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VÍRUS DENGUE SOROTIPO 3 (DENV-3) NO BRASIL: ESTUDOS SOBRE PATOGENIA, SÍTIOS DE REPLICAÇÃO, FILOGENIA E EVOLUÇÃO MOLECULAR

por

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JOSÉLIO MARIA GALVÃO DE ARAÚJO

Tese apresentada com vistas à obtenção do Título de Doutor em Ciências na área de Virologia.

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Co-Orientador(a): Prof. Dr. Hermann Gonçalves Schatzmayr

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Este trabalho é dedicado

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"Os conhecimentos nos dão meios para viver.
A sabedoria nos dá razões para viver."

(Rubem Alves)

"Atrás de uma descoberta científica sempre existe uma idéia,
e unida à ela sempre existe um sentimento,
uma beleza,
uma harmonia
e muita paciência.
Se a gente conseguir transmitir isso à sociedade,
a ciência ocupará o lugar que merece
e formará mais parte de nossa vida."

(Eloi Garcia)

"Há uma grandeza nessa visão da vida, com seus vários poderes, ter sido inspirada originalmente em umas poucas formas, ou em uma só; e isso, enquanto este planeta vai executando seus ciclos de acordo com a lei imutável da gravidade;
de um começo tão simples foram,
e estão sendo,
produzidas formas sem fim,
as mais belas e mais maravilhosas"

(Charles Darwin)

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RESUMO

Dengue é uma importante arbovirose (arthropod-borne virus) e constitui um grave problema de saúde pública não só no Brasil, mas também nos países de clima tropical. O *Aedes aegypti* é o principal vetor dos vírus dengue (DENV) e está presente na maioria dos países entre as latitudes 35ºN e 35ºS. Neste trabalho, apresentamos quatro estudos. No primeiro estudo, analisamos os níveis de RNA viral dos DENV-3 e sua correlação com o tipo de infecção (primária ou secundária) em casos fatais e não fatais por dengue, ocorridos no estado do Rio de Janeiro, 2002. O grupo de casos fatais apresentou uma média de título viral significativamente mais elevada do que o grupo de casos não fatais. Considerando que infecções primárias foram confirmadas entre os casos fatais (52,1%), a teoria da infecção seqüencial por si só não explica todos os casos graves da doença. Estes resultados sugerem que altos níveis de DENV-3 podem ter contribuído para a forma grave do dengue no Rio de Janeiro, 2002.

No segundo estudo, diferentes métodos de diagnóstico foram aplicados para investigar a presença dos DENV em amostras de tecidos humanos obtidos a partir de casos fatais (n = 29), ocorridos durante a grande epidemia em 2002 no estado do Rio de Janeiro, Brasil. A combinação de quatro métodos permitiu a confirmação da infecção por DENV-3 em 26 (89,6%) dos 29 casos suspeitos. O isolamento viral foi obtido em 2,7% (2/74) das amostras, a partir da inoculação em cultura de células C6/36. A técnica de nested RT-PCR permitiu a identificação do DENV-3 em 30,5% (22/72) das amostras analisadas. O método de RT-PCR em tempo real possuiu maior sensibilidade, detectando o RNA viral em 58,4% (45/77) dos espécimes clínicos, incluindo fígado (n=18), pulmão (n=8), baço (n=8), cérebro (n=6), rim (n=3), medula óssea (n=1) e coração (n=1). A técnica de imunohistoquímica detectou o antígeno viral em 44% (26/59) das amostras analisadas. A precisão e eficácia do RT-PCR em tempo real fez desta técnica uma ferramenta importante no diagnóstico rápido das infecções por dengue.

No terceiro estudo, revisamos a filogeografia dos três principais genótipos do DENV-3 e estimamos sua taxa de evolução, com base na análise do gene do envelope (E) de 200 isolados, provenientes de 31 países ao redor do mundo, durante um período de 50 anos (1956 - 2006). Nossa análise filogenética revelou uma subdivisão geográfica da população dos DENV-3, com grupamentos específicos em vários países. Os padrões migratórios dos principais genótipos dos DENV-3 mostraram que genótipo I circula principalmente na porção marítima do Sudeste Asiático e no Sul do Pacífico, o genótipo II permaneceu dentro das zonas continentais do Sudeste Asiático, enquanto o genótipo III foi disseminado na Ásia, Leste da África e Américas. Não foi

observada co-circulação de diferentes genótipos em uma única localidade, sugerindo que alguns fatores, além da distância geográfica, podem limitar a contínua dispersão e re-introdução de novas variantes de DENV-3. As estimativas das taxas evolutivas não revelaram diferenças significativas entre os principais genótipos do DENV-3. A média da taxa de evolução em regiões que sofreram epidemias de dengue desde a década de 70 (por exemplo, Indonésia e Tailândia) foi semelhante ao observado em regiões que presenciam estas epidemias desde a década de 90 (por exemplo, Américas). Neste estudo, estimamos o ano de origem das atuais linhagens do DENV-3 em torno de 1890, e o surgimento da atual diversidade dos seus principais genótipos entre meados de 1960-1970, coincidindo com o crescimento da população humana, urbanização, movimento humano e descrição dos primeiros casos de febre hemorrágica por DENV-3 na Ásia.

No quarto estudo, examinamos a atual classificação filogenética dos DENV-3 circulantes no Globo, com destaque para o novo genótipo (GV) descrito no Brasil. A circulação de um novo genótipo de DENV-3 foi recentemente descrito no Brasil e na Colômbia, porém, sua classificação exata tem sido controversa. Análises de distância nucleotídica do gene E apóia a subdivisão do DENV-3 em cinco linhagens distintas, denominadas genótipos (GI-GV), e confirma a classificação deste novo genótipo na América do Sul como pertencente ao GV. Distâncias genéticas extremamente baixas entre isolados brasileiros pertencentes ao GV e a amostra protótipo Philippines/L11423 isolada em 1956 levantam questões importantes sobre a origem deste genótipo na América do Sul.

ABSTRACT

Dengue is an important arbovirus (arthropod-borne virus) and constitutes a serious problem of public health not only in Brazil but also in the major tropical countries. *Aedes aegypti* is the main vector of dengue virus (DENV) and is present in most countries between latitudes 35ºN and 35ºS. In this manuscript, we present four distinct studies. In study 1, we examined levels of dengue virus type 3 RNA in association with the type of infection (primary or secondary) in patients with fatal and nonfatal outcomes in Rio de Janeiro State, 2002. Subjects with fatal outcomes had mean virus titers significantly higher than those who survived. Because primary infections were confirmed among the fatal cases (52.1%), antibody-dependent enhancement alone did not explain all the cases of severe disease in this study population. These findings suggest that high levels of DENV-3 may have contributed to the severe form of dengue in Rio de Janeiro, 2002.

In the second study, we investigate by different diagnostic methods dengue virus in human tissue specimens obtained from fatal cases (n=29) during a large-scale dengue fever epidemic in 2002 in the State of Rio de Janeiro, Brazil. The combination of four procedures provided diagnostic confirmation of DENV-3 infection in 26 (89.6%) out of the 29 suspected fatal cases. Dengue virus (DENV) was isolated from 2/74 (2.7%) tissue samples, inoculated into C6/36 cells and identified as DENV-3, nested RT-PCR accusing 22/72 (30.5%) samples as DENV-3. Real-time RT-PCR yielded the highest positivity rate, detecting viral RNA in 45/77 (58.4%) clinical specimens, including the liver (n=18), lung (n=8), spleen (n=8), brain (n=6), kidney (n=3), bone marrow (n=1) and heart (n=1). Immunohistochemical tests recognized the DENV antigen in 26/59 (44%) specimens. Given the accuracy and effectiveness of real-time RT-PCR in this investigation, this approach may play an important role for rapid diagnosis of dengue infections.

