-ELISA. Both acute and convalescent serum samples were stored at -70°C. Non-specific laboratory investigations from all patients were obtained in the hospital in which they were treated and include hemoglobin test, white blood cell (WBC) count, platelet count, liver function tests (aspartate aminotransferase [AST] and alanine aminotransferase [ALT]), renal function tests and ultrasonography (USG) of abdomen. Each sample was accompanied by an identification form containing demographic information, vital signs, physical examination, complete blood count, liver enzymes, ultrasounds and X-rays findings, history of the previous diseases and medication use.

Inclusion criteria for WHO Dengue Case Definition

Dengue confirmed cases were classified according to the 2009 WHO classification [13, 15] and grouped as follows: Dengue without warning signs (DwoWS): patients living in and/or traveling to dengue endemic area, presenting fever and two of the following symptoms: nausea, vomiting, rash, aches, pain, positive tourniquet test and leukopenia; Dengue with warning signs (DwWS): dengue patients with any of the following warning signs: abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy or restlessness, liver enlargement >2 cm, and an increase in hematocrit concurrent with rapid decrease in platelet count; Severe dengue (SD): "dengue patients presenting at least one of the following:" severe plasma leakage (leading to shock and fluid accumulation with respiratory distress), severe bleeding evaluated by clinicians, severe involvement of liver by AST or ALT>1,000U, central nervous system with impaired consciousness, and severe involvement of the heart and other organs. Non-dengue cases, excluded after a negative result by all dengue laboratorial diagnosis were considered in this study as other febrile diseases (OFD).

Dengue infection laboratorial diagnosis

Laboratory-positive dengue infection was defined as patients experiencing a febrile illness consistent with dengue according to WHO criteria and infection confirmed based on the results obtained by the laboratory diagnostic assays: RT-PCR, MAC-ELISA, NS1 ELISA and IgG-ELISA.

Serological Diagnosis

Anti-DENV immunoglobulins

The Panbio dengue IgM Capture ELISA (Panbio, Brisbane, Australia) was used for the qualitative detection of anti-DENV IgM antibodies in serum for case confirmation according to the manufacturer's instructions.

The IgG—ELISA previously described by [16] was performed for the characterization of dengue immune response as primary or secondary infections in dengue cases previously confirmed by virus isolation, RT—PCR and/or MAC-ELISA. Alternatively, the IgG Select Dx kit (Focus Diagnostics, Carlsbad, California, USA) was used for characterization of dengue immune response as primary and secondary. Briefly, the acute samples presenting an NS1 and/or RT-PCR positive result with a negative IgG detection were considered as primary infections, and acute samples NS1 and/or RT-PCR positive with a positive IgG detection were classified as secondary ones.

NS1 antigen capture ELISA

For the NS1 antigen capture, the Platelia™ Dengue NS1 Ag-ELISA kit (Biorad Laboratories, Marnes-La-Coquette, France) was used according to the manufacturer's protocol. All NS1 negative samples were submitted to a NS1 immune complex dissociation protocol previously described. [17] All samples were tested between 1-9 days after disease onset.

Molecular Methods

The viral RNA was extracted using the QIAamp Viral RNA Mini kit (Qiagen, Germany) following the manufacturer's instructions and stored at -70°C for DENV detection and typing. The RT—PCR for detecting and typing DENV was performed as described previously.[18] All negative samples by all dengue laboratorial diagnosis (OFD) were also subjected to a RT-PCR for detection of other flaviviruses and alphaviruses

according to a previous published protocol.[19] Aiming to further exclude DENV infection in all negative cases (OFD), samples were tested by using the Simplexa™ Dengue Real Time RT-PCR (Focus Diagnostics, Cypress, CA) according to the manufacturer's protocol, for viral qualitative detection and typing of DENV.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 6.0 (GraphPad Software Inc., San Diego, CA, USA). The Kruskal-Wallis non-parametric test and Dunn's Multiple Comparison Test were used to compare the differences between study groups (OFD, DwoWS and DwWS/SD). Fisher test was used to compare clinical and laboratorial characteristics between patients groups. Correlation was estimated by Spearman regression analysis. Values of $p<0.05$ were considered as significant for all statistical analysis.

Results

The 2010 Epidemic

During the 2010 epidemic occurred in Campos dos Goytacazes, RJ, a total of 49 dengue suspected cases were investigated. DENV infection was confirmed in 49.0% (24/49) of the cases and 51.0% (25/49) were characterized as OFD. By RT-PCR and MAC-ELISA, 66.7% (16/24) of the cases were confirmed and 45.8% (11/24) by NS1capture ELISA, Table 1.

All positive cases found were between the 1st and 8th of disease. According to the 2009 WHO clinical criteria, confirmed cases (n=24) were classified as DwoWS (54.0%; 13/24), DwWS (21.0%; 5/24) and SD (4.0%, 1/24), Table 2. Males were more affected

than females; however, independently of gender patients, the mean age was 34 years old. DENV-2 was the only infecting serotype identified in 66.7% (16/24) of the cases. Secondary infections were prevalent (91.1%, 18/24) when compared to the primary ones (8.9%, 3/24). Hospitalizations occurred in 45.8% (11/24) of the cases and 41.6% (10/24) of the patients presented comorbidity, Table 2.

The main signs and symptoms of patients were fever (87.5%), myalgia (87.5%), prostration (83.3%), anorexia (79.1%), retro-orbital pain 67%, arthralgia 62.5%, vomiting (50.0%), thrombocytopenia (47.0%), nausea (37.5%), rash (37.5%), itch (33.3%), diarrhea (29.1%), positive tourniquet (21.0%) and petechiae (8.3%). The most common warning signs of DwWS and SD patients were hemorrhagic manifestations (60.0%, 6/10), persistent abdominal pain (50.0%, 5/10), pleural effusion (40.0%, 4/10) and increased hematocrit with rapid decrease of platelets (40.0%, 4/10), figure 1.

It was observed that the DwWS/SD patients from the epidemic occurred in 2010 and mainly caused by DENV-2, had significantly lower leukocytes and platelets counts ($p<0.001$) when compared to OFD patients, however no statistically significant differences between monocytes count and hematocrit was observed (Figure 2). Thrombocytopenia was observed in 41.6% (10/24) of the total number confirmed dengue cases, with 20.0% (2/10) occurring on DwoWS patients and 80.0% (8/10), on DwWS/SD ones. The platelet count <50,000/mm^3 was observed in 87.5% (7/8) of the DwWS/SD patients.

The analysis of the AST revealed high levels in 33.3% (8/24) of the patients during the 2010 epidemic, of those, 62.5% (5/8) occurring on DwWS/SD patients and 37.5% (3/8) on DwoWS ones. The high AST levels on DwWS/SD patients were statistically

209 significant when compared to the OFD ($p< 0.01$) and to the DwoWS patients ($p< 0.05$),
210 Figure 3. The ALT analysis revealed elevated levels in 37.5 % (9/24) of the dengue
211 cases, 50.0% (4/8) on DwoWS patients and 50.0% (4/8) on DwWS/SD, and no
212 significant differences among the groups were observed.

213 The 2013 Epidemic

214 In 2013, a total of 104 dengue suspected were analyzed and 67.0% (70/104) were
215 confirmed and 33.0% (34/104) of the cases considered as OFD. By using MAC-ELISA,
216 62.8% (44/70) of the infections were confirmed, RT-PCR confirmed 48.5% (34/70) of
217 the cases and the NS1 capture ELISA, 38.5% (27/70), Table 1. However, when the
218 DENV-4 cases negative by NS1 ELISA were heat dissociated, the test sensitivity
219 increased in 18.5%. According to the 2009 WHO clinical criteria, confirmed cases
220 (n=70) were classified as DwoWS (63.0%; 44/70), DwWS (34.2%; 24/70) and SD
221 (1.4%, 1/70), Table 2.

222 In the 2013 epidemic, females were more affected than males; however, independently
223 of gender patients, the mean age was 37 years old. DENV-4 was the only infecting
224 serotype identified in 48.6% (34/70) of the cases. Secondary infections were prevalent
225 (84.2%, 59/70) when compared to the primary ones (15.7%, 11/70). Hospitalizations
226 occurred in 22.9% (16/70) of the cases and 38.5% (27/70) of the patients presented
227 comorbidity.

228 Moreover, the factors (abdominal pain, plasma leakage, bleeding and pleural effusion)
229 involved in a more severe disease were less frequently observed than those on the 2010
230 epidemic, Table 2.

The main signs and symptoms of patients were fever (97%), myalgia (86%), headache (80%), arthralgia (63%), nausea (54%), retro-orbital pain (50%), back pain (49%), prostration (49%), abdominal pain (37%), itch (34%), vomiting (33%), diarrhea (31%), rash (26%), anorexia (20%), dizziness (19%), asthenia (19%), thrombocytopenia (17%) and petechiae (7 %), Figure 1.

DwWS/SD patients from the epidemic occurred in 2013 and mainly caused by DENV-4, had significantly lower platelets and leukocytes counts ($p<0.001$) when compared to OFD patients. Furthermore, the differences on the platelet counts between the DwoWS and DwWS/SD were also significant ($p<0,05$), Figure 2.

The thrombocytopenia was observed in 17.1 % (12/70) of the total number of dengue cases, with 8.3% (1/12) reported on DwoWS patients and 91.7% (11/12) on DwWS/SD ones. Low platelet counts was observed in 25.0% (3/12) of patients, all with DwWS.

Both DwoWS and DwWS/SD presented lower counts than the OFD ($p< 0.001$), however no significant difference was observed in relation to the hematocrit and monocytes values, Figure 5.

During the 2013 epidemic, increased AST was observed in 42.8% (30/70) of dengue cases, 53.3 % (16/30) on patients with DwoWS and 46.6% (14/30) on DwWS ones, whereas the increased ALT levels were also observed on 42.8% (30/70) of the cases, 50% (15/30) on DwoWS cases and 50% (15/30) on DwWS ones.

Both high AST levels observed on DwoWS and DwWS/SD patients on 2013 were statistically significant when compared to the OFD ($p< 0,05$ and $p<0,001$, respectively), however no significant differences were observed between DwoWS and DwWS/SD cases, Figure 4. The ALT analysis revealed elevated levels statistically significant on

DwoWS and DwWS/SD patients on 2013 when compared to the OFD cases ($p< 0,05$ and $p<0,001$, respectively), and also between DwoWS and DwWS/SD cases ($p<0,05$), Figure 3.

In an overall overview, independently of the cases clinical classification, the lower platelet count observed in the 2010 epidemic caused by DENV-2 was statistically significant when compared to that observed on the cases occurred on the 2013 epidemic ($p<0,05$). Likewise, the lower leucocytes count observed on both epidemics were significant, when compared to the OFD cases ($p<0,001$). Moreover, in both epidemics, both AST and ALT levels were higher when compared to the OFD group, Figure 4. Independently of the epidemic year, patients presenting DwWS/SD, showed lower platelets and leucocytes counts considering both DwoWS and OFD groups ($p<0,001$) and higher AST and ALT levels, Figure 5.

Discussion

In Brazil, dengue transmission has been occurring continuously since 1986, with occurrence of outbreaks and epidemics, usually associated with the introduction of new serotypes in previously unaffected areas or serotypes re emergences. The years of 2010 and 2013 were characterized by two of the largest dengue epidemics reported in the country, when about 1 million and 1.4 million cases were reported, respectively. Moreover, despite the introduction of DENV-4 in 2010, this serotype was prevalent in 2013 and responsible mostly by mild cases.[10] In this study, dengue infection was confirmed in 61.4 % (94/153) of suspected cases occurred in two distinct epidemics in

2010 and 2013, in the city of Campos do Goytacazes, RJ, revealed the circulation of DENV-2 and DENV-4, respectively, and distinct hematological and biochemical characteristics.

According to the 2009 WHO criteria, patients were classified as DwoWS (60.6%; 57/94), DwWS (30.9%; 29/94) and SD (2.1%; 2/94). Early recognition of warning signs it is important for the appropriate management of patients in order to prevent deaths from dengue. Furhtermore, those markers are important since they occur before the onset of severe disease.[20] In 2010, when DENV-2 was the prevalent serotypes, the number of hospitalizations (45.8%) was higher than during the 2013 epidemic (22.9%), corroborating previous studies, as it has been shown that infections caused by DENV-2 and DENV-3 are two times more likely to result in a more severe disease than the infection by DENV-4.[21] Most of the hospitalizations 80.8% (21/26) occurred in the acute phase of the disease between the first and seventh day after the onset of illness and 96.2% (25/26) were due to DwWS/SD.

Considering the epidemiological situation of the country, the immune response characterization is important as secondary infections are also related to a more severe disease. In fact, in this study, 81.9% (77/94) of patients had secondary dengue infection and a high prevalence of secondary infections in the country has been reported in high proportions previously.[22-24]

When comparing the hematologic parameters in dengue patients from this study, it was observed that regardless of their clinical status, patients of the 2010 epidemic, caused mainly by DENV-2, had lower platelet counts than patients of the 2013, caused mainly

by DENV-4 (Figure 4). It was shown that DENV-2 induced more severe thrombocytopenia than DENV-4, corroborating the study by [25], which conducted a retrospective study comparing signs and symptoms of patients infected with different DENV serotypes, reporting differences in the virulence between the serotypes.

Several hypotheses have been formulated in order to elucidate the possible mechanisms responsible for thrombocytopenia in dengue.[26] suggested that DENV affect the bone marrow progenitor cells by inhibiting their function. Other studies reported that the virus indirectly diminishes the proliferative capacity of hematopoietic cells.[27] Indeed, there is evidence that DENV induced bone marrow hypoplasia in the acute phase of the disease [28], and *in vitro* studies have demonstrated that DENV-4 replicates in progenitor cells from human bone marrow by changing its capacity proliferative.[29] Other studies reported that DENV infection induces platelet consumption due to increased lysis by complement and apoptosis. Moreover, the involvement of antiplatelet antibodies has also been demonstrated.[30, 31]

In the 2010 and 2013 epidemics, leukopenia was observed in dengue patients compared to OFD cases (Figure 4). The analysis of leukocytes according to the clinical classification, independently of the epidemic year, has shown a decreased count in DwWS /SD patients compared to DwoWS, however without significant differences (Figure 5). Some studies show that in the early phase of the disease, in both primary and secondary infections, a decrease in leukocyte counts and increase of atypical lymphocytes may be related to the removal process by the bone marrow.[32]

Liver involvement is one of the characteristics of DENV infection. The liver changes, including hepatomegaly and increases in transaminases have been described in both cases of dengue fever and dengue hemorrhagic fever.[33-35] In severe dengue, the occurrence of fulminant hepatic failure has been the cause of death in children.[36] Mechanisms of liver damage in dengue may be related to the direct effects of the virus or as a consequence of the host immune response in the liver, leading to circulatory impairment, metabolic acidosis, hypotension, hypoxia and/or vascular leakage.[37] Our results showed that 41.5% (39/94) of patients had transaminase levels above normal, and from those, 51.3% (20/39) were DwWS/SD patients. Different variations of transaminase levels during dengue are not fully understood. In this study, there were no significant statistical differences in the levels of AST/ALT between epidemics.

The recognition of hematological and biochemical differences between patients with dengue fever and other diseases, it is of great importance in identifying patients who may develop severe disease. The initial clinical phase of dengue is often confused with other febrile diseases, raising doubts at the time of clinical management and surveillance of the disease. However, the use of hematological differences and cytokine levels could be used as an early tool to differentiate dengue from other febrile illnesses. Moreover, it was also demonstrated that it was possible to predict the possible onset of thrombocytopenia by using algorithms.[38]

By comparing the data analyzed in this study, we showed a clear difference in hematological and biochemical parameters between patients infected by DENV-2 and DENV-4, with different clinical forms of the disease. The recognition of these parameters helps to ensure that support measures can be taken to prevent increased

morbidity and mortality from dengue. Further studies are needed to improve those observations which may impact on the practice of medicine during dengue epidemics, especially in tropical areas where medical resources are deficient and where such epidemics may generate large economic impacts.

Authors' contributions

FBS, ELA and LJS designed the study. NRCF, VEFS, JBSS, FBN, MRQL and TCC implemented the study. NRCF, ELA, FBS, LMOP, RMRN and CFK analyzed the data and FBS, ELA and NRCF wrote the paper. All authors read and approved the final version of the paper. FBS and ELA are the guarantors of the paper.

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Competing interests

None declared.

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519 Table 1: Laboratorial confirmation of dengue suspected cases analyzed from epidemics
520 occurred in 2010 and 2013, in Campos dos Goytacazes, Rio de Janeiro, Brazil.

521

Dengue laboratorial diagnostic methods	Year	
	2010 (n=24)	2013 (n=70)
	Positive/Tested (%)	Positive/Tested (%)
MAC –ELISA	16/24 (66.7)	44/70 (62.8)
NS1 ELISA	11/24 (45.8)	27/70 (38.5)
RT-PCR [Serotypes]	16/24 (66.7) [DENV-2; 16/16]	34/70 (48.5) [DENV-4; 34/34]

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533 Table 2: Characteristics of dengue suspected cases from epidemics occurred in 2010 and

534 2013, in Campos dos Goytacazes, Rio de Janeiro, Brazil.

Cases characteristics	Epidemic Year (Total of cases analyzed)	
	2010 (*n*=49)	2013 (*n*=104)
	Positive/Tested (%)	Positive/Tested (%)
Dengue	24/49 (49.0)	70/104 (67.3)
Mean age (in years)	34	37
Gender (male:female)	29:20	23:62
Cases classification *	(*n*=24) Positive/Tested (%)	(*n*= 70) Positive/Tested (%)
DwoWS	13/24 (54.0)	44/70 (63.0)
DwWS	5/24 (21.0)	24/70 (34.2)
SD	1/24 (4.0)	1/70 (1.4)
Infecting serotype	(*n*=24) Positive/Tested (%)	(*n*= 70) Positive/Tested (%)
DENV-2	16/24 (66.7)	0/70
DENV-4	0/24	34/70 (48.6)
Type of infection	(*n*=24) Positive/Tested (%)	(*n*= 70) Positive/Tested (%)
Primary	3/24(8.9)	11/70 (15.7)
Secondary	18/24 (91.1)	59/70 (84.2)
Factors associated to a more severe disease	(*n*=24) Positive/Tested (%)	(*n*=70) Positive/Tested (%)

Hospitalization	11/24 (45.8)	16/70 (22.9)
Abdominal pain	10/24 (41.6)	15/70 (21.4)
Plasma leakage	5/24 (20.8)	3/70 (4.3)
Bleeding	6/24 (25.0)	4/70 (5.7)
Pleural effusion	4/24 (16.6)	0/70
Increased hematocrit with rapid decrease of platelets	4/24 (16.6)	4/70 (5.7)
Comorbidity:	10/24 (41.6)	27/70 (38.5)
Arterial hypertension	4/10 (40.0)	18/27 (66.7)
Diabetes mellitus	1/10 (10.0)	2/27 (7.4)
Other comorbidities	5/10 (50.0)	16/27 (59.2)

* Due to the lack of information for the 2009 WHO clinical classification, 5 cases were not classified in 2010 and 1 case was not classified in the 2013 epidemic.

545 FIGURE LEGENDS

546 Figure 1

547 Major signs and symptoms of dengue cases from the 2010 and 2013 epidemics occurred
548 in Campos dos Goytacazes, Rio de Janeiro, Brazil.

549

550 Figure 2

551 Hematological parameters of dengue positive cases according to clinical classification
552 and epidemic, Campos dos Goytacazes, Rio de Janeiro, Brazil. DwoWS: Dengue
553 without warning signs; DwWS: Dengue with warning signs ;SD: Severe dengue; OFD:
554 other febrile disease; p values: * < 0,05; ** < 0,01 and *** < 0,001 were considered
555 statistically significant. (A) Platelet, (B) Hematocrit, (C) Leucocytes and (D)
556 Monocytes.

557

558 Figure 3

559 Biochemical parameters of dengue positive cases according to the clinical classification
560 and epidemic, Campos dos Goytacazes, Rio de Janeiro, Brazil. DwoWS: Dengue
561 without warning signs; DwWS: Dengue with warning signs; SD: Severe dengue; OFD:
562 other febrile disease; AST: aspartate aminotransferase; ALT: alanine aminotransferase;
563 p values * < 0,05; ** < 0,01 and *** < 0,001 were considered statistically significant.
564 (A) AST and (B) ALT.

565

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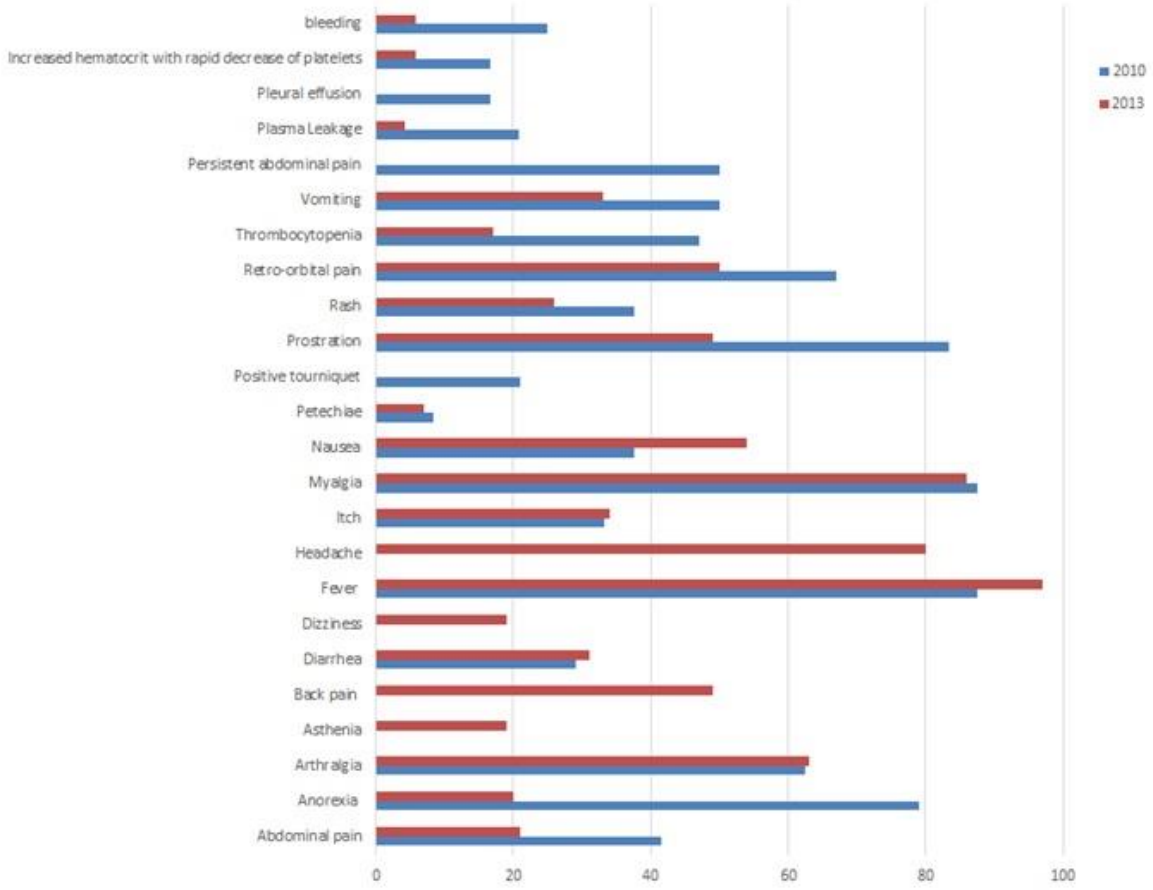
Figure 4

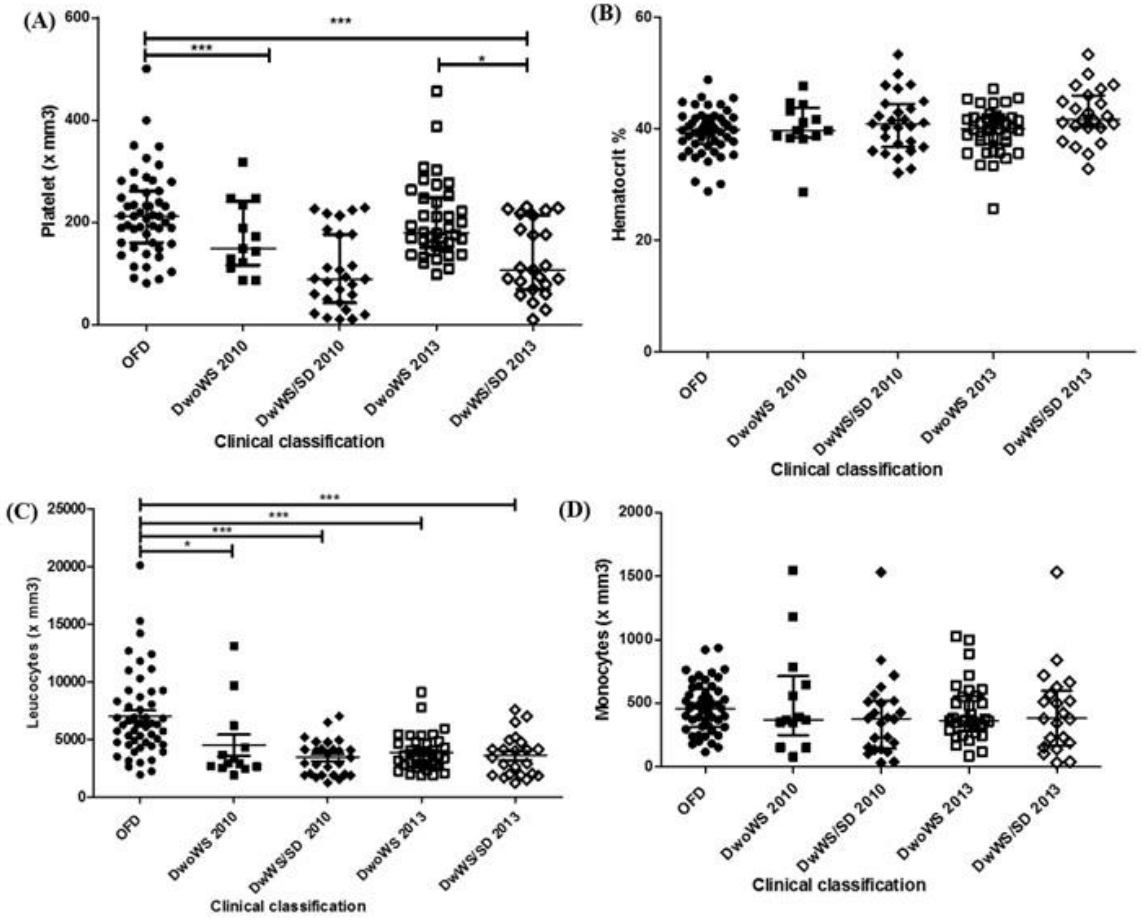
Hematological and biochemical parameters of dengue positive cases according to the epidemics, Campos dos Goytacazes, Rio de Janeiro, Brazil. OFD: other febrile disease; p values * < 0,05; ** < 0,01 and *** < 0,001 were considered statistically significant. (A) Platelets, (B) Hematocrit, (C) Leucocytes, (D) Monocytes, (E) AST and (F) ALT.

Figure 5

Hematological and biochemical parameters of dengue positive cases according to the clinical classification, Campos dos Goytacazes, Rio de Janeiro, Brazil. DwoWS: Dengue without warning signs (n=); DwWS: Dengue with warning signs; SD: Severe dengue; OFD: other febrile disease; (A) Platelets, (B) Hematocrit, (C) Leucocytes, (D) Monocytes, (E) AST: aspartate aminotransferase; (F) ALT: alanine aminotransferase p values * <0,05; ** <0,01 and *** <0,001 were considered statistically significant.



Major signs and symptoms of denque cases from the 2010 and 2013 epidemics occurred in Campos dos Goytacazes, Rio de Janeiro, Brazil.
254x190mm (72 x 72 DPI)



Hematological parameters of dengue positive cases according to clinical classification and epidemic, Campos dos Goytacazes, Rio de Janeiro, Brazil. DwoWS: Dengue without warning signs; DwWS: Dengue with warning signs ;SD: Severe dengue; OFD: other febrile disease; p values: * < 0,05; ** < 0,01 and *** < 0,001 were considered statistically significant. (A) Platelet, (B) Hematocrit, (C) Leucocytes and (D) Monocytes.
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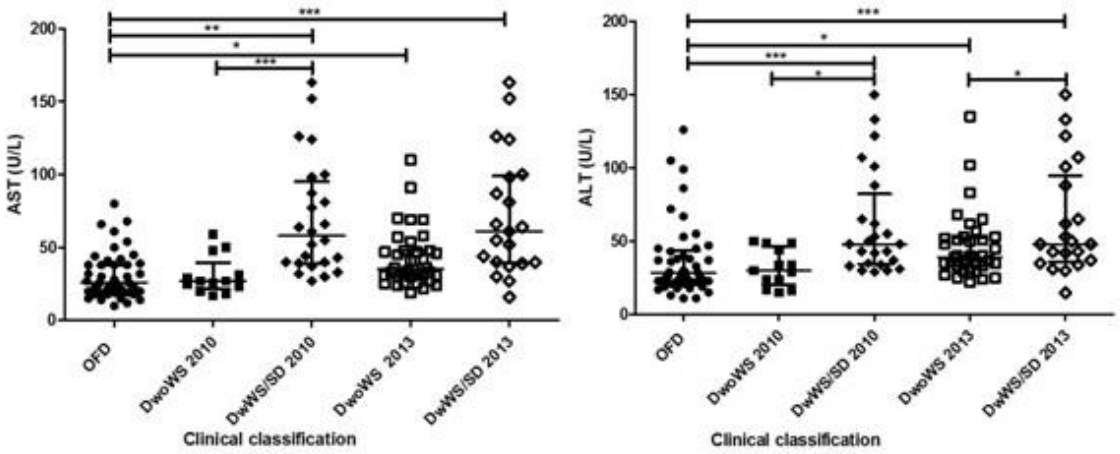
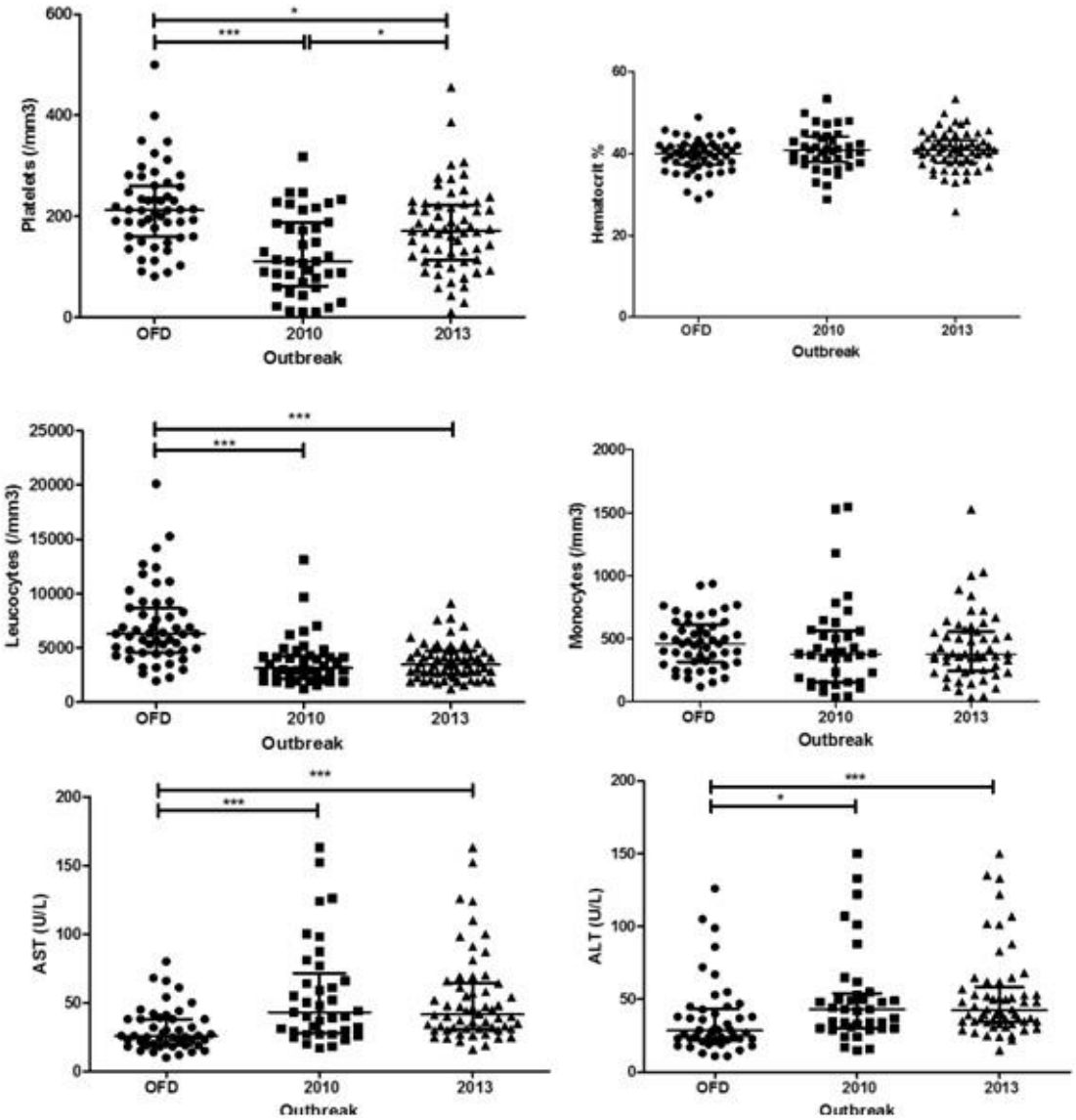
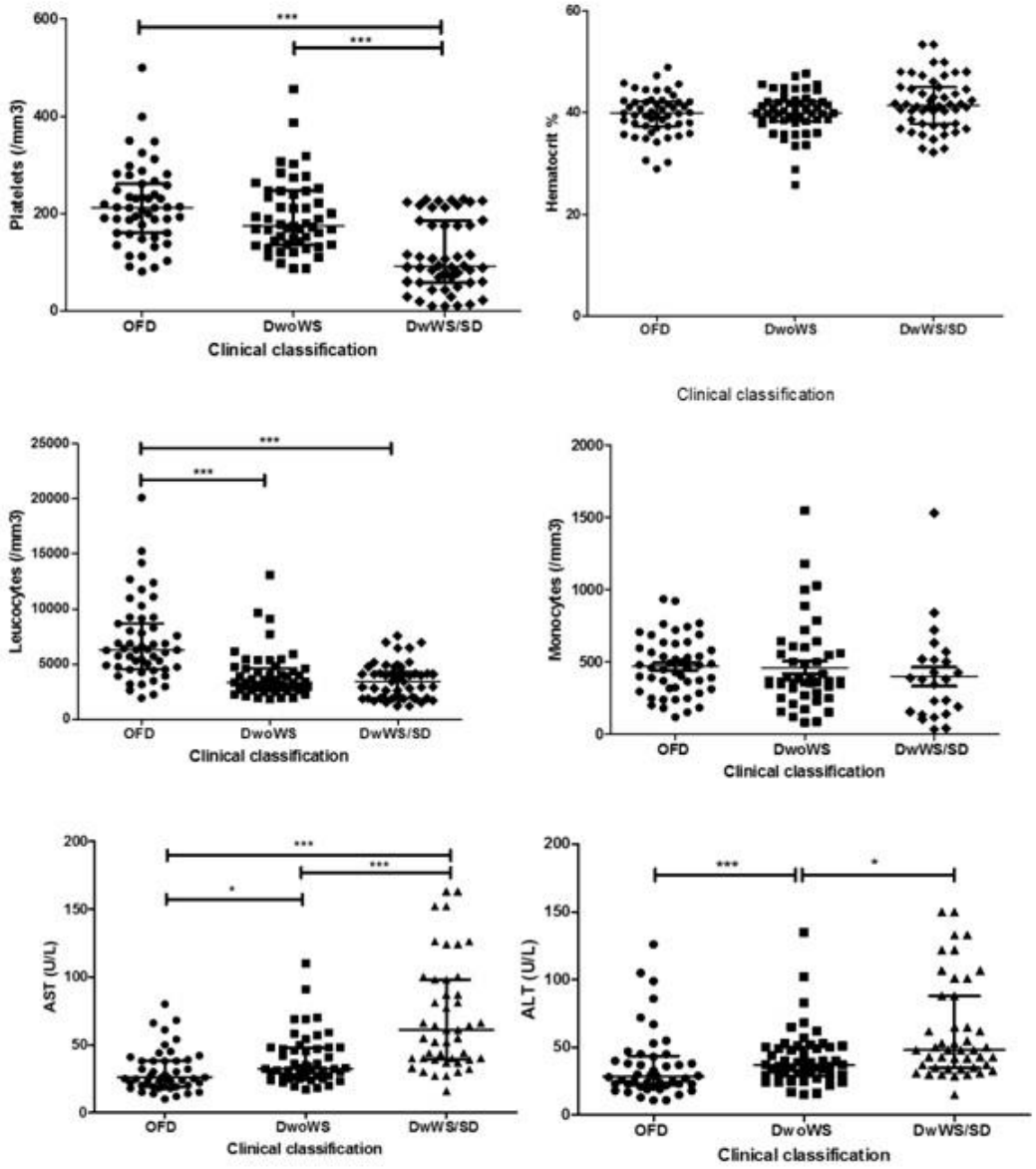


Biochemical parameters of dengue positive cases according to the clinical classification and epidemic, Campos dos Goytacazes, Rio de Janeiro, Brazil. DwoWS: Dengue without warning signs; DwWS: Dengue with warning signs; SD: Severe dengue; OFD: other febrile disease; AST: aspartate aminotransferase; ALT: alanine aminotransferase; p values * < 0,05; ** < 0,01 and *** < 0,001 were considered statistically significant. (A) AST and (B) ALT.
254x190mm (72 x 72 DPI)



Hematological and biochemical parameters of dengue positive cases according to the epidemics, Campos dos Goytacazes, Rio de Janeiro, Brazil. OFD: other febrile disease; p values * < 0,05; ** < 0,01 and *** < 0,001 were considered statistically significant. (A) Platelets, (B) Hematocrit, (C) Leucocytes, (D) Monocytes, (E) AST and (F) ALT.
254x190mm (72 x 72 DPI)



Hematological and biochemical parameters of dengue positive cases according to the clinical classification, Campos dos Goytacazes, Rio de Janeiro, Brazil. DwoWS: Dengue without warning signs (n=); DwWS: Dengue with warning signs; SD: Severe dengue; OFD: other febrile disease; (A) Platelets, (B) Hematocrit, (C) Leucocytes, (D) Monocytes, (E) AST: aspartate aminotransferase; (F) ALT: alanine aminotransferase p values * <0,05; ** <0,01 and *** <0,001 were considered statistically significant.
254x190mm (72 x 72 DPI)

4.3. *Análise dos aspectos virais através de estudos de infecção in vitro de células dendríticas:*

Artigo 6: Análise da infecção "in vitro" de células dendríticas humanas por distintas linhagens do genótipo Sudeste Asiático/Americano de DENV-2 circulantes no Brasil.

- **Objetivo específico:** Padronizar a infecção das duas linhagens de DENV-2 circulantes no Brasil em MoDCs humanas e determinar as taxas de infecção viral, expressão de moléculas fenotípicas de DC, de citotoxidade, de ativação e produção de citocinas.

Situação do manuscrito: em fase de preparação

Classificação QUALIS:

Referência: Faria NR, Torrentes-Carvalho A, Cipitelli MC, dos Santos FB, Azeredo EL, .

Resumo: Atualmente a dengue é a doença viral mais importante transmitida por artrópodes, com mais de 100 milhões de indivíduos infectados anualmente. Infecções pelos vírus dengue (DENV) apresentam um amplo espectro de manifestações clínicas que vão desde uma doença febril aguda até uma doença com risco de vida apresentando permeabilidade vascular grave. A resposta imunológica inata é a primeira linha de defesa no controle da replicação do DENV. As células dendríticas (DC) desempenham um papel crucial nas respostas imune inata e adaptativa para infecções virais e são as primeiras células infectadas por DENV. Estas células foram consideradas como sendo as principais células alvo para infecção pelos DENV. No presente estudo, foram realizadas analíses comparativas de células dendríticas (DC) infectadas com duas linhagens de DENV-2 do genótipo Sudeste Asiático. Observou-se que ambas as linhagens brasileiras de DENV2 foram capazes de infectar as DCs e induzir um padrão semelhante de citocinas e quimiocinas. Curiosamente, as DCs infectadas pela Linhagem II mostraram altos níveis de proteína NS1 quando comparadas com as DCs infectadas pela Lineage I. Embora nenhuma relação com gravidade apresentada por pacientes infectados pelos DENV-2 (Linhagens I e II) possa ser correlacionada a infecção das DCs com as distintas linhagens, este trabalho torna-se importante por ser o primeiro na literatura a utilizar distintas linhagens brasileiras

de DENV-2 na infecção de DCs. O aprofudamento dos estudos *in vitro* com diferentes sorotipos, genótipos e linhagens podem ajudar no entendimento da influência deste vírus na patogenêse da dengue.

MANUSCRITO EM PREPARAÇÃO

Analysis of the infection human dendritic cell "in vitro" by different lineages of the Southeast Asian DENV-2 genotype in Brazil.

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Abstract:

Dengue is the most important arthropod-borne viral disease with 100 million affected individuals annually. Dengue virus (DENV) infection ranges from acute febrile illness to life-threatening disease with severe vascular permeability. The innate response is the first line of defense in the control of DENV replication. Dendritic cells (DCs) play a crucial role in the innate and adaptive immune responses to viral infections and are the first infected cells by DENV. These cells were considered to be the main target cells for DENV infection. In this study, we examine infected dendritic cells (DCs) with two DENV-2 Lineages and comparative analyses were performed. We observed that both Brazilian lineages of DENV2 were able to infect DCs and induced a similar pattern of cytokine and chemokine. Interestingly, supernatants from lineage II infected DCs showed high levels of NS1 protein compared with Lineage I. Since NS1 is frequently associated with dengue severity, our results suggest that Lineage II of DENV-2 may contribute to poor clinical outcomes observed in more severe disease in part, due high amounts of NS1 protein.

Key Words: Dengue virus – dendritic cells - cytokines – Brazil

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Introduction

Dengue virus (DENV) is the most important arthropod-borne disease in tropical areas. DENV belongs to the Flaviviridae family of the flavivirus genus with four serotypes: DENV-1, DENV-2, DENV-3 and DENV-4 (Gubler et al., 2007; Halstead et al., 2007). DENV infection causes a spectrum of manifestations varying from an asymptomatic form, an acute febrile disease- Dengue Fever (DF), and in some cases can develop into a more serious condition, with plasma extravasation, thrombocytopenia and hemorrhagic manifestations which can be fatal. The innate immune response is the first line of defense against viral infections, and is of great importance in the early dengue control. During blood feeding by the vector, dendritic cells (DCs) present in the skin are the first infected cells by DENV. DCs and activated monocytes migrate from the skin to the lymph nodes facilitating the spread of the virus and infection of other cells. These cells are important in the development of adaptive immune response to the virus. The lack of animal models that reproduce immunopathology led to the development of in vitro models using primary human lineages, such as monocytes and dendritic cells (DCs). These cells are capable of producing cytokines and soluble factors when infected with DENV.

All DENV serotypes are capable of causing the broad spectrum of pathological conditions of Dengue (Kyle et al., 2008). However, the DENV serotypes are genetically diverse with at least 40% of amino acid sequence divergence. In this regard, studies have shown that DENV vary in terms of pathogenicity and virulence. Epidemiological studies as well as phylogenetic analysis allowed the characterization of different genotypes, and multiple strains. Some studies show that certain genotypes and strains of DENV-2 and DENV-3 were associated with increased virulence and epidemic potential (Leitmeyer et al., 1999; Watts et al., 1999) (Cologna et al., 2005; Leitmeyer et al. 1999; Rico-Hesse, 1990). Phylogeny analysis of DENV-2 circulating in 90's and after its re-emergence characterized two distinct lineages within the Southeast Asian genotype. In this way, strains isolated from 1990 to 2003 were classified as Southeast Asian genotype, Lineage I, and strains isolated from 2007 and 2010 as Southeast Asian genotype Lineage II. The introduction of a Lineage II in Brazil was associated with greater severity (Faria et al., 2013). In this study we evaluated the innate response induced by two distinct lineages of DENV-2. Viral replication rates and the induction of a cytokine profile after infection of dendritic cells derived from human monocytes in

vitro were analyzed and comparative analyses between DENV-2 lineages were done. Our results showed that both DENV-2 lineages were able to infect human dendritic cells but no difference was found in the viral replication ratio or in the cytokine production. Interestingly, supernatants of infected dendritic cells with DENV-2 lineage II exhibited high levels of NS1 protein. This differential NS1 profile may influence the pathogenic outcomes observed in the DENV-2 outbreaks.

Materials and Methods

Cell cultures, preparation of virus stock and virus titration

Monolayer cultures of *Aedes albopictus* C6/36 mosquito cells were grown at 28°C in L-15 Medium (Leibovitz) supplemented with 2 mM glutamine, Penicillin-Streptomycin (10.000 U/mL), 0.1% non-essential amino acids, and 10% Fetal Bovine Serum (FBS), (Gibco, Life Technologies).

The PCR-positive serum specimens were used to infect C6/36 cell cultures. Original serum of Dengue virus strain type 2 belonged to lineage I of the southeast Asian genotype (BR39145/RJ/90)was passaged seven times on C6/36 cell culture with a 8-10 days incubation period for each and the original serum belonged to lineage II (BR0337/RJ/2008) was passaged 4 times in C6/36 cell to produce stocks of high viral. Virus stock was titrated by serial dilution cultures in microtiter plates and detected by immunofluorescence as previously described (Miagostovich et al. 1993) and by Real-time PCR as previously described (Johnson et al., 2005). An uninfected flask was also maintained and the supernatant was collected to be used as a mock inoculum. A mock infection was included in each experiment. Both viral were complete sequenced as previely reported (Faria et al., 2013).

Human monocyte-derived DCs (MoDCs)

Human peripheral blood was obtained from from University Hospital Clementino Fraga Filho, Federal University of Rio de Janeiro (UFRJ), RJ. Peripheral blood mononuclear cells (PBMCs) were isolated from twelve healthy donors' buffy coats by centrifugation on a density gradient (400*g* for 30 min in Ficoll-Paque Plus, GE Heathcare Bio-Sciences AB), as described previously (Reis et al. 2007). Cells were resuspended at 10^6 cells/mL in RPMI 1640 supplemented with 200 mM glutamine, Penicillin-Streptomycin (10,000 U/mL) (Gibco, Life Technologies). Cell viability was verified in the cultures by Trypan blue exclusion and was = 95% were seeded on 2.0 x

10^6 cell/mL on 48 well plates (Nunc Thermo Fisher Scientific) and was incubated at 37°C under a humid atmosphere with 5% CO2 for 90 min. The cells where then allowed to adhere to enrich for monocytes. Non-adherent cells were gently removed by five times washing and the adherent cells were enriched monocytes were suspended in RPMI 1640 medium supplemented with 10% FBS, 500 U/mL human rGM-CSF and 500 U/mL rIL-4 (Peprotech). Cytokines were added every other day for six days. The appropriate phenotype of DC differentiation was confirmed before each experiment by flow cytometry.

Human MoDCs infection of DENV-2.

Prior to infection, total number of cells per well was determined, to estimate the multiplicity of infection (MOI) in genome equivalents per cell. For experiments comparing the DENV-2 lineage I and DENV-2 lineage II, the multiplicity of infection (MOI) was 5 (MOI of 5). After allowing adsorption of the virus to the cells for 3h at 37°C under humid atmosphere with 5% CO2, the culture supernatants were replaced with medium containing 10% FCS and further incubated for 48h. The supernatants were collected and stored at -70°C for determination of cytokine levels and cells were recovered for flow cytometry.

Detection of viral antigen and specific co-stimulatory markers by flow cytometry.

Cells were harvested by vigorous pipetting using cold wash buffer [phosphate-buffered saline (PBS) pH 7.2, 1% fetal bovine serum and 0.1% EDTA (500nM), and were and were resuspended at 1 x 10^6 cells/microtube.. The cells were then centrifuged (260 g, 7 min) and washed once more with wash buffer. Single or double cell labeling of infected and uninfected cultures was performed. For surface labelling, the cells were blocked with 5% heat inactivated human plasma in wash buffer for 30 min at 4°C and further incubated for 30 min with anti-human CD1a-PE (Iotest Immunotech, Beckman Coulter Inc.Brea, CA, USA), DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN APC) (R&D Systems), CD14-PE (DakoCytomation), HLADR-FITC (Dako Cytomation), CD40-Alexa fluor 700 (BioLegend), OX40L-PE (BD Biosciences Pharmingen) TRAIL-PE (BD Biosciences Pharmingen). Matching isotype antibodies were used as negative controls. The cells were washed and subsequently fixed with 2% paraformaldehyde. Intracellular staining was performed according to

previously described methods (Neves-Souza et al. 2005), with slight modifications. Briefly, the cells were fixed with cold 2% paraformaldehyde in PBS for 20 min and, after centrifugation, the membranes were permeabilised with 0.1% saponin in wash buffer. The cells were blocked with 5% heat-inactivated human plasma in wash buffer for 30 min at 4°C. Conjugated antibody anti-Dengue Complex (Chemicon, Millipore, Billerica, MA, USA) with anti-mouse labeled with Alexa Fluor-488 (Molecular Probes, Invitrogen) or an isotype-matched antibody diluted in wash buffer containing 0.1% saponin for 60 min at 4°C. The cells were washed once with wash buffer. The cells were acquired (10,000 events for DC-SIGN positive gate) on a FACSAria flow cytometer (BD Biosciences). Analysis was performed using FlowJo (TreeStar Inc, Ashland, OR, USA).

Cytokine detection in cell culture supernatant.

For this analysis, we used the test base set Luminex, Human Cytokine Base Kit A and specific sets for each analyte: Human TNF-α, IL-1β, IFN-γ, MCP-1/CCL2, MIP-1β/CCL4, IL-1ra e IL-6 (R&D Systems, Minneapolis, EUA).

NS1 concentration in cell culture supernatant.

Concentration of NS1 was determined utilized the recombinant NS1 protein of the DENV-2 (The Native Antigen Company - Cherwell Innovation Centre-Oxfordshire- United Kingdom), previous quantified. For determine concentration a standard curve was utilized to obtain a straight line from this equation that showed a mathematical relationship between absorbance and concentration. The recombinant NS1 protein DENV-2 and cell culture supernatant were incubated together on the same plate. We utilized the NS1 antigen capture, the Platelia™ Dengue NS1 Ag-ELISA kit (Biorad Laboratories, Marnes-La-Coquette, France) was used according to the manufacturer's protocol.

Viral RNA levels in supernatant by Real Time PCR.

The viral RNA was extracted using the QIAamp Viral RNA Mini kit (Qiagen, Germany), following the manufacturer's instructions and stored at -70°C. Viral titers were determined by Real-time PCR as previously described (Johnson et al., 2005).

Ethical Approval

The samples were collected as part of an ongoing Project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05 and CEP100/00) and by the Ethics Committee of Plataforma Brasil, FIOCRUZ (CAAE 13318113.7.0000.5248).

Statistical analyses

Data were first tested for normality with Prism version 6.0 for Windows (GraphPad Software). Flow cytometry data exhibited normal distributions, while normality was not detected for cytokine and chemokine production. To determine whether there were significant differences in viral antigen expression, data values were subjected to one-way analysis of variance followed by Tukey's Multiple comparison test. Data from cytokine and chemokine assessments were submitted to a Wilcoxon Signed rank test.

Results

Lineage I and Lineage II infection kinetics in human MoDCs.

Figure 01 A demonstrate the cellular flow cytometry profile showing size and granulosity of monocyte-derived dendritic cells (MoDCs). MoDCs that originated from healthy human PBMC donors exhibited characteristic upregulation of DC-SIGN (80%), HLA-DR (63%) and CD1a (39,3 %) respectively (Figure 1 B) and down regulation of CD14 molecules (2%) (data not shown). The MoDCs exhibit characteristics of immature DCs, which have the ability to perform endocytosis and capture antigens and are more susceptible to virus infection (Steinman & Nussenzweig 2002). MoDC cultures were incubated with lineage I and lineage II of the DENV-2 virus with equivalent dose.

Figure 1C shows percentages of viral antigen-positive cell (Ag^+) rates at 48 h post-infection (hpi). Both lineages were able to infect MoDCs. Figure 2A shows representative histograms of DENV expression in mock, DENV-2 lineage I and DENV-

2 lineage II in infected MoDCs. A significant frequency of positive cells was detected by flow cytometry in DCs infected with lineage I as compared with mock-infected ones. Similarly, DCs infected with lineage II showed a significant frequency of positive cells as compared with mock. No difference was observed in the frequency of positive cells between lineages II or I as demonstrated in Figure 2 B.

Trail and OX40L are regulated during MoDCs infection with lineage I and Lineage II of DENV-2.

OX40 L expression in DCs (Ohshima et al., 1997) may contribute to the th-2 polarisation by enhancing IL-4 and IL-13 induction and suppressing IFN-α after it binds to OX40 on T cells (Ohshima et al., 1998; Delespesse et al., 1999). TNF-related apoptosis-inducing ligand (TRAIL) is a proapoptotic molecule, which induces death of cells that express its death receptors (DR), DR4 and DR5 (Sheridan et al., 1997; Wu et al., 1997). So we investigated the expression of both molecules by infected DCs in six donors. Figure 3A demonstrate that TRAIL was expressed on infected cells in 48h pos infection but a weakly expression of OX40L was detected as compared with Mock infected cells. The frequency of TRAIL as well as OX40L among DCs were not different in infected cells with Lineage I or Lineage II as compared with Mock infected cells (Figure 3 B-C).

Viral RNA levels in supernatant and intracellular MoDCs by Real Time PCR.

We investigated the viral titers (copies/mL) of viral in MoDCs culture supernatants from infected MoDCs exposed to DENV2 lineage I or DENV2 lineage II by Real time PCR. The results revealed that DENV2 Lineage II showed an increase in RNA levels compared to DENV2 Lineage I at 48 hpi, although no statistically significant difference were observed p= 0.3222 (Figure 4 B).

NS1 concentration in MoDCs supernatant

Different dilutions were utilized with realized a standard curve, we adopted dilution 1:25 for NS1 concentration in MoDCs supernatant. The dilution 1:2 recommend of manufactures for human samples were utilized, but the cell culture infected with DENV-2 produced high levels of NS1 and the similar quantification presentation and overflow.

We analyzed the percentage of positive infected MoDCs by flow cytometry, viral titers (copies/mL) of viral in MoDCs culture supernatants from infected MoDCs exposed to DENV2 lineage I or DENV2 lineage II by Real time PCR and supernatants levels of NS1 from infected MoDCs (Figure 4A-C). No difference was observed in the percentage of positive neither in viral titers between DENV2 lineages. However, supernatants from infected MoDCs with lineage II showed increased levels of NS1 protein when compared with Lineage I (Figure 4C), and statistically significant difference were observed p= 0.0024.

Several cytokines and chemokines are present in supernatants of virus-infected MoDCs.

High level circulating of cytokines and chemokines in severe dengue present with "cytokine storm". Serum cytokines and chemokines levels can serve as a laboratory tool for predicting severe disease (John et al., 2015).

We investigated the production of cytokines and chemokines after MoDCs infected with different lineages of Southeast Asian DENV-2 genotype. Supernatant from MOCK infected MoDCs were utilized in all experiments.

We detected IL-1β, IL-1ra, IL-6, TNF-α, MCP-1/CCL2 and MIP-1β/CCL4, test base set Luminex, Human Cytokine Base Kit A and specific sets for each analyte (R&D Systems, Minneapolis, EUA).

Significant amounds of IL-1ra, IL-6, TNF-α and MIP-1β/CCL4 were detected at 48 hpi in supernatants of infected MoDCs with both lineages compared with MOCK. (Figure 5C-E-G-I). Significant amounds of MCP-1/CCL2 were only observed in supernatants of infected MoDCs with lineage I (Figure 5I).

In relation to IL1β, we found no difference in the IL-1 β levels between lineage I or lineage II as compared with MOCK (Figure 5A). Similarly, no difference was observed in the IL-1 β levels between both lineages (Figure 5B).

No significant differences in the cytokine (IL1β, IL1ra,IL-6 and TNF-α) and chemokine production (MCP-1/CCL2 and MIP-1β/CCL4) were observed in the infected MoDCs by DENV2 lineage I compared with DENV2 lineage II. (Figures 5B,5D,5F,5H,5J and 5M),

Discussion

The dendritic cells are fundamental in the induction of innate immune responses and in directing the adaptive response by antigenic presentation and cytokine production. (Lipscomb and Masten 2002). DCs are a distinct lineage of specialized phagocytic mono nuclear cells in antigenic presentation to T and B lymphocytes Once in contact with the pathogen, the DCs go through the maturation process that culminates with the efficient antigenic presentation and production of cytokines. The cytokines produced by DCs becomes part of the microenvironment and induce immune responses capable of stimulating the development of lymphocytes T effectors (Reis et al. 2006). The dengue incidence is increasing every year, since there are no available antivirals and the recently approved vaccine has not yet been released for marketing and increasing the mosquito incidence generate great concern to public health authorities.

Considering the prospects, pointed to the need for more studies about the pathogenicity and protection mechanisms related to dengue. The lack of animal models that reproduce the severe form of the disease led to the development of in vitro models using primary human lineages, such as monocytes and differentiated dendritic cells (MoDC) and further endothelial strains able to produce soluble cytokines and factors when infected with DENV. Although several "in vitro" studies have already been carried out using MoDC, most of these strains using Asian and Brazilian studies strains are scarce. After seven years without activity, this serotype re-emerge in 2007 and caused the most serious outbreak of dengue in the country so far, characterized by a greater number of hospitalizations and deaths in children in 2008 (Teixeira et al, 2009;. SVS, 2009; Macedo et al., 2013). Thus, this study aimed to compare infection of MoDCs by two strains of genotype Southeast Asia dengue 2 in vitro in attempt to identify differences and similarities between them and the probable implications in the disease severity. For this, we evaluated the frequency of DENV antigens in infected MoDCs, viral loads and NS1 levels in the culture supernadants as well as cytokine/chemokine production.

292 Previous data from our group have shown that MoDCs are targets of DENV
293 infection, with a peak at 24 h infection and significant rates of infection at 48 h post-
294 infection (Gandini et al. 2011). We observed by flow cytometry and real time PCR that
295 both DENV-2 strains are able to infect and multiply in MoDCs, corroborating previous
296 studies (Gandini et al. 2011; Silveira et al. 2011; Wu et al. 2000). No significant
297 differences were observed in viral loads between DENV-2 Lineage I and DENV-2
298 Lineage II as analized by real time PCR, despite lineage II tended to present slightly
299 increase in relation to lineage I.

300 DCs undergo a number of phenotypic changes during viral infections with
301 expression of surface molecules related to antigen presentation to T cells and also with
302 effector cell responses. TRAIL (apoptosis-inducing ligand related to tumor necrosis
303 factor) is a proapoptotic molecule that induces death of cells that express death
304 receptors (DR), DR4 and DR5 (Marsters et al. 1997). Lu et al demonstrated that
305 activated DCs express TRAIL (Lu et al. 2002). Interestingly, MoDCs express TRAIL
306 and exhibit cytotoxic activity against tumor cells (Liu et al. 2001). Little is known about
307 the role of TRAIL during DENV infection. Warke and colleagues demonstrated anti
308 viral action of this molecule in DENV infected DCs in vitro (Warke et al. 2008).
309 Interestingly, Gandini and colleagues showed higher circulating levels of TRAIL in
310 mild dengue patients (Gandini et al. 2013).

311 OX40L expressed on DC may contribute to the polarization of TH-2 immune
312 response by IL-4 increases and IL-13 induction by suppressing IFN-γ after binding to
313 OX40 T cells (Delespesse et al. 1999). OX40L expression is a fundamental requirement
314 for optimal induction of primary and memory Th-2 responses in vivo. (Jenkins et al.
315 2007, Blazquez & Berin 2008). There were no differences in the OX40L expression or
316 TRAIL expression between MoDCs infected by the lineages I and II. Although both
317 lineages were able to increase the expression of TRAIL and OX40L in infected DCs, no
318 statistical difference was detected probably due to the small number of donors tested.

319 Patients with DHF/DSS present a 'cytokine storm', with high levels of
320 circulating cytokines and chemokines. Therefore, serum cytokine and chemokine levels
321 can serve as a laboratory tool for predicting severe disease. In this context, high TNF-α
322 circulating levels are found in patients with DHF and haemorrhagic manifestations
323 (Braga et al. 2001) (Costa et al. 2013). Circulating levels of IL1β, 4, 6, 10, 18, TGF-β
324 are elevated in patients infected with different serotypes and with severe clinical

manifestations (Srikiatkhachorn and Green 2010). Besides these mediators, MCP1 / CCL2 chemokine, MIP1α / CCL3, IL8 / CXCL-8 and IP-10 / CXCL10 are important inflammatory mediators frequently associated with severity of dengue infection (Bozza et al. 2008; Lee et al. 2006).

Monocytes and macrophages produce TNF-α (Bazzoni & Beutler, 1996), which is in the form of surface molecule, and is cleaved by matrix metalloproteinases assuming the biologically active soluble form. TNF-α induce physiological changes that acts in the *clearance* of pathogens by binding to their specific receptors, particularly with the TNFR-I. While TNF-α exerts protection against pathogens by activating the leukocytes; its systemic release can activate the endothelium, resulting in endothelial permeability and plasma extravasation, leading to hypovolemic shock. During DENV infection is frequently associated with disease severity (Suharti et al. 2003). Since soluble immune mediators are involved in dengue immunopathology, we investigated the presence of cytokines and chemokines in the supernatants of infected MoDCs with both lineages of DENV-2. Cytokines and chemokines quantified in supernatants from infected MoDCs. We observed that both strains were able to induce the production of cytokines (IL-1β, IL-1ra, IL-6, TNF-α) and chemokines (MCP-1 and MIP-1β). We did not observe significant differences between strains amongst themselves, but significant differences were observed in the analysis comparing MOCK and the lineages separately. In fact, several studies have reported the production of TNF-α, IL-6 by DCs exposed to DENV (Ho et al. 2001; Libraty et al. 2001; Palmer et al. 2005). Chemokines and inflammatory mediators with important roles in the pathogenesis and immunity (Lusso 2000; Rossi and Zlotnik 2000), have also been secreted in vitro by several human cells after infection with DENV (Chen and Wang 2002; Lin et al. 2005).

The soluble receptor antagonist IL-1 (IL-1ra) is anti-inflammatory and has a role immunomodulatory dendritic cells. It acts by competing cellular receptor of IL-1 with IL-1 cytokine that is activating DCs (Blanco et al., 2008). In this study, we found no differences between IL-1ra production induced by two lineages. We note only that some donors exhibited remarkable production compared to MOCK. The cytokine IL-6 is a pleiotropic functions molecule and acts in the regulation of immunoreactivity in the acute phase response in inflammation, and hematopoiesis in oncogenesis. Scientific studies correlate cytokine IL-6 with the severity of DF/DHF (Juffrei et al., 2001; Chen et al., 2006). Several authors report that infected MoDCs are able to produce IL-6 in response to DENV-2 (Chaturvedi et al., 1999;

359 Sanchez et al., 2006). In our study, no significant differences between IL-6 production
360 induced by two lineages, only when comparing the strains with MOCK.
361 Chemokines are chemotactic cytokines that direct the flow cell or induced
362 homeostatic way. DCs change the profile of these mediators and express chemokine
363 receptors during the maturation process. In general, the chemokine MCP-1/CCL2 has
364 been correlated with severity (Lee et al., 2006) and thrombocytopenia and hypotension
365 (Bozza et al., 2008) in dengue patients. The MIP-1ß/CCL4 was associated with a good
366 prognosis in patients infected with DENV3 (Bozza et al., 2008). We observed that the
367 production of MCP-1/CCL2, MIP-1β,/CCL4 BY two strains of highly variable manner
368 between donors. The MIP-1β /CCL4 was induced at low levels in some donors, and
369 there were no differences between the production capacity of both strains, only when
370 compared to MOCK. Rabablert et al (2007) reported that an attenuated strain of DENV-
371 2 was capable of inducing mRNA expression of MIP-1β/CCL4 similarly to the wild
372 type strain in human PBMC (Rabablert et al., 2007). Nightingale et al. (2008) also
373 demonstrated the production of MIP-1ß/CCL4 by MoDCs infected with DENV-2.
374 The involvement of the NS1 protein of dengue gravity is suggested by several
375 authors (Paranavitane et al, 2014;. Beatty et al, 2015). In its secretory form NS1 carries
376 lipids it is thought to have many implications in disease pathogenesis, as lipoproteins
377 are important in coagulation pathways and are associated with vascular inflammation
378 (Gutsche et al., 2011). Beatty et al (2015), shown recently that NS1 of all four DENV
379 serotypes induced vascular leak in dengue mouse models by inducing endothelial
380 barrier dysfunction (Beatty et al., 2015). Modhiran et al (2015), also demonstrated that
381 dengue NS1 stimulated cytokine production from innate immune cells by acting through
382 Toll-like receptor (TLR)-4 (Modhiram et al., 2015).
383 In our study, analysis of the data of the NS1 protein quantitation revealed
384 increased production of MoDCs cultures infected with Lineage II than with Lineage I
385 (p=0.0024). In fact, the re-emergence of DENV-2, later described as a new strain
386 (strain II) different from the samples before circulating in Brazil was associated with
387 severity in patients with dengue (Macedo et al, 2013;. Nunes et al, 2016).
388 The secretory NS1 formed immune complexes have been shown to activate
389 complement, which is also thought to contribute to disease pathogenesis (Avirutnan et
390 al., 2006). Although the main effects of NS1 in the pathogenesis of dengue infections
391 are believed to occur due to immune complexes and anti-NS1 antibodies, recent studies

have shown that NS1 is pathogenic by itself (Beatty et al., 2015; Modhiran et al., 2015).

Finally, we observed in this study that infected MoDCs with both DENV2 lineages of DENV-2 Southeast Asia were permissive to DENV infection in vitro and were capable of producing cytokines and chemokines involved in the pathogenesis of dengue. In fact, the sequencing of both lineages demonstrated the presence of an asparagine (N) in position E390, previously characterized as a probable viral marker of DHF development (Faria et al. 2013; Leitmeyer et al. 1999). Thus, these results suggest that both DENV-2 lineages have potential in the development of more severe forms of dengue. However, factors related to the host, particularly the features derived from each donor used in this study also contribute to the variability in responses as observed by other groups (Deauvieau et al., 2007; Nightingale et al., 2008). Interestingly, infected MoDCs with the lineage II showed higher levels of NS1 suggesting an association with dengue severity. Our knowledge of virological and immunological mechanisms and relation with severe outcomes is still limited and many important issues need to be clarified. However, our results open new perspectives on the role of NS1 and of different Brazilian strains in the dengue pathogenesis important in preventing severe cases in areas of risk.

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FIGURE 1

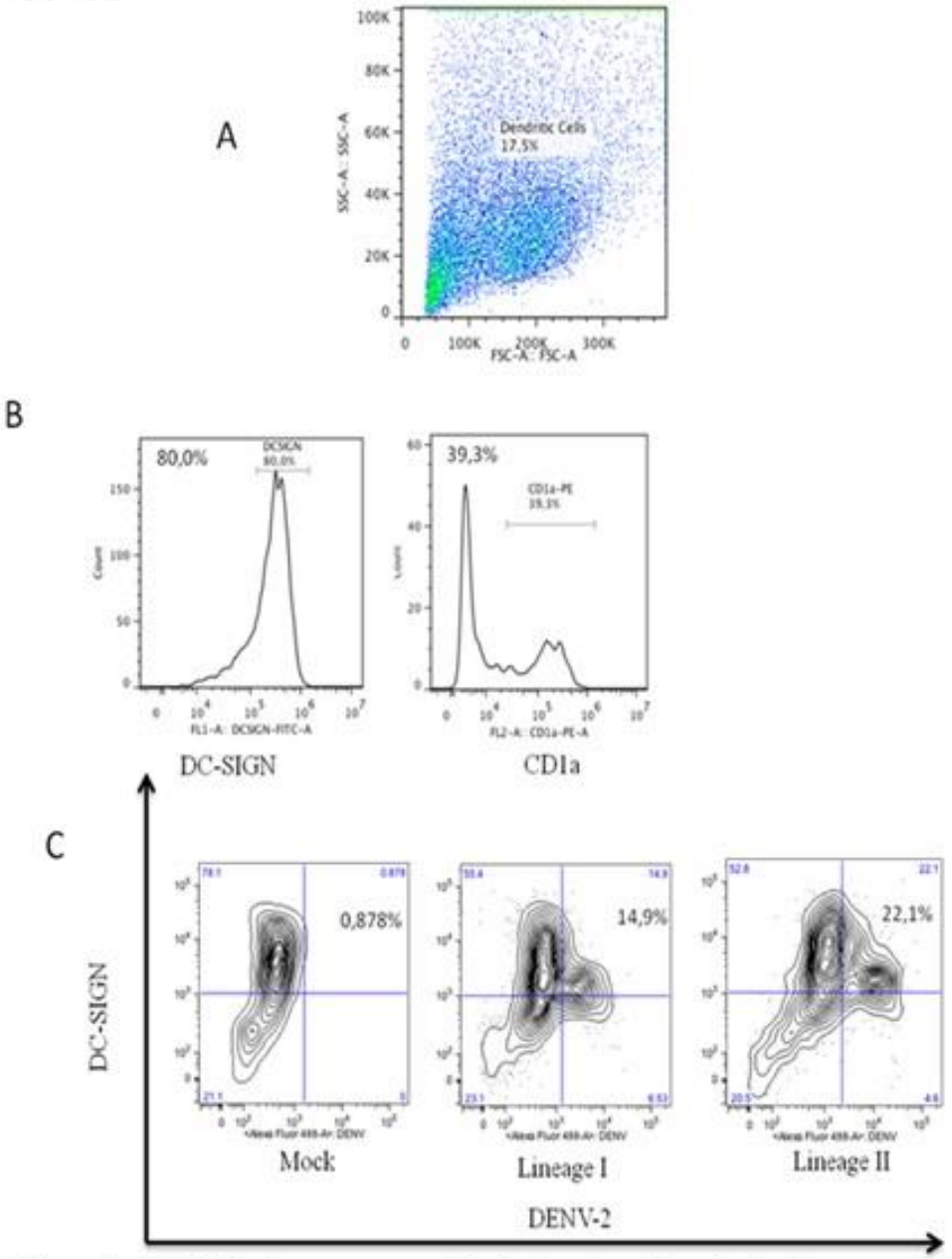
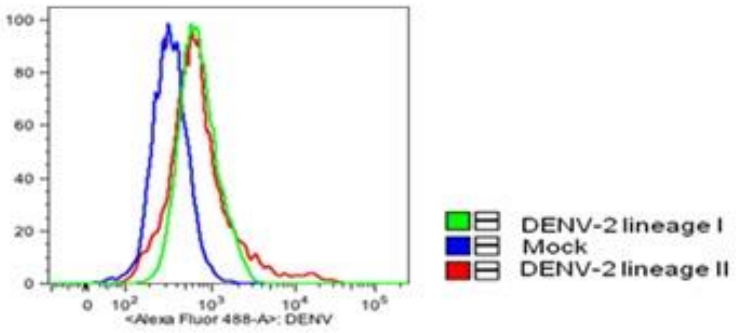


Figure 1: **(A)** Cellular flow cytometry profile showing size and granulosity of monocyte-derived dendritic cells (MoDCs). **(B)** Expression profile of different cell type markers by flow cytometry. One representative analysis is shown. C) Representative contour plots showing the percentages of double CD209+DENV-2 positive cells in mock, DENV-2 lineage I and DENV-2 lineage II, respectively.

FIGURE 2

A



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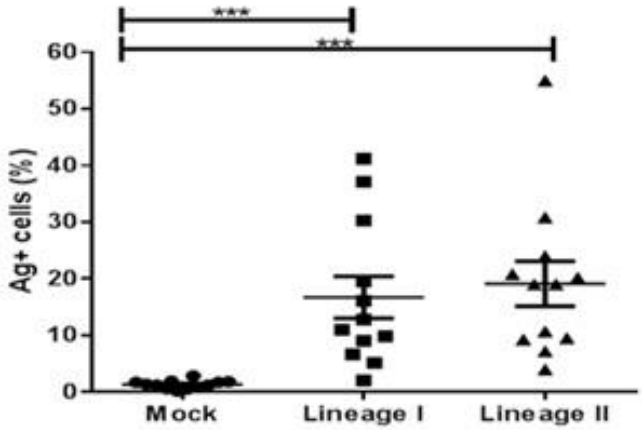

FIGURE 2: **(A)** Representative histograms of DENV expression in mock, DENV-2 lineage I and DENV-2 lineage II in infected Monocyte-derived dendritic cells (MoDCs).
(B) Frequencies of DENV positive cells among mock, lineage I and lineage II - are shown. MoDCs with DENV-2 lineage I and DENV-2 lineage II. MoDCs were infected with with DENV-2 lineage I or DENV-2 lineage II at equivalent multiplicity of infection (MOIs) or mock infected. Cells were labelled with anti – DENV using duplicates for each peripheral blood mononuclear cells-PBMCs donor. A flow cytometry patterns for viral antigens on gated MoDCs 48 h after in vitro infection. Histograms (A) demonstrate cell distribution by Alexa Fluor -488 fluorescence intensity. Cells were cultured with mock (BLUE) or with DENV-2 lineage I (GREEN) or lineage II (RED) in equivalent doses (MOI of 5). Intracellular viral antigens detection at 48 h after infection representing 12 cell donors (horizontal line: median and interquartile ranges; x axis viral inoculum; y axis: percentage of viral Ag + cells.
Statistical significance was assessed using the Tukey's multiple comparison test.
Differences between mock and DENV-2 lineage I or lineage II are given above the figures. ***p<0,001.

FIGURE 3

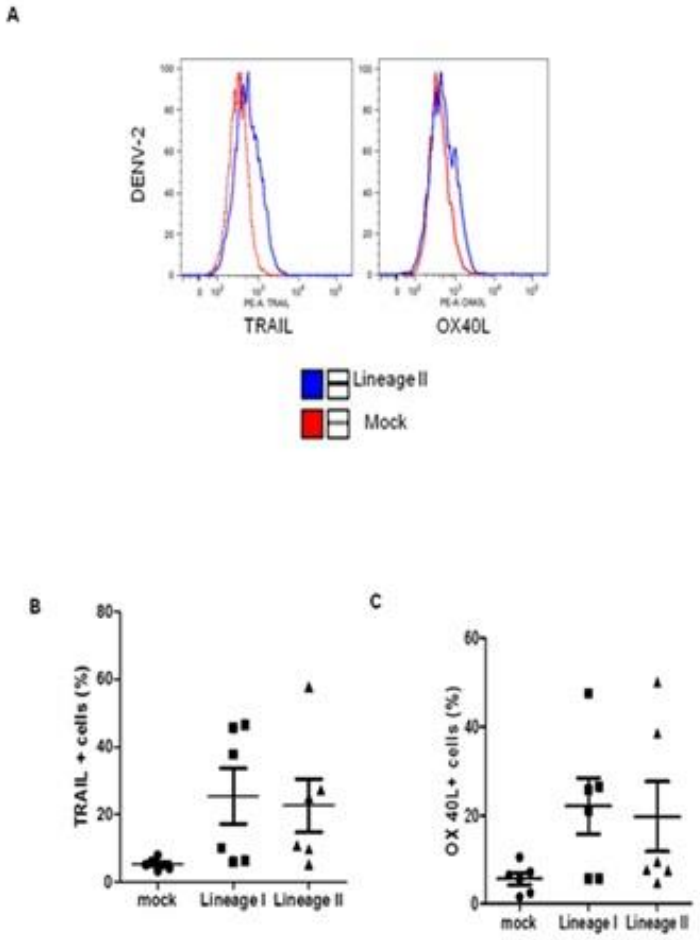

FIGURE 3: TRAIL and OX40L markers expression during DENV-2 infected Monocyte-derived dendritic cells (MoDCs). MoDCs were infected with both DENV-2 Lineages or mock infected. Cells were labelled with anti-TRAIL PE and with anti –OX40L PE monoclonal antibodies.

(A) Representative histograms of TRAIL and OX40 L expression in mock, DENV-2 lineage II in infected MoDCs.

(B) Frequencies of TRAIL positive cells among mock, lineage I and lineage II are shown.

(C) Frequencies of OX40L positive cells among mock, lineage I and lineage II are shown.

A flow cytometry patterns for TRAIL and OX40L on gated MoDCs 48 h after in vitro infection. Histograms (A) demonstrate cell distribution by PE fluorescence intensity. Cells were cultured with DENV-2 Lineage II (BLUE) or with Mock (RED) (MOI of 5). Extracellular detection at 48 h after infection representing 6 cell donors (horizontal line : median and interquartile ranges;

Statistical significance was assessed using the Tukey's multiple comparison test.

FIGURE 4

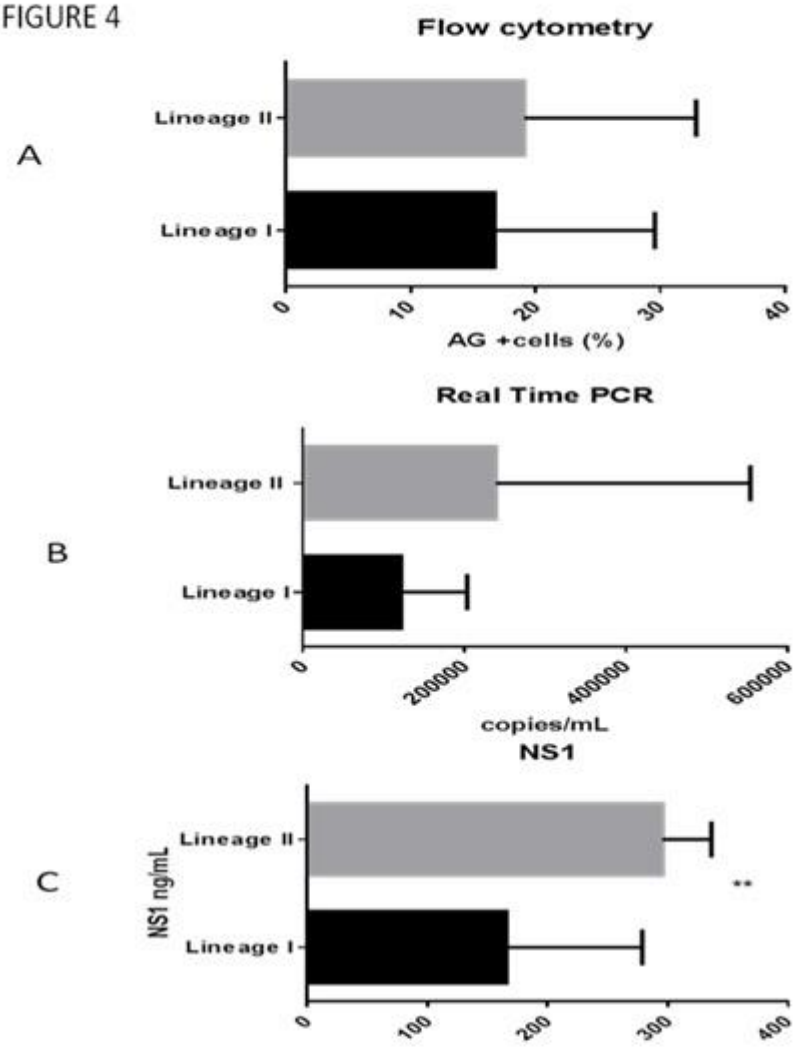

FIGURE 4 : **(A)** Percentages of infected MoDCs exposed to DENV2 lineage I or DENV2 lineage II by flow cytometry. **(B)** Quantitative PCR (qPCR): Viral titers (copies/mL) of viral in MoDCs culture supernatants from infected MoDCs exposed to DENV2 lineage I or DENV2 lineage II. **(C)** NS1 culture supernatants levels in MoDCs from infected MoDCs exposed to DENV2 lineage I or DENV2 lineage II.

Statistical significance was assessed using the Tukey's multiple comparison test.
Differences between mock and DENV-2 lineage I or lineage II are given above the figures. **p<0,001.

FIGURE 5

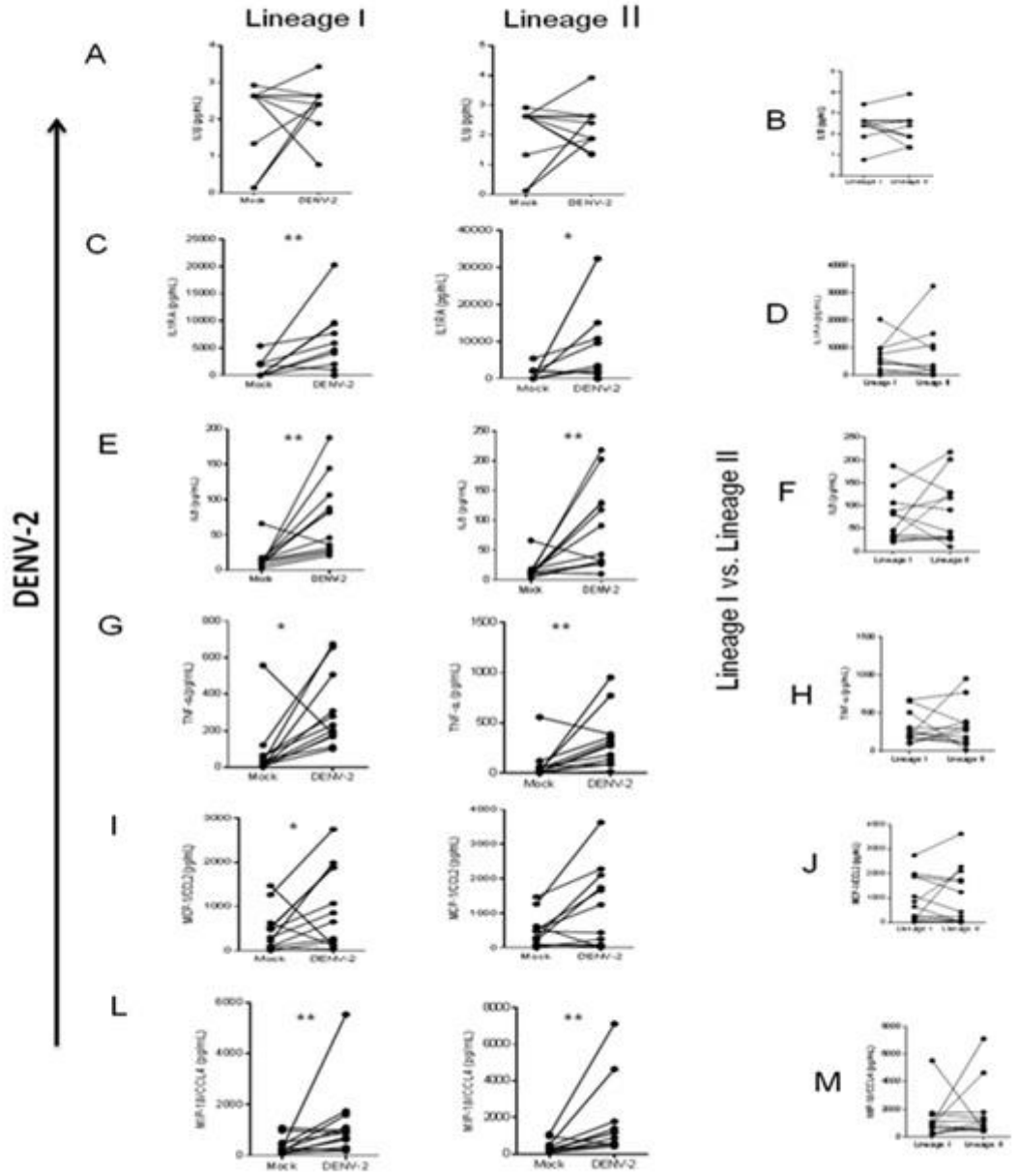
Lineage I
Lineage II
A
B
C
D
E
F
G
H
I
J
L
M
DENV-2
Lineage I vs. Lineage II
Mock
DENV-2

FIGURE 05

Multiple cytokine and chemokine induction by DENV-2 lineages after MoDCs infection. MoDCs were infected with DENV-2 lineage I or DENV-2 lineage II or mock infected. Supernadants were colleted 48 h after infection and analyzed by immunofluosrescent multiplex-bead assay.

A, C, E, G, I and L: Lines represent paired production for each donor of IL1B, IL1RA, IL6,TNF-A, MCP-1/CCL2 and MIP1B/CCL4 induced after mock and virus infection. Twelve PBMCs donors were used.

B, D, F, H, J and M: Lines represent paired production of individual tested cytokine cmpared between lineages, I and II respectively.

Statistical significance was assessed using the Wilcoxon signed rank test.

Differences between mock and DENV-2 lineage I or lineage II are given above the figures. * $p < 0.05$; **$p<0,001$.

5. Discussão

A pandemia mundial de dengue parece ter começado nas regiões da Ásia e do Pacífico, onde a primeira epidemia de dengue foi relatada, em 1779-80 (Gubler & Clark 1995). Alterações biológicas que ocorrem desde esse período provavelmente favoreceram a expansão geográfica do vetor e o aumento de densidade. O elevado número de indivíduos suscetíveis e o aumento da frequência de viagens provavelmente criaram condições que facilitaram a dispersão dos vírus (Gubler 1997; 2002). Uma série de fatores contribuíram para a emergência e re-emergência da dengue, como, por exemplo, o crescimento desordenado da população e a urbanização não planejada associada à pobreza (Gubler 2002; Guzman & Kouri 2003). O número de casos de dengue tem aumentado em todo o mundo e se espalhado por novas áreas, como Europa e América do Norte (WHO, 2009).

O Brasil é considerado um país tropical em sua totalidade, devido ao clima quente e úmido, fornecendo um ambiente receptivo e altamente favorável para a dispersão do mosquito vetor. A atividade dos DENV ocorre durante todo o ano, mas a maioria dos surtos e os níveis mais altos de infestação do vetor mostram um padrão sazonal marcado, ocorrendo durante a estação chuvosa brasileira, de dezembro a maio, que também são os meses mais quentes do ano (Siqueira *et al*., 2005).

Embora não exista nenhuma associação definitiva que comprove que os diferentes sorotipos e genótipos induzam diferentes formas clínicas apresentadas pela dengue, existem estudos que sugerem que os DENV-2 e DENV-3 causam a dengue grave mais frequentemente do que os outros sorotipos e que o DENV-4 está

associada à dengue branda (Balmaseda *et al*., 2006; Sierra *et al*., 2007). O DENV-2 foi o sorotipo mais prevalente em várias epidemias nas Américas (Brathwaite Dick *et al*., 2012) e foi classificado epidemiologicamente como o sorotipo mais relevante em todo o mundo, devido à sua capacidade de causar epidemias mais explosivas, seguido pelos DENV-3, DENV-1 e DENV-4 (Costa *et al*., 2012). O conhecimento sobre a patogênese das infecções pelos DENV é limitado, mas ambos os fatores virais e os da resposta imunológica do hospedeiro parecem estar associados à dengue grave (Romano *et al*., 2010). No entanto, os sorotipos de DENV envolvidos e o perfil genético do hospedeiro podem explicar algumas diferenças nas manifestações clínicas observadas, uma vez que polimorfismos genéticos parecem oferecer proteção ou predisposição a formas mais graves da dengue (Tan *et al*., 2009).

Diversos estudos realizados em regiões epidêmicas conduziram à geração de hipóteses sobre o desenvolvimento da dengue grave (Gubler & Clark, 1995). Eles incluem a facilitação dependente de anticorpos (Hastead, 1988), a tempestade de citocinas (Noisakran & Perng 2008), os fatores genéticos (Sierra *et al*., 2007), as características dos vírus isolados (Diamond et al., 2000; Ferreira et al., 2010), a carga viral na fase aguda (Gubler *et al*., 1981; Vaughn *et al*., 2000) e o estado nutricional do hospedeiro (Thisyakorn & Nimmannitya, 1993). Os DENV podem interagir com as células dendríticas, monócitos/macrófagos, hepatócitos e células endoteliais conduzindo à liberação exarcebada de citocinas e quimiocinas durante a dengue grave (Diamond *et al*., 2000; Acosta *et al*., 2009; Lin *et al*., 2005). No entanto, como a produção destas citocinas é induzida,o seu papel na patogênese da dengue ainda não está claro.