In the third study, we revisited the phylogeography of the three of major DENV-3 genotypes and estimated its rate of evolution, based on the analysis of the envelope (E) gene of 200 strains isolated from 31 different countries around the world over a time period of 50 years (1956 to 2006). Our phylogenetic analysis revealed a geographical subdivision of DENV-3 population in several country-specific clades. Migration patterns of the main DENV-3 genotypes showed that genotype I was mainly circumspect to the maritime portion of Southeast-Asia and South Pacific, genotype II stayed within continental areas in South-East Asia, while genotype III spread across Asia, East Africa and into the Americas. No evidence for rampant co-circulation of

distinct genotypes in a single locality was found, suggesting that some factors, other than geographic proximity, may limit the continual dispersion and reintroduction of new DENV-3 variants. Estimates of the evolutionary rate revealed no significant differences among major DENV-3 genotypes. The mean evolutionary rate of DENV-3 in areas with long-term endemic transmissions (i.e., Indonesia and Thailand) was similar to that observed in the Americas, which have been experiencing a more recent dengue spread. We estimated the origin of DENV-3 virus around 1890, and the emergence of current diversity of main DENV-3 genotypes between the middle 1960s and the middle 1970s, coinciding with human population growth, urbanization, and massive human movement, and with the description of the first cases of DENV-3 hemorrhagic fever in Asia.

In the fourth study, we re-examined the current phylogenetic classification of DENV-3 strains, with emphasis on the new genotype (GV) described in Brazil. Circulation of a new DENV-3 genotype was recently described in Brazil and Colombia, but the precise classification has been controversial. Phylogenetic and nucleotide distance analyses of the envelope (E) gene support the subdivision of DENV-3 strains into five distinct genotypes (GI to GV), confirming the classification of this new genotype in South America as GV. The extremely low genetic distances of Brazilian GV strains to the prototype Philippines/L11423 strain isolated in the 1956 GV sample raise important questions regarding the origin of this genotype in South America.

LISTA DE ILUSTRAÇÕES

Página

LISTA DE TABELAS

Página

LISTA DE ABREVIATURAS E SIGLAS

Γ ..... Gamma-distributed rate variation
aa ..... Aminoácido
ACCTRAN ..... Accelerate Character Transformations
BF ..... Bayes Factor
BI ..... Bayesian Inference
C6/36 ..... Linhagem de cultura de células de mosquito *Aedes albopictus*
CDC ..... Center for Disease Control and Prevention
cDNA ..... Ácido desoxirribonucléico complementar
DC ..... Dengue Clássico
DELTRAN ..... Delay Character Transformations
DENV ..... Vírus Dengue
DF ..... Dengue Fever
DHF ..... Dengue Hemorrhagic Fever
DNA ..... Ácido desoxirribonucléico
dNTP ..... Desoxirribonucleotídeo
DSS ..... Degue Shock Syndrome
DTT ..... Dithiotreitol
E ..... Envelope
ECP ..... Efeito Citopático
EDTA ..... Ácido etilenodiamiotetracético
EIA ..... Enzyme Immune Assay
ESS ..... Effective Sample Size
FHD ..... Febre Hemorrágica do Dengue
G ..... Genótipo
GTR ..... General Time Reversible Model
G/C .....Guanina/Citosina
HPD ..... Highest Posterior Density
I ..... Proportion of invariable sites
IF ..... Imunofluorescência
IFI ..... Imunofluorescência Indireta
IgG ..... Imunoglobulina da Classe G

kDa ..... Kilodaltons
LCR ..... Líquido Céfalo-Raquidiano
M ..... Membrana
MAP ..... Maximum Posterior Probability
MCMC ..... Bayesian Markov Chain Monte Carlo
MS ..... Ministério da Saúde
mL ..... Mililitro
ML ..... Maximum Likelihood
mM ..... Milimolar
MPRs ..... The most parsimonious reconstructions
NC ..... Não-Codificante
ng ..... Nanograma
nM ..... Nanomolar
nt ..... Nucleotídeo
OMS ..... Organização Mundial de Saúde
OPAS ..... Organização Pan Americana de Saúde
pb ..... Pares de Bases
pM ..... PicoMol
PBS ..... Salina Fosfatada Tamponada
PCR ..... Reação da Polimerase em Cadeia
PFU ..... Unidade Formadora de Placa
pH ..... Potencial Hidrogeniônico
prM ..... Pré-Membrana
RNA ..... Ácido Ribonucléico
RNAse ..... Ribonuclease
rpm ..... Rotações por Minuto
RT-PCR .....Transcrição Reversa seguida da Reação em Cadeia pela Polimerase
SCD ..... Síndrome do Choque por Dengue
SNC ..... Sistema Nervoso Central
SVS/MS ..... Secretaria de Vigilância em Saúde / Ministério da Saúde
subs/site/year ..... substitutions per site per year
TEB ..... Tris/borato/EDTA
Tmrca ..... Time of The Most Recent Common Ancestor

Tris ..... Tris (hidroximetil) aminometano

μ ..... Evolutionary rate

µL ..... Microlitro

µM ..... MicroMol

V ..... Volts

WHO .....……..... World Health Organization

ºC ..... Graus Centígrados

SUMÁRIO

Página

1. INTRODUÇÃO

O dengue é uma doença infecciosa aguda causada por um vírus de genoma RNA, do qual são reconhecidos quatro sorotipos (DENV-1, DENV-2, DENV-3 e DENV-4), transmitidos pelo *Aedes aegypti* seu principal vetor. Esta doença constitui um dos principais problemas de saúde no mundo (Gubler, 1998), pois o número de casos notificados está em ascensão, assim como a mortalidade.

Existe uma explosão de dengue? Pergunta Halstead em carta enviada ao *Lancet* em 1999. A resposta veio do britânico Jacobs, em janeiro de 2000, referindo-se à emergência do dengue como um problema global de saúde pública ao constatar que pelo menos 20 milhões de infecções ocorrem no mundo a cada ano, assim como várias centenas de milhares de casos das formas graves e potencialmente mortais como é a Febre Hemorrágica do Dengue e a Sindrome do Choque por dengue (FHD/SCD). E ainda, que o espaço geográfico do dengue tem se ampliado e o dengue hemorrágico está se apresentando em novas áreas com maior incidência (Halstead, 1999; Jacobs, 2000; Werner, 2001). Dessa forma, contribui também para o aumento da carga global de infecções transmitidas por vetores (Molyneux, 2001).

O fenômeno universal da globalização também inclui doenças infecciosas em geral e o dengue em particular (Chastel, 1997; Nuttal & Gould, 1998). É penoso reconhecer, porém há mais de dez anos já se estimava que o dengue e suas formas graves como a FHD/SCD, se reafirmariam no futuro como um problema mundial de saúde (Le Duc, 1994; Gubler & Clark, 1995). E de fato, é o que observamos hoje na maioria dos países de clima tropical ou subtropical.