5.1. Vinte anos de atividade do DENV-2 no Brasil: caracterização molecular e filogenia de cepas isoladas entre 1990 e 2010. (Artigo 1)

O primeiro isolamento do DENV-2 nas Américas ocorreu em 1953 em Trinidade (Anderson, 1956) e o primeiro caso de FHD ocorreu em 1981 em Cuba com a introdução do DENV-2 do genótipo Sudeste Asiático (Guzman *et al*., 1995). No Brasil, os primeiros casos de FHD/SCD ocorreram após a introdução do DENV-2 no Rio de Janeiro (Nogueira *et al*., 1990). A análise filogenética das amostras de DENV-2 circulantes naquela época confirmou a presença do DENV-2 genótipo Sudeste Asiático (Miagostovich *et al*., 1997; dos Santos *et al*., 2002).

Em abril de 2007, o DENV-2 remergiu no estado do Rio de Janeiro, causando, em 2008, a epidemia mais grave já documentada no Brasil (Teixeira *et al*., 2009). A análise filogenética revelou a existência de duas linhagens distintas do genótipo Sudeste Asiático, com a primeira introduzida nos anos 90 e a outra linhagem introduzida a partir da reemergência dos DENV-2 (Oliveira *et al*., 2010).

Os DENV-2 são os que apresentam a maior diversidade genética dentro dos quatro sorotipos (Rico-Hesse *et al*., 1997) e alguns dos seus genótipos têm uma ampla distribuição geográfica (Zhang *et al*., 2005). A análise genômica detalhada de 11 cepas representantes dos genótipos Sudeste Asiático e Americano do DENV-2 sugere que os determinantes de gravidade em casos de DENV-2 residem na substituição de aminoácidos na proteína E_{390} (D→N), observada no genótipo Asiático (Leitmeyer *et al*, 1999). Cologna *et al*, 2003 mostraram que as cepas de DENV-2 do genótipo Sudeste Asiático são mais adaptáveis às células humanas e às células de mosquito do que as cepas do genótipo Americano (Cologna *et al*., 2003).

Diversos estudos analisando as variações genéticas de cepas de DENV, isoladas de pacientes com dengue grave (FHD/SCD) e cepas de pacientes com dengue branda, tentaram correlacionar alterações de aminoácidos com o desenvolvimento da forma grave

da dengue, porém nenhuma destas alterações encontradas nestas cepas foi consistente para correlacionar essas alterações com a gravidade da doença (Pandey *et al*., 2000; Raekiansyah *et al.*, 2005).

Com o aumento do número de casos graves e a reemergência dos DENV-2, nós realizamos o sequenciamento e a análise filogenética a fim de compreender a evolução e caracterizar os DENV-2 brasileiros de cepas isoladas de casos de dengue clássico, FHD/SCD e dos casos fatais ocorridos desde a introdução deste sorotipo no país, em 1990, até o ano de 2010. Foram analisadas as sequências dos genes C/prM/M/E de vinte e cinco amostras e o sequenciamento completo da região codificante de nove amostras.

A análise dos genes C/prM/M/E e do genoma completo da região codificante das 34 sequências revelou a existência de dois grupos filogeneticamente distintos, um formado pelas cepas de 1990 a 2003 e outro formado pelas cepas de 2007 a 2010, corroborando estudos anteriores (Oliveira et al., 2010). As amostras isoladas de 1990-2003, pertencentes ao genótipo Sudeste Asiático, Linhagem I, apresentam similaridade com a cepa brasileira BR64022/98 e com a cepa Jamaica/83. No entanto as amostras isoladas de 2007-2010 apresentaram maior similaridade com a cepa DR59/01, da República Dominicana, representando o genótipo do Sudeste Asiático, Lineage II, corroborando com a análise realizada por Oliveira et al 2010. Um estudo realizado com cepas de DENV-2 circulantes no Paraguai também demostrou que a existencia de duas linhagens distintas dentro do genótipo do Sudeste Asiático e sugere a introdução de uma nova linhagem possivelmente associada uma mudança de sorotipo DENV-3 para DENV- 2, como observado no Brasil em 2007 e 2008 (Aquino *et al*., 2008; SVS., 2009).

A ausência da circulação dos DENV-2 nos anos anteriores a sua reemergência e a alta similaridade observada entre os vírus da Linhagem II com as cepas da República Dominicana, em 2001, permitem sugerir que a introdução desta nova linhagem foi responsável pela epidemia de 2008 no Brasil. Um estudo realizado com cepas isoladas de

uma epidemia de 2010 em São Paulo demonstrou que essas cepas formavam um grupo monofilético com as cepas isoladas no Rio de Janeiro 2007/2008 e que estas amostras estavam intimamente relacionadas com as cepas isoladas em Cuba e na República Dominicana, com uma pequena distância genética, sugerindo que esta nova linhagem introduzida no Brasil pode ter sido importada do Caribe (Romano *et al*., 2010).

Diversas variantes genéticas dos DENV têm sido implicadas com a dengue grave no passado (Rosen 1977; Gubler *et al*., 1978), mas foi através do avanço de estudos evolutivos com base na análise filogenética combinada com dados epidemiológicos dos genótipos dentro de cada sorotipo que foi possível associar a menor ou maior gravidade apresentada em indivíduos infectados pelos DENV (Rico-Hesse *et al*., 1997; 2007; Messer *et al*., 2002;2003).

Substituições que foram identificadas nas posições E_{129} (V→I) e E_{131} (L→Q) estão relacionadas com a divisão do genótipo Sudeste Asiático em dois clados distintos, resultados concordantes com os obtidosem estudos prévios que associam estas posições na proteína E como marcadores críticos na classificação genética dos DENV (Aquino *et al*., 2008; Bennett *et al*., 2006).

Todas as 34 cepas analisadas neste estudo apresentaram uma asparigina (N) na posição $E_{390,}$ previamente caracterizada como um provável determinante genético desencadeador de FHD detectado em cepas de origem asiática (Leitmeyer *et al*, 1999). Substituições em E_{390} (N→D) resultam em uma redução na replicação viral em macrófagos e células dendríticas. Já a substituição inversa (D→N) na mesma posição resulta em uma maior replicação, maturação e ativação dos macrófagos, exacerbação da resposta imune com uma maior produção de citocinas, aumento da permeabilidade vascular e, consequentemente, com maiores chances de desenvolvimento de FHD (Pryor *et al*, 2001).

As mutações no domínio III da proteína E dos flavivírus podem induzir a virulência, ou atenuação ou o escape do vírus, frente ao sistema imune (Sánchez I & Ruiz, 1996; Lin

& Wu, 2003), e no presente estudo, foram observadas alterações ao longo deste domínio (AA 297 a 394). Um caso FHD (59382/1997), que culminou na morte do paciente, mostrou diferenças de aminoácidos apenas no gene E, mas essas diferenças foram compartilhadas com outras cepas de casos FD, quando eles foram comparados com a cepa referência BR64022/98.

Neste estudo, uma substituição na prM_{39} foi observada em três cepas: 0690/2008 - isolada de um caso fatal, 55769/1996 - isolada de um caso DF e 0199/2010. Catteau et al 2003 demonstraram que a produção intracelular do ectodomínio M de todos os quatro sorotipos de DENV podem induzir a apoptose em células hospedeiras. A região carboxi-terminal da proteína prM com nove aminoácidos (AA 32-40) de alguns flavivírus, foi designada como apopto M e parece desempenhar um papel importante na indução da apoptose e efeitos citopáticos (Catteau *et al*., 2003; Marianneau *et al*., 1998).

Várias mudanças foram observadas nas proteínas não estruturais. Estudos realizados por Yábar (2000) mostraram que mutações na NS1 estão relacionadas ao desenvolvimento de FHD/SCD, quando comparadas com os casos de FD (Yábar, 2000).

O reconhecimento da diversidade genética dos DENV e suas origens fornecem subsídios para que se possam monitorar os genótipos mais virulentos na vigência de epidemias, auxiliando na adoção das medidas de controle. Esses estudos também podem ajudar no esclarecimento da patogênese do DENV e nos mecanismos associados à resposta imune do hospedeiro, bem como no entendimento da influência do vetor na seleção das variantes genéticas.

5.2. Dengue grave associada à idade e à nova linhagem do DENV-2 no Rio de Janeiro Brasil. (Artigo 2)

O DENV-2 emergiu em 2007 causando uma das mais graves epidemias em 2008 (Macedo *et al*., 2013). De acordo com a análise das cepas de DENV-2 utilizadas neste estudo (1990-2011), as amostras isoladas em 2007 foram geneticamente distintas e, apesar de pertencerem ao mesmo genótipo Sudeste Asiático, foram agrupadas em um clado distinto denominado de Linhagem II (Faria *et al*., 2013).

A falta de atividade da Linhagem I e a reemêrgencia da Linhagem II foram associadas a altos níveis de RNA circulante e maiores chances de desenvolver a forma grave da dengue durante a epidemia de 2008, corroborando com a hipótese descrita em estudos anteriores, na qual a replicação mais eficiente de determinados vírus poderia competir e deslocar aqueles que apresentam um impacto epidemiológico inferior (Vu Tu *et al*., 2010; OhAinle *et al*, 2011; Rico-Hesse, 2003; Rico-Hesse *et al*., 1997).

A associação dos níveis de viremia com a resposta imunológica do hospedeiro e sua relação com a progressão da doença permanecem contraditórias. Alvarez et al (2006) demonstraram que a infecção secundária pelo DENV-2 é comumente caracterizada por uma maior gravidade da doença e com elevado nível de viremia em pacientes previamente infectados pelos DENV-1 ou DENV-3 (Alvarez *et al*., 2006).

Uma análise restrospectiva sobre a dengue no Brasil possibilita mostrar que: (i) os casos graves e fatais começaram a ser descritosapós a introdução dos DENV-2 em 1990, (ii) com a introdução do DENV-3 ocorreu um aumento no número de óbitos e casos graves na epidemia de 2002 e (iii) durante a epidemia de 2008 com a reemêrgencia do DENV-2 a taxa de letalidade foi duas vezes maior, devido à introdução desta nova linhagem.

Em relação ao estado do Rio de Janeiro, é importante destacar, cronologicamente, as introduções e reemêrgencias dos DENVs no território fluminense: DENV-1 (1986); DENV-2 (1990); DENV-3 (2000/01); DENV-2 (2007); DENV-1 (2010) e DENV-4 (2011).

Nossas análises demonstraram que a infecção secundária independente da linhagem, não foi responsável pela gravidade da dengue. Não houve associação significativa entre os níveis mais elevados de viremia e a apresentação da doença devido ao estado imunológico dos pacientes previamente infectados com DENV-1 e / ou DENV-3, sorotipos que circularam amplamente no Rio de Janeiro, em 1986-1997 e 2001-2006, respectivamente,

antes da introdução e reemergência do DENV-2 em 1990 e 2008 (Macedo *et al*, 2013; Nogueira *et al*., 2007; SVS, 2012; Teixeira *et al*., 2009).

Quanto à faixa etária, contrastando com outras regiões endêmicas no mundo, no Brasil, desde a introdução dos DENV, a população mais acometida era a de maiores de 15 anos. No entanto, a partir da reemergência do DENV-2, ocorreu uma mudança no perfil da doença, com um maior acometimento da infecção por DENV na faixa etária menor do que 15 anos.

É pertinente registrar que durante a epidemia de 2008, o estado do Rio de Janeiro foi responsável por 37% dos casos notificados no Brasil, com 50% destes casos acomentendo menores de 15 anos, faixa etária na qual 86% dos casos evoluíram para o óbito (Teixeira *et al*., 2009). Nesta epidemiafoi observado um aumento de sete vezes no número de internações em menores de um ano de idade. Posteriormente, em 2010, , ocorreu uma nova mudança no perfil da doença com a epidemia de DENV-1 quando a faixa etária mais acometida foi a de maiores de 60 anos.

Em nossa casuística, além da associação significativa com a gravidade da doença, os níveis de viremia do RNA foram mais elevados no grupo de ≤ 15 anos idade, durante a circulação da linhagem II, sugerindo que a idade era um fator importante para o pico da viremia. A associação de uma linhagem emergente de DENV-2 com uma maior viremia também foi descrita em pacientes pediátricos internados no Vietnan (Vu TT *et al*., 2010).

No entanto é preciso considerar que outros fatores como as condições fisiológicas, neurológicas e comportamentais podem contribuir para uma taxa substancialmente mais elevada de mortalidade Neste contexto, o sexo é considerado um dos fatores de risco que tem sido associado com mortes por dengue e dengue grave. No Brasil, a distribuição de casos de dengue por sexo é proporcional, mas com um ligeiro aumento para as mulheres. Em um estudo realizado no período de 2002-2010, as mulheres representavam 55% dos casos de dengue no país (Siqueira *et al*., 2010). Em nosso estudo, a população foi homogênea para o sexo (52% mulheres e 50% homens), mas 37% das mulheres e 63% dos homens tiveram dengue grave. Embora a taxa de dengue grave em homens tenha sido maior do que nas mulheres, neste estudo, a gravidade da doença não foi significativamente associada ao sexo, corroborando com estudos anteriores (Wang *et al*., 2003).

Ainda considerando outros fatores, não foram observadas associações entre a gravidade da doença, idade ou sexo durante o período de linhagem I e a ocorrência de níveis de viremia mais elevados durante o período de linhagem II reforça a hipótese de que as cepas

com maior virulência são um fator importante para a gravidade da doença (Romano *et al*., 2010; Simmons *et al*., 2007), como observado durante a epidemia de 2008.

No Brasil, embora a circulação e a evolução das linhagens do DENV-2 tenham sido previamente relatadas (Drumond *et al*, 2013; Halstead *et al*, 1970; Rico-Hesse *et al*, 1997), este foi o primeiro estudo a correlacionar o impacto epidemiológico dessas linhagens na população brasileira. De acordo com nossos dados, a capacidade da linhagem II de se replicar em títulos mais elevados e a alta susceptibilidade aos DENV-2 em indivíduos ≤ 15 anos podem ter sido o fator-chave para a dinâmica da doença na epidemia de 2008.

O hiperendemicidade da dengue com cocirculação de múltiplos sorotipos DENV, a emergência ou reemergência de novos sorotipos, genótipos ou linhagens mais virulentas, a reposição constante de indivíduos susceptíveis devido à coorte de nascimento e a alta densidade de *Aedes aegypti* são os fatores que contribuem para o cenário da dengue grave observada nos últimos 10 anos no Brasil.

5.3. Epidemia de dengue em dois períodos específicos revela distintos aspectos epidemiológicos, clínicos e laboratoriais em um mesmo cenário: analíse das epidemias de dengue de 2010 e 2013 no Mato Grosso do Sul, Brasil. (Artigo 3)

O Brasil tem sofrido sucessivas epidemias desde a introdução dos DENV na década de 1980. Em 2013 o país sofreu uma das maiores epidemias já ocorridas no país com aproximadamente 2 milhões de casos notificados. Durante o presente estudo, foram avaliados dois períodos epidêmicos correspondentes aos anos de 2010 e 2013, no estado de MS, Brasil, onde o número de casos notificados e da gravidade da doença vem aumentando continuadamente (SVS, 2015).

Os métodos sorológicos ainda são a ferramenta mais útil para o diagnóstico da dengue durante as epidemias. O MAC-ELISA foi a técnica que mostrou maior positividade na epidemia de 2010 e neste estudo foi possível detectar anti-DENV IgM no primeiro dia de febre. O NS1 ELISA é o método sorológico mais recentemente desenvolvido que permite o diagnóstico precoce das infecções de DENV, durante a fase inicial da doença, mesmo em laboratórios com reduzidos recursos humanos e equipamentos (Andries *et al*., 2012).

Todas as amostras negativas por ELISA NS1 de ambas as epidemias foram testadas de acordo com Lima *et al* (2014), usando ácido para a dissociação de imunocomplexos. Amostras negativas para NS1 da epidemia de 2010, causada pelo DENV-1 e DENV-2, mantiveram-se negativas. No entanto, foi observado um aumento de 22% na detecção do

NS1 em amostras negativas provenientes da epidemia de 2013, causada pelo DENV-4, corroborando com os resultados descritos por Lima et al que em seu artigo publicado em 2014 demonstram que a dissociação por calor dos soros antes da realização do ensaio para a detecção da proteína NS1, aumenta a sensibilidade do teste em áreas endêmicas onde as infecções secundárias são frequentemente relatadas.

É pertinente registrar que durante o período estudado foi observada a cocirculação de dois ou mais sorotipos de DENV. Na epidemia de 2010, os DENV-1 e DENV-2 cocircularam. e, de acordo com dados do Ministério da Saúde do Brasil, somente no estado de MSforam confirmados 1.815 casos graves com 47 mortes. Quanto à epidemia de 2013, com a cocirculação dos DENV-1, DENV-2 e DENV-4, apesar do menor número de casos graves, foram confirmados 695 casos graves com 30 mortes.

A cocirculação de dois ou mais sorotipos durante a mesma epidemia tem sido identificado e é uma das principais causas da manifestação grave da dengue no sudeste da Ásia (Endy *et al*., 2002). Vários estudos têm sugerido que as infecções por DENV-2 e DENV-3 de origem asiática, como aqueles que circulam no Brasil, estão relacionadas com a gravidade da dengue (Vaughn *et al*., 2000; Nisalak *et al*., 2003). Embora o número de casos analisados referentes à epidemia em 2010 tenha sido menor do que os casos analisados da epidemia de 2013, o número de casos graves e mortes foram relativamente mais elevados em 2010. Além disso, foi observado um maior número de hospitalizações em 2010 (46%) quando comparado com 2013 (21%) (SES/MS, 2010; 2013).

De acordo com a nova classificação clínica, proposta pela OMS em 2009 (OMS, 2009), a gestão adequada dos pacientes depende do reconhecimento precoce dos sinais de alerta. Estes sinais são importantes, uma vez que ocorrem antes do início da dengue grave (Alexander *et al*., 2011). O Brasil adotou oficialmente a nova classificação em 2014. Este estudo mostrou a prevalência de 64% de dengue sem sinais de alarme (DSSA) em ambas as epidemias estudadas. Na epidemia de 2010, 47.9% dos casos eram de dengue com sinais de alarme/dengue grave (DCSA/DG), e os sorotipos detectados foram os DENV-1 e DENV-2. Na epidemia de 2013, 71.2% dos casos eram de DSSA e esta epidemia foi causada principalmente pelo DENV-4 (90,4%), corroborando com os dados do Ministério da Saúde (SVS 2013). Dor abdominal persistente (16.1%) e sangramento das mucosas (8.4%) foram as manifestações clínicas mais comuns em pacientes com DCSA, semelhante a outros estudos realizados na Ásia (Leo *et al*., 2013; Rathakrishnan *et al*., 2014) e no Brasil (Cavalcanti et al., 2014) que utilizaram a nova classificação da OMS.

Em relação aos outros sorotipos, as infecções causadas pelos DENV-4 são tipicamente associadas à apresentação clínica branda da dengue (Nishiura *et al*., 2007). Entretanto, diversos estudos têm relatado dengue grave e mortes relacionadas à infecção pelo DENV-4, principalmente em infecções secundárias (Amâncio *et al* 2014; Rico-Hesse, 2003).

Foram detectadas altas taxas de imunoglobulina G na população estudada, caracterizando uma exposição prévia aos DENV. Embora neste estudo 84% dos pacientes tenham apresentado infecção secundária, não foram observadas manifestações graves nos pacientes infectados pelos DENV-4 na epidemia de 2013. Por outro lado, na epidemia de 2010, foi relatado um maior número de casos com sinais de alarme e dengue grave, o que sugere a presença de infecção secundária não seria o único fator responsável por uma doença mais grave. Na verdade, a gravidade da doença também é causada pelo aumento da virulência (Rico-Hesse, 2003) e também pelos elevados títulos virais em pacientes com FHD (Libraty et al., 2002; Thomas et al., 2008). Além disso, infecções sequenciais por DENV-1 seguida por DENV-2 ou DENV-3, ou infecções por DENV-3 seguida por DENV-2 foram associadas as formas graves da dengue (Guzmán *et al*., 1991; Alvarez *et al*., 2006).

Um estudo anterior demonstrou quedemonstram que o aumento da gravidade nos casos de dengue pode estar relacionado com a introdução de um novo sorotipo, em áreas que circulam outros sototipos, com o aumento do número de infecções secundárias, com a presença de fatores intrínsecos relacionadas à população (Hastead, 2006). Posteriormente Macedo e colaboradores ao analisarem as epidemias de 2004-2008 no estado do Rio de Janeiro verficaram um aumento dos casos de dengue em crianças menores de 15 anos de idade, aumento de FHD e de dengue com complicações nesta faixa etária e quepacientes infectados pelo DENV-2 foram, comprovadamente, três vezes mais propensos a ter sinais e sintomas associados a uma doença mais grave (Macedo *et al*., 2013). Reforçando as assertivas acima, um estudo mais recente, também desenvolvido no Rio de Janeiro, mostrou que pacientes infectados pelos DENV-3 e DENV-2, com infecções secundárias, desenvolveram uma doença mais grave (Heringer *et al*., 2015).

No presente estudo foi observado que casos de dengue pelos DENV-1 e DENV-2, durante a epidemia de 2010, s foram mais grave do que os casos causados principalmente pelo DENV-4durante a epidemia de 2013, quando o número de hospitalização foi cerca de duas vezes menor do que a observada em 2010.

Um estudo realizado por Thomas e cols relatou que o DENV-1 induziria uma gravidade intermediária, sem extravasamento plasmático aparente, um outro estudo descrito por Huy e cols foi demostrado que infecções pelo DENV-2 estavam associadas à síndrome de

choque por dengue. Em contraste, o DENV-4 induziu uma apresentação clínica mais branda, confirmando a existência de diferenças na virulência entre os sorotipos (Thomas *et al*., 2014; Huy *et al*., 2013).

Diante do exposto, visando identificar possíveis preditores de gravidade na dengue, diversos estudos têm avaliado biomarcadores tais como viremia, citocinas e parâmetros laboratoriais (Srikiatkhachorn & Green, 2010; Oishi *et al*., 2007). Neste contexto, nosso estudo avaliou os parâmetros laboratoriais dos dois grupos das diferentes epidemias e os resultados obtidos possibilitou observar que o DENV-4 foi associado com menor gravidade na apresentação clínica da dengue. Em nosso estudo, 51.7% dos pacientes apresentaram leucopenia e 27.7% trombocitopenia, corroborando com estudos anteriores realizados no Brasil (Azin *et al*., 2012; Oliveira *et al*., 2009).

É importante ressaltar que pacientes da epidemia de 2010, com prevalência do DENV-1 e DENV-2, apresentaram baixa contagem de plaquetas, alta frequência de manifestações hemorrágicas e extravasamento de plasmático. Em comparação, com os pacientes da epidemia de 2013, infectados pelo DENV-4, que apresentaram maior contagem de plaquetas e menor frequência de sangramento. Trombocitopenia e desordens na cascata de coagulação são observadas nos casos graves de dengue e, assim, marcadores circulantes da coagulopatia, tais como o fator tecidual (tissue factor-TF), proteína necessária para a iniciação da cascata de coagulação, que estão aumentados durante a fase aguda da doença, têm sido associados com a patogênese da dengue (Wills *et al*., 2002; Huerta-Zepeda *et al*., 2008; Azeredo *et al*., 2010). Curiosamente, observou-se que os níveis circulantes de TF foram menores nos pacientes infectados com DENV-4 em comparação com pacientes infectados com DENV-2 (dados não publicados).

A participação da proteína NS1 na gravidade da dengue tem sido sugerida por vários estudos (Beatty *et al*., 2015; Libraty *et al*., 2002; Paranavitane *et al*., 2014). Na Ásia têm se demonstrado que os níveis de NS1 e/ou viremia são mais elevados em casos graves (Libraty *et al*., 2002, Duyen *et al*., 2011; Tricou *et al*., 2011). Com o objetivo de confirmar e ampliar esses resultados anteriores, analisamos os níveis circulantes de NS1 nos pacientes brasileiros a partir dessas duas epidemias na mesma cidade. Assim, foi possível verificar no presente estudo que os pacientes DCSA/DG apresentaram níveis elevados de NS1 em comparação com o grupo DSSA, independentemente do sorotipo infectante. Além disso, os pacientes da epidemia 2010, causada por DENV-1 e DENV-2, apresentaram níveis mais elevados de NS1 em comparação com aqueles da epidemia 2013 causada principalmente por DENV-4. Estudos anteriores demonstraram que indivíduos infectados por DENV-1

apresentaram níveis mais elevados de NS1 do que os infectados por DENV-2 (Duyen *et al.*, 2011; de la Cruz-Hernandez *et al*., 2013; Duong *et al*., 2011). Não foram detectadas diferenças entre o DENV-1 e DENV-2, visto que poucas amostras foram simultaneamente positivas para NS1 e RT-PCR.

Como a maioria dos pacientes apresentou infecções secundárias em ambas epidemias analisadas neste estudo, é possível especular que a proteína NS1 solúvel poderia ter formado imunocomplexos com anticorpos IgG. Assim, todas as amostras foram dissociadas por ácido de acordo com Lima et cols (2014), e a partir deste procedimento foi possível verificar que ocorreu um aumento do número de amostras positivas.

A quantificação dos níveis de NS1 circulante foram maiores nas amostras da epidemia de 2010 , quando ocorreu a co-circulação dos DENV-1 e DENV-2 em comparação com as amostras de 2013, quando o DENV-4 foi prevalente. Muitos estudos têm relatado o papel da NS1 dos DENV, como um biomarcador de gravidade, associando os altos níveis circulantes de NS1 na fase inicial da dengue com o desenvolvimento de FHD, no entanto estudos avaliando antigenenia de NS1 em casos de pacientes infectados pelos outros sorotipos e em infecções primárias ainda são escassos, e necessitam de mais estudos para avaliar se existem diferenças entre os sorotipos nos níveis circulantes de NS1 e se estes níveis aumentados estariam influenciando no desenvolvimento da forma grave da dengue (Libraty *et al*., 2002; Paranavitane *et al*., 2014).

Quanto à trombocitopenia, curiosamente encontramos uma correlação inversa entre os níveis circulantes de NS1 e a contagem de plaquetas ($p = 0,0018$, R de Spearman = - 0,377). Estudos anteriores relataram associações entre maior carga viral e trombocitopenia (Libraty *et al*., 2002; Duyen *et al*., 2011) além da destruição aumentada de plaquetas associadacom imunocomplexos contendo antígeno de DENV na superfície das plaquetas (Wang *et al*., 1995; Mitrakul *et al*., 1977). Sun e colaboradores (2007) demonstraram em estudos “in vitro” que os anticorpos contra o antígeno NS1 reagem de forma cruzada com as plaquetas conduzindo à ativação de plaquetas e a opsonização. Em nosso estudo foi identificada uma correlação inversa entre os níveis de NS1 e leucócitos, corroborando com o estudo de Paranavitane (Paranavitane *et al*., 2014). Além disso, também observamos que as contagens de monócitos foram inversamente correlacionadas com os níveis NS1 ($p = 0,0169$, R de Spearman = - 0,333).

Considerando que os monócitos são as principais células alvo para a replicação dos DENV e que estas células são fonte de citocinas inflamatórias que estão envolvidas na patogênese da dengue, em nosso estudo os pacientes da epidemia de 2010, com níveis

aumentados de NS1, apresentaram menor contagem de monócitos. Como os monócitos circulantes são ativadados durante a fase aguda da infecção por dengue (Azeredo *et al*., 2010), esse fenômeno poderia, em parte, justificar a baixa contagem de monócitos observada nesses pacientes. O reduzido número destas células pode também ser justificado pela apoptose, durante a replicação DENV nos monócitos. De fato, os monócitos CD14 + infectados com DENV-2 expressam níveis elevados de receptor de morte Fas e podem ser susceptíveis a apoptose (Torrentes-Carvalho *et al*., 2009).

Como a quantificação da viremia por métodos de RT-PCR é onerosa e nem sempre disponível nas regiões endêmicas, a quantificação da forma secretada de NS1 (sNS1) passa a ser um importante instrumento diagnóstico por proporcionar não só um diagnóstico específico de infecção DENV, mas também por prever o desenvolvimento de casos graves na fase inicial da infecção.

Quanto às enzimas hepáticas, neste estudo observamos níveis aumentados das transaminases nos pacientes infectados com dengue independente do sorotipo infectante ou epidemia estudada. Na epidemia de 2010 causada pelo DENV-1 e DENV-2, não foram observadas diferenças nos níveis de AST e ALT de acordo com a classificação clínica, enquanto que, na epidemia de 2013 causada pelo DENV-4, o grupo DCSA/DG mostrou um aumento significativo em AST e ALT, em comparação com o grupo DSSA. O envolvimento hepático é uma das características de infecção pelos DENV. Disfunção hepática, incluindo hepatomegalia e elevações das transaminases, tem sido descrita tanto em casos brandos como em casos graves podendo levar ao óbito (Souza *et al.*, 2004; Lee *et al*., 2012; Trung *et al*., 2010). De fato, um paciente infectado pelo DENV-2 que apresentou níveis elevados de ALT e de AST (5598 e 20618 UI / L, respectivamente) evoluiu para o óbito com hepatite fulminante (dados não publicados).

Apesar da utilização de todos os métodos diagnósticos disponíveis, um grande número de amostras coletadas em ambos os períodos foram negativas para DENV e, desta forma, as amostras foram classificadas como de outras doenças febris (ODF). Essas amostras também foram negativas para alfaviroses e flaviviroses, utilizando o protocolo descrito po Bronzoni *et al*., 2005, para se certificar de que realmente eram DENV negativo. Doença febril não específica pode ser causada por uma série de outras doenças infecciosas que podem ser comumente confundidas com dengue. Por exemplo, um surto de gripe que geralmente se manifestam como febre e mialgia foi relatado no mesmo período no estado do Mato Grosso do Sul – MS (SES/MS, 2014).

5.4. Hepatite fulminante associada ao vírus dengue tipo 2: relato de caso. (Artigo 4)

A disfunção hepática em formas de desarranjos nos testes de função hepática é comum e pode incluir pequenas elevações de bilirrubina, elevados níveis de transaminases e alterações nos níveis de albumina sérica. Embora na maior parte das infecções a dengue se apresente de forma assintomática, manifestações clínicas como icterícia e insuficiência hepática aguda (ALF – do inglês acute liver failure), podem eventualmente complicar o quadro clínico. A dengue tem sido apontada como uma importante causa de ALF em países endêmicos (Samanta *et al*., 2015).

Neste trabalho uma paciente jovem, sem histórico de comorbidades, evoluiu para o óbito em decorrência de hepatite fulminante causada pelo DENV-2 (Linhagem II).

Observamos altos títulos virais no plasma e nas PBMCs, corroborando com estudo descrito anteriormente, que desmonstrou que na forma grave da dengue alto nível de viremia está associado com o envolvimento de diferentes órgãos (fígado, cérebro) (Martina *et al*., 2009).

A disfunção hepática é uma característica crucial observada na infecção pelo DENV. Hepatócitos e células de Kupffer são os principais alvos na infecção pelo DENV (Marianneau *et al*., 1999; Seneviratne *et al*., 2006), como confirmado em biópsias e autópsias de casos fatais (Huerre *et al*., 2001). O aumento das transamisases é observado na infecção por dengue, porém o aumento das transaminases > 1000 UI/L é observado na dengue grave (Souza *et al*., 2004). No presente estudo a paciente apresentou altos níveis circulantes de transamisases, com aumento de 590 e 136 vezes acima dos valores normais para AST e ALT, respectivamente.

O diagnóstico diferencial com outras doenças que causam a ALF deve ser verificado, e em nosso estudo os testes para as hepatites virais, febre amarela, hantavirose e arenavirose também foram negativos.

Além das medidas de suporte as medidas específicas também devem ser adotadas no manejo de pacientes infectados pelos DENV com ALF, as medidas específicas previamente descritas, em diversos estudos como o tratamento intravenoso com N-acetilcisteína (Kumarasena *et al*, 2010; Senanayake *et al* 2013). Além disso, alguns cuidados devem ser tomados em relação ao diagnóstico e ao uso de drogas que podem agravar a lesão hepática (Jorup-Rönström *et al.,* 1986; Maddox *et al*., 2010).

5.5. Análise das alterações clínicas e laboratorias relacionadas com a gravidade de casos de dengue: um estudo comparativo entre sorotipos 2 e 4 no Brasil. (Artigo 5)

No Brasil, a transmissão da dengue vem ocorrendo de forma continuada desde 1986, intercalando-se com a ocorrência de epidemias, geralmente associadas com a introdução de novos sorotipos em áreas anteriormente indenes. A maior epidemia ocorreu em 2013, com aproximadamente 1.4 milhões de casos notificados, com o predomínio do DENV-4, mas com a maioria dos casos brandos (SVS, 2014). Este estudo foi realizado com 153 pacientes com sinais e sintomas compatíveis com dengue, em amostras provenientes das epidemias de 2010 e 2013 ocorridas no município de Campos dos Goytacazes, no estado do Rio de Janeiro, Brasil. A infecção foi confirmada em 61.4% (94/153) dos pacientes com suspeita de dengue, por uma ou mais técnicas de diagnóstico de rotina do Laboratório de Flavivírus, Instituto Oswaldo Cruz, FIOCRUZ. A análise pela técnica de RT-PCR detectou o DENV-2 na epidemia em 2010 e o DENV-4 na epidemia em 2013.

De acordo com a nova classificação da WHO 2009, os pacientes foram classificados como 60.6% (57/94) DSSA, 30.9% (29/94) DCSA, 2.1% (2/94) DG e 6.3% (6/94) não puderam ser classificados. O reconhecimento precoce dos sinais de alarme torna-se importante para o manejo adequado dos pacientes, como o objetivo de evitar as mortes pela dengue. Estes sinais são importantes, uma vez que surgem antes do estabelecimento da forma grave da doença (Alexander et al., 2011). Em 2010 o número de hospitalizações (45.8%) foi maior do que na epidemia de 2013 (22.9%), resultado semelhante aos obtidos em estudo prévio que mostraram que infecções causadas pelos DENV-2 e DENV-3 apresentam duas vezes mais probabilidade de desenvolver a forma grave da doença do que infecções pelos DENV-4 (Fried *et al*., 2010). Um total de 80.8 % (21/26) das internações hospitalares ocorreu na fase aguda da doença, entre os primeiro e sétimo dia após do início da doença, enquanto que em 19.2% (5/26) com mais de 7 dias de doença. Das 27.6% (26/94) internações ocorridas 96.2% (25/26) eram DCSA/DG e 3.8 % (1/26) eram DSSA.

Considerando a situação epidemiológica do país, procedemos à classificação da resposta imune dos pacientes. Em nossa casuística, 81.9% (77/94) dos pacientes apresentaram infecção secundária. De fato a infecção secundária tem sido relatada em altas proporções em estudos realizados no país (Honório *et al*., 2009; Guilarde *et al*., 2008; Nogueira *et al*., 2005).

Ao compararmos as alterações hematológicas dos pacientes, observamos que independente da forma clínica apresentada os pacientes infectados pelo DENV-2, na epidemia de 2010 apresentaram trombocitopenia mais acentuada do que os pacientes

provenientes da epidemia de 2013 que foi causada pelo DENV-4, corroborando com os resultados obtidos nos estudos de Thomas et al (2008) e Faria et al (2016).

Diversas hipóteses vêm sendo formuladas visando à elucidação dos prováveis mecanismos responsáveis pela trombocitopenia na dengue. Murgue et al (1997) sugeriram que o DENV afetaria as células progenitoras da medula óssea inibindo suas funções. Outros estudos descreveram que mecanismos indiretos ao vírus, diminuiriam a capacidade proliferativa das células hematopoiéticas (Basu *et al*., 2008). De fato, existem evidências de que o DENV induz hipoplasia da medula óssea na fase aguda da doença (Tsai *et al*., 2011), já que estudos *in vitro* demonstraram que o DENV-4 se replica em células progenitoras da medula óssea humana alterando a sua capacidade proliferativa (Nakao *et al*., 1989). Adicionalmente, estudos têm demonstrado que a infecção pelo DENV pode induzir o consumo plaquetário em decorrência de uma maior lise causada pelo complemento e apoptose, além do mecanismo relacionado aos anticorpos antiplaquetários (Lin *et al*., 2006; Hottz *et al*., 2013).

Pacientes infectados pelo DENV apresentaram leucopenia independente do sorotipo infectante e da epidemia estudada, quando comparados com pacientes ODF. Jamel e colaboradores demonstraram que no início da doença, tanto em infecções primárias quanto secundárias, ocorre uma queda na contagem de leucócitos concomitante a queda de linfócitos T e aumento de linfócitos atípicos, evento que podem estar relacionado ao processo de supressão da medula óssea durante a fase aguda da doença (Jameel *et al*., 2012).

O envolvimento hepático é uma das características da infecção pelo DENV. As alterações hepáticas, incluindo hepatomegalia e aumentos das transaminases já foram descritas tanto em casos de dengue clássica como de febre hemorrágica do dengue (Nguyen *et al*., 1997; Trung *et al*., 2010; Lee *et al*., 2012;). Na dengue grave, a ocorrência de insuficiência hepática fulminante tem sido causa de morte em crianças e adultos (Roy et al., 2013). Os mecanismos de lesão hepática na dengue podem estar relacionados aos efeitos diretos do vírus ou por consequência da resposta imune do hospedeiro no tecido hepático, levando ao comprometimento circulatório, acidose metabólica, hipóxia por hipotensão e/ou derrame vascular (Itha *et al.,* 2005) Nossos resultados demostraram que cerca de 41.5% (39/94) dos pacientes apresentaram níveis de transaminases acima dos valores normais, destes 51.3% (20/39) eram de pacientes DCSA/DG. As diferentes variações nos níveis das transaminases durante a dengue não estão completamente elucidadas. No presente estudo

não foram observadas diferenças estatísticas significativas nos níveis de AST/ALT entre as epidemias.

O reconhecimento das diferenças hematológicas e bioquímicas entre pacientes com dengue e com outras doenças febris, é estrategicamente um grande aliado na identificação de pacientes com potencial de desenvolver a forma grave da doença. A fase clínica inicial da dengue muitas vezes é confundida com outras doenças febris, gerando dúvidas no momento da gestão clínica e vigilância da doença. Neste sentido, Tanner et al. (2008) demostraram que diferenças hematológicas poderiam ser usadas como ferramentas para diferenciar a dengue de outras doenças febris em amostras colhidas precocemente. Curiosamente, eles também demostraram que é possível prever o eventual aparecimento de trombocitopenia através da utilização de algoritmos.

Ao comparar os dados analisados neste estudo observamos uma clara diferença nos parâmetros hematológicos e bioquímicos entre os pacientes com diferentes formas clínicas da doença e pacientes portadores de outras doenças febris (ODF). O reconhecimento destes parâmetros contribui para que medidas de suporte possam ser tomadas a fim de evitar o aumento da morbidade e mortalidade pela dengue. É necessário um estudo mais aprofundado para esclarecer essas questões tão importantes, pois as respostas têm grande impacto na prática da medicina em epidemias de dengue distribuídas em todo o mundo, principalmente em áreas tropicais onde tais epidemias geram grandes impactos sócio-econômicos.

5.6. Análise da infecção “in vitro” de células dendríticas humanas por distintas linhagens do genótipo Sudeste Asiático/Americano de DENV-2 circulantes no Brasil. (Artigo 6)

As DCs sao fundamentais na indução da resposta imune inata e direcionam as resposta adaptativas através da apresentação de antígenos e da produção de citocinas (Lipscomb and Masten, 2002). As DCs constituem uma distinta linhagem celulas monucleares fagociticas especializadas na apresentaçao antigenica aos linfócitos T e B. Uma vez em contato com o patogeno, as DCs passam pelo processo de maturaçao que culmina com a eficiente apresentaçao antigenica e produçao de citocinas. As citocinas

produzidas pelas DCs se tornan parte do microambiente que induz respostas imunes capazes de estimular o desenvolvimento de linfocitos T efetores (Reis et al., 2006).

Devido ao aumento no número de casos graves associados à infecção pelos DENV. A utilização de métodos "in vitro" tornou-se uma importante ferramenta para o entendimento da patogênese da dengue. A falta de modelos animais que reproduzam a forma grave da doença levou ao desenvolvimento de modelos *in vitro* utilizando linhagens humanas primárias, como monócitos e células dendríticas diferenciadas (MoDC) e ainda linhagens endoteliais, capazes de produzir citocinas e fatores solúveis quando infectadas pelo DENV. No presente estudo, duas linhagens de DENV-2 originárias de pacientes brasileiros foram testadas quanto a capacidade de replicaçao em MoDC em PBMCs de doares saudáveis. Embora vários estudos *in vitro* já tenham sido realizados utilizando MoDC, a maioria destes utilizam cepas asiáticas (Gandini *et al*., 2011) e, estudos com cepas brasileiras, são escassos.

Em 2008, o Brasil experimentou mais grave epidemia de dengue (Teixeira *et al*., 2009). Após a re-emergência do DENV-2 em 2007, ocorreu uma mudança no perfil da doença, um estudo realizado com amostras dos 20 anos de circulação dos DENV-2 no Brasil, detectou a presença de uma nova linhagem do genótipo Sudeste Asiático do DENV-2, essas amostras pertencem a uma linhagem diferente das primeiras amostras introduzidas no Brasil nos anos de 1990 (linhagem I) (Faria *et al.,* 2013). A partir desses achados nós realizamos a infecção *"in vitro"* de MoDCs, utilizando as duas diferentes linhagens de DENV-2 do genótipo Sudeste Asiático com intuito de identificar diferenças e semelhanças entres elas. Dados prévios do nosso grupo demonstraram que as DCs são alvos da infecção pelo DENV, com pico de infecção em 24 h e significativas taxas de infecção 48 h após infecção (Gandini *et al*., 2011). Desta forma, para as comparações entre as taxas de replicação/detecção das cepas virais nas células, o ponto de 48 horas após a infecção foi escolhido.

Observamos através de citometria de fluxo e de real time PCR que as ambas as linhagens de DENV-2 são capazes de infectar e multiplicar em MoDCs, corroborando estudos prévios (Gandini *et al*., 2011; Silveira *et al*., 2011; Wu *et al*., 2000).

As DCs passam por várias mudanças feno típicas durante as infeções virais com expressão de moléculas de superfície relacionadas a apresentação antigénica as células T e também com as respostas celulares efetoras . TRAIL (ligante indutor de apoptose relacionado ao fator de necrose tumoral), é uma molécula proapoptótica, que induz a morte de células que expressam os seus receptores de morte (DR), DR4 e DR5 (Sheridan *et al*, 1997; Wu *et al*., 1997). Lu e colaboradores demostraram que as DCs expressam TRAIL quando ativadas (Lu *et al*., 2002). Interessantemente, MoDCs expressam TRAIL e exibem atividade citotóxica contra células tumorais (Liu *et al*., 2001). Pouco se sabe sobre o papel do TRAIL durante a infecção pelo DENV. Warke e colaboradores demonstraram ação anti viral desta molécula em DCs infectadas com DENV in vitro (Warke *et al*., 2008). Interessantemente, Gandini e colaboradores demonstraram maiores níveis circulantes de TRAIL em pacientes brandos (Gandini *et al*., 2013).

OX40L expresso em DCs (Oshima *et al*., 1997), pode contribuir para a polarização da resposta imunológica TH-2 o através do aumento da indução de IL-4 e IL-13, suprimindo o IFN-γ depois da ligação ao OX40 em células T (Ohshima *et al*., 1998; Delespesse *et al*., 1999). Por fim, não observamos diferenças na expressão de OX40L ou na expressão de TRAIL entre as MoDCs infectadas pelas linhagens I e II. Embora, ambas linhagens tenha sido capazes de aumentar a expressão de TRAIL e OX40L nas DCs infectadas, não observamos diferença estatística provavelmente devido ao pequeno numero de doadores testados.

Maiores níveis circulantes de mediadores inflamatórios (citocinas e quimiocinas) são detectados em pacientes graves, sugerindo que as formas graves da doença, se manifestam em um contexto de amplificação da produção de citocina, chamada de tempestade de citocinas, que atinge o epitélio endotelial causando aumento da permeabilidade vascular (Costa et al., 2013). Neste context, altos níveis de TNF-α sao encontrados na FHD e nos pacientes apresentando manifestações hemorrágicas (Braga et al., 2001) (Costa et al., 2013). Os níveis circulantes das IL1β, 4, 6, 10 18, TGF-β também encontram-se elevados em pacientes infectados com diferentes sorotipos e manifestações clínicas graves (Srikiatkhachorn and Green, 2010). Além destes mediadores, as quimiocinas MCP1/CCL2, MIP1α/CCL3, IL8/CXCL-8 e IP-10/CXCL10 são importantes

mediadores inflamatórios e são frequentemente associadas com a gravidade da infecção pelo dengue em pacientes (Bozza et al., 2008; Lee et al., 2006).

As citocinas e quimiocinas foram quantificadas nos sobrenadantes das culturas de MoDCs infectadas. Nós observamos que ambas as linhagens foram capazes de induzir a produção de citocinas (IL-1β, IL-1ra, IL-6, TNF-α) e de quimiocinas (MCP-1 e MIP-1β). Não observamos diferenças significativas entre as linhagens entre si, e diferenças significativas só foram observadas nas análises entre o MOCK e as linhagens separadamente. De fato, diversos estudos reportaram produção de TNF- α, IL-6 por DCs expostas ao DENV (Ho *et al*., 2001; Libraty *et al*., 2001; Palmer *et al*., 2005). Quimiocinas e outros mediadores inflamatórios com importantes papéis na patogênese e imunidade (Lusso, 2000; Rossi and Zlotnik, 2000) também foram secretados in vitro por diversas células humanas depois da infecção pelo DENV (Chen and Wang, 2002; Lin *et al*., 2005).

A quantificação da proteína NS1 foi realizada nos sobrenadantes das culturas infectadas, e após análise observamos níveis mais elevados na linhagem II comparados à linhagem I, apresentando (p=0.0024). Esses resultados corroboram nossos dados prévios. De fato, demonstramos que pacientes com dengue apresentando manifestações clinicas mais graves, originários da epidemia de 2010 e circulação dos sorotipos DENV 1 e 2 apresentaram altos níveis circulantes de NS1 (Faria *et al*., 2016). Além disso, um caso fatal em que isolamos cepa DENV-2 e posteriormente sequenciamos e caracterizamos a linhagem II apresentou maiores níveis circulantes de NS1 (275 ng/mL) (manuscrito submetido).

Estudos prévios tem demonstrado o envolvimento da proteína NS1 na gravidade da dengue (Paranavitane *et al*, 2014;. Beatty *et al*, 2015). Na sua forma secretora a NS1 (sNS1) transporta lipídeos, este fato tem gerado muitas especulações entre diversos grupos de pesquisa, tem se pensado que esse transporte tem muitas implicações na patogênese da doença, pois as lipoproteínas são importantes nas vias de coagulação e estão associadas com a inflamação vascular (Gutsche *et al*., 2011). Beatty et al (2015), mostraram recentemente que a NS1 de todos os quatro sorotipos dos DENV, induziram um extravazamento vascular em um modelo animal (camundongos) infectados pelos DENV

pela indução da disfunção da barreira endotelial (Beatty *et al*., 2015). Modhiran *et al* (2015), também demonstraram que a NS1 do DENV estimulou a produção de citocinas a partir de células da imunidade inata, agindo através de receptor Toll-like (TLR) -4 (Modhiram *et al*., 2015).

Por fim, observamos neste estudo que as MoDCs infectadas com as linhagens I e II do DENV-2 Sudeste Asiático foram permissivas à infecção pelo DENV in vitro e capazes de produzir citocinas e quimiocinas envolvidas com a patogênese da dengue. De fato, o sequenciamento de ambas linhagens demostrou a presença de uma asparagina (N) na posição E390, previamente caracterizada como um provável marcador viral de FHD em cepas de origem asiática (Faria et al., 2013; Leitmeyer et al., 1999). Desta forma, estes resultados sugerem que ambas linhagens de DENV-2 possuem potencial no desenvolvimento das formas mais graves de dengue. No entanto, fatores decorrentes do hospedeiro, em especial as características decorrentes de cada doador utilizado neste estudo também contribuem para a variabilidade nas repostas conforme observado por outros grupos (Deauvieau *et al*., 2007; Nightingale *et al*., 2008). Interessantemente, culturas infectados com a linhagem II apresentaram maiores níveis de NS1 em concordancia com o papel desta proteina na imunopatologia da dengue. Nosso conhecimento sobre mecanismos virologicos e imunológicos e o defecho na gravidade da doença é ainda limitado e vários aspectos importantes precisam ser esclarecidos. No entando, acreditamos que nossos resultados abrem novas perspectivas sobre o papel da NS1 e de diferentes cepas brasileiras na patogenese da doença que poderao ser importantes na prevensao de casos graves em áreas de risco.

6. Conclusões

6.1. Vinte anos de atividade do DENV-2 no Brasil: caracterização molecular e filogenia de cepas isoladas entre 1990 e 2010.

- A análise filogenética das cepas de DENV-2 isoladas no país nos últimos 20 anos demonstrou a circulação de um único genótipo (Sudeste Asiático) de DENV-2, ressaltando a importância deste tipo de estudo na identificação de genótipos comumente associados a gravidade da dengue ;
- As cepas de DENV-2 do período 1990-2003 pertencem ao genótipo do Sudeste Asiático, Linhagem I enquanto que as cepas isoladas após a reemergência deste sorotipo em 2007 pertencem ao genótipo Sudeste Asiático, Linhagem II, a presença de diferentes linhagens nos períodos estudos indica que ocorreu uma substituição de linhagens e que as mesmas não cocircularam nos períodos epidêmicos;
- As cepas de 1990 a 2003 analisadas neste estudo foram mais similares às cepas de referência BR64022/98 e Jamaica/83, enquanto que as cepas isoladas, entre 2007 e 2010, foram mais similares com a cepa DR59/01 proveniente da República Dominicana;
- O percentual de similaridade entre as cepas de DENV-2 do período de 2007 - 2010 e a cepa isolada na República Dominicana em 2001 e Porto Rico em 2005, combinado ao percentual de divergência com as cepas introduzidas no país na década de 90 sugerem que estes vírus não sofreram uma evolução local, mas que foram introduzidos, provavelmente importados do Caribe;
- Não foi possível associar as alterações de aminoácidos encontradas nos isolados de DENV-2 com as diferentes formas clínicas apresentadas em especial como quadro mais grave da doença;

6.2. Dengue grave associada à idade e à nova linhagem do DENV-2 no Rio de Janeiro Brasil.

- Altos níveis de viremia e de gravidade da doença foram observados em indivíduos infectados pela Linhagem II, resultado concordante com os disponíveis na literatura;
- É possível que a sequência de introdução ou reemergencia dos diferentes sorotipos de DENV possa ter influenciado na gravidade da doença;
- Com a reemergencia do DENV-2 linhagem II foi possível observar um aumento em duas vezes na ocorrência de casos graves;
- A presença de infecção secundária não foi correlacionada com a gravidade da doença;
- Indivíduos com idade ≤ 15anos e infectados com DENV-2 do genótipo Sudeste Asiático linhagem II, apresentaram altos níveis de viremia , sugerindo que esses altos níveis estariam associados ao aumento da gravidade da dengue em indivíduos infectados pela nova linhagem e com idade ≤ 15anos ;fecção pela nova linhagem introdução da nova linhagem;
- Os níveis de viremia em indivíduos infectados pela linhagem II foram maiores do que os níveis encontrados nos indivíduos infectados pela linhagem I, ressaltando a importância da quantificação precoce de níveis de viremia em associação ao desenvolvimento das formas graves da dengue.

6.3. Epidemia de dengue em dois períodos distintos revelam aspectos epidemiológicos, laboratoriais e clínicos em um mesmo cenário: análise das epidemias ocorridas em Mato Grosso do Sul e, 2010 e 2013.

- O MAC-ELISA foi a técnica que confirmou o maior número de casos de dengue, reforçando a importância do teste sorológico no diagnóstico confirmatório;
- A dissociação ácida das amostras da epidemia de 2013, aumentou a detecção do antígeno NS1 em 22%, procedimento oque influenciou no diagnóstico sorológico de pacientes infectados principalmente com DENV-4;
- Pacientes infectados pelos DENV-1/2 (2010) apresentaram menores contagens de plaquetas e leucócitos, maior número de hospitalizações, casos graves e maior frequência de sangramentos, quando comparados com pacientes infectados pelo DENV-4 (2013), sugerindo a associação dos DENV-1/DENV-2 com a gravidade da doença;
- Altos níveis circulantes de NS1 foram observados nos pacientes DENV-1/2 e casos graves. Além disso, uma correlação inversa entre os níveis de NS1 e a contagem de plaquetas, leucócitos e monócitos foi encontrada, confirmando a associação da proteína com a gravidade da doença;
- Os níveis de transaminases foram mais elevados em pacientes dengue com sinais de alarme/dengue grave, confirmando o envolvimento hepático na infecção pelos DENV.

6.4. Análise de alterações clínicas e laboratoriais relacionadas com a gravidade de casos de dengue: um estudo comparativo entre sorotipos 2 e 4 no Brasil

- A epidemia de 2010 apresentou o maior número de hospitalizações (45.8%), quando comparada com a epidemia de 2013 (22.9%) provavelmente devido ao sorotipo infectante (DENV-2) que circulou na epidemia de 2010 e que comumente está associado ao desenvolvimento da dengue grave, enquanto que na epidemia de 2013, causada pelo DENV-4, a maioria dos casos foram associados ao dengue brando, sugerindo que o DENV-4 geralmente está relacionado as manifestações clínicas mais leves;

- Pacientes infectados pelo DENV apresentaram altos níveis significativos dos parâmetros bioquímicos e baixos níveis hematológicos quando comparados com aqueles apresentados pelos pacientes ODF, dados que comprovam que as análises bioquímicas e hematológicas podem ser ferramentas utéis na diferenciação da infecção pelos DENVs de outras doenças febris agudas;

- Pacientes infectados pelo DENV-2 apresentaram baixas contagens de plaquetas quando comparadas com pacientes infectados pelo DENV-4, ressaltando que baixa contagem de plaquetas e infecção pelo DENV-2 geralmente estão associadas a manifestações clínicas mais graves;

- Embora pacientes DCSA apresentassem menor contagem de leucócitos quando comparados com pacientes DSSA não houve diferença significativa entre os dois grupos, mostrando que a baixa contagem de leucócitos é observada na maioria dos pacientes infectados pelo DENV independente da classificação clínica.

6.5. Análise da infecção “in vitro” de células dendríticas humanas por distintas linhagens do genótipo Sudeste Asiático/Americano de DENV-2 circulantes no Brasil.

- O DENV-2 linhagem I e o DENV-2 linhagem II do genótipo Sudeste Asiático/Americano são capazes de infectar e multiplicar em MoDCs, ressaltando a importância dos estudos “in vitro” utilizando cepas circulantes na natureza;
- Ambas as linhagens foram capazes de induzir a produção de citocinas e quimicionas, mostrando a importância dos estudos “in vitro” para um melhor entendimento da patogênese da dengue;
- Sobrenadantes das culturas infectadas com DENV-2 linhagem II apresentaram maiores níveis de NS1 em concordância com dados prévios da literatura que demonstraram a associação da NS1 e da linhagem de DENV-2 com a gravidade da dengue.

7. Perspectivas

O aumento da frequência e da intensidade das epidemias de dengue ocorridas nos últimos anos e a recente introdução dos vírus Chikungunya (CHIKV) e vírus Zika (ZIKV), devem se a vários fatores, principalmente aos associados à intensificação do processo de urbanização, à falta ou irregularidade do abastecimento de água potável e à deficiência na coleta de lixo. O reconhecimento dos sorotipos/genótipos dos DENV, ZIKV e CHIKV e suas origens fornecem subsídios para que se possam monitorar as cepas mais virulentas na vigência de epidemias auxiliando na adoção das medidas de controle. A rápida evolução do DENV, CHIKV e ZIKV indica que a análise genética viral constitui um componente essencial para o entendimento de suas epidemiologias, caracterizando fatores que levam à transmissão e dispersão viral (Heesterbeek *et al*. 2015).

A identificação de marcadores associados à gravidade e à letalidade por DENV, ZIKV e CHIKV poderá auxiliar nos estudos de imunopatológicos e, consequentemente, no impacto dos surtos e epidemias em uma dada comunidade. Visamos, portanto, realizar a caracterização molecular, análise filogenética e estudos "in vitro" de cepas de DENV, ZIKV e CHIKV detectadas a partir de casos ocorridos no país. Além disso, associar sorotipos/genótipos com as manifestações clínicas, laboratoriais e imunológicas apresentadas pelos pacientes. Constituem se como perspectivas, identificar possíveis biomarcadores relacionados ao hospedeiro e aos vírus capazes de predizer um risco aumentado de desenvolvimento de formas graves da doença e de, consequentemente, contribuir para as decisões terapêuticas mais precoces. A identificação destas características poderá nortear o desenho racional de terapias antivirais/ e ou imunomodulatórias, que possam também contribuir para a prevenção de formas graves apresentadas pela infecção pelos DENV, ZIKV e CHIKV, tendo em vista as várias lacunas ainda existentes no conhecimento acerca, principalmente, das duas últimas arboviroses de

impacto. O impacto das epidemias causadas pelos DENV, CHIKV e ZIKV para a infraestrutura da saúde pública brasileira, turismo e economia está aumentando e já foi reportado (Teixeira *et al*., 2013).

Diante da problemática que atualmente vive o país, com a ocorrência de epidemias simultâneas das três arboviroses e o desconhecimento de como está cocirculação poderá influenciar na gravidade da doença, mais estudos são necessários, especialmente sobre a co-infecção e o efeito da infecção sequencial com diferentes vírus. Nossos estudos poderão auxiliar no esclarecimento da patogênese induzida por estas três arboviroses, nos mecanismos associados à resposta imune do hospedeiro bem como, no entendimento da influência do vetor na seleção das variantes genéticas.

8. Referências Bibliográficas

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9. Anexos

9.1. Outras produções geradas durante a tese:

Artigo 7: Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance.

Artigo 8: Evaluation of a generic RT-nested-PCR for detection of flaviviruses in suspected fatal cases of dengue infection, Rio de Janeiro, Brazil.

Artigo 9:A review on dengue diagnosis and epidemiology by a regional reference laboratory from 1986 to 2011, Rio de Janeiro, Brazil.

Artigo 10: Impact of the emergence and re-emergence of different dengueviruses' serotypes in Rio de Janeiro, Brazil, 2010 to 2012.

Artigo 11: Insights of the genetic diversity of DENV-1 detected in Brazil in 25 years: Analysis of the envelope domain III allows lineages characterization.

Capítulo de livro: Advances in Medicine and Biology - Chapter Title: Molecular Biology Approaches For Dengue Diagnosis And Research in Brazil: an overview.

Artigo 7: Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance. (Artigo 9)

Classificação QUALIS: Aréa de Medicina II – B2

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Resumo: In Niterói, state of Rio de Janeiro, dengue virus type 4 (DENV-4) was isolated for the first time in March 2011. We analysed the laboratory findings of the first cases and evaluated the use of molecular techniques for the detection of DENV-4 in Aedes aegypti that were field-caught. Conventional reverse transcriptase-polymerase chain reaction (RT-PCR) and Simplexa™ Dengue real-time RT-PCR confirmed DENV-4 infection in all cases. Additionally, DENV-4 was confirmed in a female Ae. aegypti with 1.08 x 10(3) copies/mL of virus, as determined by quantitative real-time RT-PCR. This is the first time the Simplexa™ Dengue real-time assay has been used for the classification of cases of infection and for entomological investigations. The use of these molecular techniques was shown to be important for the surveillance of dengue in humans and vectors.

Dengue virus type 4 in Niterói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance

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In Niterói, state of Rio de Janeiro, dengue virus type 4 (DENV-4) was isolated for the first time in March 2011. We analysed the laboratory findings of the first cases and evaluated the use of molecular techniques for the detection of DENV-4 in Aedes aegypti *that were field-caught. Conventional reverse transcriptase-polymerase chain reaction (RT-PCR) and Simplexa™ Dengue real-time RT-PCR confirmed DENV-4 infection in all cases. Additionally, DENV-4 was confirmed in a female* Ae. aegypti *with 1.08 x 10^1 copies/mL of virus, as determined by quantitative real-time RT-PCR. This is the first time the Simplexa™ Dengue real-time assay has been used for the classification of cases of infection and for entomological investigations. The use of these molecular techniques was shown to be important for the surveillance of dengue in humans and vectors.*

Key words: dengue virus type 4 - *Aedes aegypti* - RT-PCR - real-time RT-PCR - Simplexa™ Dengue real-time RT-PCR

Dengue is widespread in the tropical and sub-tropical areas of Asia, Africa and the Americas and the transmission of the virus is primarily associated with *Aedes aegypti*. In Brazil, a dengue outbreak that was caused by dengue virus (DENV) type 1 and DENV-4 was reported in 1981-1982 in a city in the Amazon Region (Osanai et al. 1983). However, it was only after DENV-1 was introduced in Rio de Janeiro (RJ) in 1986 (Schatzmayr et al. 1986) that the disease became a nationwide public health problem. Additionally, a virological and entomological program was established to monitor DENV in human sera and vectors in 1986 (Nogueira et al. 1988, 1999, Lourenço-de-Oliveira et al. 2002). RJ has assumed an important role in the epidemiology of dengue, with the first case of DENV-2 identified in 1990 (Nogueira et al. 1993) and the first case of DENV-3 identified in 2000 (Nogueira et al. 2001).

DENV-4 was reintroduced into Brazil in 2010 in the municipalities of Boa Vista and Canta, state of Roraima (RR) (Temporão et al. 2011). The virus then spread to the different regions of Brazil. Cases of infection have been identified in northern, northeastern and southeastern Brazil (MS/SVS 2011). Sequencing of the viral isolate genomes revealed that the DENV-4 Brazilian strains belonged to genotype II (de Sousa et al. 2011). In RJ, the first DENV-4 cases that were detected occurred in the Cafubá neighbourhood. Cafubá is located in the oceanic region of the municipality of Niterói which is located in the metropolitan region of RJ, Brazil (Nogueira & Eppinghaus 2011).

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The entomological surveillance of DENV in adult and immature mosquito stages is an important tool for the early prediction of dengue epidemics. Additionally, the virological surveillance of field-caught dengue vectors using molecular techniques, such as conventional reverse transcriptase-polymerase chain reaction (RT-PCR), has been useful for the rapid detection of dengue outbreaks in endemic regions and/or for the detection of the introduction of novel DENV variants (Chow et al. 1998, Pinheiro et al. 2005, Mendez et al. 2006, Chen et al. 2010, Guedes et al. 2010).

In this study, we aimed to characterise the first DENV-4 cases and demonstrate the role of rapid molecular techniques, such as conventional RT-PCR and real-time RT-PCR, in the entomological surveillance of the newly introduced DENV-4 variant in vector populations from Niterói after it was isolated from humans. Furthermore, we evaluated a real-time PCR commercial kit that has recently become available for the detection and typing of DENV in serum samples and mosquito macerates.

Human serum samples were obtained during a surveillance program of the Laboratory of Flavivirus, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (Fiocruz), RJ. This program was an on-going project that was approved by the Fiocruz Ethical Committee in Research (CEP 274/05, resolution CSN196/96), Ministry of Health.

The investigation of DENV-4 cases was performed during the RJ DENV-1 epidemic in 2011. The first two confirmed cases were two sisters who lived in the Cafubá neighbourhood of Niterói. Both experienced an onset of symptoms on March 6 2011 (Nogueira & Eppinghaus 2011). We investigated nine other suspected cases of individuals who experienced an onset of symptoms from March 23 2011-April 11 2011 and who lived in Cafubá, São Francisco, São Domingos and Engenho do Mato (Fig. 1A).

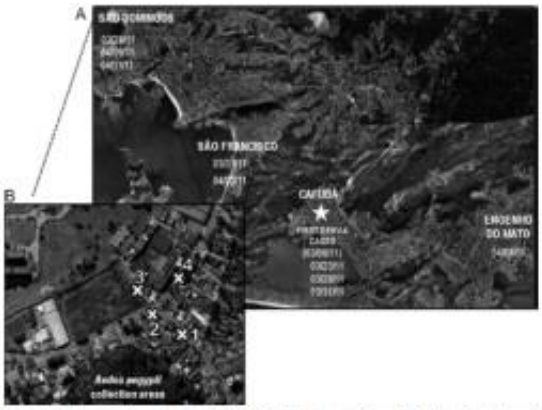

Fig. 1: dengue virus type 4 (DENV-4) introduction in Niterói, state of Rio de Janeiro, Brazil, 2011. A: Niterói neighbourhoods with DENV-4 confirmed cases. Dates shown are DENV-4 confirmed cases in the different neighbourhoods; B: entomological surveillance in the São Domingos neighbourhood. White crosses represent the four collection sites within an area with confirmed DENV-4.

Ae. aegypti adult mosquitoes were collected at nine residential and non-residential sites (São Domingos) where DENV-4 human cases had been confirmed. The collection of the mosquitoes was performed at four sites (Fig. 1B) on May 4 2011, using battery-operated aspirators. Mosquitoes were anaesthetised at 4ºC, identified, sexed and stored in liquid nitrogen on the same day of collection. Seventy-two *Ae. aegypti* (33 females and 39 males) adult mosquitoes were collected. Of these mosquitoes, 47 (18 females and 29 males) were collected from a single site in a village-like residential area (site #1) that was comprised of six houses. *Ae. aegypti* were individually macerated in 1 mL of Leibovitz L-15 medium (Sigma) with antibiotics (penicillin-streptomycin, 10,000 units; Invitrogen) and centrifuged (6,000 rpm at 4ºC for 30 min). The supernatant was then transferred to an Eppendorf tube that contained 100 mL of streptomycin/fungizone and penicillin. The tube was kept in an ice bath for 1 h and centrifuged (3,000 rpm at 4ºC for 15 min). The supernatant was then transferred to an Eppendorf tube that contained 0.3 mL of foetal calf serum (Invitrogen) and frozen (-70ºC).

Virus isolation was performed by inoculating the C6/36 *Aedes albopictus* cell line (Igarashi 1978) and the viral isolates were identified by an indirect fluorescent antibody test using serotype-specific monoclonal antibodies (Gubler et al. 1984). Infected supernatant was clarified by centrifugation and the virus stocks were stored in 1-mL aliquots at -70ºC until use.

Viral RNA was directly extracted from mosquito macerates using the QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. The RNA was stored at -70ºC for DENV detection and genotyping.

RT-PCR for the detection and genotyping of DENV was performed as described previously (Lanciotti et al. 1992). DNA products of a size unique to DENV-4 (392 bp) were amplified and then analysed by agarose gel electrophoresis and ethidium bromide staining.

For the quantification of the virus, the RNA that was isolated from the individually macerated *Ae. aegypti* mosquitoes was subjected to a quantitative real-time RT-PCR according to the protocol described by Drosten et al. (2002).

Simplexa™ Dengue real-time RT-PCR - For the qualitative detection and typing of the viral isolates, the RNA from individually macerated *Ae. aegypti* mosquitoes was subjected to the Simplexa™ Dengue real-time RT-PCR assay (Focus Diagnostics, Cypress, CA) according to the manufacturer's protocol. The assay uses the 3M Integrated Cycler instrument for the in vitro detection and genotyping of DENV-1 through DENV-4. The assay is based on a real-time RT-PCR that detects DENV-1 and DENV-4 in one reaction and detects DENV-2 and DENV-3 in a separate reaction. The assay uses bi-functional fluorescent primer-probes and reverse primers for the following specific regions of DENV: DENV-1 (NS5 gene), DENV-2 (NS3 gene), DENV-3 (NS5 gene) and DENV-4 (capsid gene). An internal RNA control is used to monitor the efficiency of the extraction process and to detect RT-PCR inhibition. In real-time RT-PCR, a positive reaction is detected by the accumulation of a fluorescent signal. The cycle threshold (Ct) is defined as the number of cycles that are required for the fluorescent signal to cross a particular threshold exceeding the background level. Ct values are inversely proportional to the amount of target nucleic acid present in the sample. Therefore, the lower Ct value, the greater the amount of target nucleic acid that is present in the sample. The Simplexa™ Dengue real-time RT-PCR assay stipulates 40 cycles of amplification. The Simplexa™ Dengue kits from Focus Diagnostics were kindly provided for the evaluation. The evaluation was performed for research purposes only and the authors have no competing financial interests.

Anti-dengue IgM antibodies in human serum were measured using the commercially available Panbio Dengue IgM capture ELISA. The results were classified as positive, negative or equivocal according to the manufacturer's instructions.

For the NS1 antigen capture, two commercial kits were used for the analysis of human serum and macerates. The Platelia™ Dengue NS1 Ag ELISA (Biorad Laboratories, Marnes-La-Coquette, France) is a one-step, sandwich format microplate enzyme immunoassay that is used to detect the DENV NS1 antigen in human serum or plasma. The Dengue NS1 Ag STRIP (Biorad Laboratories, Marnes-La-Coquette, France) is an immunochromatographic test for the rapid detection of the NS1 antigen. This assay was performed according to the manufacturer's protocol.

During March and April 2011, a total of 11 DENV-4 cases were confirmed using the following routine laboratory diagnostic techniques: MAC-ELISA, NS1 capture ELISA and rapid test, virus isolation and conventional RT-PCR. Two of the first cases were previously analysed, both consisting of individuals who experienced an onset of symptoms on March 6 2011 (Nogueira & Eppinghaus 2011). Three of the other cases occurred in the same neighbourhood as the first cases (Cafubá), three cases occurred in São Domingos, two in São Francisco

and one occurred in Engenho do Mato (Fig. 1A). The age of the patients ranged from 14-46 years (mean ± 24.7 years). Six patients were male and three were female. All patients had acute infections (up to 4 days of infection) and two of the nine patients (22.2%) were positive by MAC-ELISA. The isolation of virus was possible in five out of nine patients (55.5%) and all patients were positive by conventional RT-PCR. Four patients (44.4%) were positive when analysed by both the NS1 capture ELISA and the NS1 Ag Strip test. We further analysed all cases using the Simplexa™ Dengue real-time RT-PCR assay and all of the cases (9/9) were confirmed as DENV-4 using this method (Table). Ct values that were obtained from the DENV-4 human samples ranged from 16.8-35.1 (mean ± 25.0) (Fig. 2).

Because of the establishment of a sentinel network for DENV surveillance, which includes blood collections from febrile cases for virus detection, in 1986 in Niterói, it was possible to detect the introduction of DENV-2 and DENV-4 into the human population early, in 1990 and 2011, respectively (Nogueira et al. 1990, Nogueira & Epppinhaus 2011). Immediately after DENV-4 was isolated, an intensive study was conducted to monitor the distribution of the virus. Nine other DENV-4 cases from the neighbourhoods of Cafubá, São Francisco, São Domingos and Engenho do Mato were confirmed by laboratory analysis (Fig. 1A).

The development of conventional RT-PCR and real-time RT-PCR techniques has significantly reduced the processing time required to permit the detection of both the virus in the early stages of the infection in humans and its transmission by viral vectors. The DENV-4 cases that were investigated in this study were initially detected by conventional RT-PCR, which is established as a routine diagnostic test for all suspected acute dengue cases. Conventional RT-PCR results are usually released 24-48 h after samples are received in the laboratory. Additionally, for novel serotypes, all DENV-4 cases were re-tested separately using genotyping primers to confirm the new genotype. Concomitantly, cases were subjected to MAC-ELISA, NS1 ELISA and virus isolation. Because the cases studied consisted of patients in the acute phase of disease, MAC-ELISA was able to confirm only two out of nine cases. Samples were obtained from both of these patients within four days after the onset of symptoms. Samples from all of the other cases were acquired within the first and second days after the onset of symptoms. The most common technique used to serologically diagnose dengue is still based on the detection of anti-DENV IgM using MAC-ELISA (Huang et al. 2001). However, one of the limitations of this method is that there are variations in the detection rate during the acute phase of disease.

A previous study showed that the NS1 capture ELISA has a higher detection rate during the first four days after the onset of symptoms compared with the MAC-ELISA (Lima et al. 2010). In this study, both of the NS1 tests confirmed four out of the nine cases up until the fourth day after the onset of symptoms. During the acute phase of disease, the NS1 protein exists as a secreted and membrane-associated protein. Both forms of the protein have been demonstrated to be immunogenic (Young et al. 2000). High levels of NS1 have been demonstrated to circulate during the acute phase of dengue infection and they are found in the serum of patients with both primary and secondary DENV infections until the ninth day after the onset of symptoms (Young et al. 2000).

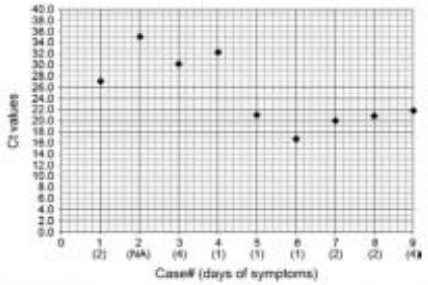

Fig. 2: Simplexa™ Dengue real-time reverse transcriptase-polymerase chain reaction amplification on dengue type 4 cases (n = 9) from Niterói, state of Rio de Janeiro, Brazil according to the number of days after the onset of the symptoms. Cycle threshold (Ct) values are shown. NA: not available.

TABLE

Human dengue virus type 4 cases laboratorial investigation in Niterói, state of Rio de Janeiro, Brazil

	Routine laboratorial diagnosis methodologies positive/tested					Newly available diagnosis methodology positive/tested
Dengue cases	MAC-ELISA	Virus isolation	NS1 ELISA	NS1 Ag STRIP	Conventional RT-PCR	Simplexa™ Dengue real-time RT-PCR
Males (n = 6)	2/6	4/6	3/6	2/6	6/6	6/6
Females (n = 3)	0/3	1/3	1/3	2/3	3/3	3/3
Total [n (%)]	2/9 (22.2)	5/9 (55.5)	4/9 (44.4)	4/9 (44.4)	9/9 (100)	9/9 (100)

RT-PCR: reverse transcriptase-polymerase chain reaction.

Despite the increased time required to produce a final result, virus isolation is still the "gold-standard" technique for the diagnosis of dengue infection. After the inoculation of C6/36 cells with the viral isolates, DENV-4 could be recovered and genotyped in five of the human cases. This technique is important because virus can be isolated from the supernatant and used for molecular epidemiologic studies by partial or complete viral genome sequencing. Sequencing and phylogenetic analysis have characterised Brazilian DENV-4 as belonging to genotype II (de Sousa et al. 2011).

All individual macerates were initially subjected to conventional RT-PCR, virus isolation and Simplexa™ Dengue real-time RT-PCR. Of the 19 total adult mosquitoes (13 males and 6 females) that were collected, DENV-4 was identified by conventional RT-PCR in a single female *Ae. aegypti* mosquito (1/72; 1.4%) that was captured in one of the residences (15.2). Due to the nature of the genetic material of these mosquitoes, there were many non-specific bands that were visualised on the agarose gel (Fig. 3); therefore, all of the macerates were separately retested using conventional RT-PCR with all four typing primers (TS1, TS2, TS3 and TS4). The same *Ae. aegypti* female, designated 15.2.4/11, was also the only mosquito that was found to be positive for DENV-4 when all macerates were subjected to the Simplexa™ Dengue real-time RT-PCR assay and the mosquito was identified as having a Ct value of 23.5 (Fig. 4). No viruses were recovered from any of the 72 macerates when viral isolation using C6/36 cells was attempted. Real-time RT-PCR detected 1.08 x 10^3 copies/mL of DENV-4 in the macerate from the single *Ae. aegypti* female that was naturally infected.

A single *Ae. aegypti* female collected at a residence in site #1 was identified to be positive for DENV-4 infection by molecular techniques. Additionally, we performed both a NS1 capture ELISA and a NS1 Ag Strip test on all of the 47 macerates that were available from the same location. The same female mosquito (15.2.4/11) was positive by both of the NS1 tests. Interestingly, both tests also detected NS1 in a macerate from an *Ae. aegpypti* male mosquito (15.2.3). The use of the NS1 antigen capture kit for the detection of DENV antigens from *Ae. aegypti* mosquitoes has recently been demonstrated (Tan et al. 2011). However, none of the other techniques that were available could confirm infection or verify the infecting serotype. The transovarial transmission of DENV, which occurs when the virus is transmitted to the progeny of an infected female, has been reported previously (Khin & Khin 1983, Joshi et al. 2002, Le Goff et al. 2011).

DENV detection rates from *Aedes* mosquitoes by RT-PCR may vary depending on the geographical location, epidemiological background or the vector population. In Taiwan, only 0.2% of *Ae. aegypti* females that were analysed were positive for DENV (Chen et al. 2010). However, it has been shown that 16.1% of the *Ae. aegypti* females that were collected from Mexican schools were infected with DENV (García-Rejón et al. 2011). In Brazil, previous studies showed that 17% of the *Ae. aegypti* mosquitoes were infected in a DENV-3 surveillance program that was initiated during an epidemic in the city of Manaus, located in the northern region of Brazil (Pinheiro et al. 2005). Conversely, only 0.1% of adult mosquitoes were found to be infected with DENV-3 in an entomological surveillance study performed in RJ during the inter-epidemic year of 2006 (unpublished observations). In Recife, located in northeastern Brazil, 10% of the tested pools were infected and, despite the

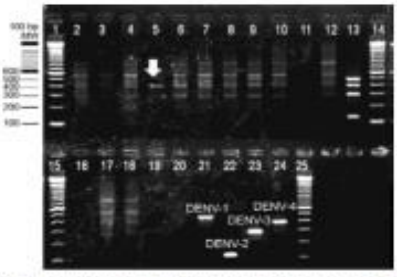

Fig. 3: conventional reverse transcriptase-polymerase chain reaction agarose gel electrophoresis analysis from *Aedes aegypti* adult mosquitoes, individually macerated from the entomological surveillance performed in nine residential and non-residential locations in the São Domingos neighbourhood, Niterói, state of Rio de Janeiro, Brazil in 2011. Lanes 1, 14, 15, 25: 100 bp molecular weight (Invitrogen); 2-12, 16-19: *Ae. aegypti* macerates; 5: dengue virus type 4 (DENV-4) positive *Ae. aegypti* individually macerated; 13: DENV-1-4 positive controls mix; 20: negative control (water); 21-24: DENV-1-4 positive controls, respectively.

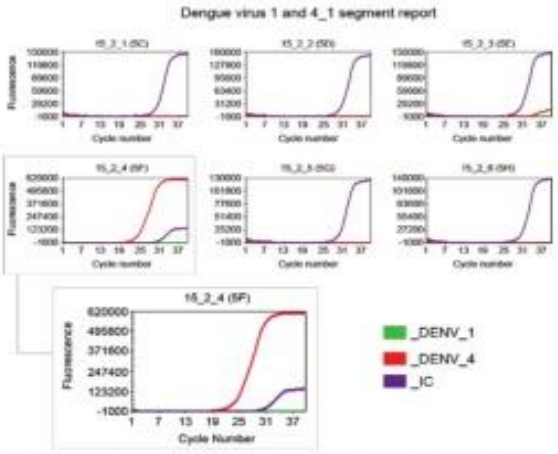

Fig. 4: Simplexa™ Dengue real-time reverse transcriptase-polymerase chain reaction amplification on *Aedes aegypti* mosquitoes collected in the neighbourhood of São Domingos, Niterói, state of Rio de Janeiro, Brazil and individually macerated. Experiment report sheet after reaction. Samples 5C-E, G, H: negative *Ae. aegypti* macerates samples #15.4.1/11, #15.4.2/11, #15.4.3/11, #15.4.5/11 and #15.4.6/11, respectively; 5F: *Ae. aegypti* female #15.2.4/11 positive for dengue virus type 4 (DENV-4) at a cycle threshold of 23.5; red line: DENV-4 probe fluorophore CFR610; purple line: internal control (IC); green line: probe fluorophore Q670.

predominance of DENV-3 in human cases of dengue infection, both DENV-2 and DENV-1 were also detected in mosquitoes (Guedes et al. 2010).

During an entomological surveillance program performed in RJ by our group in 2001, DENV-1 was also detected in *Ae. aegypti* mosquitoes when the presence of DENV-3 was being investigated. Likewise, DENV-1 was also detected during a DENV-4 surveillance study in RR in 2010 (MG de Castro et al., unpublished observations). It has been suggested that a predominant serotype may persist for one or two years until it is replaced by a new serotype (Chow et al. 1998). In the present study, infection with DENV-4 in humans and mosquitoes was confirmed during an explosive DENV-1 epidemic in RJ, as well as in other Brazilian states. During this epidemic, approximately 87% of the confirmed dengue cases that were reported in 2011 were confirmed to be DENV-1 infections (MS/SVS 2011).

Real-time RT-PCR methods have been established as a more rapid and sensitive technique for the detection and quantification of DENV in clinical samples (Drosten et al. 2002, Lai et al. 2007). In this study, we used quantitative real-time RT-PCR to quantify the DENV-4 viral titre (1.08×10^3 copies/mL) from a single *Ae. aegypti* female mosquito that was naturally infected and individually macerated.

We also evaluated, for the first time, the effectiveness of the Simplexa™ Dengue real-time RT-PCR kit for the detection and genotyping of DENV in both human cases and *Ae. aegypti* samples. All of the DENV-4 human cases that were analysed in this study were confirmed using a commercial real-time RT-PCR kit. The Ct values that were observed ranged from 16.8-35.1 (mean ± 25.0). The Ct values in a real-time PCR assay are inversely proportional to the amount of target nucleic acid that is present in the sample. Because all of the samples were obtained during the acute phase of disease (2 samples within 4 days of the onset of symptoms, 3 samples within 2 days of the onset of symptoms, 3 samples within 1 day of the onset of symptoms and 1 sample in which the timing was unknown), high viraemia levels would be expected, resulting in lower Ct values (Fig. 2). From all of the *Ae. aegypti* macerates that were subjected to the Simplexa™ Dengue real-time RT-PCR analysis, only female 15.2.4/11 was positive for DENV-4 and was observed to have a low Ct value (23.5). This suggests that there was a high viral load in the single female (Fig. 3).

Despite the confirmation of DENV-4 cases in RJ, a major DENV-1 epidemic was established at the same time. In December 2011, a new DENV-4 case was identified in Niterói and confirmed by the laboratory methods that were available. This case was identified eight months after the first cases were confirmed. Therefore, our overall results with regard to the laboratory diagnosis and entomological surveillance of dengue using molecular techniques, such as conventional RT-PCR and real-time RT-PCR, show that these approaches are fast, reliable, sensitive and specific for dengue serotype surveillance. Furthermore, these techniques were found to still be effective when a new serotype is introduced or when a serotype re-emerges during a dengue epidemic of a different serotype.

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Artigo 8: Evaluation of a generic RT-nested-PCR for detection of flaviviruses in suspected fatal cases of dengue infection, Rio de Janeiro, Brazil.

Situação do manuscrito: Publicado

Referência: de Araújo JM, Gomes GM, **da Costa Faria NR**, de Araújo ES, de Filippis AM, dos Santos FB, Schatzmayr HG, Nogueira RM.

Resumo: Flaviviruses are significant causes of disease worldwide and can be classified serologically into several antigenic complexes. The purpose of the present study was to evaluate the effectiveness of a generic RT-nested-PCR for detection of flavivirus during a dengue outbreak in Brazil in 2008. A total of 105 serum samples were collected from patients with fatal outcome and examined by generic RT-PCR, conventional RT-PCR, and IgM serology. The generic RT-PCR confirmed 19 of 105 (18%) cases. Conventional RT-PCR performed on 105 serum samples detected 45 (42.8%) dengue virus infections. The IgM serology confirmed 44 of 102 (43.1%) cases. The infecting serotype was identified by generic RT-PCR in 19 cases (18 DENV-2 and 1 DENV-3) and by conventional RT-PCR in 45 cases (40 DENV-2 and 5 DENV-3). In addition, we analyzed the performance of the generic and conventional RT-PCRs and IgM serology on serum samples stratified by the day of onset of symptoms. Our results indicate that different methods should be included in flavivirus surveillance programs, including virological and serological approaches.

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Evaluation of a generic RT-nested-PCR for detection of flaviviruses in suspected fatal cases of dengue infection, Rio de Janeiro, Brazil

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ABSTRACT

Flaviviruses are significant causes of disease worldwide and can be classified serologically into several antigenic complexes. The purpose of the present study was to evaluate the effectiveness of a generic RT-nested-PCR for detection of flavivirus during a dengue outbreak in Brazil in 2008. A total of 105 serum samples were collected from patients with fatal outcome and examined by generic RT-PCR, conventional RT-PCR, and IgM serology. The generic RT-PCR confirmed 19 of 105 (18%) cases. Conventional RT-PCR performed on 105 serum samples detected 45 (42.8%) dengue virus infections. The IgM serology confirmed 44 of 102 (43.1%) cases. The infecting serotype was identified by generic RT-PCR in 19 cases (18 DENV-2 and 1 DENV-3) and by conventional RT-PCR in 45 cases (40 DENV-2 and 5 DENV-3). In addition, we analyzed the performance of the generic and conventional RT-PCRs and IgM serology on serum samples stratified by the day of onset of symptoms. Our results indicate that different methods should be included in flavivirus surveillance programs, including virological and serological approaches.

1. Introduction

The genus *Flavivirus* is characterized by a single-stranded plus-sense RNA genome of approximately 11 kb, constituted by a single open reading frame (ORF) flanked by an untranslated region (UTR) in the 5′ and 3′ termini (Lanciotti et al., 2000). The ORF codes for three structural proteins are capsid (C), membrane (prM/M), and envelope (E), while those for seven non-structural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990).

The genus *Flavivirus* includes more than 70 arthropod-borne viruses that can cause severe encephalitis, hemorrhagic fever, and febrile illness in humans (Monath et al., 1996). Mosquito-borne flaviviruses represent a serious public health issue in Brazil, with Dengue viruses (DENV), Saint Louis Encephalitis virus (SLEV), Bussuquara virus, Cacipacore virus, Iguape virus, Ilheus virus, Rocio virus and Yellow Fever virus (YFV) being isolated from mosquitoes, animals, or humans (Figueiredo, 2000).

In South America, the West Nile virus (WNV) has been reported in neighboring countries of Brazil, including Venezuela (Bosch et al., 2007), Colombia (Berrocal et al., 2006), and Argentina (Morales et al., 2006). For these reasons, the implementation of virological methods may play an important role for rapid diagnosis of new flaviviruses in the country. The purpose of the present study was to evaluate the effectiveness of a generic RT-nested-PCR for detection of flavivirus during a dengue outbreak in Brazil in 2008.

2. Materials and methods

2.1. Patients and samples

The 105 suspected dengue fatal cases included in this study had acute febrile illness with two or more of the following clinical symptoms: headache, retrobulbar pain, myalgia, arthralgia, rash, and hemorrhage. All samples were received refrigerated and separately from private and public hospitals in the metropolitan area of Rio de Janeiro city and stored at −70 °C until tested. All samples were collected between January and May 2008 from patients ranging in age from 5 days to 90 years old. Ethical clearance was obtained with the approval resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil.

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[1] In memoriam.

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2.2. RNA extraction

Viral RNA for the conventional RT-PCR and generic RT-PCR assays was extracted from 140 μL serum samples by the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA), in accordance with the manufacturer's suggested protocol. RNA was eluted in 60 μL of buffer AVE and stored at −70 °C.

2.3. Conventional reverse transcriptase PCR assay

The conventional RT-PCR protocol for DENV detection and typing was performed on 105 serum samples, as described previously by Lanciotti et al. (1992). Briefly, cDNA copies of a portion of the viral genome (capsid/prM) were produced and amplified using two consensus primers designed to anneal to any of the four dengue virus types. Second-round amplification with type-specific primers results in different bands of DNA, characteristic of each dengue virus type, which can be observed directly in 1% agarose gel stained with ethidium bromide. Positive and negative controls were included in all steps.

2.4. Generic reverse transcriptase PCR assay

The generic RT-PCR protocol for *Flavivirus* detection was performed on 105 serum samples, as described previously by Sánchez-Seco et al. (2005). Degenerated primers were designed based on conserved motifs in a region of gene NS5. Positive controls tested by this method were DENV-1, DENV-2, and DENV-3 (obtained from Flavivirus Laboratory, Fiocruz, Brazil), and YFV (17D) (obtained from Biomanguinhos, Fiocruz, Brazil).

2.5. Nucleotide sequencing and phylogenetic analyses

Products from the second round of generic amplification were purified using the PCR purification kit or gel extraction kit (Qiagen, US). Sequencing reactions on both strands were performed with the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, US), and analyzed using an ABI Prism 3730 Sequencer (Applied Biosystems, US).

Electropherograms were visualized by Chromas software version 1.45 (Technelysium Pty. Ltd., Queensland, Australia). Nucleotide sequences were aligned and analyzed using the Clustal X program (Thompson et al., 1997) and later edited by hand. All alignments are available from the authors upon request.

Representative sequences from 10 flaviviruses and arboviruses were retrieved from Genbank (www.ncbi.nlm.nih.gov), including DENV1-4, SLEV, YFV, WNV, Tick-borne encephalitis virus, Powassan virus, and Japanese encephalitis virus. A phylogenetic tree was constructed using MEGA 4 software (Tamura et al., 2007), using the Neighbor Joining (NJ) method and the Tamura Nei model. A bootstrap of 1000 replications was used to estimate the reliability of the predicted tree.