Os fatores de maior importância para a extensão e o aumento das epidemias de dengue estão relacionados com mudanças na ecologia humana, as quais propiciam um maior contato com o *Ae. aegypti*. Nessa complexa interação participam fatores virais, do hospedeiro, do vetor, do ambiente e do clima (Monath, 1994). Todos estes fatores são importantes, porém os fatores sociais e a qualidade de vida das populações são determinantes (Marzochi, 1994).

Ressaltou W. Ledermann: "Não esqueçamos que o patógeno interage com o homem – e os animais – através do meio ambiente ou entorno, tendo como única arma a adaptação ou a mutação. O homem pode higienizar o meio, erradicar ou limitar os vetores e usar armas (antimicrobianos, soros e vacinas) contra os micróbios, porém, por melhor que a conduta humana consiga controlar seu entorno, há fatores naturais que escapam à sua vontade, como os fatores climáticos" (Ledermann, 1999).

Entre os fatores virais que influem na emergência ou reemergência de doenças, são consideradas as variações e evoluções dos próprios vírus. Isso pode determinar a emergência de

uma nova doença viral, o aumento da patogenicidade dos vírus já conhecidos ou alterações antigênicas sofridas por um determinado vírus que o permitam infectar populações já imunes a cepas progenitoras do mesmo vírus (Murphy & Nathanson, 1994).

As mesmas cepas virais podem ter variações genéticas e não se exclui a possibilidade de que aumentam sua virulência ou patogenicidade. Por exemplo, em uma epidemia pelo DENV-2, na Tailândia, observou-se que amostras de pacientes com DC compararando-se com casos de FHD apresentavam substituições do aminoácido de I (isoleucina) para R (arginina) na proteína prM (pré-membrana), enquanto que nos isolados de pacientes com SCD houve a substituição do aminoácido D (ácido aspártico) para G (glicina) na proteína não-estrutural 1 (NS1) do DENV-2 (Igarashi, 1997).

Com o objetivo de contribuir para a compreensão dos fatores virais relacionados à patogenia, sítios de replicação e evolução molecular dos vírus dengue tipo 3, apresentamos neste trabalho os resultados de quatro estudos: 1) Quantificação dos vírus dengue tipo 3 (DENV-3) em casos fatais e casos clássicos, com o objetivo de correlacionar a viremia e a gravidade da doença. 2) Pesquisa dos vírus dengue em diferentes tecidos provenientes de casos fatais, com vistas a investigar os diferentes sítios de replicação viral. 3) Estudo sobre os aspectos evolutivos do DENV-3 no Globo; e 4) Filogenia dos DENV-3 circulantes no Brasil.

A seguir, descrevemos alguns aspectos gerais sobre os vírus dengue – da classificação atual à prevenção e controle – fornecendo base para a compreensão dos temas abordados neste estudo.

1.1 Classificação

Os vírus dengue pertencem à família *Flaviviridae* e ao gênero *Flavivirus.* Do ponto de vista epidemiológico, os DENV são classificados como arbovírus, sendo mantidos na natureza por um ciclo de transmissão envolvendo hospedeiros vertebrados e mosquitos hematófagos do gênero *Aedes*, sendo o homem, o único hospedeiro capaz de desenvolver as formas clínicas da infecção (Gubler, 2002).

São vírus RNA fita simples, polaridade positiva e com propriedades antigênicas distintas, caracterizando quatro sorotipos específicos denominados DENV-1, DENV-2, DENV-3 e DENV-4 (Sabin, 1952; Hammon *et al*., 1960; Westaway *et al*., 1985).

1.2 Morfologia

São vírus esféricos, envelopados e com cerca de 40 a 50 nanômetros de diâmetro. O virion consiste de RNA de fita simples (ssRNA) de polaridade positiva e envolto por um nucleocapsídeo de simetria icosaédrica, composto por uma única proteína, a proteína de capsídeo (C) e circundada por uma bicamada lipídica associada às proteínas de membrana (M) e envelope (E) (Figura 1.1).

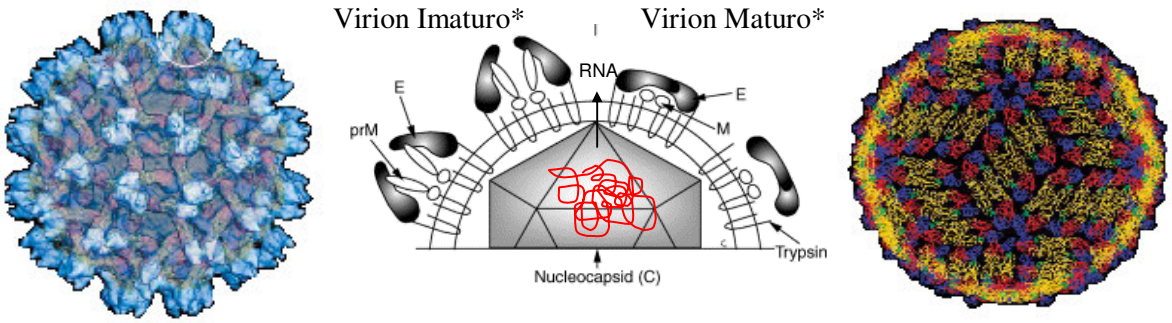


Figura 1.1: Partícula viral e composição dos DENV. Partícula imatura com projeções (à esquerda). À direita, partícula matura lisa e ao centro a composição esquemática dos DENV (M – Proteína de membrana, prM – Proteína pré-membrana, E – Proteína de envelope). As cores representam domínios diferentes (adaptado de Heinz & Allison, 2001; Kuhn *et al*., 2002 e Mackenzie *et al*., 2004).

1.3 Características físico-químicas

A densidade de flutuação dos *Flavivirus* é de 1.22 – 1.24 g/cm em Cloreto de Césio e 1.18 – 1.20 g/cm em sacarose. O coeficiente de sedimentação para os DENV em sacarose é de 175-218 S (Brinton, 1986). Os vírus são rapidamente inativados a 50^{o}C com infectividade decrescendo 50% a cada 10 minutos nesta temperatura. E também sensível à inativação por raios ultravioletas, detergentes iônicos e não iônicos e digestão por tripsina. A infectividade dos DENV é mais estável em pH 7 a 9 e mantendo-se por 5 anos a –70^{o}C ou liofilizado e conservado a 4^{o}C (Guzman, 1980; Brinton, 1986).

1.4 Estrutura do genoma

O RNA fita simples de polaridade positiva é infeccioso, portando-se como um RNA mensageiro (RNAm) quando utilizado em experimentos em condições adequadas. O genoma dos *Flavivirus* possui cerca de 11.000 nucleotídeos (nt) e apenas uma fase aberta de leitura *(ORF*)

codificando uma poliproteína que é posteriormente clivada em proteínas estruturais (C, prM, M e E) e não estruturais (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) (Chambers *et al.*, 1990) (Figura 1.2).

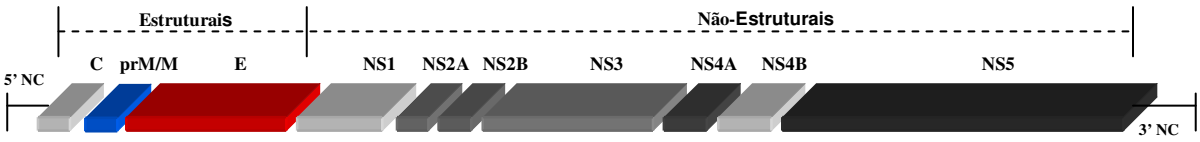


Figura 1.2: Organização do genoma dos *Flavivirus* (adaptado de Chambers *et al.*, 1990).

1.5 Replicação

Os DENV podem infectar muitos tipos de células, incluindo tecidos do sistema vascular, muscular e hematológico, causando diversos sinais clínicos e patológicos (Seneviratne *et al.,* 2006). Estudos com pacientes que desenvolveram FHD/SCD, revelaram a presença do antígeno (Ag) e do RNA viral em tecidos como o fígado, baço, cérebro, linfonodos, timo, rins, pulmões, coração, pele e medula óssea (Bhamarapravati *et al.,* 1967; Boonpucknavig *et al.,* 1979; Yoskan *et al.,* 1983; Hall *et al.,* 1991; Bhoopat *et al.,* 1996; Miagostovich *et al.,* 1997; Jessie *et al.,* 2004; Araújo *et al.,* 2009c).

Wu e colaboradores demonstraram a replicação dos DENV em células de Langerhans, células dérmicas e dendríticas intersticiais (Wu *et al.,* 2000).

O processo de interação vírus-célula tem início com a ligação do DENV a receptores presentes na superfície celular do hospedeiro, endocitose das partículas virais, que é mediada pela rede de clatrinas, formando a vesícula endocítica. À medida que a vesícula vai sendo transportada, seu interior é gradualmente acidificado. Ao atingir a faixa de pH 6.2 – 6.0 (Post, 1996), ocorre uma mudança conformacional das proteínas E do envelope viral. Essa mudança conformacional desencadeia a fusão do envelope viral com a membrana da vesícula endocítica (Monath & Heinz, 1996; Rice, 1996), e subseqüente liberação do nucleocapsídeo no citoplasma da célula. Nesse ambiente, o genoma viral é traduzido como um RNAm da célula numa atividade poliribossomal, inicialmente livre no citoplasma, dando início à síntese da proteína C.

Continuando a tradução do RNA viral (RNAv), quando são incorporados os aminoácidos hidrofóbicos da seqüência sinal da poliproteína, esta seqüência é reconhecida pela proteína reconhecedora de sinal (PRS) constitutiva da célula. A PRS interage com o complexo formado pelo peptídeo nascente, ribossoma e RNAm e bloqueia a tradução, enquanto desloca o conjunto até encontrar o seu receptor (receptor de proteína reconhecedora de sinal – RPRS). Esse receptor

está ancorado na face citoplasmática do retículo endoplasmático. A síntese da proteína continua e o transporte é iniciado para o lúmem do retículo (Albert *et al*., 1994; Lehninger *et al.,* 1997).

As proteínas NS2B e NS3 neo-formadas, agregam-se em heterodímeros com atividade de serina protease que, clivando a extensão subseqüente da poliproteína, liberam as NS4A, NS4B e NS5. Os domínios conservados na região N-terminal da proteína NS5 são reconhecidos pela proteína NS3, para a formação de um complexo que, em seguida, interage com a proteína NS2A. Quando ligada ao complexo, a proteína NS2A é capaz de reconhecer a porção 3'NC do RNA viral (RNAv). O complexo NS2A-RNA-NS3-NS5 é transportado até à membrana do retículo endoplasmático onde está inserida a proteína NS4A que, por sua vez, está associada à proteína NS1 (Lindenbach & Rice, 1997; 1999). Como conseqüência da interação dessas proteínas, ocorre a alteração alostérica na proteína NS5, provavelmente, no domínio de interação com o RNA, dando origem ao chamado complexo de replicação (RC). O RC promove a síntese de novas moléculas de RNA.

Os novos RNAv produzidos, depois de traduzidos, geram proteínas que se acumulam. Dentre essas, a proteína C que, devido à sua natureza altamente básica, interage com os RNAv, formando as estruturas precursoras dos nucleocapsídeos (Rice, 1996). Essas, por sua vez, são deslocadas para a membrana do complexo de Golgi. Nesse processo de construção dos DENV, os nucleocapsídeos neo-formados interagem com as espículas prM e E, que estão inseridas na membrana dessa organela, com imediata liberação das novas partículas para o lúmem do complexo de Golgi (Mackenzie & Westaway, 2001; Beeck *et al.,* 2003) ou para o interior das vesículas pós-Golgi. As proteínas prM e E foram instaladas no retículo endoplasmático sob a forma de dímeros (Murphy, 1980; Wengler & Wengler, 1989; Allison *et al*., 1995). A clivagem de prM em M parece ser catalisada nas vesículas pós-Golgi (Muylaert *et al.,* 1997), por proteases do tipo furina ou de atividade semelhante (Stadler *et al*., 1997). Essa clivagem distingue os virions das partículas de vírus incompletas (Shapiro *et al.,* 1972). As partículas virais, assim neo-formadas, são transportadas em vesículas até à membrana plasmática e, por processo semelhante à exocitose, são liberadas para o ambiente extracelular.

A seguir, descrevemos algumas características e funções de cada proteína dos DENV.

A proteína C (13,0 - 14 kDa) é uma proteína não glicosilada. Ao interagir com o RNA genômico, forma o nucleocapsídeo que interage com a proteína E (Murphy, 1980; Mackenzie & Westaway, 2001).

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). Sob a forma de prM, a proteína E não exerce atividade fusogênica (Stadler *et al.,* 1997; Heinz & Allison, 2001).

A proteína E (51,0 - 60 kDa) é uma glicoproteína com atividade fusogênica dependente de pH (Post, 1996). Forma projeções de 5-10nm de comprimento com terminações arredondadas de cerca de 2nm de diâmetro, ao longo da superfície externa do vírus. É a principal e maior proteína estrutural do vírus, sendo responsável por atividades biológicas do ciclo viral, tais como a montagem da partícula, a interação com receptores celulares e a fusão de membrana; além de ser o principal alvo para anticorpos neutralizantes e possuir atividade hemaglutinante.

As análises estruturais da proteína E têm definido três domínios estabilizados por ligações dissulfeto. O domínio I, região central da molécula, compreende os aminoácidos da região amino (N-) terminal da glicoproteína e está relacionado com eventos de endocitose viral. O domínio II contém epítopos neutralizantes específicos de sorotipo e de reação cruzada com outros membros da família *Flaviviridae*. O domínio III está composto de um "*loop*" livre de ligação dissulfeto e contém sítio de glicosilação no aminoácido 157 da proteína (Chambers *et al.,* 1990; Allison *et al.,* 2001).

A proteína NS1 (48,0 kDa) é associada ao folheto interno da membrana e é uma subunidade do complexo de replicação (Lindenbach & Rice, 1997). A NS2A é uma proteína de transmembrana de 20,0 kDa. Esta proteína reconhece a porção 3'NTR do RNA genômico e é uma subunidade do complexo de replicação (Khromykh *et al.,* 1999). A proteína NS2B (14,5 kDa), quando ligada à NS3, exerce uma atividade proteolítica na biossíntese viral (Rice, 1996).

A proteína NS3 (70,0 kDa) catalisa a clivagem da porção amino terminal da proteína C e das junções NS2B-NS3, NS3-NS4A e NS4B-NS5 (Rice, 1996); reconhece a porção N-terminal da proteína NS5 para montagem do complexo de replicação (Lindenbach & Rice, 1997; 1999); é a maior proteína viral e é altamente conservada entre os *Flavivirus*.