2.6. Serological test

Dengue IgM-capture enzyme-linked immunosorbent assay (ELISA) (PanBio, Brisbane, Australia) was performed on 102 serum samples, according to the manufacturer's instructions.

3. Results

A total of 105 serum samples were collected from patients with fatal outcome and examined by generic RT-PCR, conventional RT-PCR, and IgM serology to evaluate the effectiveness of the generic RT-PCR for use as a diagnostic tool for flaviviruses. Dengue infection was confirmed in 60% (63/105) of cases by the combined results obtained by generic RT-PCR, conventional RT-PCR, and IgM serology. The generic RT-PCR confirmed 19 of 105 (18%) cases. All positive controls tested by generic RT-PCR (DENV1-3 and YFV) rendered positive amplification (Fig. 1). Conventional RT-PCR performed on 105 serum samples detected 45 (42.8%) DENV infections. From those 45 cases positive by RT-PCR, 8 (17.7%) were positive only by this method. The IgM serology confirmed 44 of 102 (43.1%) cases. From those 44 cases positive by IgM serology, 18 (40.9%) were positive only by this method.

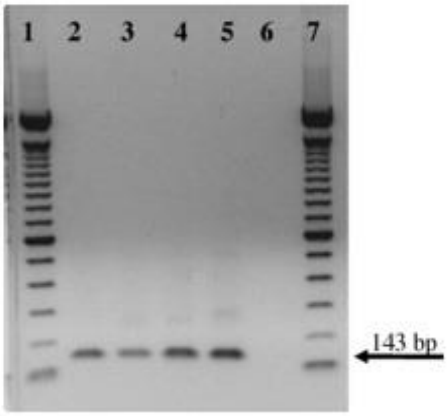

Fig. 1. Agarose gel electrophoresis of generic RT-PCR amplicons. Line 1, 100-bp DNA ladder; lines 2–5, positive controls; line 2, DENV-1; line 3, DENV-2; line 4, DENV-3; line 5, YFV (17D); line 6, negative control; line 7, 100-bp DNA ladder.

The infecting serotype was identified by generic RT-PCR in 19 cases (18 DENV-2 and 1 DENV-3) and by conventional RT-PCR in 45 cases (40 DENV-2 and 5 DENV-3). All 19 cases positive by generic RT-PCR were confirmed by the conventional RT-PCR. The performance of the generic RT-PCR and conventional RT-PCR (RT-PCRs) and IgM serology in serum samples, stratified by the day of onset of symptoms, is shown in Fig. 2. As expected, the molecular methods were more efficient until the fifth day of illness, and IgM serology was more efficient after the eighth day of illness (Fig. 2).

Double-stranded DNA products amplified in the generic nested PCR were directly sequenced as described in Section 2.5. The dengue sequences obtained in this study were submitted to GenBank (accession nos. FJ390025, FJ390026, FJ390027, FJ392583, FJ392584, FJ392585, FJ392586, FJ392587, FJ392588, FJ392589, FJ392590, FJ392591, FJ392592, FJ392593, FJ392594, FJ392595, FJ392596, FJ392597, and FJ392598). DENV-2 and DENV-3 were identified by phylogenetic analysis (Fig. 3), confirming the results obtained by conventional RT-PCR.

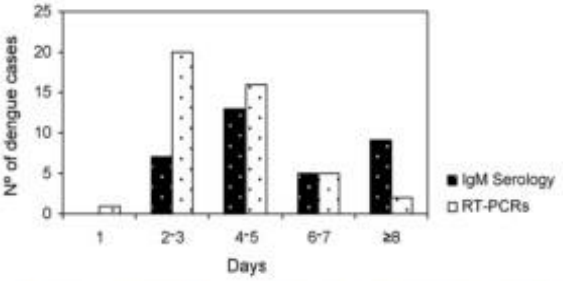

Fig. 2. Detection of dengue virus by day of illness by IgM serology and molecular methods (conventional RT-PCR and generic RT-PCR) in fatal cases during an outbreak in Rio de Janeiro, Brazil, 2008.

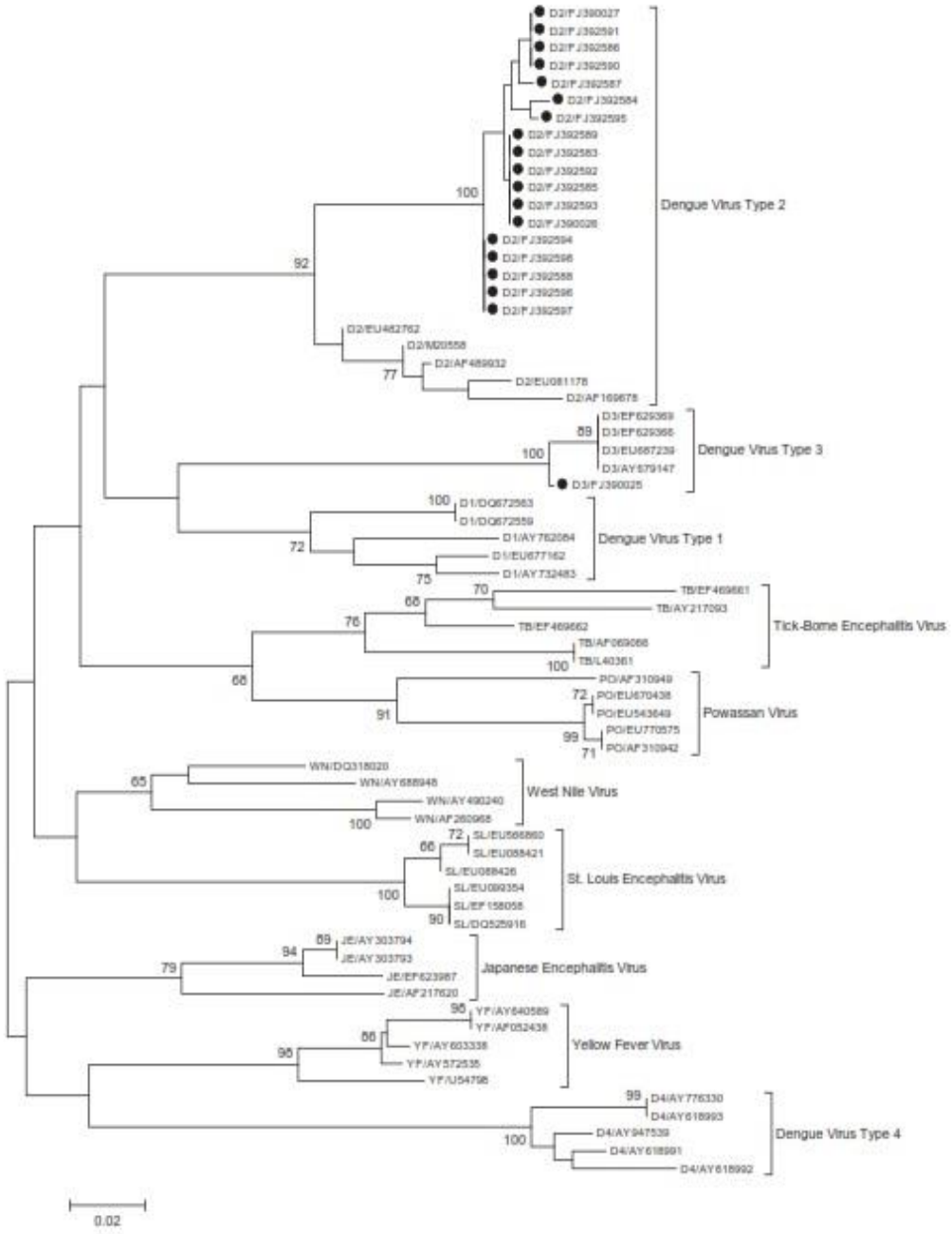

Fig. 3. Phylogeny of 67 flavivirus sequences using 143 nucleotides from the NS5 gene. Strains are denoted by accession numbers from Genbank and viruses are indicated by brackets. Horizontal branch lengths are drawn to scale. Bootstrap values of statistical support for major branches are shown as percentage equivalents.

4. Discussion

The State of Rio de Janeiro is an important epidemiological site for the introduction of flaviviruses, as observed previously for DENV-1 (1986), DENV-2 (1990), and DENV-3 (2000) (Schatzmayr et al., 1986; Miagostovich et al., 1993; Nogueira et al., 2005). Currently, the emergence of new flaviviruses has become an important issue of great public health concern due to the recent incursion and continued transmission of WNV in the Americas (Hayes et al., 2005; Berrocal et al., 2006; Morales et al., 2006; Bosch et al., 2007). In Brazil, the first isolation of SLEV from a human case that was thought to be dengue occurred in the city of São Pedro, State of São Paulo (Rocco et al., 2005). Previous to this case, only two SLEV human infections had been reported in Brazil, both in the Amazon basin (Vasconcelos et al., 1998; Figueiredo, 2000). However, an outbreak of SLEV occurred concomitantly with a large DENV-3 outbreak in São José do Rio Preto, State of São Paulo. During this outbreak, some SLEV patients showed hemorrhagic manifestations

identified by a positive tourniquet test, petechiae, and bleeding (Mondini et al., 2007). Sporadic cases of encephalitis, like those caused by the enzootic flaviviruses, require effective surveillance programs to identify areas of transmission and to enable the immediate implementation of procedures aimed at reducing transmission to humans, including vector control and vaccination.

Several RT-PCRs have been developed for detection of flavivirus RNA by using different pairs of primers for differentiating between species of viruses (Eldadah et al., 1991), including flavi-universal primers for mosquito-borne flaviviruses (Tanaka, 1993) and seven published primers pairs permitting complete detection of the *Flavivirus* genus (Scaramozzino et al., 2001; Chang et al., 1994; Chow et al., 1993; Fulop et al., 1993; Kuno, 1998; Meiyu et al., 1997; Pierre et al., 1994). The generic RT-PCR applied in this study is a very useful method for detection of a wide spectrum of flaviviruses (Sánchez-Seco et al., 2005) and can be used as a complementary method for diagnosing these viruses in Brazil. The amplified flavivirus is identified by sequencing the resulting fragment, and the sequence obtained is compared with those of known flaviviruses in order to identify with precision the detected virus, as shown in Fig. 3. Here, this assay was thoroughly evaluated using a large number of clinical samples during a dengue outbreak in Rio de Janeiro in 2008. The comparison of generic RT-PCR with conventional RT-PCR revealed lower sensitivity of generic RT-PCR, which may be attributed to degenerated primers. As expected, the application of IgM serology showed the importance of serodiagnosis after the eighth day of illness (Fig. 2). These results clearly indicate that different methods should be included in flavivirus surveillance programs, including virological and serological approaches.

Phylogenetic trees of flaviviruses derived from NS5 gene sequences have been described previously (Kuno, 1998; Lanciotti et al., 1999; Scaramozzino et al., 2001; Sánchez-Seco et al., 2005). Furthermore, the phylogenetic tree designed by comparison of the amplification of the nested products in the NS5 location (143 bp) allowed finding all of the complexes of medical interest: the DENV, JEV, YFV, and TBE groups. By using this method, it was possible to identify DENV in 19 patient samples (Fig. 3).

Finally, this generic RT-PCR strategy coupled with DNA sequencing represents a valuable tool for the molecular diagnosis of flaviviruses. However, a definitive identification obviously requires both complete sequencing and the appropriate expertise in flavivirus identification.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

Acknowledgments

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Artigo 9: A review on dengue diagnosis and epidemiology by a regional reference laboratory from 1986 to 2011, Rio de Janeiro, Brazil.

Situação do manuscrito: Artigo publicado no Dengue Bulletin

Classificação QUALIS: Aréa de Medicina II – não se aplica

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Resumo: Dengue fever (DF) activity in Brazil during the past 25 years has been evidenced by a large number of cases in most states. Dengue viruses 1 to 3 (DENV-1, DENV-2 and DENV-3) were introduced in Rio de Janeiro in 1986, 1990 and 2000, respectively. In 2010, DENV-4 re-emerged 28 years after its first isolation. DENV-1 caused an explosive "virgin soil" epidemic in 1986–1987. The introduction of DENV-2 in 1990 caused the first cases of dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Introduction of DENV-3 caused severe epidemics in 2002, with the largest number of DF/DHF cases and deaths. In 2007–2008, the country experienced the most severe epidemic in terms of morbidity and mortality and severe cases in children. Phylogeny performed on DENV-2 identified distinct lineages of the Asian–American genotype. In 2009 and 2010, DENV-1 re-emerged and was prevalent in many Brazilian states. Phylogenetic studies also demonstrated distinct lineages of DENV-1. Since 1986, when virus isolation and immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) were first used, laboratory diagnosis has played an important role in disease surveillance and epidemiology. After the introduction of DENV-2 in 1990, the characterization of immune response performed by the haemagglutination inhibition test was replaced by IgG-ELISA. In the 1990s, real-time reverse transcriptase-polymerase chain reaction (rtRT-PCR) and sequencing were used for nucleic acid detection and characterization. rtRT-PCR and immunohistochemistry proved to be essential for the confirmation and study of fatal dengue cases. NS1 capture tests were used for the early diagnosis of DENV infections after 2007. Since the introduction of DENV, a total of 47 346 suspected dengue cases were

received by the Laboratory of Flavivirus, IOC/FIOCRUZ, Rio de Janeiro, a regional reference laboratory for dengue diagnosis for the Brazilian Ministry of Health, from March 1986 to December 2011. The authors' experience has shown that the implementation of new diagnostic techniques over the years has constituted important and reliable tools for dengue surveillance in Brazil.

A review on dengue diagnosis and epidemiology by a regional reference laboratory from 1986 to 2011, Rio de Janeiro, Brazil

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Abstract

Dengue fever (DF) activity in Brazil during the past 25 years has been evidenced by a large number of cases in most states. Dengue viruses 1 to 3 (DENV-1, DENV-2 and DENV-3) were introduced in Rio de Janeiro in 1986, 1990 and 2000, respectively. In 2010, DENV-4 re-emerged 28 years after its first isolation. DENV-1 caused an explosive "virgin soil" epidemic in 1986–1987. The introduction of DENV-2 in 1990 caused the first cases of dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Introduction of DENV-3 caused severe epidemics in 2002, with the largest number of DF/DHF cases and deaths. In 2007–2008, the country experienced the most severe epidemic in terms of morbidity and mortality and severe cases in children. Phylogeny performed on DENV-2 identified distinct lineages of the Asian–American genotype. In 2009 and 2010, DENV-1 re-emerged and was prevalent in many Brazilian states. Phylogenetic studies also demonstrated distinct lineages of DENV-1. Since 1986, when virus isolation and immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) were first used, laboratory diagnosis has played an important role in disease surveillance and epidemiology. After the introduction of DENV-2 in 1990, the characterization of immune response performed by the haemagglutination inhibition test was replaced by IgG-ELISA. In the 1990s, real-time reverse transcriptase-polymerase chain reaction (rtRT-PCR) and sequencing were used for nucleic acid detection and characterization. rtRT-PCR and immunohistochemistry proved to be essential for the confirmation and study of fatal dengue cases. NS1 capture tests were used for the early diagnosis of DENV infections after 2007. Since the introduction of DENV, a total of 47 346 suspected dengue cases were received by the Laboratory of Flavivirus, IOC/FIOCRUZ, Rio de Janeiro, a regional reference laboratory for dengue diagnosis for the Brazilian Ministry of Health, from March 1986 to December 2011. The authors' experience has shown that the implementation of new diagnostic techniques over the years has constituted important and reliable tools for dengue surveillance in Brazil.

Keywords: Brazil; Dengue virus; Diagnosis; Epidemiology.

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Introduction

Currently, an estimated 2.5 billion people living in urban areas in tropical and subtropical countries in South-East Asia, the Pacific and the Americas are at the risk of infection with a dengue virus (DENV).[1] In 2012, the geographical distribution of dengue covered more than 125 countries, and the Americas have hyperendemic dengue with indigenous transmission in almost all countries.[2] In 2012, Brazil reported 565 510 dengue cases, 4055 severe cases and 284 deaths, or 52% of all dengue cases reported in the Americas.[3] By the second epidemiological week of 2013, the country had already reported 25 879 dengue cases and five deaths.[4]

The state of Rio de Janeiro (RJ) has a land mass of 43 780 172 km^2 and 15 989 929 inhabitants, is divided politically and administratively into 92 municipalities in distinct geographical regions,[5] and has experienced dengue epidemics in the last 25 years. It is an important tourist centre, with a major human population passing through its international airport.

Dengue was first recognized in RJ 90 years ago, when Antonio Pedro described a disease clinically resembling dengue fever in the city of Niteroi.[6] *Aedes aegypti* is considered the main vector for DENV in Brazil,[7] despite the presence of *Aedes albopictus*. After the eradication of *Aedes aegypti* in 1950, the country became dengue free. However, the disease re-emerged with re-infestation of the country with *Aedes aegypti*. Since then, the disease incidence and *vector* abundance follow seasonal patterns in RJ, with peaks during the summer when both the rainfall and temperatures are high.[8,9] The Laboratory of Flavivirus at the Oswaldo Cruz Institute, as a regional reference laboratory for dengue diagnosis for the Brazilian Ministry of Health, supports the dengue surveillance programme in the state.

Despite the outbreak caused by dengue virus serotypes DENV-1 and DENV-4 in Boa Vista, state of Roraima, in 1981–1982, and the reintroduction of *Aedes aegypti* in the 1970s, the minimal circulation of arboviruses in Rio de Janeiro[10] did not encourage research in this field, particularly in light of the state's public health priorities.[11,12] However, Dr Schatzmayr, a renowned virologist at the Oswaldo Cruz Institute, envisaged dengue as a potential threat to RJ and attended an International Dengue Course in Venezuela in March 1986, sponsored by the Pan American Health Organization. By April, serology and virus isolation were established at the Laboratory of Flavivirus at the Oswaldo Cruz Institute. In the same month, the first DENV-1 case was isolated during an outbreak of exanthematic disease in the city of Nova Iguaçu,[13] and, since then, RJ has become an important centre of epidemiological studies of dengue, with the introduction of DENV-2 in 1990[14] and DENV-3 in 2000.[15] DENV-3 was prevalent in the majority of Brazilian states from 2002 to 2006 and, from 2007 to 2008, DENV-3 was displaced by DENV-2. In 2008, Brazil experienced a severe dengue epidemic, with 806 036 reported cases, and RJ alone accounted for 255 818 cases. In 2009, DENV-1 re-emerged in the south-east region and was detected in 50.4% of isolated viruses, displacing

DENV-2 and DENV-3.[16] Despite the introduction of DENV-4 in RJ in 2010 and its subsequent spread to other states of the country, DENV-1 was the most prevalent dengue virus and was responsible for epidemics, with more than 2 million reported cases in Brazil in 2010 and 2011.[17,18]

The dramatic increase of dengue cases in Brazil has led to the establishment and consolidation of a National Dengue Diagnosis Network in 1989,[11] to monitor DENV transmission and spread. Since then, DENV surveillance has been accepted as one of the most important tools for the prediction of dengue epidemics.[19] Each Brazilian state has a Central Laboratory (LACEN), where samples from suspected dengue cases at health centres and public hospitals are tested. The national network is supported by regional reference laboratories responsible for the five Brazilian regions (North, North-east, Midwest, South and South-east): Evandro Chagas Institute (IEC); the National Reference Laboratory, Adolfo Lutz Institute (IAL); LACEN/Distrito Federal; LACEN/Recife; and the Laboratory of Flavivirus at the Oswaldo Cruz Institute (FIOCRUZ/RJ).[20] The latter maintains a surveillance programme in the state of RJ, to detect the role of the state in the introduction and spread of the disease. This paper reports and analyses the epidemiological and laboratory aspects of data obtained during DENV surveillance performed by a regional reference laboratory in RJ, Brazil, between 1986 and 2011.

Materials and methods

Suspected dengue cases

The specimens analysed in this study were collected between March 1986 and December 2011. Suspected dengue cases (n = 47 346) were received during a surveillance programme performed by the Laboratory of Flavivirus, IOC /FIOCRUZ, regional reference laboratory for the Brazilian Ministry of Health, located in RJ. Acute serum samples (collected ≤7 days after the onset of the symptoms) were stored at –70 °C and submitted for virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR) and NS1 antigen-capture enzyme-linked immunosorbent assay (ELISA). Convalescent samples (collected >7 days after the onset of symptoms) were stored at –20 °C and used for immunoglobulin M (IgM) antibody-capture (MAC)-ELISA, haemagglutination inhibition (HI) and immunoglobulin G (IgG) antibody-capture ELISA (IgG-ELISA) tests.

Virus isolation

Virus isolation was performed by inoculation into C6/36 cells,[21] and isolates were identified by indirect fluorescent antibody test, using DENV type-specific monoclonal antibodies.[22]

Generic reverse transcriptase-nested polymerase chain reaction (generic RT-nested-PCR)

Generic RT-nested-PCR for flavivirus detection was performed as described previously,[28] as an alternative molecular tool to detect and confirm DENV infection during the epidemic that occurred in 2008.

Ethics statement

The samples were drawn from a collection belonging to an ongoing project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil.

Results and discussion

DENV infection was first confirmed in the state of RJ in April 1986, when DENV-1 was isolated during an outbreak of exanthematic disease in the city of Nova Iguaçú.[13] From 1986 to 1987, DENV-1 was the only DENV type isolated and was detected in 46.8% of cases, with 95% of isolated viruses producing a cytopathic effect in C6/36 cells.[29] Anti-DENV IgM antibody was detected in 926 DENV-1 cases during the early phase of the disease (day 2) and persisted for 3 months after the onset of symptoms.[30] About 59% of cases analysed by virus isolation and/or MAC-ELISA were confirmed, and the infecting DENV type was detected in 80% of isolations performed ≤4 days after the onset of the symptoms.[31] At that time, the evaluation of clinically based dengue surveillance had demonstrated the need for laboratory-based surveillance.[32] A serological survey estimated that, owing to intense DENV transmission from 1986 to 1987, more than one million persons were infected with DENV-1.[33]

In April 1990, 4 years after DENV-1 had first been isolated, DENV-2 was isolated from a 56-year-old patient with dengue in the city of Niteroi;[14] however, the first death due to dengue shock syndrome (DSS) occurred in June 1991,[34] after the introduction of DENV-2. The introduction of DENV-2 in 1990 resulted in establishment of the HI test for characterization of immune response, as secondary infections led to hospitalizations and severe cases. In fact, the predominant circulation of DENV-2 from August 1990 to 1991 was associated with more hospitalizations,[30] and dengue haemorrhagic fever (DHF) cases were analysed.[35] A seroepidemiological survey conducted in schoolchildren inhabiting the city of Niteroi, RJ, after sequential epidemics caused by DENV-1 and DENV-2, characterized secondary DENV infection in 66% of the children tested by HI test.[36] However, owing to its laborious and time-consuming characteristics, the HI test was replaced with IgG-ELISA in 1995. The protocol described by Miagostovich et al.[25] showed that IgG titres were reliably associated with primary and secondary infections for characterization of the patients' immune response.

found in the samples from fatal cases. Moreover, as more than half of the fatal cases were primary infections, antibody enhancement alone would not have explained the deaths.[45] The rtRT-PCR also yielded the highest rate of positivity in detecting DENV-3 RNA in tissues from those fatal cases.[46] This approach may play an important role for rapid diagnosis of dengue infections, given its accuracy and effectiveness.

DENV-3 was prevalent in RJ and most Brazilian states from 2002 to 2006; however, in 2007, DENV-2 re-emerged and displaced DENV-3. The re-emergence of DENV-2 in 2008 caused the most severe epidemic reported in Brazil and, in RJ, a total of 255 818 cases were reported. Increased disease severity and deaths were observed in children aged 15 years and under.[47] A combination of factors, such as climate, mosquito abundance, susceptible population because of previous low transmission, or viral evolution, could explain the severity of this epidemic.[48] In fact, a phylogenetic analysis of DENV-2 circulating in the 1990s, and after its re-emergence in 2007, identified two distinct lineages within the south-east Asian genotype.[49] Moreover, a study with DENV-2 strains isolated over 20 years confirmed these observations and further characterized these strains.[50]

A recent report by the authors' study group described the results of active DENV surveillance in RJ during an inter-epidemic period (2004–2005): DENV-3 circulation in 2006, re-emergence of DENV-2 in 2007, and a severe epidemic caused by DENV-2 in 2008. From 2004 to 2006, DENV-2 was not evidenced and its detection rate increased from 10.7% in 2007 to 66.6% in 2008. Confirmation of DENV cases by any of the methodologies used (MAC-ELISA, RT-PCR and virus isolation) ranged from 2.3% in 2004 to 20.7% in 2006, characterizing an inter-epidemic period with case confirmations of 34.4% and 46.8% in 2007 and 2008, respectively. In this study, 69 fatal cases were confirmed and 79.7% were due to DENV-2.[51]

Suspected dengue cases in 2008 were tested by a generic RT-nested PCR, as an alternative molecular tool to detect and confirm DENV infection. By using this approach, 18% of the suspected cases were confirmed. Despite its usefulness in detecting a wide range of *Flavivirus*, this method was shown to be less sensitive than the conventional RT-PCR that confirmed 42.8% of the cases, maybe because of the use of degenerated primers by the protocol.[52] Another inconvenience is the need for sequencing amplified RNA.

The NS1 antigen-capture ELISA was established in late 2007 as an alternative approach for the early diagnosis of DENV infections. As previously discussed, the most used techniques for dengue serodiagnosis are based on anti-DENV IgM and IgG detection by using MAC-ELISA and IgG-ELISA. However, one of the limitations consists of the variations on the detection rate during the acute phase of the disease. The Brazilian Ministry of Health established this new approach in sentinel clinics throughout the country after the 2008 dengue epidemic, although this occurred before the evaluation of available commercial tests. Evaluation of the sensitivity and specificity of three commercially available dengue NS1 antigen kits was performed and demonstrated its potential use for early laboratory confirmation of acute DENV infection. The highest sensitivity (89.6%) was obtained by the NS1 Ag Strip (Biorad

Laboratories); however, a lower sensitivity was observed for DENV-3 cases for all three kits.[53] The usefulness of the NS1 tests was also described as an alternative tool to confirm DENV infection on tissue specimens from fatal cases.[54]

In 2009, DENV-1 re-emerged in the south-east region of Brazil and was detected in 50.4% of isolated viruses, thereby displacing DENV-2 and DENV-3. Despite the introduction of DENV-4 in Roraima state in 2010,[55] and its subsequent spread to other states in the country, DENV-1 remained the most prevalent DENV type and was responsible for epidemics with more than two million cases in Brazil in 2010 and 2011.[17,18] In the state of RJ, a total of 29 824 dengue cases were reported in 2010, an increase of 274.5% when compared to 2009. Distinct from what was observed in the whole country, DENV-2 was still prevalent in the state and was detected in fatal-case patients who presented with comorbidities.[18] For the first time, distinct lineages of DENV-1 were reported in RJ in 2010 and 2011.[56]

The first DENV-4 cases reported in RJ in 2011 occurred in two young sisters living in Niterói,[57] and, at the same time, this DENV type was recovered from an individual *Aedes aegypti* female collected in the field during entomological surveillance. Using molecular techniques, DENV-4 was identified and quantified in a single specimen of vector.[58] Phylogenetic studies on Brazilian DENV-4 characterized those viruses as belonging to genotype II,[55,59] and a recent report on DENV-4 isolated in RJ describes the emergence of genotypes I and IIb in the state.[60]

As reported here, dengue has become a major public health problem in RJ, owing to many factors such as the human-host susceptibility, virus emergence and re-emergence and shifts in circulating DENV types/genotypes, vector abundance, and environmental factors. Since the establishment of dengue activity in RJ and Brazil, laboratory diagnosis of dengue has proven to be imperative in surveillance, by serving as an early warning tool. In this scenario, the implementation of the National Dengue Network in the country, and establishment of the reference laboratories for dengue diagnosis have constituted an important effort aiming to help control the disease.

Overall, during the last 25 years (March 1986 to December 2011), a total of 47 346 suspected dengue cases were received in the Laboratory of Flavivirus, IOC/FIOCRUZ, and 41 614 (87.89%) were subjected to one or more of the routine diagnosis techniques available (MAC-ELISA, IgG-ELISA, HI, virus isolation, RT-PCR and NS1 ELISA). The yearly distribution of suspected dengue cases diagnosed by the different methodologies from 1986 to 2011 is shown in Table 1 and Figure 1.

A total of 32 374 cases (77.8% of the total) were tested by MAC-ELISA; 25 037 (60.2%) were subjected to virus isolation, 181 cases (0.4%) to HI test and 829 cases (2.0%) to IgG-ELISA. RT-PCR was performed in 7441 cases (17.9%) and the NS1 antigen-capture ELISA in 1124 cases (2.7%). The distribution of diagnosis and confirmation of suspected dengue cases, according to the methodology implemented and used over the 25-year period, is shown in Table 1.

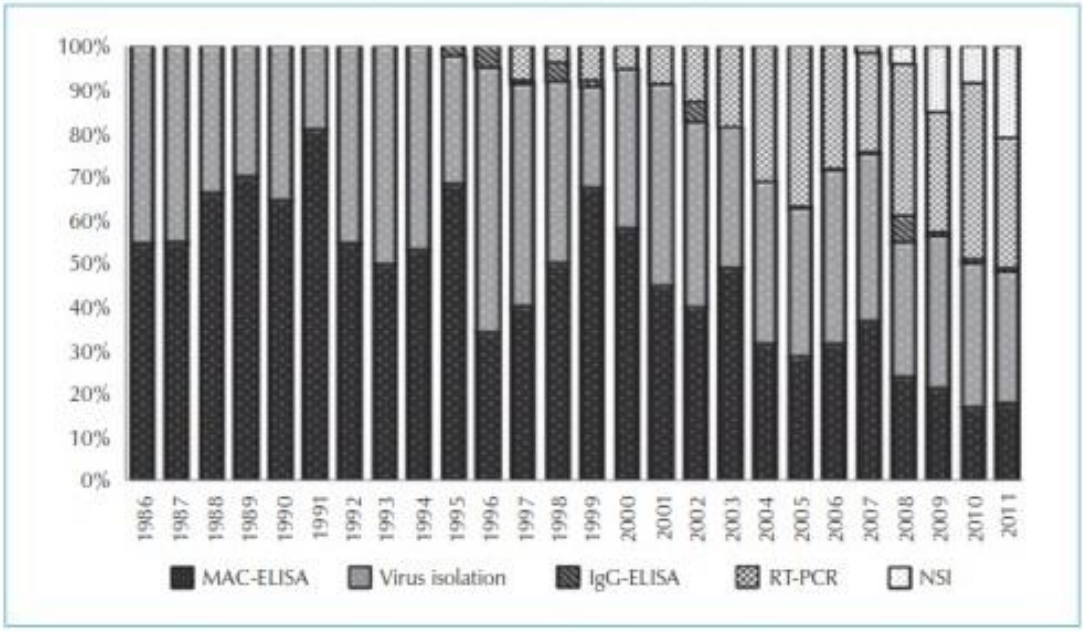
Table 1: Suspected dengue cases confirmed by different methodologies used in routine diagnosis, 1986–2011

Year	MAC-ELISA	Virus isolation		HI		IgG-ELISA		RT-PCR		NS1 capture ELISA
	Positive/ tested	Positive/ tested	DENV types	Tested	Immune response	Tested	Immune response	Positive/ tested	DENV types	Positive/ tested
1986	252/1538	594/1303	594 DENV-1							
1987	623/1544	445/1217	445 DENV-1							
1988	30/391	22/149	22 DENV-1							
1989	57/412	39/177	39 DBNV-1							
1990	446/2669	396/1473	234 DBNV-1, 162 DENV-2	155	43 P, 113 S					
1991	1059/3508	56/841	30 DBNV-1, 26 DENV-2	26	7 P, 19 S					
1992	0/95	0/79	0							
1993	7/119	0/120	0							
1994	79/380	10/334	10 DBNV-2							
1995	1291/4597	129/1974	7 DENV-1, 122 DENV-2			155	77 P, 64 S			
1996	301/630	85/1118	43 DENV-1, 42 DENV-2			92	50 P, 34 S			
1997	150/756	39/953	33 DBNV-1, 6 DENV-2			18	15 P, 3 S	51/145	30 DENV-1, 21 DENV-2	
1998	714/2921	417/2437	388 DENV-1, 19 DENV-2			241	185 P, 54 S	114/229	101 DBNV-1, 13 DENV-2	
1999	234/1097	18/376	12 DENV-1, 6 DENV-2			26	19 P, 7 S	21/126	19 DENV-1, 2 DENV-2	
2000	395/1946	76/1224	19 DENV-1, 32 DENV-2, 1 DENV-3					26/178	19 DENV-1, 6 DENV-2, 1 DENV-3	
2001	1048/2644	498/2732	330 DENV-1, 116 DENV-2, 52 DBNV-3					120/516	49 DENV-1, 32 DENV-2, 39 DENV-3	

Year	MAC-ELISA	Virus isolation		HI		IgG-ELISA		RT-PCR		NS1 capture ELISA
	Positive/ tested	Positive/ tested	DENV types	Tested	Immune response	Tested	Immune response	Positive/ tested	DENV types	Positive/ tested
2002	602/1104	276/1172	7 DENV-1, 4 DENV-2, 265 DENV-3			125	64 P, 45 S	148/353	148 DENV-3	
2003	244/730	50/481	1 DENV-2, 49 DENV-3					41/278	1 DENV-2, 40 DENV-3	
2004	1/143	9/170	1 DINV-1, 8 DENV-3					17/141	1 DENV-1, 16 DENV-3	
2005	80/595	28/711	28 DINV-3			6	6 P	80/771	80 DENV-3	
2006	309/1271	25/1611	25 DINV-3			12	7 P, 5 S	143/1133	143 DINV-3	
2007	901/2015	133/2117	10 DENV-2, 123 DENV-3			19	8 P, 10 S	311/1252	1 DENV-1, 31 DENV-2, 279 DINV-3	71/85
2008	170/321	45/411	33 DINV-2, 12 DINV-3			75	20P, 55 S	179/467	133 DINV-2, 46 DENV-3	27/55
2009	20/128	12/209	7 DENV-1, 13 DENV-2			5	1 P, 4 S	21/167	13 DENV-2, 1 DENV-3	6/90
2010	97/253	99/488	31 DENV-1, 67 DINV-2, 1 DENV-3			16	8 P, 8 S	220/596	50 DENV-1, 158 DINV-2, 12 DENV-3	44/126
2011	237/650	215/1106	157 DINV-1, 49 DENV-2, 9 DENV-4			31	13P, 18 S	348/1089	248 DENV-1, 85 DENV-2, 1 DENV-3, 14 DENV-4	227/768
Total (%)	934/3247 4 (28.8)	376/2598 3 (14.9)	2,409 DENV-1, 530 DENV-2, 564 DENV-3, 9 DINV-4	181	50 P, 132 S	821	473 P, 307 S	1840/7441 (24.7)	518 DENV-1, 496 DINV-2, 806 DENV-3, 14 DENV-4	375/1124 (33.4)

DENV: dengue virus; HI: haemagglutination inhibition test; IgG-ELISA: immunoglobulin G antibody-capture enzyme-linked immunosorbent assay; MAC-ELISA: immunoglobulin M antibody-capture enzyme-linked immunosorbent assay; P: primary infection; RT-PCR: reverse transcriptase polymerase chain reaction; S: secondary infection.

Figure 1: Yearly distribution of diagnosis of suspected dengue cases by different methodologies, 1986–2011



In a retrospective analysis, the overall case confirmation, independent of the methodology used, was 33.8%; however, case confirmation during epidemic years was 59.7% and 58% in the DENV-1 epidemic that occurred in 1986 and 1987, respectively; 52.8% during the DENV-3 epidemic in 2002; 52% during the DENV-2 epidemic in 2008; and 47% and 45% during the DENV-1 epidemic in 2010 and 2011, respectively (see Figure 2).

The implementation of RT-PCR in 1997 constituted an important advance in diagnosis, by detecting the infecting DENV type and identifying DENV in cases that were negative by virus isolation. In some years, RT-PCR identified the infecting DENV type in 40% of cases where the virus was isolated. Virus isolation and RT-PCR identified the infecting DENV type in a total of 4990 dengue cases, and characterized epidemics caused by DENV-1 in 1986 and 1998, caused by the co-circulation of DENV-1 and DENV-2 in 1990, and caused by the co-circulation of DENV-1, DENV-2 and DENV-3 in 2001 (see Table 1, Figure 3). An important benefit of the virus isolation is that low-passage viruses are available for molecular characterization and phylogenetic studies.

Despite the limitations and distinct sensitivities and laboriousness that some diagnostic techniques may present, their contribution to disease surveillance is clear. In the authors' experience, implementation of new techniques may improve diagnosis, increasing viral detection and case confirmation during dengue epidemic and inter-epidemic periods.

Figure 2: Suspected dengue cases confirmed by any of the routine diagnosis methods used, 1986–2011

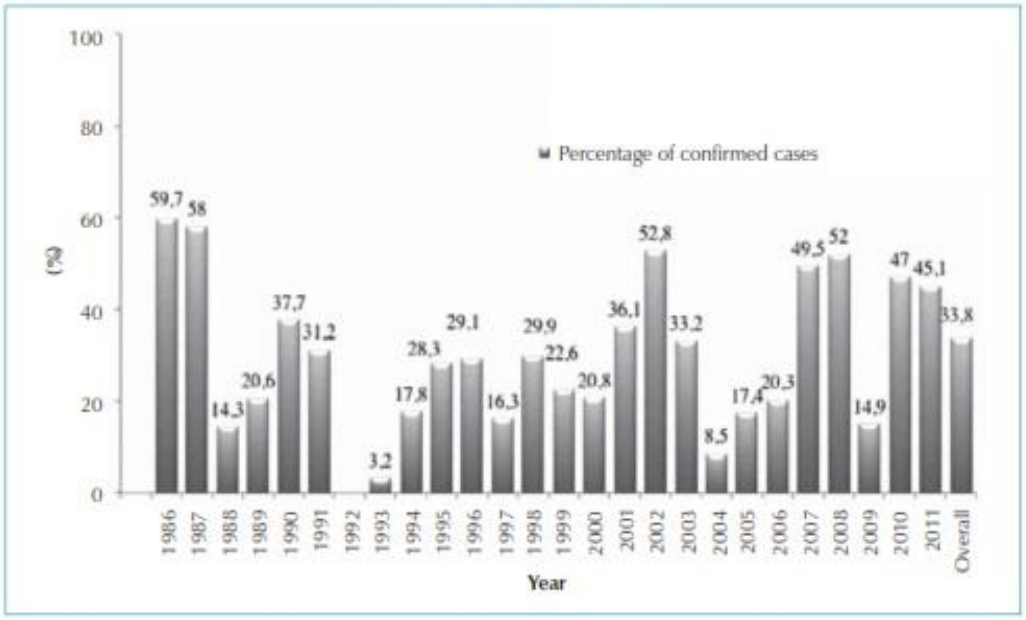
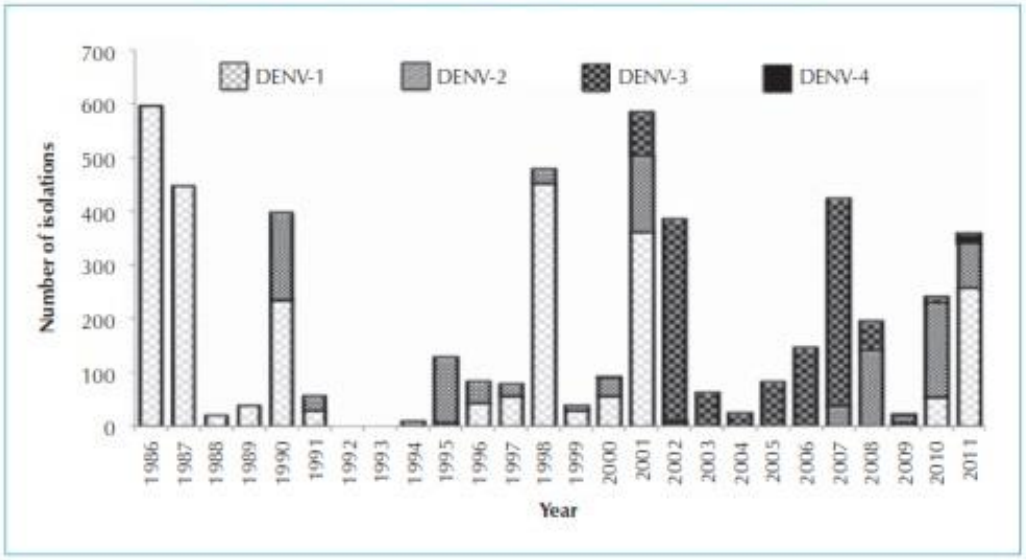


Figure 3: Dengue virus serotypes identified by virus isolation and/or RT-PCR by the Laboratory of Flavivirus, IOC/FIOCRUZ, 1986–2011



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Artigo 10: Impact of the emergence and re-emergence of different dengue viruses' serotypes in Rio de Janeiro, Brazil, 2010 to 2012.

Situação do manuscrito: Publicado na Transactions of the Royal Society of Tropical Medicine and Hygiene

Classifiação QUALIS: Área de Medicina II – B1

Referência: Heringer M, Nogueira RM, de Filippis AM, Lima MR, **Faria NR**, Nunes PC, Nogueira FB, dos Santos FB.

Resumo: BACKGROUND: Rio de Janeiro (RJ) has been of major importance for the epidemiology of dengue viruses (DENVs) in Brazil. After the DENV 1-4 introductions in 1986, 1990, 2000 and 2011, respectively, the state has suffered explosive epidemics. We aimed to describe laboratorial, epidemiological and clinical aspects due to the emergence and re-emergence of distinct DENV in a 2-year period.

METHODS:

Suspected dengue cases (n=2833), including 190 fatal cases, were submitted to virus isolation, RT-PCR and non-structural 1 (NS1) antigen capture ELISA, IgM antibody-capture (MAC)-ELISA and IgG-ELISA.

RESULTS:

Case confirmation was 47.5%. MAC-ELISA confirmed 32.6% of the cases, RT-PCR confirmed 56.3%; DENV was recovered in 33.1% of samples inoculated and NS1 ELISA confirmed 27.5% of the cases. DENV-2 was prevalent in 2010, DENV-1 in 2011 and DENV-4 in 2012. Individuals infected by DENV-3 and over 65 years-old, and children 15 years-old and under infected by DENV-2 had a significantly higher risk of developing a severe disease. Fatal cases confirmed (n=67) were due to DENV-1 (26.8%), DENV-2 (14.9%), DENV-3 (2.9%) and DENV-4 (7.4%).

CONCLUSIONS:

It has been shown here that viral emergences or re-emergences may play different roles

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ORIGINAL ARTICLE

Impact of the emergence and re-emergence of different dengue viruses' serotypes in Rio de Janeiro, Brazil, 2010 to 2012

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Background: Rio de Janeiro (RJ) has been of major importance for the epidemiology of dengue viruses (DENVs) in Brazil. After the DENV 1–4 introductions in 1986, 1990, 2000 and 2011, respectively, the state has suffered explosive epidemics. We aimed to describe laboratorial, epidemiological and clinical aspects due to the emergence and re-emergence of distinct DENV in a 2-year period.

Methods: Suspected dengue cases (n=2833), including 190 fatal cases, were submitted to virus isolation, RT-PCR and non-structural 1 (NS1) antigen capture ELISA, IgM antibody-capture (MAC)-ELISA and IgG-ELISA.

Results: Case confirmation was 47.5%. MAC-ELISA confirmed 32.6% of the cases, RT-PCR confirmed 56.3%; DENV was recovered in 33.1% of samples inoculated and NS1 ELISA confirmed 27.5% of the cases. DENV-2 was prevalent in 2010, DENV-1 in 2011 and DENV-4 in 2012. Individuals infected by DENV-3 and over 65 years-old, and children 15 years-old and under infected by DENV-2 had a significantly higher risk of developing a severe disease. Fatal cases confirmed (n=67) were due to DENV-1 (26.8%), DENV-2 (14.9%), DENV-3 (2.9%) and DENV-4 (7.4%).

Conclusions: It has been shown here that viral emergences or re-emergences may play different roles in the disease epidemiology, especially when many serotypes co-circulate.