A proteína NS4A (16 kDa) promove a interação do complexo NS5-NS3-NS2A-RNA à proteína NS1, que se encontra no lúmen do retículo endoplasmático, além de ser uma subunidade do complexo de replicação (Lindenbach & Rice, 1999). A proteína NS4B (24,0 kDa) encontra-se dispersa na membrana citoplasmática e, possivelmente, no núcleo. Sua função ainda é desconhecida (Westaway *et al.,* 1997).

A proteína NS5 (105,0 kDa) é uma subunidade do complexo de replicação e apresenta em sua seqüência o domínio característico de RNA polimerase RNA dependente (Khromykh *et al.,* 1999).

Quando a infecção por *Flavivirus* ocorre em células deficientes de furinas, as novas partículas liberadas apresentam prM na sua constituição e, dessa forma, são capazes de desencadear a atividade fusogênica mediada pelas espículas E, quando expostas ao ambiente de pH ácido (Stadler *et al.,* 1997).

A análise da seqüência nucleotídica e da provável estrutura secundária da região não traduzível (NTR), do terminal 3´ do RNAv, revelou a existência de uma seqüência conservada de, aproximadamente, 90 bases, em forma de "*hairpin*" (Hahn *et al.,* 1987). A necessidade dessa estrutura para a replicação viral foi demonstrada por Bredenbeek *et al.* (2003), com experimentos de deleção. Próximo à região do "*hairpin*" do genoma dos *Flavivirus*, existem duas regiões conservadas, denominadas CS1 (26nts) e CS2 (24nts), separadas entre si por 22 nucleotídeos. Todos os *Flavivirus* apresentam uma região conservada, denominada CS, que está localizada poucos nucleotídeos após o códon inicial da tradução.

Hahn *et al.* (1987) relataram a existencia de uma complementariedade entre as seqüências conservadas dos terminais 5´(CS) e a 3´(CS1), resultando na interação intramolecular no RNA dos flavivírus, com a ciclização do RNAv em uma estrutura semelhante a uma "*panhandle*". Os mesmos pesquisadores sugeriram que essa estrutura estaria envolvida na modulação da tradução dos genomas virais, em células infectadas por flavivírus. Esse tipo de pareamento foi, posteriormente, demonstrado por Khromykh *et al.* (2001) como sendo essencial para a replicação do genoma dos vírus Kunjin.

Ensaios "*in vitro*", realizados por You *et al.* (2001), demostraram o mesmo tipo de fenômeno para os DENV. Essa região de pareamento apresenta uma mesma seqüência de oito nucleotídeos e, provavelmente, é reconhecida pelos elementos da maquinaria de replicação do RNA. É possível que a função das outras bases da região de pareamento seja estabilizar a molécula, de forma que a dupla fita, formada pelo core de oito nucleotídeos, seja reconhecida pela replicase (Corver *et al.,* 2003).

1.6 Diversidade genética

Baseados nas diferenças genéticas detectadas inicialmente por *fingerprinting* (Trent *et al*., 1983) e, mais recentemente, por sequenciamento do genoma viral, os quatro sorotipos de DENV foram agrupados em diversos genótipos. Atualmente, na tentativa de esclarecer e unificar a atual classificação de genótipos, estudos de filogenia tem demonstrado 5 genótipos para o DENV-1 (genótipos I-V) (Ong *et al.,* 2008), 6 genótipos para o DENV-2 (genótipos Asiático 1, Asiático 2, Asiático/Americano, Americano, Cosmopolita e Selvagem) (Ong *et al.,* 2008; Zaki *et al.,* 2008),

5 genótipos para o DENV-3 (genótipos I-V) (Araújo *et al.,* 2009a) e 3 genótipos para o DENV-4 (genótipos Indonésia, Malásia e Sudeste Asiático) (Rico-Hesse, 2003).

1.7 Dengue Clássico e Dengue Hemorrágico

As características clínicas variam em intensidade de acordo com as características do hospedeiro e do vírus. O período de incubação é em média de 4 a 7 dias. As infecções pelos DENV apresentam um amplo espectro clínico, variando desde infecções assintomáticas a formas graves denominadas febre hemorrágica do dengue e síndrome do choque por dengue (FHD/SCD). A maioria dos pacientes apresenta a forma branda da doença, conhecida como dengue clássico (DC) (WHO, 1997).

A fase aguda da doença pode variar de 3 a 7 dias, mas a fase de convalescença pode ser prolongada por semanas e pode estar associada a fraqueza e/ou depressão, especialmente nos adultos. Ao final do quadro febril, exantema com aspecto maculo-papular pode ser observado em 30% dos casos, às vezes com aparência escarlatiniforme nas áreas de confluência acompanhado de prurido generalizado. Descamação nas regiões palmares e plantares podem ocorrer (Souza, 1992; Gubler, 1998).

Segundo a Organização Mundial de Saúde (OMS) a FHD apresenta-se com diferentes graus de gravidade: I, II, III e IV e é caracterizada por uma diátese hemorrágica, aumentando a permeabilidade vascular e hipovolemia.

Laboratorialmente caracteriza-se por apresentar contagem de plaquetas inferior a 100.000/mm^3 e hemoconcentração, refletida por um aumento de 20% do hematócrito. Os graus I e II são considerados formas mais brandas da FHD, enquanto os graus III e IV representam a evolução da doença para o estágio de choque (WHO, 1997).

A Síndrome do Choque por Dengue (SCD) resulta em uma perda crítica do plasma com sinais de insuficiência circulatória tais como: pele fria e congestionada, inquietação e baixa pressão do pulso (<20 mm Hg). O choque é curto e pode levar o paciente a óbito em um período de 12 a 24 horas, caso não seja iniciado o tratamento apropriado. A convalescença de pacientes com FHD/SCD é rápida e sem maiores complicações (WHO, 1997).

Os critérios da Organização Mundial de Saúde (OMS) para a distinção de DC e os quatro graus de FHD estão resumidos na Tabela 1.1

Tabela 1.1: Critérios para a distinção do dengue clássico (DC) dos graus de febre hemorrágica do dengue (FHD).

	Extravasamento do plasma[1]	Plaquetas (μl)	Insuficiência circulatória	Teste do torniquete	Sangramento
DC	Ausente	Variável	Ausente	Variável	Ás vezes
FHD grau I	Presente	< 100.000	Ausente	Positivo	Ausente
FHD grau II	Presente	< 100.000	Ausente	Positivo	Presente
FHD grau III	Presente	< 100.000	PP[2] < 20mmHg	Variável	Ás vezes
FHD grau IV	Presente	< 100.000	PA[3] não detectável	Variável	Ás vezes

[1] Identificado por um hematócrito 20% acima do normal.
[2] Pressão do pulso.
[3] Pressão arterial.

Os critérios atuais propostos pela OMS tiveram como base a experiência pediátrica das epidemias de dengue no Sudeste Asiático. No entanto, com a disseminação da doença para países tropicais e subtropicais do globo, observaram-se diferentes perfis clínicos e epidemiológicos.

Bandyopadhyay e colaboradores (2006) em uma revisão de trinta e sete trabalhos discutiram a experiência e as dificuldades na aplicação dos critérios da OMS em diferentes países. Muitos estudos aplicaram rigorosamente os critérios da OMS, entretanto, outros grupos, devido às dificuldades em classificar seus pacientes entre os quatro critérios da FHD, optaram pela utilização de uma classificação modificada. Em casos de FHD, a trombocitopenia foi observada em 8,6-96%, extravasamento de plasma em 6-95% e manifestações hemorrágicas em 22-93%. Os autores observaram que a dificuldade da classificação da FHD pode ser devido a: não repetição de testes ou exames físicos no tempo apropriado; falta da administração de fluidos intravenosos e uma considerável sobreposição de manifestações clínicas por diferentes sorotipos (Bandyopadhyay *et al.,* 2006).

Contemplando ainda este aspecto, outros trabalhos sugerem uma forma simplificada para classificar as diferentes formas clínicas do dengue (Balmaseda *et al.,* 2005; Deen *et al.,* 2006; Rigau-Pérez, 2006).

No Brasil, o Ministério da Saúde (MS) tem considerado as seguintes apresentações do dengue: dengue clássica (DC), febre hemorrágica da dengue (FHD) e dengue com complicações (DCC) (MS, 2008).

A dengue clássica é caracterizada por febre, início abrupto, associada a cefaléia, prostração, mialgia, artralgia, dor retroorbitária, exantema maculopapular acompanhado ou não de prurido. Anorexia, náuseas, vômitos e diarréia podem ser observados. No final do período febril, podem ser observadas manifestações hemorrágicas como epistaxe, petéquias, gengivorragia, metrorragia.

As manifestações clínicas iniciais da dengue hemorrágica são as mesmas descritas para a dengue clássica, até que ocorra a defervescência da febre, entre o terceiro e o sétimo dia, e a síndrome se instale. Evidenciam-se o surgimento de manifestações hemorrágicas espontâneas ou provocadas, trombocitopenia (plaquetas <100.000/mm^3) e perda de plasma para o terceiro espaço.

Dengue com complicações são casos que não se enquadram nos critérios de FHD, e quando a classificação de dengue clássica é insatisfatória. Nessa situação, a presença de um dos achados a seguir caracteriza o quadro: alterações graves do sistema nervoso; disfunção cardiorrespiratória; insuficiência hepática; plaquetopenia igual ou inferior a 50.000/mm^3; hemorragia digestiva; derrames cavitários; leucometria global igual ou inferior a 1.000/mm^3 e óbito (MS, 2008).

Manifestações clínicas do sistema nervoso, presentes tanto em adultos como em crianças, que podem surgir no decorrer do período febril ou mais tardiamente na convalescença, incluem: delírio, sonolência, coma, depressão, irritabilidade, psicose, demência, amnésia, sinais meníngeos, paresias, paralisias, polineuropatias, síndrome de Reye, síndrome de Guillain-Barré e encefalite (MS, 2008).

Os dados de anamnese e exame físico são utilizados para estadiar os casos e para orientar as medidas terapêuticas cabíveis, dividido em quatro níveis denominados grupos variando de A-D. Resumidamente, são apresentados características relacionadas à cada grupo, encontrando-se em anexo a conduta para cada um deles. No grupo A, incluem-se os casos com sinais e sintomas de dengue, ausência de manifestações hemorrágicas e de sinais de alerta. Nestes casos estaria indicado hidratação oral e acompanhamento ambulatorial. O grupo B comporta os casos com manifestações hemorrágicas induzidas (prova do laço) ou espontâneas sem repercussão hemodinâmica e ausência de sinais de alerta, podendo o paciente ser tratado ambulatorialmente dependendo dos exames complementares. Nos grupos C e D estão incluídos os casos mais graves, com sinais de alerta e/ou choque. Exigem hidratação venosa imediata e observação em leito ou hospitalar (MS, 2005).

A dengue é uma doença dinâmica, o que permite que o paciente evolua de um estágio a outro rapidamente. O manejo adequado dos pacientes depende do reconhecimento precoce de sinais de alerta, do contínuo monitoramento e reestadiamento dos casos e da pronta reposição hídrica. Os sinais de alerta e o agravamento do quadro costumam ocorrer na fase de remissão da febre (MS, 2005).

Não há tratamento específico para a dengue, o que o torna eminentemente sintomático ou preventivo das possíveis complicações. As drogas antivirais, o interferon alfa e a gamaglobulina, testada até o momento, não apresentaram resultados satisfatórios que subsidiem sua indicação terapêutica.

Manifestações não usuais, ocorrendo nas formas clássica e hemorrágica da doença tem sido descritas, inclusive no Brasil (Chimelli *et al*., 1990; Lum *et al*., 1993, 1996; Patey *et al*., 1993; Row *et al*., 1996; Miagostovich *et al*., 1997; Angibaud *et al*., 2001; Nogueira *et al*., 2002; Araújo *et al.,* 2009c). Elevação dos níveis séricos das aminotransferases (AST e ALT) e falência hepática fulminante foram demonstradas (Kuo *et al*., 1992; Nguyen *et al*., 1997; Souza *et al*.,2005).

1.8 Patogenia

A inexistência de um modelo animal que reproduza clinicamente a infecção tem dificultado a compreensão da patogenia do dengue, levando a hipóteses que tentam associar diferentes fatores de risco à gravidade da doença. Embora nenhuma destas hipóteses seja excludente, a mais difundida é a teoria da infecção seqüencial, também conhecida como "*teoria immune-enhancement*", que preconiza uma associação entre infecções secundárias e o aparecimento de FHD/SCD (Halstead, 1988; Thein *et al*., 1997; McBride & Bielefeldt-Ohmann, 2000; Vaughn *et al*., 2000).

Segundo Halstead (1988), 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 na infecção celular e replicação viral. Essas células infectadas liberariam na corrente sanguínea mediadores vasoativos capazes de aumentar a permeabilidade vascular, ativação do sistema complemento e da tromboplastina tissular.

Casos de FHD/SCD resultantes de infecções primárias tem sugerido que variações da virulência entre amostras de DENV poderiam ser responsáveis pela variabilidade na expressão clínica da doença (Rosen, 1977; Araújo *et al.,* 2009b). As teorias da infecção seqüencial e da virulência viral serão discutidas com maior profundidade na seção discussão (item 4).

Fatores de risco individuais, epidemiológicos e virais também têm sido considerados na patogenia do dengue. A hipótese integral, proposta por Kouri *et al*. (1987), sugere que a interação

entre esses fatores, determine as condições para o aparecimento das formas mais graves da doença.

Outra teoria sob investigação é a da gravidade do dengue por poliformismo genético, no qual envolvem fatores individuais como polimorfismos de genes relacionados com o sistema imunológico. Estes fatores individuais podem determinar o curso da infecção por DENV e sua gravidade. Muitos estudos demonstram o papel de fatores genéticos na patogenia de doenças infecciosas. Para os *Flavivirus*, o aumento na expressão de moléculas HLA (Antígenos Leucocitários Humanos, do inglês "*Human Leukocyte Antigen*") de classe I e II em células infectadas, e o nível de resposta imunológica contra epítopos virais, podem também ser responsáveis pela imunopatologia da infecção (Polizel *et al.,* 2004).