Keywords: Brazil, Dengue, Fatal cases, Laboratorial diagnosis, Surveillance

Introduction

Dengue viruses (DENV 1–4) belong to the family *Flaviviridae* and the genus *Flavivirus*.[1] WHO estimates that between 70 and 500 million people are infected with DENV annually worldwide.[2] In Brazil, reinfestation by vectors in the 1970s led to epidemics in 1981–1982 in Boa Vista, Roraima.[3] In 1986, dengue became a public health problem in the country, when the DENV-1 was identified in the serum of patients in an epidemic in the state of Rio de Janeiro (RJ).[4] The introduction of DENV-2 in 1990, also in the state of RJ,[5] led to an increase in the disease severity and the first dengue hemorrhagic fever (DHF) cases were reported in the country.[6] The introduction of DENV-3 occurred in the municipality of Nova Iguaçu, RJ and the emergence of this new serotype caused one of the most severe epidemics reported in the country.[7,8] In 2007–2008, the country experienced the most severe epidemic in terms of morbidity and mortality and severe cases in children due to the re-emergence of DENV-2. A total of 255 818 cases were reported in RJ.[9–11] In 2009, DENV-1 re-emerged in the southeast region of the country and it was this serotype detected in 50.4% of the viral isolations, displacing DENV-2 and DENV-3.[12] In July 2010, DENV-4 was isolated in Roraima,[13] 28 years after its first detection in that same state and soon this serotype spread to other states, including RJ.[14] Despite the epidemic caused by DENV-1, DENV-4 could be isolated during the disease surveillance supported by the laboratorial diagnosis performed.

Dengue has become a major public health problem in RJ due to many factors such as the human host susceptibility, virus emergences, re-emergences and serotype shifts, vector abundance and environmental factors. Since the establishment of dengue activity in Brazil, the laboratorial diagnosis has proven to be imperative for disease surveillance and in many occasions playing a role as an early warning tool. The existence of an ongoing program of virological surveillance aims to detect and monitor the activity of DENV serotypes in the state, where the four serotypes co-circulate.

This study aimed to evaluate the epidemiological, laboratorial and clinical impact of the emergence and re-emergence of different DENV serotypes in the state of RJ, from suspected dengue cases received by the Laboratory of Flavivirus-Regional Reference

Laboratory of the Oswaldo Cruz Institute, FIOCRUZ at RJ from 2010 to 2012.

Materials and methods

Dengue suspected cases

The specimens analyzed in this study comprised the period of January 2010 to December 2012. Dengue suspected case samples (n=2833) were received during a Surveillance Program performed by the Laboratory of Flavivirus, IOC/FIOCRUZ, Regional Reference Laboratory for the Brazilian Ministry of Health, located in RJ. Acute serum samples (up to the 7th day after the onset of the symptoms) stored at -70°C were submitted to virus isolation, RT-PCR and NS1 antigen capture ELISA. Convalescent samples (more than 7 days after the onset of the symptoms) stored at -20°C were tested by MAC-ELISA and IgG-ELISA.

Dengue cases classification

Dengue cases were classified as dengue (cases with or without warning signs) and severe dengue, according to the WHO classification,[15] with modifications due to the lack of information in some cases. The clinical manifestations described in the epidemiological records from each patient were analyzed and positive dengue cases presenting fever with one or more of the following symptoms: hemorrhagic manifestations, central nervous system involvement, shock and transaminases alterations, were considered as severe dengue cases in this study.

Virus isolation

Virus isolation was performed by inoculation into C6/36 *Aedes albopictus* cell line[16] and isolates were identified by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies.[17]

Serological diagnostics

MAC-ELISA: the Panbio dengue IgM Capture ELISA (E-DEN01M) was used for the qualitative detection of anti-DENV IgM antibodies in serum for case confirmation according to the manufacturer's instructions.

IgG antibody detection ELISA (IgG-ELISA): the IgG-ELISA previously described was performed for the characterization of dengue immune response as primary or secondary infections,[18] in dengue cases previously confirmed by virus isolation, RT-PCR and/or MAC-ELISA.

NS1 antigen capture ELISA: for the NS1 antigen capture, the Platelia™ Dengue NS1 Ag-ELISA kit (Biorad Laboratories, Marnes-La-Coquette, France) was used according to the manufacturer's protocol.

Molecular methods

Viral RNA was extracted from sera using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and stored at -70 C for DENV typing.

RT-PCR for detecting and typing DENV was performed as described previously.[19]

The χ^2 and/or Fisher exact tests were used to assess the significance by using Epi Info 7.0.9.34 (CDC, Atlanta, GA, USA).

Results

The highest percentage of the 2833 cases analyzed in this study were from the metropolitan region of RJ (67.2%), and the counties most affected by DENV activity in 2010–2012 were RJ and Niterói, followed by Campos dos Goytacazes located in the north region of the state.

Dengue was confirmed in 47.5% (1323/2833) of the cases analyzed considering any method used for diagnosis. By RT-PCR, 56.3% (1022/1814) of the cases were confirmed; viral isolation confirmed 33.1% (520/1569) of the cases inoculated, MAC-ELISA confirmed 32.6% (376/1153) and NS1-ELISA 27.5% (547/1989). DENV 1–4 were identified by virus isolation and/or RT-PCR in 362 (27.3%), 238 (17.9%), 11 (0.83%) and 423 (31.9%) cases, respectively, out of the 752 samples tested.

In 2010, DENV-2 was the prevalent serotype identified (153/216, 70.8%), however, in 2011 DENV-1 re-emerged and became the serotype responsible for most cases confirmed (253/514, 49.2%). In April of that same year, the first DENV-4 isolations were reported in the state, from cases occurring in the city of Niterói, located in the Metropolitan Region. The introduction of DENV-4 in 2011 in RJ resulted in the emergence of this serotype in 2012; it was responsible for 67.9% (411/605) of dengue confirmed cases and, for the first time, the co-circulation of the four serotypes was reported in the state (Figure 1).

A significant increase ($p<0.05$) was observed in children under 15 years-old among the dengue suspected cases as well as in the confirmed cases (131/247, 52.9%), mainly from 2010 to 2011. An increased case confirmation was also reported in the age group over 65 years ($p<0.001$) from 2011 to 2012 (Table 1).

Overall, dengue classification was performed in 1265 confirmed cases: 6.9% (88) were characterized as severe dengue and 93.1% (1177) as dengue fever. There were fewer severe dengue cases observed among young adults, regardless of the year analyzed. However, a higher number of severe cases among children under 15 years-old was observed in 2010, and in 2011 in the age group over 55 years (Figure 2).

A relationship between the disease severity and type of infection (primary or secondary) was shown (Table 2). Individuals infected by DENV-3 and over 64 years-old had a significantly higher risk of developing severe disease. Furthermore, children 15 years-old and under also had a higher risk for severe disease among patients infected with DENV-2 ($p<0.05$, Table 3).

During the study, the fatal cases of confirmed dengue (n=67) were due to DENV-1 in 26.8% (18) of the cases, to DENV-2 in 14.9% (10), to DENV-3 in 2.9% (2) and to DENV-4 in 7.4% (5). Moreover, fatal cases were more frequently observed in children 15 years and under in 2010 and 2011 (Table 4). Immune response characterization was possible in 67.1% (45/67) fatal cases and 40.0% (18) were due to primary infections and 60% (27) due to secondary ones. In the 18 fatal cases due to DENV-1, eight were from primary infections and six from secondary ones. In nine fatal

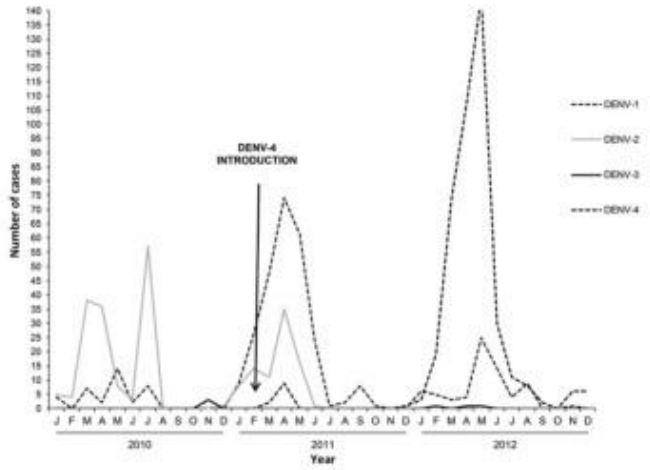

Figure 1. Monthly distribution of dengue viruses serotypes detected by viral isolation and/or RT-PCR in the State of Rio de Janeiro, Brazil, from 2010 to 2012.

Table 1. Age distribution of dengue suspected cases analyzed and confirmed by any methodology used in the laboratory, Rio de Janeiro, Brazil, 2010-2012

	2010		2011		2012	
	Positive cases/Total analyzed (%)					
Age group (years)						
0–5	6/24	(25)	29/87	(33.3)	21/58	(36.2)
6–10	10/30	(33.3)	53/84	(63.9)	30/55	(54.5)
10–15	15/32	(26.8)	49/76	(64.4)	69/117	(58.9)
16–20	13/33	(39.3)	49/105	(46.6)	51/98	(52.0)
21–25	18/35	(51.4)	53/111	(47.7)	58/122	(47.5)
26–30	11/32	(34.3)	46/104	(44.2)	57/121	(47.1)
31–35	13/39	(33.3)	37/87	(42.5)	46/107	(42.9)
36–40	11/27	(40.7)	25/81	(30.8)	59/110	(53.6)
41–45	10/33	(60.6)	35/79	(44.3)	44/90	(48.8)
46–50	26/36	(55.5)	35/66	(53.0)	44/87	(50.5)
51–55	15/31	(48.3)	34/65	(52.3)	31/60	(51.6)
56–60	8/21	(38.0)	14/35	(40.0)	32/57	(56.1)
61–65	8/18	(44.4)	13/26	(50.0)	18/37	(48.6)
>65	6/21	(28.5)	10/24	(41.6)	27/52	(51.9)
Total	174/412	(42.2)	482/1030	(46.7)	587/1111	(52.8)

DENV-2 cases, three were characterized as the primary infection and six as secondary. Fatal cases of DENV-3 and DENV-4 (one each) were due to secondary infections. In 19 cases (42.2%), the infecting serotype identification was not possible, but the immune response was characterized as secondary infection in 13 of those.

Discussion

The Laboratory of Flavivirus at the Oswaldo Cruz Institute, as a Regional Reference Laboratory for Dengue Diagnosis for the Brazilian Ministry of Health, has supported the dengue surveillance program in the state of RJ, since the first dengue case confirmation

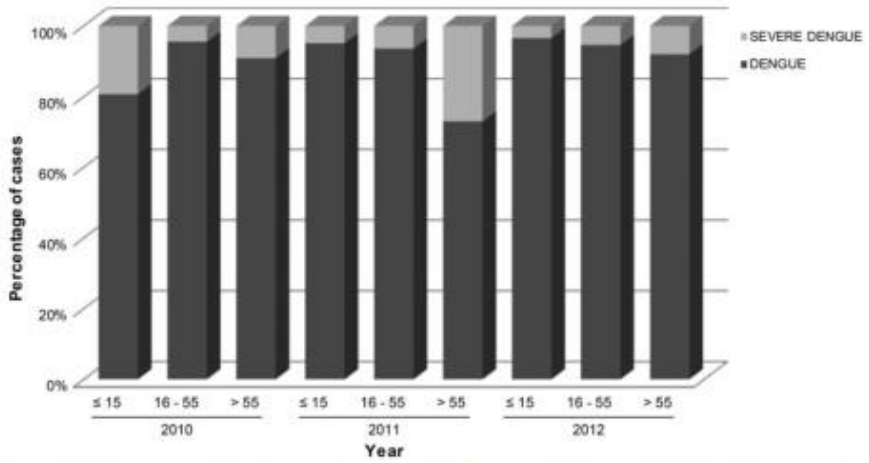

Figure 2. Yearly distribution of dengue and severe dengue cases by age group and year of occurrence, Rio de Janeiro, Brazil, 2010 to 2012.

Table 2. Analysis of factors associated to dengue cases severity in Rio de Janeiro, Brazil from 2010–2012

Factors	Dengue		Severe dengue		p-value	OR (95% CI)
	n	%	n	%		
Age group (years)						
≤15	261	91.3	25	8.7	NS	0.77 (0.47–1.26)
>15–64	839	94.2	52	5.8		
≥65	45	84.5	7	13.5	0.02	2.51 (0.98–6.15)
Serotype						
DENV-1	339	95.0	18	5.0	NS	1.96 (0.87–4.50)
DENV-2	196	94.7	11	5.3	NS	2.07 (0.82–5.23)
DENV-3	6	75.0	2	25.0	0.02	12.3 (1.52–80.98)
DENV-4	406	97.4	11	2.6	NS	0.77 (0.47–1.26)
Type of infection						
Primary	19	79.2	5	20.8		
Secondary	14	32.6	29	67.4	<0.001	7.87 (2.15–30.50)

NS: not significant.

in April 1986, when DENV-1 was first isolated during an outbreak of exanthematic disease in the city of Nova Iguaçu.[4,20] Since then, RJ has been important to the epidemiology of dengue, with the introduction of DENV-2 in 1990[5] and DENV-3 in 2000.[7,8]

Despite the introduction of DENV-4 in Roraima in 2010 and its subsequent spread to other states of the country, DENV-1 was the most prevalent serotype and responsible for epidemics with more than 2 million cases reported in Brazil in 2010 and 2011. However, in RJ, 2010 was characterized by low activity compared to subsequent years, with 29 824 notifications. In 2011, the number of cases increased by over 100% (76 404 cases) and in 2012, an increase of over 200% compared to 2010, was observed with 184 123 cases reported.[21] As observed in other biennia epidemics, most cases occurred in the first months of the year, during the warmer and rainy season.

In 2010, dengue suspected cases received by the Laboratory of Flavivirus, IOC/FIOCRUZ constituted 1.6% of reported cases in the state. In 2011 and 2012, 1.4% and 0.6% of the reported cases in the state of RJ, respectively, were analyzed in the laboratory and this sampling reflected the characteristics of the epidemics.

Table 3. Age and infecting serotype paired analysis and its association to the disease severity in cases studied in Rio de Janeiro, Brazil, 2010–2012

Age group (years)/infecting serotype	Dengue		Severe dengue		OR	(95% CI)	p value
	n	%	n	%			
≤15							
DENV-1	79	93.0	6	7.0	2.08	0.44–10.0	NS
DENV-2	32	86.5	5	13.5	4.27	0.82–24.2	0.04
DENV-3	1	100	NA	NA	NA	NA	NA
DENV-4	82	96.5	3	3.5	NA	NA	NA
16–65							
DENV-1	230	96.2	9	3.8	1.05	0.52–4.33	NS
DENV-2	153	96.8	5	3.2	1.06	0.38–3.21	NS
DENV-3	5	83.3	2	16.7	15.30	1.74–114.1	<0.01
DENV-4	306	98.0	8	2.0	NA	NA	NA
>65							
DENV-1	12	85.7	2	14.3	0.38	0.03–4.32	NS
DENV-2	5	100	NA	NA	NA	NA	NA
DENV-4	15	100	NA	NA	NA	NA	NA

NA: not available; NS: not significant.

Table 4. Age and dengue infecting serotype distribution in fatal cases occurring from 2010 to 2012 in the state of Rio de Janeiro, Brazil

	2010	2011	2012
	Confirmed deaths/deaths analysed (%)		
Age group (years)			
≤15	6/18 (33.3)	10/14 (71.4)	3/12 (25)
16–65	6/33 (18.1)	17/38 (44.7)	14/45 (31.1)
>65	1/5 (20.0)	3/5 (60.0)	2/20 (10.0)
Serotype	Deaths with serotype identified (%)		
DENV-1	3/14 (21.4)	15/33 (45.4)	NI
DENV-2	6/14 (42.8)	3/33 (9.0)	1 (5.0)
DENV-3	NI	1 (3.0)	1 (5.0)
DENV-4	NI	NI	5 (25.0)

NI: not identified.

The metropolitan area was responsible for most cases occurring during the studied period, mainly in the municipalities of RJ and Niterói. This region has a high population density and high-traffic, which partly explains the concentration of the cases studied.[22]

Almost half of the suspected dengue cases analyzed in this study (47.5%; 1323/2833) were confirmed by using any of the methods performed in the laboratory for dengue diagnosis. There was a significant increase ($p<0.001$) in the rate of confirmation of these cases during the study period, mainly in the municipalities of RJ and Niterói, accompanying the increase in notifications during the whole period in the state. In 2011, the number of cases in the state accounted for 21.6% of the number of notified cases in the country.

In a retrospective study, confirmation of dengue cases by the Laboratory of Flavivirus, IOC/FIOCRUZ over a period of 25 years, using any of the methods available, was 33.8%.[23]

Serological methods are still the most useful tool for the diagnosis of the disease during epidemics, as observed since its establishment in 1986 in RJ. In that year, 68.7% of cases tested by MAC-ELISA were confirmed.[24] In this study, 32.6% were confirmed between 2010 and 2012. In the study by Macedo et al. performed in suspected cases occurring between 2004 and 2008, the test confirmed 23% of the cases analyzed.[25]

In 33.1% of the cases analyzed this study, the four DENV serotypes were identified by virus isolation using mosquitoes cell cultures. In 1986/1987 when the first DENV-1 outbreak in virgin soil was characterized, the percentage of virus isolation was 41.2%. The co-circulation of this serotype with DENV-2 in 1990/1991 was identified by virus isolation in 16.7% of the cases tested. In 1995/1996 and 1998, DENV-1 and DENV-2 were the serotype isolated in 9.8% and 18.4% of the patients, respectively.[26]

Between 2004 and 2006, the low activity of DENV was characterized by the low percentage of viral isolation (from 0.35 to 1.1%). However, the re-emergence of DENV-2 resulted in a higher percentage of viral isolation in 2007 and 2008 (5.7% and 10%, respectively).[25] Despite the emergence of DENV-1 in 2009, this study demonstrated that DENV-2 was still the prevalent serotype isolated in 70.8% of the cases tested in 2010. The re-emergence of DENV-1 was evident in 2011, when this serotype was responsible for 49.2% of confirmed cases.

The introduction of DENV-4 in the state of Roraima in 2010 warned of the possibility of a new outbreak of dengue in Brazil.[13] In 2011, the first isolation of DENV-4 in RJ, from cases that occurred in the city of Niterói resulted in the emergence of this serotype in the state in 2012, characterized by isolation of this serotype in 69.3% of confirmed cases. That same year, the co-circulation of the four DENV serotypes was reported for the first time in the state.

Although the virus isolation method is considered the gold standard for diagnosis of dengue fever, the sensitivity of molecular detection of the viral genome offers a great advantage in epidemic periods, as shown by the results presented here. It allowed the viral detection in 56.3% of cases tested in the period, with 91.0% (183/201) of case confirmation in 2010. However, it is noteworthy that the high positivity rate obtained in this study may be the result of the selection criteria of the samples currently adopted in LABFLA/IOC, FIOCRUZ for performing RT-PCR, which includes the samples testing in the acute febrile phase (up to the 5th day of symptoms) and in many cases, previously screened and positive for NS1 antigen. However, previous studies have demonstrated the usefulness of RT-PCR in confirming cases and identifying the infecting serotype when virus isolation was not possible.[23,27,28]

The NS1 antigen capture test is a more recently developed serological method which enables the early diagnosis of DENV infections, during the initial stage of the disease, even in laboratories with limited human resources and equipment.[29] The hexameric form of the NS1 protein is highly conserved in the four serotypes and found circulating in the serum of patients from the first to ninth day after the onset of fever.[30–32] In the Laboratory at FIOCRUZ, this test was first introduced in 2007 and evaluated as an alternative diagnostic method presenting a sensitivity of 89.6% in confirmation of DENV 1–3 cases.[32] In 2008, the Brazilian Ministry of Health established the NS1 antigen capture test in sentinel units for early identification of suspected cases in several states. That same year, the ELISA Platelia™ Dengue NS1 Ag-ELISA confirmed 49% (27/55) of the cases analyzed in LABFLA / IOC, FIOCRUZ, however in this study, the confirmation of cases were 36.5, 29.8 and 25% in 2010, 2011 and 2012, respectively.

The introduction of a new serotype of DENV is associated with the occurrence of major epidemics and increased proportion of severe cases,[33] more frequent in individuals susceptible to the new serotype circulating. Due to the lack of information on some patients' records, the classification of cases according to the new WHO criteria[15] was not possible. In this study, hemorrhage, shock, increased transaminases levels and central nervous system involvement was considered for severe case classification. From 2010 to 2012, 6.9% of the cases confirmed were classified as severe dengue cases and this observation is in agreement with those reported for the state of RJ.

Our results suggested a significantly higher risk for developing severe disease among children 15 years-old and under in 2010, and individuals over 65 years-old in 2011, while in 2012 there was a more homogeneous distribution among age groups. However, disregarding the period studied there was a significant difference between age groups. It has been demonstrated that individuals infected with DENV-3 had a greater chance of presenting signs and symptoms associated with severity (OR=12.30, 95% CI 1.52–80.98, $p<0.05$), however this should be carefully addressed due to the small number of DENV-3 cases represented in this study. It also demonstrated an increased risk of severity in individuals older than 64 years (OR=2.51, 95% CI 0.98–6.15, $p<0.05$). Additionally, it demonstrated an increased risk of severe dengue in secondary cases.

The higher fatal case confirmation in this study was observed in 2011, when a prevalent circulation of DENV-1 was observed in the state. Despite this, an increased number of deaths from secondary infections and due to DENV-2 were reported. However, an increase in deaths due to primary infection was also described.

As previously described, the four DENV serotypes were reported simultaneously for the first time in RJ, however one of the limitations of the study was the low number of DENV-3 cases identified during the period.

Dengue has become a major public health problem in RJ due to many factors such as the susceptibility of the human host, emergence and re-emergences with alternating circulation of serotypes, high rate of infestation of mosquito vectors and environmental factors. In this scenario, it also became evident that laboratory diagnosis has a major role in disease surveillance, both in inter-epidemic periods and epidemics, and acting preventively as a tool for early detection of dengue cases. Furthermore, it has been shown here that viral emergences or re-emergences may play different roles in the disease epidemiology, especially when many serotypes co-circulate.

Authors' contributions: FBS, RMRN, AMBP designed the study. MH, MRQL, NRCF, PCGN, FBN, implemented the study. FBS, MH, RMRN and AMBP analyzed the data and FBS and MH wrote the paper. All authors read and approved the final version of the paper. FBS is the guarantor of the paper.

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Competing interests: None declared.

Ethical approval: The samples belong to a collection from an ongoing Project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health-Brazil.

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Artigo 11: Insights of the genetic diversity of DENV-1 detected in Brazil in 25 years: Analysis of the envelope domain III allows lineages characterization.

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Resumo: Dengue virus type 1 (DENV-1) was first isolated in Brazil in 1986 in the state of Rio de Janeiro (RJ) and during 25 years, this serotype emerged and re-emerged causing explosive epidemics in the country. Here, we aimed to present the phylogeny and molecular characterization based on the envelope gene (E) of DENV-1 (n=48) isolated during epidemics occurred from 1986 to 2011. Six full coding region genomes of DENV-1 were fully sequenced and possible genomic recombination events were analyzed. The results showed that the Brazilian DENV-1 isolates analyzed belong to genotype V (Americas/Africa), but grouping into distinct clades. Three groups were identified, one dating from 1986 to 2002 (lineage 1a), a second group isolated from 2009 to 2011 and a representative strain isolated in 2002 (lineage 2), and a group of strains isolated from 2010 to 2011 (lineage 1b). The lineages 1a and 1b were more closely related to the American strains, while lineage 2 to the Asian strains. Amino acids (aa) substitutions were observed in the domains I and III of the E protein and were associated to the lineages segregation. A substitution on E297 differentiated the lineage 1a from the lineages 1b and 2. Substitutions on E338, E394 (domain III), E428 and E436 (stem region) differentiated lineages 1a, 1b and 2. With the exception of the C gene, all the others genes analyzed allowed the DENV-1 classification into the distinct genotypes. Interestingly, the E gene's domain III and stem regions alone were able to characterize the distinct lineages, as observed by the analysis of the entire E gene and the complete coding region. No recombinant events were detected, but a strain belonging to lineage 1a was closely related to a known recombinant strain (AF513110/BR/2001).

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Insights of the genetic diversity of DENV-1 detected in Brazil in 25 years: Analysis of the envelope domain III allows lineages characterization

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ABSTRACT

Dengue virus type 1 (DENV-1) was first isolated in Brazil in 1986 in the state of Rio de Janeiro (RJ) and during 25 years, this serotype emerged and re-emerged causing explosive epidemics in the country. Here, we aimed to present the phylogeny and molecular characterization based on the envelope gene (E) of DENV-1 ($n = 48$) isolated during epidemics occurred from 1986 to 2011. Six full coding region genomes of DENV-1 were fully sequenced and possible genomic recombination events were analyzed. The results showed that the Brazilian DENV-1 isolates analyzed belong to genotype V (Americas/Africa), but grouping into distinct clades. Three groups were identified, one dating from 1986 to 2002 (lineage 1a), a second group isolated from 2009 to 2011 and a representative strain isolated in 2002 (lineage 2), and a group of strains isolated from 2010 to 2011 (lineage 1b). The lineages 1a and 1b were more closely related to the American strains, while lineage 2 to the Asian strains. Amino acids (aa) substitutions were observed in the domains I and III of the E protein and were associated to the lineages segregation. A substitution on E_{287} differentiated the lineage 1a from the lineages 1b and 2. Substitutions on E_{338}, E_{394} (domain III), E_{428} and E_{436} (stem region) differentiated lineages 1a, 1b and 2. With the exception of the C gene, all the others genes analyzed allowed the DENV-1 classification into the distinct genotypes. Interestingly, the E gene's domain III and stem regions alone were able to characterize the distinct lineages, as observed by the analysis of the entire E gene and the complete coding region. No recombinant events were detected, but a strain belonging to lineage 1a was closely related to a known recombinant strain (AF513110/BR/2001).

1. Introduction

Dengue viruses (DENV 1–4) belong to the *Flaviviridae* family and *Flavivirus* genus and exist in either sylvatic or human transmission cycles, most prevalent in tropical and subtropical areas (Vasilakis et al., 2011). The disease has become a major public health problem with relevant social and economical impact due to the increased geographic extension, number of cases and disease severity (Guzman and Harris, 2015). The viral genome of approximately 11 kb in size, encodes three structural proteins (capsid [C], membrane [M] and envelope [E], seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) and is flanked by approximately 100 nucleotides (nts) at the 5′ untranslated region (UTR) and 388–462 nts at the 3′ UTR (Chambers et al., 1990; Shurtleff et al., 2001; Miller et al., 2010).

The four DENV serotypes share a 65–70% genome sequence homology and are clustered into different genotypes due to high mutation rates (Holmes and Twiddy, 2003). DENV-1 falls into five distinct genotypes designated as genotype I (Southeast Asia, China and East Africa), genotype II (Thailand), genotype III (Malaysia), genotype IV (South Pacific) and genotype V (Americas/Africa) and, the existence of lineages with distinct geographic and temporal relationships, have been reported previously in the Americas (Myat Thu et al., 2005; Kukreti et al., 2009) and Asia (Zhang et al., 2005, 2014; Carrillo-Valenzo et al., 2010; Duong et al., 2013; Lambrechts et al., 2012; Shin et al., 2013). The term "lineage" has been used to characterize those viruses clustered in clades in a taxonomic level beneath genotype (Mendez et al., 2010). Furthermore, those genetically distinct lineages may temporally emerge or disappear on a regular basis (Drumond et al., 2012;

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Choudhury et al., 2014) however, the mechanisms involving lineage's replacement, emergences or disappearances are not fully known.

DENV evolution has a major impact on its virulence to humans and epidemiology of the disease worldwide. Although antigenic and genetic differences in viral strains are evident, the lack of an animal model of the disease, difficulties the studies on DENV pathogenesis. The patients immune status and genetic characteristics of the host are also determinants for the disease outcome. However, phylogenetic studies also allow the correlation between specific genotypes and disease severity. Although genetic variants of DENV have been implicated in disease severity, it was with the advances in evolutionary studies based on phylogenetic analysis combined to epidemiological data that, genotypes within each serotype may be associated with a greater or lesser disease severity.

Phylogenetic studies based on either partial and/or complete viral genomic sequences have contributed for the understanding of DENV evolution and diversity, including recombination events (Weaver and Vasilakis, 2009). Evidences for DENV-1 recombination have been reported previously (Tolou et al., 2001; dos Santos et al., 2002; Aaskov et al., 2007; Chen et al., 2008; Weaver and Vasilakis, 2009; Carvalho et al., 2010; Shin et al., 2013; Chu et al., 2013).

In Brazil, more than eight million dengue cases were reported over the past 29 years (Silva, 2013; SVS/MS, 2013, 2014) and, the state of Rio de Janeiro in particular, has been important for the disease epidemiology, since DENV-1 introduction and spread in 1986. After epidemics caused by the introduction of DENV-2 in 1990, DENV-3 in 2000 and the re-emergence of DENV-2 in 2008, DENV-1 re-emerged in 2009, displacing DENV-2 and DENV-3 and caused more than 1 million probable cases in 2010 (reviewed in dos Santos et al., 2013). The phylogeny of DENV-1 recently re-emerged characterized the existence of multiple lineages for the first time in the country (dos Santos et al., 2011). However, a more detailed analysis, broader characterization of strains and possible recombination events within this serotype, was not performed. Therefore, herein, we aimed to perform the phylogeny, molecular characterization and analysis of possible recombination events of based on the analysis of the E gene (1,485 nucleotides [nts]) and complete coding region (10,176 nts) of Brazilian DENV-1 isolates over the 25 years (1986–2011).

2. Materials and methods

2.1. Ethical statement

The strains analyzed in this study belong to a previously gathered collection from the Flavivirus Laboratory, IOC/FIOCRUZ, Rio de Janeiro, Brazil, obtained from human serum from an ongoing Project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil. Samples were chosen anonymously, based on the laboratorial results and clinical manifestations available on the Laboratory database.

2.2. Dengue viral strains

The DENV-1 strains ($n = 48$) analyzed were detected in serum samples from patients positive for dengue by virus isolation and/or RT-PCR, received at the Flavivirus Laboratory (LABFLA), IOC/FIOCRUZ, Regional Reference Center for Dengue and Yellow Fever Diagnosis from epidemics occurred between 1986 and 2011. Viral strains were selected according to the year of isolation, state of origin and clinical manifestation presented, according to the WHO (2009) classification: dengue without warning signs (DWWS), dengue with warning signs (DWS) and severe dengue (SD). The Brazilian states represented were: Rio de Janeiro (RJ), Minas Gerais (MG), Espírito Santo (ES), Mato Grosso do Sul (MS), Ceará (CE), Alagoas (AL) Rio Grande do Norte (RN) and Piaui (PI). Forty-three strains were derived after one or two passages of the original isolate on cell culture and five were analyzed directly from the serum sample. Virus isolation was performed by inoculation into C6/36 *Aedes albopictus* cell line (Igarashi, 1978) and isolates were identified by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies (Gubler et al., 1984).

2.3. Viral RNA extraction

Viral RNA was extracted from 140 μL of supernatant from cultures isolated or serum using the QIAmp Viral Mini Kit (Qiagen, Inc., Germany) according to the protocol described by the manufacturer and stored at −70 °C.

2.4. Reverse transcription followed by the polymerase chain reaction (RT-PCR)

The methodology described by Lanciotti et al. (1992) was used to confirm the positivity of DENV-1 strains. This protocol detects all four serotypes simultaneously in a semi-nested procedure, generating amplification products with specific size in base pairs (bp) of each DENV serotype.

2.5. Dengue virus genome amplification, sequencing and recombination analysis

For E gene sequencing of DENV-1, primers sets 1–4 were used to amplify overlapping fragments of approximately 900 bp and 16 sets to amplify the complete genome coding region (Table 2). Five microliters of the extracted RNA was reverse transcribed into cDNA and amplified for sequencing using AccessQuick™ RT-PCR System (Promega Corporation, Wisconsin, USA). The thermocycling parameters consisted of one cycle of reverse transcription (42 °C/60 min), followed by 40 cycles of denaturation (94 °C/35 s), annealing (63 °C/1 min) and extension (72 °C/2 min), ending with a final extension cycle (72 °C/10 min), in a GeneAmp® PCR System 9700 (Applied Biosystems®, California, USA). PCR products were sequenced in both directions using the BigDye Terminator Cycle Sequencing Ready Reaction version 3.1 kit (Applied Biosystems®, California, USA) and the thermocycling conditions consisted of 35 cycles of denaturation (94 °C/1 min), annealing (63 °C/2 min) and extension (72 °C/3 min). Sequencing was performed on an ABI 3730 DNA Analyzer, Applied Biosystems®, California, USA (Otto et al., 2008) and the sequences generated were deposited on GenBank (Table 1). The sequences analysis was performed using the Chromas® 1.45 (http://www.technelysium.com.au/chromas14x.html). The percentages of nts and amino acid (aa) identities were determined using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html.), sequences' identity was performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and alignments using CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylog enetic trees were constructed using the MEGA 6 (http://www.megasoftware.net/), by the "Maximum-Like lihood" method, according to the Tamura-Nei 93 model and Gamma distribution (TN93 + G), with a bootstrap of 1000 replications. The trees were built based on the analysis of the best-fit model for each dataset, as provided by the software. Six discrete Gamma categories were used to model evolutionary rate differences among sites. The Brazilian strain AF226685.2/BR/90 was considered as a reference sequence for comparison purposes and strains representative from the five genotypes available in

Table 1
DENV-1 strains used in this study for partial (E gene; $n = 43$) and complete coding region sequencing ($n = 6$) of the genome.

ID/state/year of isolation	Origin of strain	Passage in C6/36 culture	Case Classification (WHO, 2009)	Genes sequenced	Genbank accession number	References
31708/RJ/1986	Isolated (C6/36)	1st	DWWS	C/prM/M/E	HQ026760	dos Santos et al. (2011)
31807/RJ/1986	Isolated (C6/36)	1st	DWWS	C/prM/M/E	JN122280	dos Santos et al. (2011)
36034/RJ/1988	Isolated (C6/36)	2nd	DWWS	(C → NS5)	KF072761	This study
38159/RJ/1989	Isolated (C6/36)	1st	DWWS	(C → NS5)	KF072762	This study
45907/MS/1991	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072791	This study
58485/MG/1997	Isolated (C6/36)	1st	SD	C/prM/M/E	KF072769	This study
58522/MG/1997	Isolated (C6/36)	1st	NI	C/prM/M/E	KF072770	This study
58540/RJ/1997	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF072771	This study
58610/RJ/1997	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072772	This study
58724/RN/1997	Isolated (C6/36)	1st	NI	C/prM/M/E	KF072773	This study
60068/RJ/1998	Isolated (C6/36)	1st	SD	C/prM/M/E	KF072774	This study
60443/CE/1998	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072775	This study
60606/MG/1998	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF072776	This study
60619/MG/1998	Isolated (C6/36)	1st	SD	C/prM/M/E	KF072777	This study
62114/ES/1998	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072778	This study
62189/ES/1998	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF072779	This study
64450/RJ/1999	Isolated (C6/36)	2nd	DWS	C/prM/M/E	KF072780	This study
64451/RJ/1999	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072781	This study
64616/RJ/1999	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072792	This study
66508/RJ/2000	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072782	This study
66694/ES/2000	Isolated (C6/36)	1st	NI	(C → NS5)	KF072763	This study
67993/PI/2000	Isolated (C6/36)	1st	NI	(C → NS5)	KF072787	This study
68826/RJ/2001	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072764	This study
71602/RJ/2001	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF072788	This study
75424/ES/2002	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072783	This study
15_2010/MS/2010	Serum		DWWS	C/prM/M/E	HQ896612	dos Santos et al. (2011)
876_2010/MS/2010	Isolated (C6/36)	1st	NI	C/prM/M/E	KF072768	This study
2676_2010/RJ/2010	Isolated (C6/36)	2nd	Fatal case	C/prM/M/E	KF072786	This study
1141_2011/AL/2010	Isolated (C6/36)	1st	NI	C/prM/M/E	JQ015184	This study
1142_2011/AL/2010	Isolated (C6/36)	1st	NI	C/prM/M/E	JQ015185	This study
122_2011/RJ/2011	Isolated (C6/36)	1st	DWWS	C/prM/M/E	JN122281	dos Santos et al. (2011)
169_2011/RJ/2011	Isolated (C6/36)	1st	Fatal case	C/prM/M/E	KF072765	This study
325_2011/RJ/2011	Isolated (C6/36)	1st	SD	C/prM/M/E	KF072766	This study
1049_2011/RJ/2011	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF072790	This study
1266_2011/RJ/2011	Isolated (C6/36)	1st	Fatal case	(C → NS5)	KF072760	This study
73834/ES/2001	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072789	This study
55_2009/ES/2009	Isolated (C6/36)	1st	NI	C/prM/M/E	HM043709	dos Santos et al. (2011)
1433_2009/RJ/2009	Serum		DWWS	C/prM/M/E	HQ026761	dos Santos et al. (2011)
1435_2009/RJ/2009	Serum		DWWS	C/prM/M/E	HM043710	dos Santos et al. (2011)
19_2010/RJ/2010	Serum		DWWS	C/prM/M/E	HQ026762	dos Santos et al. (2011)
20_2010/RJ/2010	Serum		DWWS	C/prM/M/E	HQ896613	dos Santos et al. (2011)
188_2010/RJ/2010	Isolated (C6/36)	1st	DWS	C/prM/M/E	HQ896614	dos Santos et al. (2011)
242_2010/RJ/2010	Isolated (C6/36)	1st	DWWS	(C → NS5)	KF072759	This study
516_2010/ES/2010	Isolated (C6/36)	1st	Fatal case	C/prM/M/E	KF072785	This study
568_2010/ES/2010	Isolated (C6/36)	1st	Fatal case	C/prM/M/E	KF719187	This study
594_2011/CE/2010	Isolated (C6/36)	1st	NI	C/prM/M/E	JN982362	This study
512_2011/RJ/2011	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF072767	This study
514_2011/RJ/2011	Isolated (C6/36)	1st	Fatal case	C/prM/M/E	KF072784	This study

ID: Identification; BR: Brazil; RJ: Rio de Janeiro, ES: Espírito Santo; MG: Minas Gerais; MS: Mato Grosso do Sul; EC: Ceará; AL: Alagoas; PI: Piauí; NI: No Information; SD: severe dengue; DWWS: dengue without warning signs; DWS: dengue with warning signs; C: capsid; prM: pre-membrane; M: membrane; E: envelope; NS5: nonstructural protein 5; WHO: World Health Organization.

Genbank (www.ncbi.nlm.nih.gov) were also used for the analysis. The analysis of the aa substitutions in the E gene was performed using the Bioinformatics Toolkit (http://toolkit.tuebingen.mpg.de/). The Visual Molecular Dynamics Program (Humphrey et al., 1996; http://www.ks.uiuc.edu/Research/vmd/) was used for the aa localization on the E protein. The analysis to detected possible recombination events among DENV-1 isolates was performed using the RDP and BOOTSCAN methods in the Recombination Detection Program 3 (Martin et al., 2010; http://darwin.uvigo.es/rdp/rdp.html), by using default parameters.

3. Results

Aiming to perform the molecular characterization and phylogenetic analysis of DENV-1 during 25 years of viral activity in Brazil (1986–2011), viral strains ($n = 48$) isolated from patients presenting different disease manifestations were sequenced. Partial

Table 2
Oligonucleotide primers used for the amplification of the E gene and the complete coding region of Brazilian DENV-1 strains.

ID primer	Primer sense A (5′-3′)	Primer antisense B (5′-3′)	Position in the genome	Amplicon (bp)	Tm (°C) A/B
1	TTA GTC TAC GTG GAC CGA CAA GAA	GCC TAT TCC CAC GCA TCG	6–938	932	62/63
2	TGA CCT ATG GGA CGT GTT CTC A	CAG TCC AAT GTG AGG GCT CC	660–1469	809	63/63
3	GAC GCG AAC TTT GTG TGT CG	GGC GCA TCT GTT CCT TCG TA	1193–1900	707	63/64
4	CTG GGA TCA CAA GAA GGA GCA	CCA ATG GCT GCT GAC AGT CTT	1691–2539	848	63/63
5	GGG ATT AAA TTC AAG GAG CAC G	ACT TGC CTA GAT GCC ATG GC	2332–3217	885	62/62
6	TGT GTG ACC ACC GGC TAA TG	CAT TGC AAG TCC ATC CCC TAG	2952–3842	890	63/62
7	CCT AGC CTT GAT GGC TAC TCT CA	GCC CAA CAG TCC TCT TTG CA	3697–4596	899	62/64
8	CCA CTT TGC TGG CAG TCT CA	TGT AAT TGG GAA CTC TCA CTG GG	4431–5335	904	63/62
9	CTC CCA CAA GAG TTG TCG CC	TCC CCG TCT ATT GCT GCA CT	5187–6068	881	63/63
10	TGC CCA AAG GAG AGG AAG AAT	GTC CTG TGG AGT GCG CTG T	5884–6751	867	62/63
11	TGA CGC TGT TCT TCC TAT CAG G	CCA GAA TTT TCC TGG AGA TCC C	6579–7447	868	62/63
12	GTG GAC GGG ATT GTT GCA AT	CCC TCC ATG TTT CCT TTG CA	7268–8160	892	63/63
13	CTG AGA AAT GTG ACA CCC TTC TGT	TTG GTT TTC ATC AAC GAA CAC TG	7983–8802	819	62/62
14	CAC ACC CTT CGG ACA ACA GA	CCA TGT TTT TCC AAC CAG TCA AG	8808–9500	892	62/63
15	AAA TGG AAC CGT GAT GGA TGT T	TCT CAT TCC CGA TGA GCC TT	9325–10183	858	63/62
16	GTG GAA TAG GGT TTG GAT AGA GGA A	TTC TGT GCC TGG AAT GAT GCT	10006–10675	669	63/63

[*] According to the DENV-1 strain Genbank Accession number AF513110.

genome sequencing (C/prM/M/E genes; 2325 nts) was performed in the 48 DENV-1 strains and full-length genome sequencing (complete coding region; 10,176 nts) was performed in 6 strains. A total of 22 DENV-1 strains were from DWWS cases, 7 from DWS, 4 SD and 6 from fatal cases. The geographical origin of the strains represented the Southeast region – Rio de Janeiro (RJ), Minas Gerais (MG) and Espírito Santo (ES); Northeast region-Alagoas (AL), Ceará (CE), Piauí (PI) and Rio Grande do Norte (RN); and the Midwest region – Mato Grosso do Sul (MS), Table 1.

3.1. Phylogeny and molecular characterization of DENV-1 strains based on the analysis of the E gene

Phylogenetic analysis based on the E gene of the 48 DENV-1 showed that all strains analyzed belong to genotype V (Americas/Africa), but the DENV-1 isolated during this serotype's reemergence (2009–2011), grouped into distinct clades. The groups were differentiated into two lineages, one sub-divided into two lineages. Samples representing the DENV-1 introduction in Brazil (1986–2002) and strains isolated from 2010 to 2011 were grouped in lineage 1. Those two groups were phylogenetically related, but one sub-division was evidenced and those considered as lineages 1a and 1b, respectively. Another distinct group represented by strains isolated from 2009 to 2011, and a strain representative of 2001, were considered as lineage 2, Fig. 1.

The E gene sequences alignment demonstrated conserved nts substitutions among strains of the same lineage, and it was responsible for the changes of some aa in domains I and III of the E gene. In the domain I, a substitution in E_{207} (T → M) was observed between lineage 1a and the lineages 1b and 2, respectively. Four substitutions were detected in domain III, at positions E_{338} (S → L), E_{394} (K → R), E_{428} (V → L) and E_{436} (V → I). The substitutions occurred at positions E_{338}, E_{428} and E_{436} were conserved on strains from lineage 2, while strains from lineage 1be, showed conserved modifications in the position E_{394} (Table 3). The domain III and stem region of the E gene presented the highest number of substitutions causing changes in conserved aa among the strains and, the phylogenetic analysis based on this domain was sufficient to segregate the different lineages (Table 3 and Fig. 2). The aa replacements in the ectodomain of the E protein is represented in a three-dimensional model (Fig. 3). The nts sequences identity of the E gene from 48 DENV-1 strains ranged from 95.6% to 97.2% between lineages 1a and 2, from 96.6% to 98.1%, between lineages 1a and 1b and, from 95.2% to 96.2% between lineages 2 and 1b. Comparing those with genotypes I, II, III and IV, the nts divergences observed, ranged from 6.3 to 8.7%.