Green & Rothman (2006) demonstraram efeitos opostos dos alelos HLA de classe I, incluindo um papel protetor do HLA A33 e um patogênico para o HLA A24 em vietnamitas com dengue. Polizel e colaboradores (2004) demonstraram uma alta freqüência de antígenos HLA-DQ1 entre pacientes com FD na população branca do Sul do Brasil. Estudos adicionais demonstraram um aumento significativo do alelo TNF-308A em pacientes com dengue e, conseqüentemente, um aumento dos níveis de Fator de Necrose Tumoral alfa (TNF-alfa), provavelmente relacionados à permeabilidade vascular e hemorragia (Fernandez-Mestre *et al.,* 2004).

Outra teoria discutida na imunopatogenia do dengue é a do "Pecado Original". Existe uma forte evidência "*in vivo*" da ativação de células T CD4 e CD8 durante a infecção por DENV, sendo que, tal ativação seria mais intensa em pacientes graves quando comparados a pacientes com a forma branda da doença. Sugere-se que a doença possa ser causada pela ativação dessas células. O nível de citocinas como TNF alfa, assim como a magnitude das respostas via células T, estariam correlacionadas com a gravidade da doença (Mentor & Kurane, 1997; Carvalho, 2008).

A partir da replicação viral via ADE (do inglês "*antibody-dependent enhancement*") em monócitos e macrófagos, antígenos virais são apresentados e reconhecidos por moléculas na superfície de linfócitos. Esta replicação é acompanhada por uma ativação de linfócitos T que, durante a infecção primária, se expandem e apresentam uma maior afinidade pelos epítopos presentes no sorotipo infectante, ocasionando a formação de células de memória para este sorotipo. Entretanto, na infecção secundária por outro sorotipo, as células de memória sensibilizadas durante uma infecção prévia, seriam ativadas e se expandiriam mais rapidamente que as células virgens específicas para o sorotipo infectante. Os clones de células de memória teriam menor afinidade ao sorotipo presente e, consequentemente, não exerceriam suas funções

efetoras em eliminar o vírus. Porém, teriam alta capacidade em produzir mediadores inflamatórios e poderiam, de fato, promover a imunopatogenia do dengue (Mongkolsapaya *et al.,* 2003). A liberação de citocinas pró-inflamatórias por essas células, como IFN-gama e IFN-alfa, podem agir diretamente sobre o endotélio vascular e resultar no extravasamento de plasma, característico das infecções graves por DENV (Pang *et al.,* 2007).

A teoria do mimetismo molecular propõe que a patogênese do dengue seja resultado de uma reação autoimune. O desenvolvimento de anticorpos de reatividade cruzada ao plasminogênio (devido a uma similaridade em 20 aminoácidos da glicoproteína do envelope viral e uma família de fatores da coagulação) poderia estar relacionado com a hemorragia do dengue. O aumento da destruição de plaquetas ou a diminuição na sua produção poderia resultar em trombocitopenia (Rothman, 2004).

Entre os estudos sobre a compreensão da imunopatologia do dengue, estão incluídas as pesquisas com células dendríticas. Estas células são células-alvo primárias, as mais precoces participantes na infecção natural pelos DENV (Libraty *et al.,* 2001; Kurane, 2007). Em seu interior se produzem partículas virais, ocorre hipertrofia do retículo endoplasmático, aumento da mitocôndria e se expressam os marcadores de amadurecimento: B7-1, B7-2, HLA-DR, CD11, CD83, bem como indução da produção de TNF alfa e IFN alfa (Ho *et al.,* 2001).

O DENV conduz ao amadurecimento e à ativação das células dendríticas, à expressão de moléculas classe II do sistema HLA e outras moléculas co-estimuladoras, e à produção de citocinas. Isso ocorre não só nas células infectadas, mas também nas que as rodeiam. A presença ou ausência relativa de IFN no microambiente celular modula a intensidade da imunidade celular (Librati *et al.,* 2001; Green & Rothman, 2006).

Enquanto as células dendríticas vão se deslocando para os vasos linfáticos e os gânglios linfáticos, onde geralmente ocorre o contato com as células T, os vírus que penetram a derme são reconhecidos pelos macrófagos ali existentes, bem como pelas células do endotélio vascular. Os linfócitos T CD4 são os primeiros a serem ativados, com produção de interferom gama e interleucina 2, que conduzem o seu crescimento e extensão mediante estimulação autócrina e parácrina (Torres, 2005; Kurane, 2007).

Os clones de células T CD4+ específicos para dengue têm sido estudados por Gagnon *et al.* (1999, 2001). Esses autores demonstraram que o IFN-gama é a citocina produzida com maior intensidade (resposta tipo 1 ou T_H1) pela totalidade das células, seguida de TNF alfa e beta, com intensidade variável. As interleucinas 2 e 4 (Il-2 e Il-4) são produzidas em menor intensidade e nem por todos os clones. A resposta de células T CD4+ ao DENV tem-se demonstrado ser

específica de sorotipo e também de reação cruzada com outros sorotipos, com atividade principalmente não citolítica, embora possa ser citolítica direta mediante perforinas ou mediada pela molécula Faz, capaz de induzir apoptose (Green & Rothman, 2006).

Os linfócitos T CD4+ reconhecem as proteínas C, E, NS1-2A e NS3 dos DENV e, como mencionado anteriormente, as citocinas produzidas foram IFN gama, IL-2 e TNF alfa e beta. O TNF conduz ao recrutamento e ativação de leucócitos (monócitos e outros), bem como das células de adesão, com produção de IL-8 e outras citocinas. A ativação dos linfócitos CD8+ ocorre posteriormente. A resposta das células T CD8+ ao DENV são específicas para cada sorotipo, e também de reação cruzada contra outros sorotipos; sua ação é citolítica direta, produz citocinas Il-2 e IFN gama e reconhece as proteínas virais prM, E, NS1-2A e NS3 (Gagnon *et al.,* 2002; Pang *et al.,* 2007).

As citocinas produzidas por células T CD4+ e CD8+ atuariam diretamente sobre a célula endotelial, bem como o TNF alfa liberado pelos monócitos ativados antes de serem objeto de citólise pelos linfócitos T. O sistema do complemento, ativado pelos imunocomplexos (vírus-anticorpos heterotípicos), liberaria anafilatoxinas (C3a, C5a). Em conjunto, causariam o extravasamento plasmático, elemento fundamental da FHD.

É aceita a existência de um processo imunológico na patogenia da FHD/SCD (Pang & Lam, 1983). Mas qual é o limite entre o fisiológico e o patológico? Quando termina a reação imune que protege e começa a resposta patogênica?

Os estudos realizados em crianças com FD e FHD parecem conduzir à idéia de que as diferenças na ativação de células T durante a infecção por DENV são mais quantitativas do que qualitativas, e que essa ativação é por antígenos convencionais e não por um superantígeno viral (Gagnon *et al.,* 2001). Uma participação crucial, segundo Rothman (2001), teria os linfócitos T específicos de DENV de reação cruzada de sorotipos, pois, de acordo com seus níveis em um momento determinado, cumpririam a função positiva de antagonismo, e em outro momento predominaria a função de ativação com produção exagerada de citocinas conducentes à expansão e provável indução de apoptose.

Para alguns pesquisadores, a imunopatogenia da FHD/SCD estaria no conjunto de respostas anormais que diminuem a ação contra o vírus, ao mesmo tempo em que causam uma superprodução de citocinas que afetam monócitos, células endoteliais, hepatócitos e outras células do hospedeiro (Lei *et al.,* 2001). Outros atribuem a FHD a uma desregulação da resposta imunitária de tipo transitória (Chaturvedi et al., 2000; Pacsa et al., 2000; Mustafa et al., 2001;

Yang et al., 2001), expressa por: superprodução de citocinas; mudança da resposta tipo T_H1 para resposta T_H2; inversão da relação CD4/CD8.