3.2. Phylogeny and molecular characterization of DENV-1 strains based on the complete genome's coding region (C → NS5)

The phylogenetic analysis based on the sequences of the entire coding region of six DENV-1 strains, corroborated the analysis of the E gene and characterized those strains as belonging to genotype V (Americas/Africa), Fig. 4. In order to determine which DENV-1 genes could be used to differentiate the distinct lineages, phylogenetic trees were constructed based on the C, prM/M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 genes. Except by the C gene analysis, all genes allowed the genotype V characterization. The analysis of the E, NS3 and NS5 genes enabled the DENV-1 grouping into two different lineages (lineages 1 and 2). However, only the analysis of the E gene was able to characterize the sub-lineages 1a and 1b, with bootstrap support ⩾75%.

The comparison of the 3,392 aa corresponding to the 10,176 nts of the six Brazilian DENV-1 to the strain AF226685.2/BR/90, showed 82 aa substitutions along the entire region (Table S1). The strains 36034/RJ/1988 and 38159/RJ/1989 showed six and seven substitutions, respectively. The strains 66694/ES/2000 and 68826/RJ/2001, belonging to the lineage 1a, showed a higher number of substitutions, nineteen and sixteen, respectively. In the strain 1266_2011/RJ/2011, representative of the lineage 1b, a total of 26 aa substitutions were identified and the strain 242_2010/RJ/2010, representative of lineage 2, presented 43 substitutions. Most substitutions were unique to the sequences, but sixteen were conserved among the strains studied. In the strains isolated in 2000 and 2001 (lineage 1a), nine substitutions were detected in NS1, NS4B and NS5 genes. Three substitutions were found in strains representative of lineage 1b and 2 in E_{207} (T → M), $NS2A_{67}$ (K → R) and $NS5_{678}$ (I → T). The nucleotides identity of the six complete coding region sequences ranged from 96.1% to 96.5% between lineages 1a and 2; from 97.3% to 97.9%, between lineages 1a and 1b and 95.4%, between lineages 1b and 2.

The analysis of the E gene from Brazilian strains belonging to genotype V and other genotypes available in GenBank, showed no evidence of recombination. However, the analysis of the complete coding region identified a possible recombination event in the strain AF513110.1/BR/2001 isolated in the state of Paraná in 2001. The strain 36034/BR/RJ/1988 isolated in RJ in 1988 was more closely related to that possible recombinant, indicated by *P* values

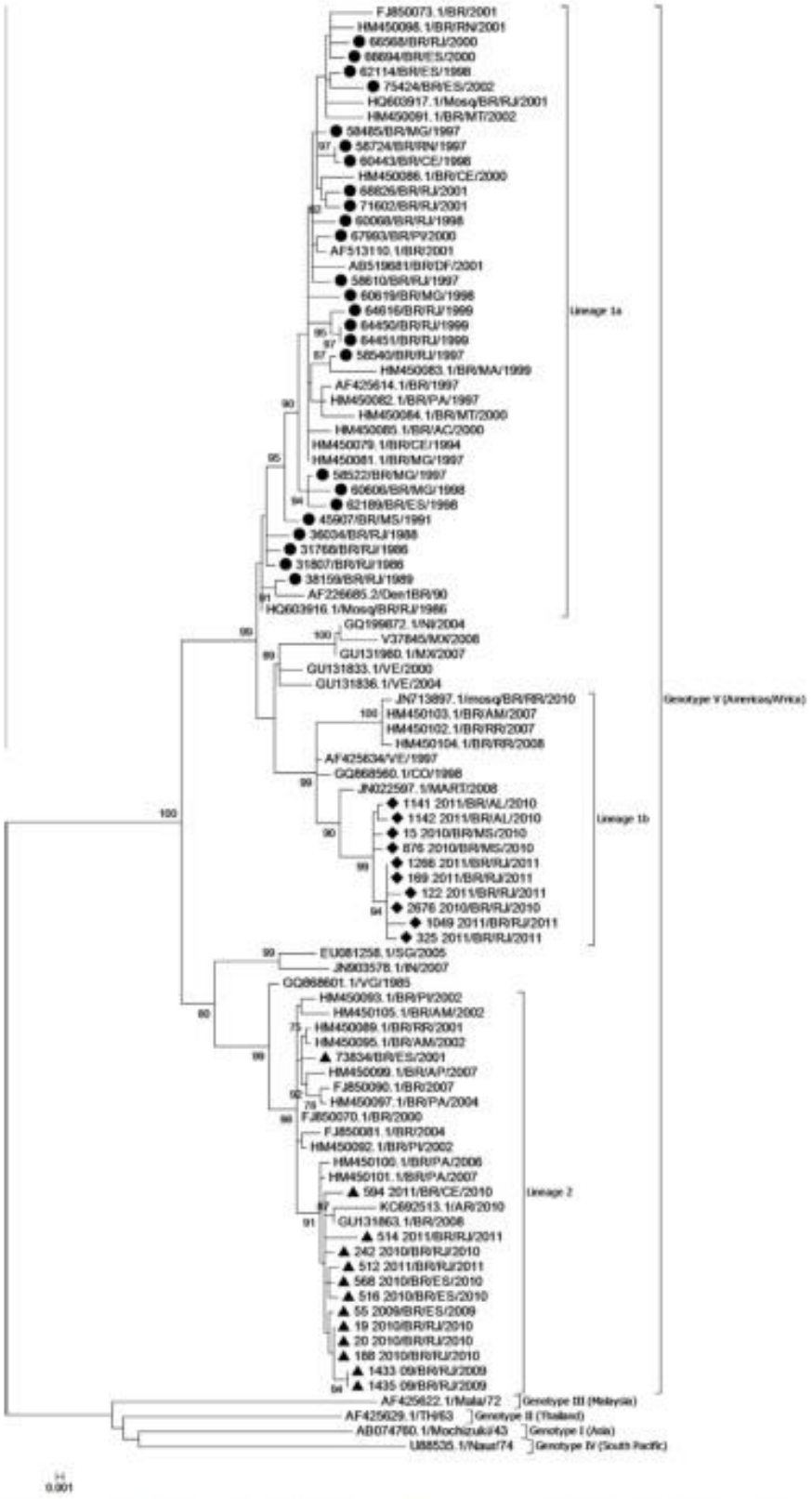

Fig. 1. Phylogenetic analysis based on the E gene of 48 Brazilian DENV-1 isolates during the epidemic periods (1986–2011). Maximum-likelihood method, TN93 + G model with 6 discrete Gamma categories (parameters = 0.2630). The DENV-1 sequences analyzed are represented as: circle (lineage 1a), triangle (lineage 1b) and diamond (lineage 2). DENV strains were named as follows: GenBank accession number/country/year.

of 6.39×10^{-06}, 5.75×10^{-05}, obtained by the RDP and BootScan methods, respectively. The region indicated as recombinant, ranges from nts position 8037 to 8677 (aa 2679–2893) within the NS5 gene, and overlaps the coding region for the FtsJ methyltransferase (aa 2548–2714) and Flavi_NS5 (aa 2743–3386).

4. Discussion

In Brazil, DENV-1 was first detected in 1982 in Roraima during an outbreak that did not spread (Osanai et al., 1983). However, in 1986 the introduction of this serotype in Rio de Janeiro resulted in more than 60,000 cases and the spread of the virus (Schatzmayr et al., 1986) establishing a new national public health concern (Nogueira et al., 2007; Rodriguez-Barraquer et al., 2011). In 2009, DENV-1 reemerged and caused a major DENV-1 epidemic in 2010 and, for the first time, distinct viral lineages were reported (dos Santos et al., 2011).

DENV are highly diverse (Wang et al., 2002; Aaskov et al., 2006; Thai et al., 2012) and this genetic diversity has been described in different genotypes within each serotype (Rico-Hesse, 1990, 2003; Weaver and Vasilakis, 2009). The genotype V of DENV-1 has been previously investigated and the circulation and replacements of lineages have been reported in Brazil and in other American countries (Mendez et al., 2010; dos Santos et al., 2011; Yamanaka et al., 2011; Carneiro et al., 2012; Drumond et al., 2012; Muñoz-Jórdan et al., 2013; Añez and Rios, 2013; Dash et al., 2015). It has been shown that this serotype had three introductions events in Brazil (Drumond et al., 2012).

In this study, the analysis of 48 sequences of DENV-1 representing strains isolated in 25 years in Brazil, characterized distinct lineages and showed that, lineages 1a and 1b are more closely related to DENV-1 of Latin American origin and lineage 2 with viruses of Asian origin. Aa substitutions were observed in all sequences analyzed, mainly in the domain III of the E gene and corroborate

Table 3
Amino acid substitutions predicted to differentiate the distinct Brazilian DENV- lineages based on analysis of the envelope (E) gene.

ID state year of isolation	E gene (position)/substitution of amino acid					Genotype Rico-Hesse (2003), Weaver and Vasilakis (2009)	Lineage
	Domain I	Domain III					
	E_{207}	E_{318}	E_{394}	E_{428}	E_{438}		
31768/RJ/1986	T	S	K	V	V	Americas/Africa (V)	Lineage 1a
31807/RJ/1986	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
36034/RJ/1988	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
38159/RJ/1989	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
45907/MS/1991	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58485/MG/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58522/MG/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58540/RJ/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58610/RJ/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58724/RN/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
60068/RJ/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
60443/CE/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
60606/MG/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
60619/MG/1998	.	L	.	.	.	Americas/Africa (V)	Lineage 1a
62114/ES/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
62189/ES/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
64450/RJ/1999	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
64451/RJ/1999	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
64616/RJ/1999	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
66568/RJ/2000	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
66694/ES/2000	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
67993/PI/2000	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
68826/RJ/2001	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
71602/RJ/2001	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
75424/ES/2002	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
15_2010/MS/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
876_2010/MS/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
2676_2010/RJ/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
1141_2011/AL/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
1142_2011/AL/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
122_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
169_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
325_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
1049_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
1266_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
73834/ES/2001	M	L	.	L	I	Americas/Africa (V)	Lineage 2
55_2009/ES/2009	M	L	.	L	I	Americas/Africa (V)	Lineage 2
1433_09/RJ/2009	M	L	.	L	I	Americas/Africa (V)	Lineage 2
1435_09/RJ/2009	M	L	.	L	I	Americas/Africa (V)	Lineage 2
19_2010/RJ/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
20_2010/RJ/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
188_2010/RJ/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
242_2010/RJ/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
516_2010/ES/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
568_2010/ES/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
594_2011/CE/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
512_2011/RJ/2011	M	L	.	L	I	Americas/Africa (V)	Lineage 2
514_2011/RJ/2011	M	L	.	L	I	Americas/Africa (V)	Lineage 2

T: threonine; S: serine; K: lysine; V: valine; M: methionine; L: leucine; R: arginine; I: isoleucine.

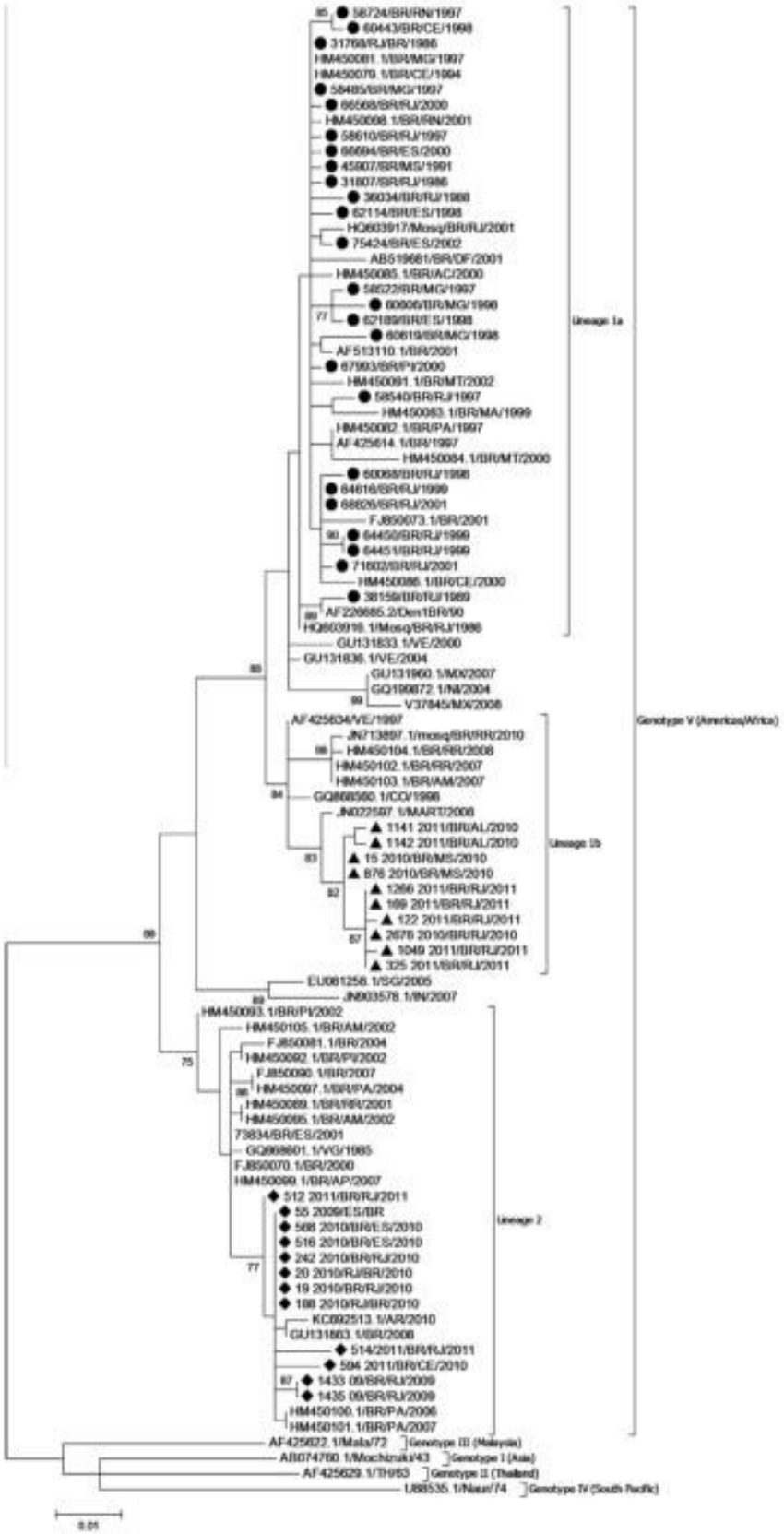

Fig. 2. Phylogenetic analysis based on the coding sequence for the domain III and stem region of the E gene of Brazilian DENV-1 isolates during the epidemic periods (1986–2011). Maximum-likelihood method, TN93 + G model with six discrete Gamma categories (parameters: =0.2941). The DENV-1 sequences analyzed are represented as: circle (lineage 1a), triangle (lineage 1b) and diamond (lineage 2). DENV strains were named as follows: GenBank accession number/country/year.

previous observations (Carneiro et al., 2012; Drumond et al., 2012). Substitutions on domain III may alter the viral particle immunogenicity, may influence pathogenicity and determine the receptor specificity, type of vector, host and cell tropism (Chen et al., 1996; dos Santos et al., 2002; Goncalvez et al., 2002; Drumond et al., 2012; Carneiro et al., 2012). As some regions in domain III are target for neutralizing antibodies in humans, mutations in this region could have important functional consequences (Thai et al., 2012). Carneiro et al. (2012) described aa substitutions at the position E_{338} in four DENV-1 strains from severe dengue patients, however in this study, substitutions at this position were observed in strains isolated from patients with all clinical presentations. Substitution at E_{196} (M → V) and E_{206} (T → P), considered as virulence markers (Bordignon et al., 2008), were not identified in the strains analyzed in this study.

Three substitutions were observed in prM, but at prM_{26}, a single strain representative of the lineage 2, presented a valine instead of an alanine and this observation has been described previously when Indian strains were compared to strains globally (Kukreti et al., 2009). Substitutions at positions $NS1_{84}$ and $NS1_{82}$ identified in two strains from lineage 1a are located immediately by the linear epitope ^{111}HKYSWK. As the NS1 glycoprotein is involved in the early stages of viral replication, it would be important to determine whether those aa differences among the strains studied, would affect the antigenicity and the biological activity of NS1 (dos Santos et al., 2002). Suzuki and collaborators (2007) analyzing a mutant DENV-1 NS1 demonstrated that a tyrosine (Y) residue at position $NS1_{247}$, highly conserved in DENV-1, would be important for NS1 function. In this study, a substitution was observed in a strain from lineage 1b, where the tyrosine (Y) was replaced by a phenylalanine (F). Substitutions at $NS3_{358}$, $NS3_{437}$, $NS3_{465}$ and $NS3_{474}$ were reported on the strains analyzed, and three of them were reported in a strain representative of the lineage 2. At $NS3_{465}$, an asparagine residue was detected in the six complete genomes analyzed, differentiating those, from the reference strain BR/90 (dos Santos et al., 2002). In NS4B, the only conserved substitutions observed were on $NS4B_{54}$ (R → H) and $NS4B_{90}$ (R → L) in two strains isolated in 2000–2001, representative of lineage 1b. This protein has strong interactions with NS1 and is associated with viral replication (Lindenbach et al., 2007). Substitutions at the NS5 protein can directly affect viral replication, since this protein acts as a RNA-dependent RNA polymerase and, along with NS3, can act by stimulating both NTPase activity of NS3 and RTPase (Lindenbach et al., 2007). The substitutions at $NS5_{114}$ (I → V) and $NS5_{784}$ (I → V) observed in this study were prevalent among the strains, and different from BR/90. Two strains isolated in 2000–2001 belonging to lineage 1a, shared aa substitutions at $NS5_{620}$ (L → F) and $NS5_{635}$ (T → S), not detected in the other strains. Although several aa substitutions were observed among the strains studied, no changes in the aa's biochemical character were identified.

The divergence analysis showed that the strains are quite similar among the groups, and that the lineages 1a and 1b are more closely related when compared to lineage 2, as those are phylogenetically separated and supported (Fig. 4). The strains from lineage 2 analyzed here, shared a high similarity with strains of Asian origin.

Phylogeny based on the C gene did not allow genotyping of the strains studied. The lineages separation on 1a, 1b and 2 was only possible by analyzing the E gene or the entire genome coding region. Moreover, the same grouping was observed by analyzing only the domain III and stem region from the E protein, supporting the fact that the substitutions on this region are associated to the lineages separation.

Previous studies describe recombination among DENV-1 strains (Tolou et al., 2001; Aaskov et al., 2007; Carvalho et al., 2010; Shin et al., 2013; Chu et al., 2013), however no evidence of recombination was observed in the analysis of the 48 Brazilian strains in this study.

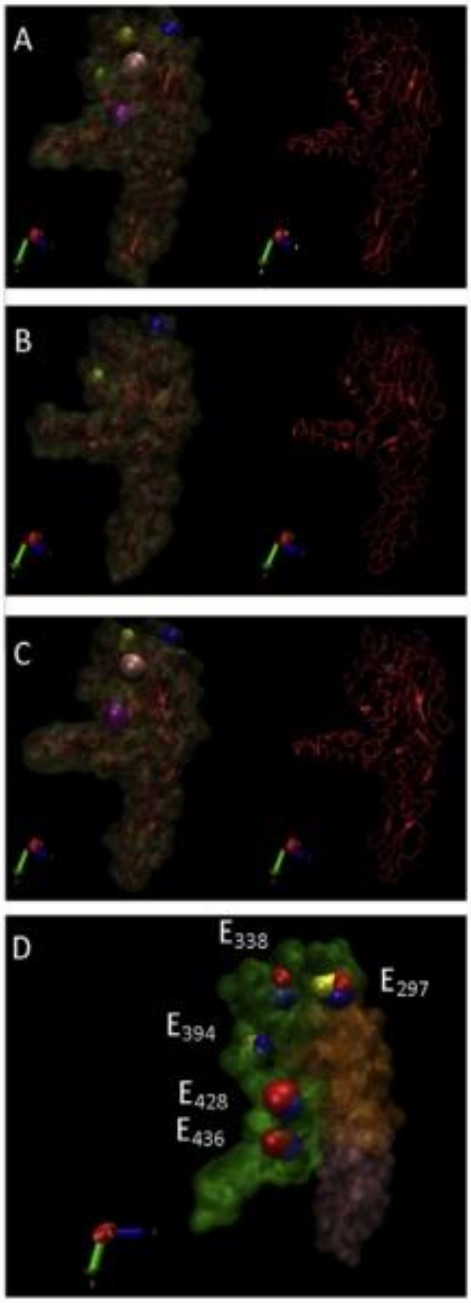

Fig. 3. Three-dimensional structure of DENV-1 E protein. Positions with conserved amino acids in domain I and III in lineage 1a (A), lineage 1b (B) and lineage 2 (C). (D) Representation constructed using as 4CCT template (Kostyuchenko et al., 2013) available from PDB (Protein Data Bank). Residues highlighted represent the amino acids found in the lineages: E_{297} (blue), E_{338} (yellow), E_{394} (green), E_{428} (pink), E_{436} (purple). Protein with a superposition of lineages 1a (blue), 1b (yellow) and 2 (red). Domains I, II and III are in orange, pink and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

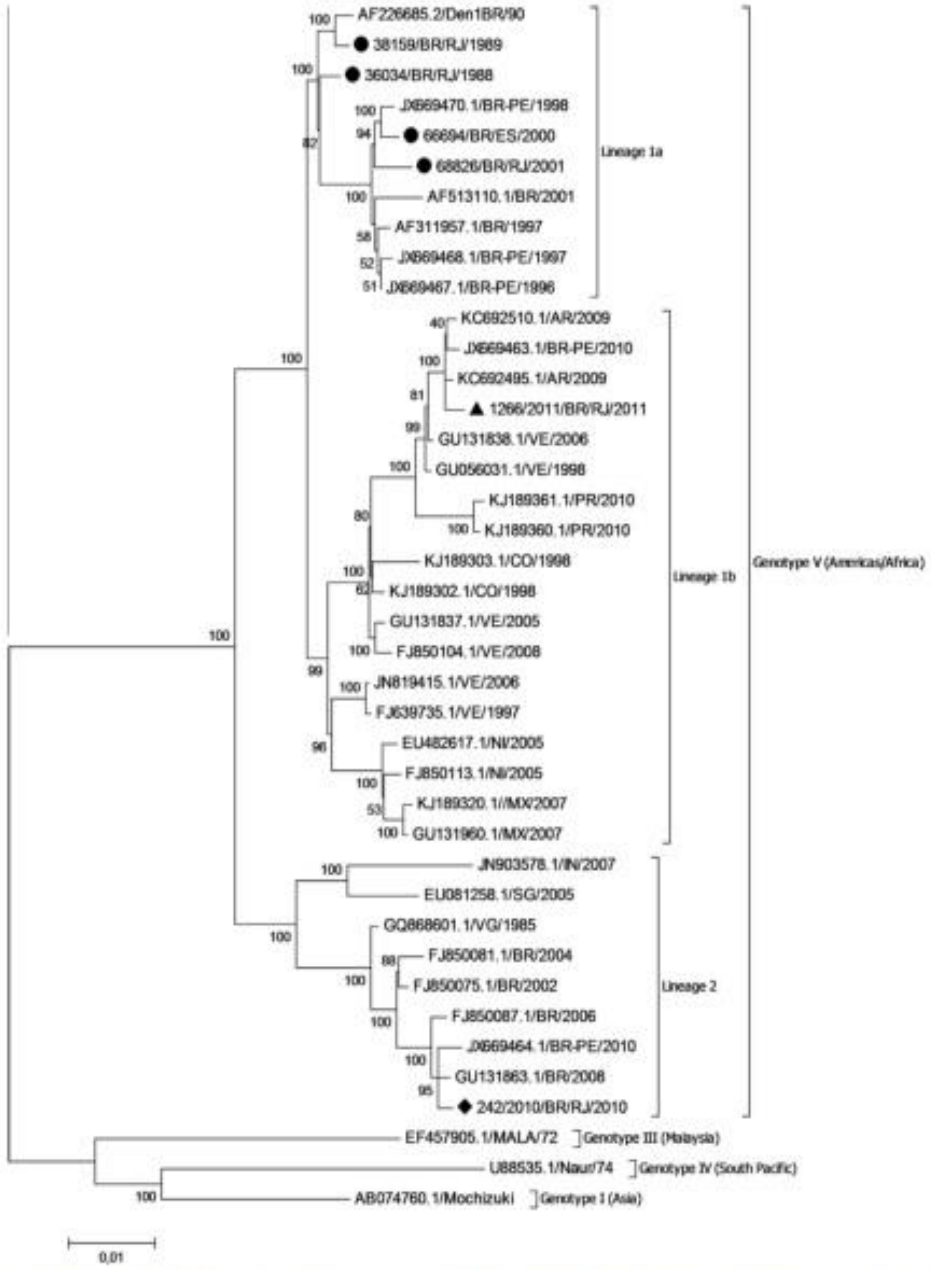

Fig. 4. Phylogenetic analysis based on the complete coding region of 8 Brazilian DENV-1 isolates during the epidemic periods (1986–2011). Maximum-Likelihood method, TN93 + G model with six discrete Gamma categories (parameters: =0.2104). The DENV-1 sequences analyzed are represented as: circle (lineage 1a), triangle (lineage 1b) and diamond (lineage 2). DENV strains were named as follows: GenBank accession number/country/year.

The co-circulation of two distinct lineages in Brazil in recent years has been characterized and, the involvement of both lineages on mild, severe and fatal cases demonstrate that, those are not restrict to a particular disease outcome. It is unknown whether the explosive outbreaks occurred in Brazil in 2009 and 2010, mainly caused by DENV-1, was due to the lineages circulation. The susceptibility of the population to a serotype that did not circulate intensively for nearly a decade, and the occurrence of secondary infections in a hyperendemic country, could have also played a role in the disease epidemiology. Due to Brazil's geography and dengue epidemiology, the surveillance of viral populations, lineages and genotypes is necessary, as the impact of those lineages replacements and co-circulation are yet not fully understood.

5. Conclusions

The phylogenetic analysis of Brazilian DENV-1 confirmed the circulation of the genotype V in the country, and the analysis of the domain III of the E gene and stem region was sufficient to differentiate the distinct lineages identified. A single substitution at

E_{297} was able to differentiate the strains belonging to lineage 1a from lineages 1b and 2. Moreover, substitutions at positions E_{338}, E_{394}, E_{428} and E_{436} were responsible for the segregation of lineages 1b and 2. Lineages 1a and 1b were more closely related to strains of American origin and lineage 2 to the strains of Asian origin. The circulation of multiple lineages in the country in 25 years was reported, with co-circulation and replacement, over time. No recombination events on the strains analyzed in this study was reported.

Author contributions

FBS, RMRN and AMB designed the study. FBN, NRCF, JBSS and PCGN implemented the study. FBN, FBS and RMRN analyzed the data. FBN and FBS wrote the paper. All authors read and approved the final version of the paper.

Competing interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.07.007.

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Capítulo de livro: Molecular Biology Approaches For Dengue Diagnosis And Research in Brazil: an overview.

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Resumo: Dengue is a major public health in tropical and subtropical regions of the world and currently there are no specific therapies and vaccines available. In Brazil, explosive epidemics have been occurring since the 80's and over the years, the dramatic increase of dengue cases in the country has led to the establishment of a National Dengue Diagnosis Network in 1989 to monitor dengue viruses (DENV) transmission and spread as surveillance has been accepted as one of the most reliable tools for the prediction of dengue epidemics. The implementation of molecular techniques in the 90's was imperative for DENV diagnosis. The use of conventional reverse transcriptase-polymerase chain reaction (RT-PCR) technique, such as the one described by Lanciotti and colleagues and suggested by Pan American Health Organization is the most widely used protocol. The two steps semi-nested RT-PCR was used to confirm cases, deaths and for the surveillance of DENV in *Aedes aegypti* mosquitoes and it has contributed to the diagnosis during 28 years of dengue activity in Brazil. A generic RT-PCR that detects dengue and other flaviviruses was also used in some opportunities. Several protocols for real time RT-PCR (rtRT-PCR) using specific primers and probes for each serotype are available. The use of fluorescent probes allows the detection of the amplified products in real time, faster, with lower risk for contamination and higher sensitivity, without the need for electrophoresis. The technique is one of the most reliable for the quantification of viral load and, studies on patient's viremia and its association to a more severe disease and death was performed. Furthermore, rtRT-PCR protocols were also useful for viral detection and quantification on *Aedes aegypti* saliva. The molecular surveillance of DENV is very important to observe the introduction, spread and shifts of potentially virulent strains, genotypes and lineages as well as to assess their impact on the population during an outbreak. The partial and/or complete DENV genome sequencing has allowed studies on DENV

genotypes surveillance and recombination by phylogenentic studies and molecular characterization. During an infection the host can present variants of a same infecting serotype, which may not be detected by the conventional Sanger's nucleotide sequencing method. By the recent advent of next generation sequencing (NGS), viral populations within a host are now more easily studied. Despite the several molecular tools available for DENV diagnosis and investigation, the establishment of new ones may improve even more the sensitivity of the viral detection and quality of data generated. Our experience has shown that the implementation of new techniques over the years has constituted an important strategy for the disease surveillance and research in Brazil.

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Chapter 1

MOLECULAR BIOLOGY APPROACHES FOR DENGUE DIAGNOSIS AND RESEARCH IN BRAZIL: AN OVERVIEW

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ABSTRACT

Dengue is a major public health in tropical and subtropical regions of the world and currently there are no specific therapies and vaccines available. In Brazil, explosive epidemics have been occurring since the 80's and over the years, the dramatic increase of dengue cases in the country has led to the establishment of a National Dengue Diagnosis Network in 1989 to monitor dengue viruses (DENV) transmission and spread as surveillance has been accepted as one of the most reliable tools for the prediction of dengue epidemics. The implementation of molecular techniques in the 90's was imperative for DENV diagnosis. The use of conventional reverse transcriptase-polymerase chain reaction (RT-PCR) technique, such as the one described by Lanciotti and colleagues and suggested by Pan American Health Organization is the most widely

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used protocol. The two steps semi-nested RT-PCR was used to confirm cases, deaths and for the surveillance of DENV in *Aedes aegypti* mosquitoes and it has contributed to the diagnosis during 28 years of dengue activity in Brazil. A generic RT-PCR that detects dengue and other flaviviruses was also used in some opportunities. Several protocols for real time RT-PCR (rtRT-PCR) using specific primers and probes for each serotype are available. The use of fluorescent probes allows the detection of the amplified products in real time, faster, with lower risk for contamination and higher sensitivity, without the need for electrophoresis. The technique is one of the most reliable for the quantification of viral load and, studies on patient's viremia and its association to a more severe disease and death was performed. Furthermore, rtRT-PCR protocols were also useful for viral detection and quantification on *Aedes aegypti* saliva. The molecular surveillance of DENV is very important to observe the introduction, spread and shifts of potentially virulent strains, genotypes and lineages as well as to assess their impact on the population during an outbreak. The partial and/or complete DENV genome sequencing has allowed studies on DENV genotypes surveillance and recombination by phylogenentic studies and molecular characterization. During an infection the host can present variants of a same infecting serotype, which may not be detected by the conventional Sanger's nucleotide sequencing method. By the recent advent of next generation sequencing (NGS), viral populations within a host are now more easily studied. Despite the several molecular tools available for DENV diagnosis and investigation, the establishment of new ones may improve even more the sensitivity of the viral detection and quality of data generated. Our experience has shown that the implementation of new techniques over the years has constituted an important strategy for the disease surveillance and research in Brazil.

Keywords: Dengue, Molecular Biology, Surveillance, Diagnosis, Research, Brazil.

INTRODUCTION

Dengue viruses (DENV) exists in either sylvatic or human transmission cycles, which are most prevalent in tropical and subtropical areas, and it has become a major public health problem with relevant social an economical effect due to the increased geographic extension, number of cases and disease severity (Guzman & Harris, 2015).

DENV 1 to 4 belong to the family *Flaviviridae* and the genus *Flavivirus* (Lindenbach & Rice, 2007) and cause one of the most important infectious diseases in the world in terms of morbidity and mortality. In the last 50 years, the disease has gradually reached the status of a pandemic, hospitalizing more than 5 million children and resulting in more than 70,000 deaths (WHO, 2013). In Brazil, more than eight million cases have been reported since the 80's, with the years of 2002, 2008, 2010 and 2013 the most critical for the country (Silva Jr., 2013). The viral genome from 10.6 to 11 kb in size, encodes three structural proteins (capsid [C], membrane [M] and envelope [E] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) and is flanked by approximately 100 nucleotides (nts) at 5′ untranslated region (UTR) and 388–462 nts at the 3′ UTR (Shurtleff et al., 2001; Miller et al., 2010).

The dramatic increase of dengue cases in Brazil has led to the establishment and consolidation of a National Dengue Diagnosis Network in 1989 to monitor DENV transmission and spread as DENV surveillance has been accepted as one of the most

important tools for the prediction of dengue epidemics. The laboratorial diagnosis can be carried out using several approaches for the detection of the virus, viral RNA, antigens or specific antibodies, or even a combination of these methods (revised in Lima et al., 2014). The implementation of molecular techniques in the 90's constituted an important advance in diagnosis for detecting the infecting DENVserotypes, identifying the viruses in cases negative by virus isolation, molecular characterizing and genotyping circulating strains and accessing its impact over the population. In this chapter, we aim to present an overview on the molecular biology approaches most commonly used for dengue surveillance in Brazil, presenting some experiences on its use for laboratorial diagnosis, molecular characterization, phylogeny and viral research.

VIRAL RNA EXTRACTION FOR MOLECULAR DETECTION

The extraction and purification of nucleic acids (RNA/DNA) in biological samples are important steps for efficacy in molecular detection and many protocols have been described. The first step is cell lysis, which is the disruption of the cell membrane that can be achieved by using chemical or physical methods that disrupt hydrophobic associations and destroy the lipid bilayer. Among the substances used are detergents, chaotropic agents, enzymes and acids and basic agents. In the physical methods, the cells may be subjected to mechanical shock, freeze-thaw, sonication and homogenization. The nucleic acid purification is performed to exclude cell debris and proteins and, organic solvents, such as phenol and chloroform, are the most commonly used. The precipitation step usually uses alcohol to decrease the water molecules around the nucleic acid.

The protocol described by Boom in 1990 is widely used for DNA/RNA extraction from samples of blood or urine, on a small scale. It uses conventional silica and can be performed manually or automated. The method is based on the lysing and nuclease inactivating properties of the chaotropic agent guanidine thiocyanate together with the binding properties of nucleic acids to the silica particles or diatoms in the presence of this agent. After the implementation of the molecular biology methods in the 90's in Brazil, this protocol was widely used to extract DENV RNA from clinical samples of dengue suspected cases. However, currently, there are several commercial kits for nucleic acid extraction based on organic extraction, silica-based technology, magnetic separation and ion change technology, used in routine and research laboratories.

MOLECULAR BIOLOGY APPLIED TO LABORATORIAL DIAGNOSIS AND RESEARCH: POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) is based on the *in vitro* enzymatic amplification, of specific sequences of DNA (target) directed by oligonucleotide primers that results in the generation of an exponential number of copies. The technique consists in repeated cycles of nucleic acid denaturation, primers hybridization on the target and chain synthesis by a DNA polymerase. The DNA denaturation occurs at an elevated temperature, in order to convert a double-stranded nucleic acid to single-stranded. The hybridization of the primers occurs when

the temperature is decreased below the dissociation temperature of the primers and the synthesis of the new DNA strand is performed by a thermostable DNA polymerase that incorporates the deoxynucleotide triphosphates in the free 3-OH end of the DNA duplex formed between the DNA-target and the initiator. The nucleotide sequence of the new chain is complementary to the target sequence and the number of copies of the generated product increases exponentially. Other variations of the polymerase chain reaction have also been developed, for instance, the RT-PCR, Semi-Nested-PCR and Nested PCR, Multiplex-PCR and Real-Time PCR (rtPCR).

The reverse transcriptase-polymerase chain reaction (RT-PCR) is used for the amplification of RNA, and to do so, the synthesis of a complementary DNA (cDNA) to RNA is needed by using a reverse transcriptase (RT) enzyme. The semi-nested- PCR and nested PCR result in amplicons obtained from a PCR reamplification. Semi-nested PCR uses a primer already employed previously in the PCR reaction while, the Nested- PCR uses for the amplification, a new pair of primers, different from those used previously in the PCR reaction. Those methods result in increased sensitivity and specificity, however it can also increase the risk of contamination by the manipulation of amplicons. In a multiplex-PCR, two or more primers are used, allowing the detection of different viruses and genotypes, however it may result in a lower sensitivity.

The RT-PCR protocol described by Lanciotti et al. (1992) and suggested by Pan American Health Organization is the most widely used protocol for dengue molecular diagnosis. The viral RNA may be detected in serum samples, plasma, infected cells, mosquitoes, fresh tissues and tissues formalin-fixed. In a first step, consensus primers (D1 and D2) are used to anneal to C and prM genes of any of the four DENV serotypes and amplify a 511-bp product in a RT reaction. After a second round of amplification (nested PCR) with type-specific primers (TS1 to TS4, for DENV-1 to 4, respectively), DNA products specific for DENV type are generated.

The protocol by Lanciotti et al. (1992) was established in 1995 in Brazil as a rapid and specific molecular approach for detecting and typing DENV in the Regional Reference Laboratory at the Oswaldo Cruz Institute, Rio de Janeiro and allowed the identification of DENV-1 and DENV-2 in 41% of previously confirmed dengue cases (Miagostovich et al., 1997). In Brazil, DENV-3 was first isolated in 2000 from a patient and from naturally infected *Aedes aegypti* collected during an entomological surveillance and by using RT-PCR and/or virus isolation, DENV-3 was identified in 13% of the cases. The RT-PCR played a definitive role in surveillance as a rapid diagnostic tool that guided the implementation of control measures as local authorities were notified of confirmed DENV-3 cases. Furthermore, in combination with virus isolation, RT-PCR increased case confirmation due to DENV infections. The implementation of RT-PCR in 1995 constituted an important advance in diagnosis by detecting the infecting DENV type and identifying DENV in cases negative by virus isolation. In some years, RT-PCR identified the infecting DENV type in 40% of cases where the virus was not isolated. From 1997 to 2011, virus isolation and RT-PCR identified the infecting DENV type in a total of 4,990 dengue cases. In the same period, RT-PCR confirmed 24.7% (1,840/7,441) of the dengue cases analyzed (reviewed in dos Santos et al., 2013). The first DENV-4 cases reported in Rio de Janeiro in 2011 were recovered by using RT-PCR from infected patients and from an individual *Aedes aegypti* female collected in the field (Castro et al., 2012). In a recent study analyzing the epidemiological and laboratorial aspects of the emergence an re-emergence of the disctinct DENV seroytpes in Rio de Janeiro,

from 2010 to 2012, the RT-PCR confirmed 56.3% (1,022/1,814) of the cases tested and when the co-circulation of the four serotypes was reported for the first time in the State (Heringer et al., 2015).

However, other RT-PCR protocols for molecular detection of DENV were previously described and are available. The protocol described by Morita et al. (1994), for instance, used serum from dengue infected patients directly subjected to the reverse transcription and amplification, without RNA extraction, and the viral was detected in 3 hours, suggesting that the direct RT-PCR procedure greatly facilitates the rapid diagnosis of dengue infection. Harris et al. (1998) adapted the nested RT-PCR protocol described by Lanciotti et al. (1992), for a multiplex reaction in a single tube in order to maximize the simplicity and minimize the risk of sample cross-contamination. The same showed an equivalent sensitivity to the two-step protocol. Wang et al. (2000) developed a competitive RT-PCR method to quantify the RNA of the four DENV serotypes. A pair of primers was designed in a highly conserved region of the C gene and the assay sensitivity was estimated to be 10-50 copies of RNA per reaction.

REAL-TIME PCR

The Real-Time PCR (rtPCR) is an amplification system based on the conventional PCR approach, but with an analysis in real time, where primers and specific probes for each DENV serotype or hybridizing to a conserved region of all serotypes may be used. The use of fluorescence in probe, allows the detection of the products, as they are produced, in real time, without the need of a post-reaction electrophoresis. The emission of fluorescent compounds generates a signal that increases in direct proportion to the amount of the PCR product. The point that detects the cycle in which the reaction reaches the threshold of the exponential phase, is called Ct (Cycle Threshold), and allows the exact nucleic acid quantification. A low Ct value indicates a higher concentration of the initial target. The amplification efficiency in rtPCR should be 90 to 100%, and can be calculated using data collected from a standard curve using the following formula: Efficiency = [10 (-1/slope)] – 1. The slope is the real indicator of amplification and represents the angular coefficient of the straight line formed by the points of the standard curve. A high efficiency is associated with approximately 3.32 slope for each 10-fold dilution of the target.

Two distinct systems are commonly used. The SYBR Green binds nonspecifically within the double-stranded DNA and the TaqMan system, uses probes doubly marked by distinct fluorescent dyes, a reporter (eg, FAM) and a quencher (eg, TAMRA). When the two dyes are close, and the probe still intact, the quencher absorbs the energy released by reporter, thus avoiding the emission of fluorescence. The Taq polymerase 5'exonuclease activity degrades the probe during the PCR amplifiction, separating the fluorophores, resulting in the release of fluorescence.

In 2003, the rtRT-PCR was established in the Regional Reference Laboratory at the Oswaldo Cruz Institute, Rio de Janeiro as a novel research tool for DENV detection and quantification in dengue suspected cases. The rtRT-PCR also yielded the highest positivity rate in detecting DENV-3 RNA in tissues from those fatal cases (de Araújo et al., 2009a). The amount of DENV-3 RNA in 42 patients with fatal and non-fatal outcomes during the 2002 epidemic in Rio de Janeiro and its correlation with primary or secondary infection was

evaluated and a significantly higher virus titer was found in the samples from fatal cases. Moreover, as more than half of fatal cases were primary infections, antibody enhancement alone would not explain the deaths (de Araújo et al., 2009b).

A SYBR Green onestep rtRT-PCR protocol described by Dos Santos et al. (2008) was developed for the early diagnosis of dengue, and presented a higher sensitivity compared to serology, standard virus isolation and the qualitative RT-PCR. However, despite a lower cost, the assay is less specific than the TaqMan system. Several commercial rtRT-PCR kits for the detection of DENV in clinical samples are available and have been evaluated previously (Najioullah et al., 2014).

Protocols detecting one DENV serotype (de Oliveira Poersch et al., 2005) or all four serotypes in a single reaction have been described (Johnson et al. 2005; Kong et al., 2006; Santiago et al., 2013). A study performed in dengue suspected cases assisted in hospitals and clinics from Goiânia, Central Brazil, evaluated a commercial rtRT-PCR kit that proved to be feasible for confirming dengue cases in a timely manner, however stressed that the cost would pose a burden (Levi et al., 2007). In Rio de Janeiro, Brazil, a study by de Araújo et al. (2009a) reported an increased sensitivity of rtRT-PCR in confirming dengue infection in tissues from fatal cases during a DENV-3 epidemic occurred in 2002. Viral genome quantification is also possible by using rtRT-PCR protocols and dengue viral quantitation by using rtRT-PCR was used to determine the correlation between the amount of viral particles and the disease severity. In that same epidemic, de Araújo et al., (2009b) reported a higher viral load in fatal cases due to DENV-3 when compared to classic dengue cases. In an investigation performed in Sao Paulo, Brazil, DENV-2 and DENV-3 were the serotypes recovered in the saliva and urine from patients by rtRT-PCR (Poloni et al., 2010). An rtRT-PCR procedure for detection and quantification of DENV- 1 to -3 successfully identified distinct DENV strains in clinical samples and mosquitoes in Brazil (Conceição et al., 2010). By using rtRT-PCR, Nunes (2012) correlated DENV viral load with disease severity in patients infected by two distinct DENV-2 lineages from the Southeast Asian genotype circulating in Brazil in 90's and 2000's. Patients infected by lineage II had a viral load significantly higher than patients infected by the lineage I, moreover, severe cases from lineage II had a viral load higher than the classic cases form that same lineage. The diagnostic performance of a rtRT-PCR protocol and of serological methods for dengue diagnosis was evaluated in a real epidemic occurred in Belo Horizonte, Minas Gerais, Brazil (Ferraz et al., 2013). The rtRT-PCR protocol by Johnson et al. (2005) was used along with other diagnostic tests during dengue cases surveillance performed in Rio Grande do Sul, South Brazil, from 2007 to 2013, to characterize the epidemiological patterns of the disease in a region with recent autochtonous cases (Tumioto et al., 2014).

GENETIC VARIABILITY AND PHYLOGENY OF DENGUE VIRUSES: TARGET GENES AND RESOURCES

RNA viruses have genetic variability due to the high degree of mutation associated with the error-prone RNA-dependent RNA polymerase, which does not have proof reading capacity and is known to produce approximately one mutation per round of genome replication (Steinhauer et al.1992; Drake, 1993). In DENV, this results in the existence of genetic variability of the four antigenically distinct serotypes (Holmes & Twiddy, 2003). The first genetic evidence for differences between DENV serotypes were determined by RNA

fingerprinting technique, using enzymes to digest the RNA strain (Rico-Hesse, 2003; Chen & Vasilakis, 2011). However, with the improvement of molecular techniques, whole genome sequences could be generated by the reverse transcription of the viral RNA into a complementary DNA (cDNA) and multiple round of amplification by RT-PCR. The sequencing of the viral genome allowed, with greater efficiency and accuracy, the characterization of DENV strains and improved evolutionary relationships between serotypes. The term "genotype" was defined as the grouping of DENV with nucleotide divergence ≥6% for a given region of the genome (Rico-Hesse, 1990; Chen & Vasilakis, 2011). The four DENV seroytpes share amino acids similarities ranging from 60-70% over the E gene and within the same serotype homologous amino acids present in a ratio of 90% or more. However, within each serotype differences of 6% and 3% at the nucleotide and amino acid, respectively are observed (Pierson & Diamond, 2013). Given the reported genetic diversity, genomic sequencing techniques aim to analyze the nucleotide sequences and characterize genotypes and possible viral variants within the same genotype.

The advances on molecular techniques, reagents and bioinformatic softwares allowed the analysis of longer fragments and larger sequences subsets, more and more frequently availble on public gene banks online such as the GenBank® (http://www.ncbi.nlm.nih.gov, European Nucleotide Archive (http://www.ebi.ac.uk/ena) and DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The bioinformatics tools have also contributed in phylogeographic studies allowing analysis on the DENV evolutionary patterns and dynamics. Some softwares for editing sequences include, for instance, Chromas® 2.4.3 (http://www.technelysium.com.au) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), for the analysis of nucleotide and amino acid identities, BLAST (http://blast.ncbi.nlm.nih.gov/ Blast.cgi), nucleotide and amino acid alignments, CLUSTAL OMEGA (http://www.ebi.ac.uk/Tools/msa/clustalo/) and MAFFT (http://www.ebi. ac.uk/Tools/msa/mafft/), phylogeny by MEGA 6 (http://www.megasoftware.net; Tamura et al., 2013), PhyML3.0 (http://www.atgc-montpellier.fr/phyml/; Guindon et al., 2010), MrBayes (http://mrbayes.sourceforge.net/; Ronquist and Huelsenbeck, 2003), evolutionary and phylogeographic analysis, BEAST (http://beast.bio.ed.ac.uk/; Drummond et al., 2012).

DENGUE VIRUSES GENOTYPES

Currently, DENV genotyping and phylogenetic analysis are based on the genomic sequencing of the E gene, the most widely used, since this structural gene is the more likely to go under nucleotide and amino acid changes, as is associated with the immunogenicity of the virus particle and the host antibody response. The complete genome analysis is usually performed when a more detailed molecular characterization is intended, allowing the analysis of other structural and non-structural genes besides the untranslated regions (UTR's), involved in viral replication (Rico-Hesse, 2003; Weaver & Vasilakis, 2009).

Phylogenetic studies based either on the partial and/or complete viral genomic sequences have contributed for the understanding of DENV evolution and diversity, including recombination events. The phylogeny beased on the analysis of the E/NS1 junction or E gene characterized five genotypes for DENV-1: Genotype I, representing strains from Southeast

Asia, China and East Africa; Genotype II, representing strains from Thailand collected in the 1950s and 1960s; Genotype III, representing a wild strain isolated in Malaysia; Genotype IV, representing strains from Western Pacific Islands and Australia; and Genotype V, representing all strains from the Americas, West Africa, and some strains from Asia (Rico-Hesse, 1990; Weaver & Vasilakis, 2009; Chen & Vasilakis, 2011).

The nucleotide sequence analysis of the E gene characterized six genotypes for DENV-2: Asian 1, represented by strains form Malaysia and Thailand, Asian 2 represented by strains from Vietnam, China, Taiwan, Sri Lanka and the Philippines; Cosmopolitan Genotype, including strains of wide geographical distribution and strains from Australia, East and West Africa, the Indian and Pacific Islands, Indian Subcontinent and the Middle East; American Genotype, represented by strains from Latin America and the oldest strains of the Caribbean, Indian Subcontinent and the Pacific Islands from the 1950s and 1960s; Southeast Asian/American Genotype, including strains from Thailand, Vietnam and from the Americas in the last two decades, approximately; and Wild Genotype, representing strains of human, wild mosquitoes, or sentinel monkeys in West Africa and South East Asia (Rico-Hesse et al., 1997; Twiddy et al., 2002; Weaver & Vasilakis, 2009; Chen & Vasilakis, 2011).

Originally, four distinct genotypes were characterized for DENV-3 (Lanciotti et al., 1994) and were corroborated by other analysis based on the partial or complete genome sequencing (Chao et al. 2005; King et al., 2008; Araújo et al., 2009a; Amarilla et al. 2009; Ramirez et al., 2010; Sharma et al., 2011). However, a detailed analysis of DENV-3 sequences available on GenBank identified five genoytpes as follows: Genotype I, represented by Southeast Asia, mainly Indonesia, Singapore, Malaysia, the Philippines, Taiwan and South Pacific Islands; Genotype II, representing strains from Thailand (1962 until recently), a single strain from Singapore (1995), one from Indonesia (1998), Taiwan, Vietnam, Bangladesh, Cambodia, China, Japan and Myanmar; Genotype III, represented by strains from Sri Lanka, India, Japan, Taiwan, Singapore, Samoa, East Africa, Central and South America, the Caribbean and a couple of imported strains to Europe; Genotype IV, represented by strains from Puerto Rico (1960s -1970s) and Tahiti and Genotype V, represented by the DENV-3 prototype strain from the Philippines (H87), Japan, China (1987-2009) and strains from Brazil sampled in the early 2000s (Chen & Vasilakis, 2011).

For DENV-4, only two distinct genotypes were initially characterized by the analysis of limited E gene sequences (Lanciotti et al., 1997). However, broader analysis reported four genotypes: Genotype I, represented by strains from Philippines, Thailand, Vietnam, Myanmar, Malaysia, Sri Lanka, India and imported cases in Japan, China and Brazil, including the This genotype includes the prototype isolated in the Philippines in 1956 (H241); Genotype II, represented by strains from Southeast Asia, China, Western Pacific Ocean Islands, Australia, the Caribbean, Americas and Easter Island; Genotype III, represented by Thai strains (1997- 2001) and Genotype IV, represented by sylvatic strains isolated from sentinel monkeys in Malaysia in 1970's (Klungthong et al., 2004; Villabona-Arenas et al., 2011; Chen & Vasilakis, 2011).

The existence of lineages within the distinct DENV genotypes, presenting distinct geographic and temporal relationships has been reported (Myat Thu et al., 2005; Kukreti et al., 2009) in the Americas and Asia (Zhang et al., 2005; Carrillo-Valenzo et al., 2010; Duong et al., 2013; Lambrechts et al., 2012; Shin et al., 2013; Zhang et al., 2014) and, the term "lineage" has been used to characterize those viruses clustered in clades in a taxonomic level beneath genotype (Mendez et al., 2010). Those genetically distinct lineages may temporally

emerge or disappear on a regular basis (Drumond et al., 2012; Choudhury et al., 2014) however, the mechanisms involving lineages replacement, emergences or disappearances are not fully known.

The DENV evolution has had a major impact on its virulence to humans and epidemiology of the disease worldwide. Phylogenetic studies allow the correlation between specific genotypes and disease severity. Despite the complex set of factors that contribute to the epidemiology, studies have suggested that specific viral structures may contribute to the increased replication in human target cells and enhance the transmission by the vector mosquito. Moreover, the patients immune status and possibly genetic characteristics of the host may also be implicated in the disease severity (Rico-Hesse, 2003).

DENGUE MOLECULAR EPIDEMIOLOGY IN BRAZIL

In Brazil, more than eight million dengue cases were reported over the past 29 years (Silva Jr, 2013) since DENV-1 introduction and spread in 1986. After epidemics caused by the introduction of DENV-2 in 1990, DENV-3 in 2000 and the re-emergence of DENV-2 in 2008, DENV-1 re-emerged in 2009, displacing DENV-2 and DENV-3 and caused more than 1 million probable cases in 2010 (reviewed in dos Santos et al., 2013). Even after the DENV-4 introduction in 2010 in a fully susceptible country, DENV-1 was responsible for outbreaks over two million suspected cases between 2010 and 2011. Only in 2015, a total of 1,006,414 cases and 377 deaths were reported in the country until May (SVS, 2015). The hyperendemic scenario in Brazil, with the co-circulation of the for DENV serotypes, resulting in distinct epidemiological patters with and increased number of cases, severe disease in children, high hospitalization rates in elderly enphazises the need for serotypes/genotypes surveillance as some are associated with greater disease severity, as those of Asian origin. DENV molecular characterization and phylogenetic studies has been performed and described by many research groups in Brazil, by the partial (E gene) or complete genome sequencing (dos Santos et al., 2002a; Miagostovich et al., 2006; Araújo et al., 2009a; Carvalho et al., 2010; dos Santos et al., 2011; Faria et al., 2013; Drumond et al., 2012; 2013).

Molecular Epidemiology of Brazilian DENV-1

DENV-1 was first introduced in Brazil, by the state of Rio de Janeiro in 1986, resulting in the spread of the virus and consequently epidemics in several states of the country. All published studies so far describe the detection of the genotype V (Americas/Africa), the only circulating since its introduction, Figure 1. A study by dos Santos et al. (2002b) analyzing the complete genome sequences of DENV-1 isolates from 1990 and 2001 in Brazil, structural genes remarkably conserved among the strains isolated in northeastern Brazil in 1997 (BR/97), in the southern region in 2001 (BR/01) and in Rio de Janeiro in 1990 (BR/90), while the non-structural genes presented 27 amino acid changes, previously identified in strains from Nauru Island, Asia and Africa. Among the amino acid changes, nine were unique to strains BR/97 and BR/01, suggesting a local evolution of the Brazilian strains. A comparative analysis of DENV-1 complete genome sequences circulating in the Americas, including two

Brazilian strains isolated in Brasilia, Federal District, in 2001 and demonstrated the existence of a Latin American monophyletic cluster, subdivided into two sub-clusters. Furthermore, three recombinant events were reported and the study suggested that possible recombination between DENV-1 could be underestimated (Carvalho et al., 2010). In the early 2000s, DENV-1 circulated silently in Brazil, re-emerging in 2009 and accounting for a serious epidemic in 2010. During this period, dos Santos et al. (2011) performing molecular epidemiological surveillance of DENV-1 isolated in the state of Rio de Janeiro, reported for the first time, the circulation of three distinct viral lineages. The analysis of 10 isolates showed that DENV-1 circulating during the period of this serotype re-emergence (2009-2011) still belonged to genotype V (Americas/Africa), but clustering in two distinct clades, different from that observed by the strains circulating in the 80's. Those observations were confirmed by Drumond et al. (2012) when analyzing the population dynamics from DENV-1 genotype V in Brazil. The co-circulation and replacement of distinct lineages was confirmed and it was shown that DENV-1 was introduced during four different events. The first DENV-1 introduction occurred in 1984-85, the second in 1997-99, and the third and fourth from 2004 to 2007. Morever, a potentially recombinant strain was reported and the study reinforced the importance of molecular surveillance of circulating genotypes in the country. The study by Carneiro et al. (2012) reported the DENV-1 variability by analyzing the genes encoding the structural proteins C/prM/M/E of 34 strains isolated during epidemics occurred in Brazil from 1994 to 2011, confirmed the circulation of genotype V and also reported the different introductions and lineages replacements. The temporal divergence analysis indicated that the circulating lineages in Brazil emerged from an ancestral lineage approximately 44.35 years ago. Although studies on circulating DENV-1 in Brazil are complementary, indicating the same genotype and reaffirming circulation, co-circulation and lineages replacements, Nogueira (2013) conducted a retrospective study on DENV-1 circulating in 25 years in Brazil in the states of Rio de Janeiro, Espirito Santo, Minas Gerais, Mato Grosso do Sul, Alagoas, Ceará, Piauí and Rio Grande do Norte. Phylogeny and molecular characterization based on analysis of E gene of 48 DENV-1 strains and the complete coding region of six, confirmed genotype V as previously reported. The three clades characterized compromised strains isolated from 1986 to 2002 (lineage 1a), a second clade represented by viruses isolated from 2009 to 2011 and a representative strain isolated in 2002 (lineage 2) and a clade represented by strains isolated from 2010 and 2011 (lineage 1b). Furthermore, the lineages 1a and 1b were more related with the American strains and lineage 2 with Asian strains. Amino acid substitutions were observed in ectodomains I and III of the E protein and were associated to the lineages separation. The phylogenetic analysis based on the nucleotide sequence of the ectodomain III and steam region was sufficient for differentiation of the three lineages, greatly contributing to the genotypes surveillance by using a target of approximately 500 base pair fragment. The role of air transportation in DENV-1 and 3 dissemination in Brazil, was recently stressed by analyzing complete viral genomes (n=287), including Brazilian DENV-1 isolates (n=42). The three lineages from genotype V characterized were estimated to have been introduced in the country in 1985, 1995 and 2006 and it suggested that lineages I and II originated from Caribbean and lineage III from Venezuela (Nunes et al., 2014).

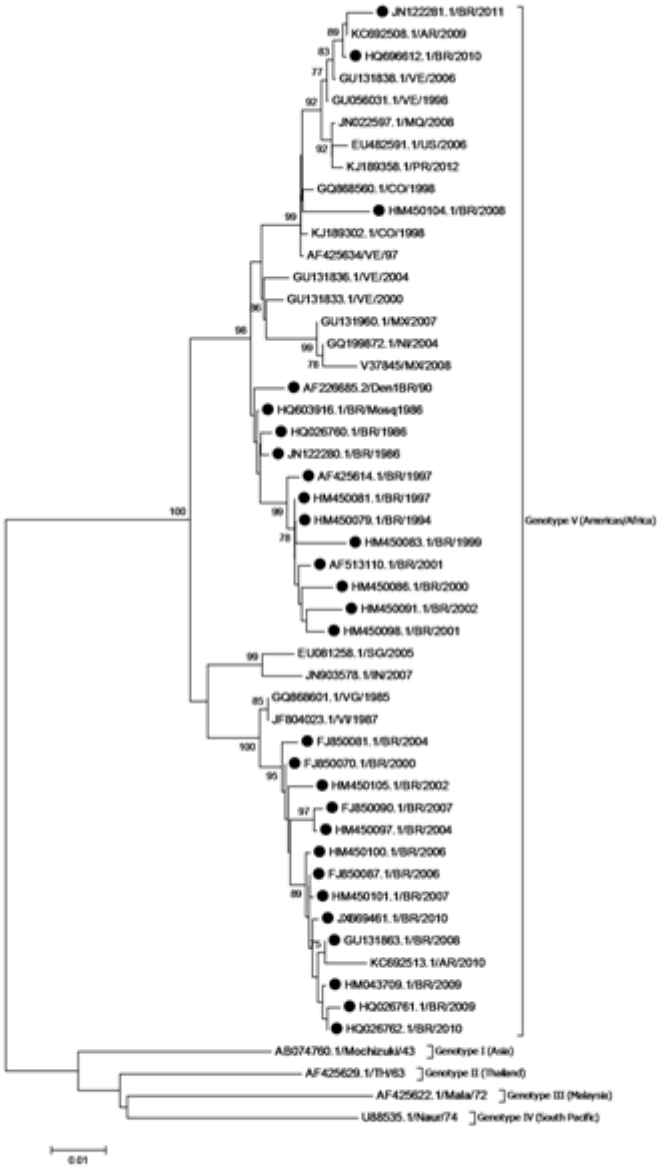

Figure 1. Phylogenetic analysis based on the E gene of representative DENV-1 strains. Neighbor joining method, TN93+G model with correction value of 0.26. Bootstrap test (1000 replicates) is shown next to the branches. Black circles represent the Brazilian DENV-1. DENV strains were named as follows: GenBank accession number/country/year.

Molecular Epidemiology of Brazilian DENV-2

In Brazil, DENV-2 was first isolated in the city of Niterói, Rio de Janeiro in 1990, and first cases of dengue hemorrhagic fever/dengue shock syndrome (FHD/DSS) (Nogueira et al., 1990) were reported. By sequencing the E gene from DENV-2 isolates from distinct geogaphical regions in Brazil from 1990 to 1995, Miagostovich et al. (1998) reported the circulation of the genotype III (Southeast Asia-American genotype), a genotype associated with increase disease severity. Those data were corroborated by dos Santos et al. (2002a) by analysing the full length genome of a brazilian DENV-2 isolated in 1998 in the the state of Rio de Janeiro. After a low circulation with other DENV serotypes, DENV-2 reemerged in 2007 causing a large epidemic in 2008 in Brazil, with 632,680 dengue cases and 561 deaths (SVS, 2014a; 2014b). The epidemic was characterized by an increase number of severe cases in children under 15 years old (Teixeira et al., 2009). Phylogeny of DENV-2 strains isolated during the re-emergence period (2007-2008) showed the same genotype (Southeast Asia-American) circulating since this serotype introduction in 1990, howerver the emerging viruses grouped in a distinct monophyletic group. The circulating viruses in the 1990s were classified as lineage I and those from the re-emergence period (2007-2008), as lineage II (Oliveira et al., 2010). Analyzing the E gene of DENV-2 isolated from eighteen patients, Romano et al. (2010) reported the reintroduction of this serotype on the coast of São Paulo and confirmed the circulating genotype. Furthermore, it was shown that the strains were related to those from Rio de Janeiro isolated in 2007 and 2008 and, the most common ancestor dated from about 40 years ago. The Southeast region of the country was strongly affected during the re-emergence of DENV-2, and as studies performed in Rio de Janeiro and São Paulo, Dettogni et al. (2012) characterized the strains isolated in the state of Espirito Santo in 2009, confirming the previous observations. The molecular characterization and phylogeny of 34 strains isolated during the twenty years of DENV-2 activity (1990 to 2010), representing six states in Brazil, identified two epidemiologically distinct groups: one represented by strains isolated from 1990 to 2003 and of isolates from 2007 to 2010. The analysis of the E gene and the complete coding region of strains representative of distinct clinical manifestations, showed no mutations on the viral genome associated to the disease severity, however all strains presented an asparagine (N) in position E_{390}, a genetic marker for virulence. The Southeast Asia-American genotype was confirmed as the only detected in 20 years and the viruses isolated between 1990 to 2003 were grouped in the lineage I and isolates as from the re-emergence in 2007 grouped in the lineage II, corroborating Oliveira et al. (2010) and Romano et al. (2010). Moreover, strains from lineage II had a high similarity with the strain isolated from the Dominican Republic in 2001 and, the high divergence with the strains isolated in the 1990's, suggested that the new strain did not evolved locally, but was probably introduced from the Caribbean (Faria et al., 2013). The study by Drumond et al. (2013) described the dynamics, molecular characterization and phylogeny of the DENV-2 lineages belonging to the Southeast Asia-American genotype based on whole genomes from viruses isolated in in Sao Jose do Rio Preto, Sao Paulo in 2008. Brazilian strains were subdivided into three lineages grouped into two clades. It was estimated that lineage 1, represented by strains with broader geographic region and higher genetic diversity, was introduced in 1988-1989. Lineage 2 was estimated to have been introduced in Northern Brazil, probably from Puerto Rico between 1998 and 2000

and lineage 3, the latest to emerge in the country, during parallel events. A report by Nunes et al. (2014), described the results on the analysis of the complete genomes of 294 DENV-2 clearly evidencing three different lineages from this serotype in Brazil. The estimated time between the introductions of different lineages is eight years (between the lines I and II) and approximately seven years (between strains II and III), and the lineage II replaced by lineage III. The results also highlighted the northern region of Brazil as a entrance and dispersal door of this serotype in the country, especially those originating from the Caribbean. Barcelos Figueiredo et al. (2014) reconstructed the phylogeographic history of DENV-2 in Brazil by analyzing the gene E of viruses isolated during an outbreak in the state of Piauí, in 2006-2007, and pointed out that the introduction and spread of those viruses in Brazil followed one Caribbean route. As Drummond et al. (2013), the authors demonstrated that lineage 2 (represented by strains of period 2007-2011) may have been introduced in Brazil from the Caribbean islands through two parallel events: one in the Southeast (lineage 2a) and another in the North (lineage 2b). This study confirmed the occurrence of the first co-circulation spatiotemporal of two DENV-2 different lineages probably introduced independently to the state of Piaui. The Caribbean islands are the main source of DENV-2 in Brazil and the Northeast seems to be an important route of introduction and spread of this virus in the country. Figure 2 presents the phylogenetic analysis on representative strains of DENV-2, circulating in Brazil.

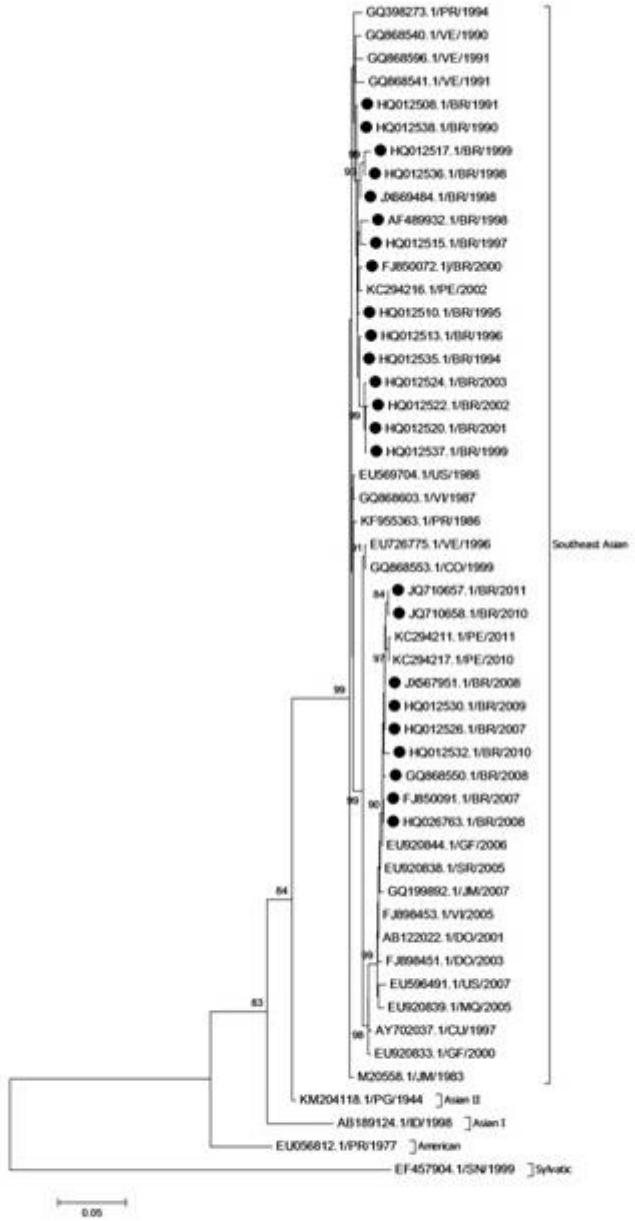

Figure 2. Phylogenetic analysis based on the E gene of representative DENV-2 strains. Neighbor joining method, TN93+G model with correction value of 0.24. Bootstrap test (1000 replicates) is shown next to the branches. Black circles represent the Brazilian DENV-2. DENV strains were named as follows: GenBank accession number/country/year.

Molecular Epidemiology of Brazilian DENV-3

The DENV-3 was first isolated in the municipality of Nova Iguaçu, Rio de Janeiro in the end of 2000 and the introduction of this new serotype resulted in the following year, in co-circulation of the three serotypes (DENV-1, DENV-2 and DENV-3), and in the largest and most severe epidemic reported in the country so far in the year 2002 (De Simone et al., 2004; Nogueira et al., 2002).

The genetic characterization of a DENV-3 isolated in Rio de Janeiro in 2001 was performed and published by Miagostovich et al. (2002). In 2006, the first complete genome genetic characterization of a DENV-3 isolated from the liver of a fatal case occurred in 2002 in Rio de Janeiro was reported and the phylogeny confirmed the genotype III (Indian Subcontinent). The genome molecular characterization identified an insertion of 11 nucleotides in the 3 'UTR of the viral genome, charcteristic of DENV-3 of Asian origin and also found in strains isolated in Martinique (Miagostovich et al., 2006). In the same year, Aquino et al. (2006) published a study on the molecular epidemiology of DENV-3 from Brazil and Paraguay isolated between 2002 and 2004, based on the analysis of the E gene and 5' and 3' UTRs. Genotype III was confirmed and reported as a common genotype in the Americas, probably originating in Southeast Asia.The 5' and 3'UTRs were highly conserved between samples studied, but two Brazilian isolates of the North region presented an eight nucleotide deletion compared to the other 26 virus analyzed. Phylogenetic analysis indicated that the two strains isolated in the North in 2002 and 2004 were more related to isolates from Martinique than to the Brazilian strains isolated in Rio de Janeiro and it was suggested that those strains did not evolved locally, but were probably introduced from the Caribbean, indicating at least two DENV-3 introductions in Brazil. In 2008, Barcelos Figueiredo et al. (2008) by the phylogenetic analysis based on a conserved fragments of the genome and on a more variable region reported the circulation of a new DENV-3 genotype (genotype I) in strains isolated in the state of Minas Gerais from DF, DHF and fatal cases. In the same year, a study by Nogueira et al. (2008) described the co-circulation of genotypes III and V in North Brazil (Acre and Rondônia) during epidemics occurred from 2002 to 2004. Araújo et al. (2009b) performed the evolutionary history reconstruction and phylogeography of the three major DENV-3 genotypes, estimating their dispersion, based on the analysis of the E gene of 200 strains isolated from 31 different countries around the world in a 50-year period (1956-2006). The phylogenetic analysis revealed a geographical subdivision of DENV-3 in several specific clades in each country. Migration patterns of the main DENV-3 genotypes showed that genotype I was mostly circulating in the maritime region of Southeast Asia and the South Pacific, genotype II remained within continental areas of Southeast Asia, while genotype III was propagated throughout Asia, East Africa and the Americas. Estimates of evolutionary rates revealed no significant differences between the main DENV-3 genotypes and the average rate of evolution in endemic areas with long-term transmission was similar to that observed in the Americas, which have suffered a more recent spread of dengue. Estimated origins of DENV-3 was around 1890, and the emergence of the current diversity of the major genotypes, between 1960 and 1970, coinciding with the population growth, urbanization and the description of the first cases of DHF by DENV-3 in Asia. Due to the controversy in the DENV-3 genoytpes classification described in Brazil, genotype I by Barcelos Figueiredo et

al. (2008) and genotype V by Nogueira et al. (2008), Araújo et al. (2009a) performed a DENV-3 phylogenetic perspective study circulating in the country. The phylogenetic analysis and nucleotide distance based on the E gene of 103 strains from 30 different countries around the world, supported the DENV-3 subdivision into five distinct genotype and confirmed the classification of the new genotype from South America as genotype V. Alfonso et al. (2012) analyzed the phylogenetic relationships of DENV-3 isolated in Brazil and Paraguay with viruses isolated around the world, and the dynamics of evolutionary divergence of the serotype based on the complete coding region. The data obtained indicated that two groups of the genotype III circulated during 2002-2009 in Brazil, being separated as two sub-lines A and B, suggesting different introduction events different regions of the country. In the same year, de Araújo et al. (2012) published a study on the origin and evolution of DENV-3 genotype III in Brazil by analyzing the E gene of 107 sequences isolated from 2001 to 2009 in several regions of the country. The analysis revealed that at least four introductions of genotype III occurred in Brazil, represented by the presence of four distinct phylogenetic lineages and highlighted the importance of the Caribbean to the frequent introduction of strains in Brazil, and suggested that the North and Southeast regions are of great importance to the introduction and spread of DENV-3 strains. Aware that the DENV genetic diversity plays an important role in the disease immunopathogenesis, and that the polymorphisms associated with adaptive response is relevant to vaccine development, Villabona-Arenas et (2013) reported the adaptive changes of DENV-3 during an epidemic in Sao Jose do Rio Preto, in 2006 and 2007. In order to characterize the role of possible genetic changes that would interfere with the immune escape, the study sequenced the entire coding region from thirty-three DENV-3 isolates. Based on the evolutionary history, at least two different DENV-3 introductions were identified in the city, and ten polymorphic sites over significant positive pressure detected were detected. Furthermore, the major polymorphic sites were found in the E, NS1, NS2A and NS5 genes, suggesting that those could be experiencing recent adaptive changes. Figure 3 presents the phylogeny based on the E gene analysis from representative Brazilian DENV-3 strains.

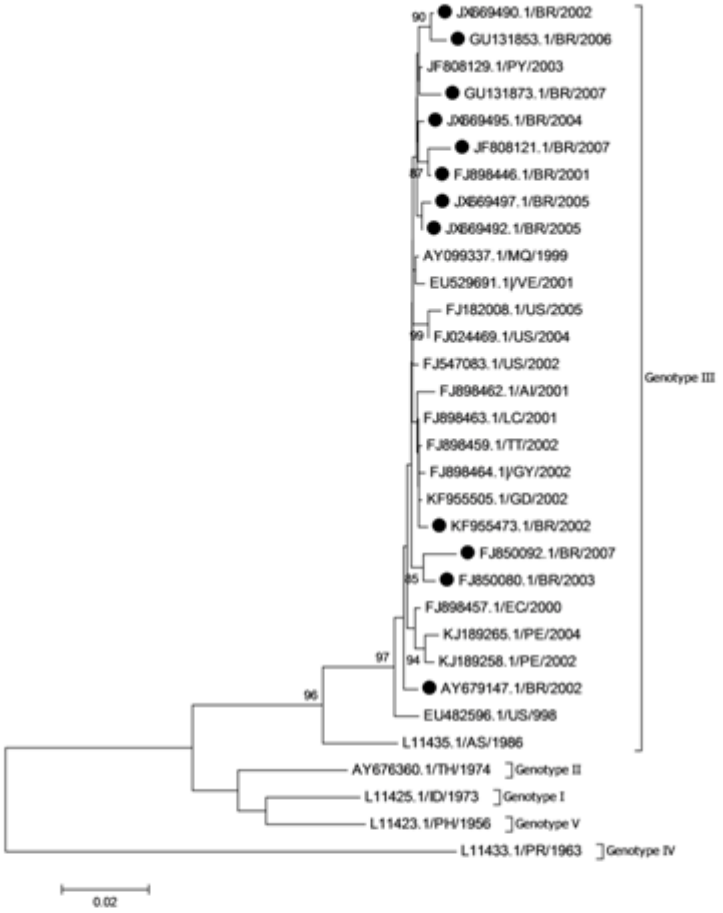

Figure 3. Phylogenetic analysis based on the E gene of representative DENV-3 strains. Neighbor joining method, TN93+G model with correction value of 0.22. Bootstrap test (1000 replicates) is shown next to the branches. Black circles represent the Brazilian DENV-3. DENV strains were named as follows: GenBank accession number country/year.

Molecular Epidemiology of Brazilian DENV-4

The DENV-4 reemerged in July of 2010 in the city of Roraima, northern Brazil, after about 30 years since its introduction in 1981-82 (Temporao et al., 2011). This serotype spread throughout the country and in 2011, co-circulated with DENV-1, causing an epidemic of 764,032 reported cases. In that same year, de Souza et al. (2011) analyzed the phylogenetic relationships based on the E gene of six DENV-4 strains isolated in the states of São Paulo, Paraná and Rio Grande do Sul and reported the genotype II circulating in the country. Nunes

et al. (2012) studied the phylogeography of DENV-4 in Brazil based on the complete genome of sixteen isolates circulating in the North (Roraima, Amazonas, Pará) and Northeast (Bahia) regions, from 2010 and 2011. It was shown that, despite the confimation of genotype II, one DENV-4 strain isolated in Bahia belonged to genotype I. Furthermore, it was also revealed that genotype II was introduced more than three times in the last decade, two from Venezuela to Roraima and from Colombia to the Amazon. Analyzing ten DENV-4 isolates from Rio de Janeiro in 2011 and 2012, Campos et al. (2013) confirmed the circulation of genotype I in one strain and genotype IIb on the remainig strains, which were closely related to those isolated in Roraima in 2010 and Sao Paulo in 2011. The co-circulation of two distinct DENV-4 genotypes was demonstrated in country (Figure 4) and corroborated by the recent results described by Pinho et al. (2015), also analyzing strains from Bahia. It was shown that the Brazilian strains were similar to the circulating strains in Vietnam between 2004 and 2008, suggesting that those were derived from this variant of genotype I.

Figure 4. Phylogenetic analysis based on the E gene of representative DENV-4 strains. Neighbor joining method, TN93+G model with correction value of 0.29. Bootstrap test (1000 replicates) is shown next to the branches. Black circles represent the Brazilian DENV-4. DENV strains were named as follows: GenBank accession number/country/year.

MOLECULAR BIOLOGY APPROACHES APPLIED TO DENGUE VIRUS SURVEILLANCE IN MOSQUITOES VECTOR

The broad spread of the mosquito vector *Aedes aegypti* has made dengue the most important human arbovirus disease in the world. Besides the man-mosquito-man cycle of dengue transmission, virus maybe vertically transmitted from a female infected to her offspring and by veneral transmission among mosquitoes. It is possible that the transovarial transmission represents a way of maintenance of the DENV in nature, because the virus can persist until the seventh generation in the mosquitoes tissues (Kow et al. 2001; Joshi et al. 2002; Le Goff et al. 2011).

The viral detection in mosquitoes highlights the position of the vector as a required element in the transmission cycle of DENV during an epidemic (Liotta et al. 2005) and the entomological surveillance of those viruses by rapid methods of detection and characterization is an important tool not only to help to predict the occurrence of explosive epidemics but also to detect the introduction of new virus and/or genotypes. DENV detection rates from *Aedes* mosquitoes by RT-PCR may vary depending on the geographical location, epidemiological background or the vector popula- tion. For instance, during a surveillance performed in Taiwan, only 0.2% of *Aedes aegypti* females were positive for DENV (Chen et al. 2010). On the other hand, in a surveillance performed in Mexico, 16.1% of the *Aedes aegypti* females collected in schools were infected with DENV (García-Rejón et al. 2011).

In Brazil, during an entomological surveillance performed in RJ in 2001, DENV-1 was detected in *Aedes aegypti* mosquitoes by RT-PCR, when the presence of DENV-3 was being investigated. Previous studies showed that 17% of the *Aedes aegypti* mosquitoes were infected in a DENV-3 surveillance performed in Manaus, North region of Brazil (Pinheiro et al. 2005). However, in Rio de Janeiro only 0.1% of adult mosquitoes was infected by DENV-3 during an entomological surveillance performed during the inter-epidemic year of 2006. A semi Nested RT-PCR specific to genus Flavivirus was performed during an entomological surveillance in 1,700 mosquitoes collected in the Northeast, Southeast and South regions of Brazil, from 1999 to 2005. DENV-1 to 3 were detected in 3.8% of the pools analyzed and one in a pool of *Haemagogus leucocelaenus* suggesting that this virus could be involved in a sylvatic cycle. DENV-3 was found infecting pools of Aedes *albopictus* larvae and phylogeny identified the DENV-3 genotype III (de Figueiredo et al., 2010). In Recife, Northeastern Brazil, despite the predominance of DENV-3 in human cases, 10% of the tested pools were infected, and both DENV-2 and DENV-1 were detected in mosquitoes (Guedes et al. 2010). During the DENV-4 entomological surveillance performed by RT-PCR in Roraima, North Brazil in 2010, after this serotype emergence, DENV-1 was also detected (Castro MG, personal communication). By using rtRT-PCR, Maciel-de-Freitas (2011) analyzed the survival rate, longevity and fecundity of *Aedes aegypti* females orally challenged with DENV-2 and demonstrated that infected mosquitoes from a long-established laboratory colony presented more viral RNA copies at death than those from the F1-generation of a field population from Rio de Janeiro, Brazil. In 2008, during an entomological surveillance performed in the district of Tancredo Neves Manaus, a total of 260 *Aedes* mosquitoes was tested by Nested RT-PCR. One *Aedes aegypti* pool was positive for DENV-4 and the genome sequencing characterized the DENV-4 genotype (Figueiredo et al., 2013). A study by Castro

et al. (2012) characterized the first DENV- 4 cases occurred in Rio de Janeiro in 2011, and demonstrated the role of rapid molecular techniques, such as conventional RT-PCR and rtRT-PCR, in the entomological surveillance of the newly introduced DENV-4 in vector populations after it was isolated from humans. The rtRT-PCR was used to quantify the DENV-4 viral titre (1.08 x 10^3 copies/mL) from a single *Aedes aegypti* female mosquito that was naturally infected and individually macerated. In the study, the effectiveness of the Simplexa™ Dengue rtRT-PCR kit comercially available, for the detection and typing of DENV in *Aedes aegypti*, was also evaluated. A single female was positive for DENV-4 with a low Ct value (23.5), suggesting a high viral load. Natural vertical transmission of DENVV-2 and DENV-3 in *Aedes aegypti* and *Aedes albopictus* was reported in a study performed in 40 neighborhoods in Fortaleza, Ceará, Brazil from March 2007 to July 2009. A total of 3,417 *Aedes aegypti* and 336 *Aedes albopictus* was analyzed by virus isolation, RT-PCR and genome sequencing. The study reported *Aedes albopictus* infected by DENV-3, *Aedes aegypti* infected by DENV-2 and *Aedes albopictus* co-infected by DENV-2 and DENV-3 (Martins et al., 2012). Aiming to contribute for DENV phylogenetic and vector-virus-human host studies, de Castro et al. (2013) sequenced the entire genome of one DENV-3 isolated from naturally infected *Aedes aegypti* from RJ in 2001 and characterized the 3' UTR from strains isolated from mosquitoes and humans. The phylogeny characterized the DENV-3 isolated from both vector and human host as belonging to Genotype III despite the differences observed on the 3' UTR. In the DENV-3 isolated from the mosquito vector, an eight nucleotides deletion was observed within the eleven nucleotides insertion on the variable region from the 3´UTR. A study evaluated whether those distinct DENV-3 variants presenting those characteristics showed differences on the virus-vector interaction by determining the vector competence of two populations of *Aedes aegypti*. The results suggested that the experimental population proved to be competent to transmit the two DENV-3 strains. However, the viral dissemination in the mosquitoes body presented heterogeneously, suggesting that there are advantages for the strain with eleven nucleotides insertion in the 3'UTR, once it disseminated more rapidly (Chouin-Carneiro, 2014). Previous studies have suggested that the sequence and secondary structures of the 5´and 3´UTR of flaviviruses play an important role in viral replication and differences in these regions may influence viral virulence. Moreover, mutations and deletions within these regions may alter infectivity and reduce efficiency of viral replication. A study aiming to compare the DENV susceptibility of field-captured *Aedes aegypti* from nine distinct geographic areas of the city of Belo Horizonte in 2009 and 2011, analyzed adult females experimentally infected with DENV-2 and the virus was detected by rtPCR in body and head samples. Infection rate ranged from from 40% to 82.5% in 2009 and 60% to 100% in 2011, vector competence ranged from 25% to 77.5% in 2009 and 25% to 80% in 2011and disseminated infection rates oscillated from 68.7% to 100% in 2009 and 38.4% to 86.8 in 2011, concluding that *Aedes aegypti* populations from Belo Horizonte exhibited wide variation in vector competence to transmit DENV (Gonçalves et al., 2014). The natural transovarial transmission of DENV in *Aedes aegypti* from Cuiabá, Mato Grosso, Brazil was evaluated by using a multiplex semi-nested RT-PCR to detect the four DENV, Yellow Fever virus, West Nile virus and Saint Louis encephalitis virus in mosquitoes collected in 2012. DENV-4 was the only flavivirus detected in 16.0% of the mosquitoes pools tested, including males. Furthermore, the nucleotide sequences presented 96-100% homology with the DENV-4 strains form genotype II detected in Manaus, Amazonas (da Cruz et al., 2015).

As shown previously, the molecular techniques used in several entomological surveillances were found be effective even when a new serotype was introduced or when a serotype re-emerged during a dengue epidemic of a different serotype.

NEW APPROACHES FOR DENGUE DIAGNOSIS AND RESEARCH: APTAMERS

With the technical development and new tools availability, several approaches for dengue diagnosis and research are currently under investigation. Some promising tools rely on molecular biology-based techniques. Aptamers, for instance, are single-stranded oligonucleotides that bind to its target with high affinity and specificity. In 1990, both the Gold (Tuerk & Gold, 1990) and Szostak groups (Ellington & Szostak, 1990), described a new method using combinatorial chemistry library of nucleic acids (DNA and RNA) to select RNA oligonucleotides that firm and selectively linked to certain targets. The technique known as "Systematic Evolution of Ligands by Exponential Enrichment" - SELEX - allows obtaining high affinity and specificity primers for any class of organic molecules. The oligonucleotide ligands generated by this technique are called aptamers derived from the Latin "*aptus"*, able to bind to and *"mers"* derived from the Greek, meaning particle. The most revolutionary aspect of SELEX is the selection of binders by the use of oligonucleotide libraries chemically synthesized without the constraint of having to be selected, or produced in a living organism (Stoltenburg et al., 2007). In general, the SELEX process comprises three steps that are repeated to seek more nucleotides capable of binding to the target. In the first step, library selection begins with library generation of single stranded random nucleotides. In a second step, oligos must be incubated with a target molecule in the presence of a buffer and the desired temperature. These factors control the stringency of the binding process of the nucleic acid molecules of interest. Finally, in the third step, the library amplified by PCR to create a new library. This new and enriched oligonucleotide pool will be used in the binding reaction with the target in the next cycle. The last cycle is stopped after amplification and PCR products are cloned to characterize the aptamers. Aptamers have been mainly developed for applications such as diagnostics or therapeutics (Bunka & Stockley, 2006; Song et al., 2012). For dengue a modular biosensor that is able to quickly identify sequences associated with the virus genome has been developed. The biosensor was able to detect sequences derived from each of the four DENV serotypes with a high degree of specificity. Along with sequences specific for each serotype, a sequence for pan-Dengue detection of all serotypes was produced (Fletcher et al., 2012). Furthermore, several DNA aptamers that bind to various important arboviruses with potential diagnostic biosensor applications haave been developed (Bruno et al., 2012). The aptamers enables direct application in the detection and quantification of molecules and its advantages over alternative methods include being a relatively simple technique, requiring simple equipment for its isolation, large number of alternative molecules that can be sorted and its chemical simplicity (James, 2000).

NEXT GENERATION SEQUENCING

As previously reported, RNA viruses are known to rapidly adapt facing selective pressure from the host immune system and/or antivirals mainly due to the error-prone characteristic of the RNA-dependent RNA polymerase. More recently, using next-generation sequencing (NGS), the generated viral populations can be identified and quantified. NGS and also known as deep sequencing has recently emerged as one of the most promising tools for genome sequencing, evolution, ecology, discovery and transcriptomics (Tang & Chiu, 2010; Radford et al., 2012; Chiu, 2013).

This platform involves the analysis of millions of nucleic acid–derived sequences present in clinical samples to detect sequences related to particular pathogens. Due to its unbiased nature, NGS allows the identification of new viruses, emerging viruses, which can be highly diverse, with high rates of mutation and recombination. Moreover, one can now accurately quantify distinct viral populations and track the changes to these populations over the course of a single infection (Chiu, 2013). Currently, there are many NGS platforms to choose from, depending on the read length and depth, such as, the 454 Sequencing (Roche Diagnostics), SOLiD sequencing (Life Technologies), Illumina and Ion Torrent. A recent study compared three NGS platforms for metagenomic sequencing and identification of pathogens in blood and reported that the Roche-454 Titanium platform detected DENV at titers as low as $1X10^{2.5}$ pfu/mL, corresponding to maximum $5.4X10^{4}$ genome copies/ml. The Ion Torrent and Illumina platforms, enabled detection of viral genomes at concentrations as low as $1X10^{4}$ genome copies/mL and platform-specific biases were evident in sequences read distributions and viral genome coverage. Overall, the platforms performed well for the pathogen identification from blood. However, unlike the identification, the pathogen characterization is likely to require higher titers, multiple libraries and/or multiple sequencing runs (Frey et al., 2014). The NGS approach has many advantages over more traditional methods for virus detection, such as PCR, ELISA and virus isolation. Those may include speed, the ability to detect non-culturable pathogens or an unknown pathogen in a clinical sample. However, difficulties associated to the intensive computational analysis, data storage, lack of reference genomes for comparison in some cases, the need of high coverage to detect some pathogens at very low levels in a clinical specimen and high cost, are some challenges still to be faced.

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