O estudo de pacientes com FHD tem demonstrado que a fuga plasmática está associada a níveis elevados de citocinas no plasma: o TNF alfa e beta mostraram-se elevados na totalidade dos doentes, o IFN gama foi identificado em menos de 50% das amostras estudadas, enquanto Il-2 e Il-4 estiveram presentes apenas em algumas amostras. TNF alfa e IL-4 tiveram maior expressão em pacientes com FHD do que com FD ou outras doenças febris (Gagnon *et al.,* 2002; Azeredo *et al.,* 2001; Juffrie *et al.,* 2001).

As citocinas são proteínas que compartilham um grande número de propriedades: são produzidas durante o desenvolvimento da imunidade natural e específica, mediando e regulando as respostas imunológicas e inflamatórias determinadas por diversos tipos de células, e são muito pleiotrópicas. A maioria das citocinas tem efeitos redundantes. Unem-se a receptores específicos na superfície das células-alvo, com ações sobre: as células que as produzem (autócrinas); células próximas (parácrinas); células distantes, quando são secretadas na circulação (hormonais).

A infecção por DENV é capaz de induzir fatores celulares, tais como o MIP-1 alfa e o IMP beta, cujos genes também se expressam em pacientes com FHD e que são verdadeiras quimiocinas que participam na imunopatogenia das infecções por dengue (Spain-Santana *et al.,* 2001).

O microambiente das citocinas representa um dos fatores mais importantes na indução de uma resposta imune, tipo 1 (T_H1) ou tipo 2 (T_H2).

Desde 1987 (Coffman) e 1991 (Romagnani) se conhece a existência de duas subpopulações linfocitárias de células T CD4, caracterizadas pela produção de perfis de citocinas diferentes: IFN gama e IL-2 pelas T_H1 e Il-4, Il-5, Il-10 pelas T_H2, implicando a polarização para uma resposta de preferência celular ou humoral. Hoje se reconhece que esses padrões não são exclusivos destas células, pois têm sido encontradas outras subpopulações CD4 produtoras de citocinas, entre as quais encontram-se: T_HP (produzem só Il-2), T_HO (produzem ambos os padrões) e T_H3 (só produzem TGF beta), as quais, ao serem ativadas, podem influenciar na indução da resposta.

Em contrapartida, o padrão T_H1/T_H2 das CD4+ é encontrado também nas CD8+, denominando-se Tc1 e Tc2, respectivamente. Além disso, tem-se demonstrado que existe uma regulação cruzada dessas subpopulações, mediadas pelos perfis de citocinas produzidas: inibição das T_H2 pelo IFN gama e das T_H1 pelo Il-10.

A subpopulação T_H1 tem uma intensa atividade na ativação de macrófagos, bem como sobre a ativação de células NK e LAK, atividade citotóxica e hipersensibilidade retardada. É a responsável pela resposta tipo 1 ou resposta dominante de IFN gama, vinculada diretamente à morte de patógenos intracelulares, em parte pela síntese de óxido nítrico, bem como à introdução de moléculas CMH-I e CMH-II em uma grande variedade de tipos celulares, e à maturação de células B para a secreção de imunoglobulinas.

A subpopulação T_H2 ajuda os linfócitos B na produção de IgE e IgG4, participa na ativação e diferenciação de eosinófilos e mastócitos, bem como na hipersensibilidade imediata.

Em pacientes com infecção heterotípica por DENV-2 tem-se estudado a amplificação dependente dos anticorpos (ADA) e sua relação com a resposta T_H1. O soro imune contra DENV-1, à diluição 1:5, mostrou neutralização de DENV-2 em todas as amostras, e a diluição de 1:25 mostrou neutralização de DENV-2 em 2/3 das amostras. Em ambos os casos verificou-se resposta T_H1 com produção de IFN gama. Ao contrário, à diluição de 1:250 identificaram-se títulos subneutralizantes contra DENV-2 e franca amplificação da infecção dependente de anticorpos, com aumento do índice de proliferação e diminuição da produção de IFN gama. A adição de IFN-gama fez diminuir a ADA e, reciprocamente, a administração de anticorpos contra IFN gama foi capaz de aumentá-la (Yang *et al.,* 2001).

A supressão da resposta T_H1 estaria relacionada à imunoamplificação (ou amplificação da infecção mediada por anticorpos) a partir da infecção heterotípica e à supressão da produção de interferon gama (Lei *et al.,* 2001). Foi sugerido que a ausência de interleucina-12 (Pacsa *et al.,* 2000), bem como o incremento dos níveis das interleucinas 13 e 18 (Mustafá *et al.,* 2001), poderia ser responsável pela mudança do tipo de resposta imune de T_H1 a T_H2.

Chaturvedi e seus colaboradores têm contribuído, durante anos, para a referida hipótese da desregulação imunológica e mudança de resposta T_H1 (tipo 1) para T_H2 (tipo 2), só que consideram que a causa desse distúrbio é a produção de uma citocina única e que a resposta do organismo relacionada à patogenia do dengue deveu-se ao fator citotóxico humano e à capacidade do paciente produzir anticorpos específicos. Da variabilidade nos níveis de anticorpos contra essa citocina depende a expressão clínica do dengue: associada a níveis elevados na FD, baixos na FHD e ausentes em quase todos os pacientes com SCD (Chaturvedi *et al.,* 2001).

Há necessidade de maiores informações sobre as implicações que, para os pacientes com FHD, têm a inversão da relação CD4/CD8, de caráter transitório. Ambas as moléculas, incluídas nas chamadas "acessórias", têm a função de contribuir subsidiariamente ao desenvolvimento de uma resposta imune efetiva; a relevância funcional que hoje se lhes atribui é muito maior do que

indica a denominação de “acessórias”, pois representam um segundo sinal, sem cuja presença não ocorre a interação do antígeno com o receptor linfocitário, e sem sua participação não se completam os mecanismos efetores. CD4 é o marcador do linfócito TH (colaborador ou auxiliar), enquanto CD8 é o marcador dos linfócitos Tc (citotóxicos ou citolíticos) e Ts (supressores), dos quais o mecanismo de ação não é bem conhecido ainda; considera-se que possuam ação reguladora da resposta imune e suas duas vertentes principais, humoral e celular, e estejam associados ao desenvolvimento da tolerância.

Tampouco tem sido suficientemente documentada a participação, na patogenia da FHD, dos linfócitos nulos ou terceira população, chamados assim por não possuírem marcadores nem de linfócitos T nem linfócitos B, mais conhecidos como células exterminadoras naturais ou células NK (do inglês *Natural Killer Cell*). Expressam em sua superfície as moléculas CD16 e CD56, e para sua ação citolótica não requerem moléculas do CMH na célula-alvo. Embora sua principal atividade seja atuar no controle do crescimento de células tumorais, também são responsáveis por um dos mecanismos de defesa relacionados a células infectadas por diversos agentes biológicos, incluindo os vírus, bem como pela produção de linfocinas tais como Il-2, Il-3, GM-CSF, TNF alfa e IFN gama, fundamentalmente. A infecção por DENV não pode ser uma exceção (Torres, 2005).

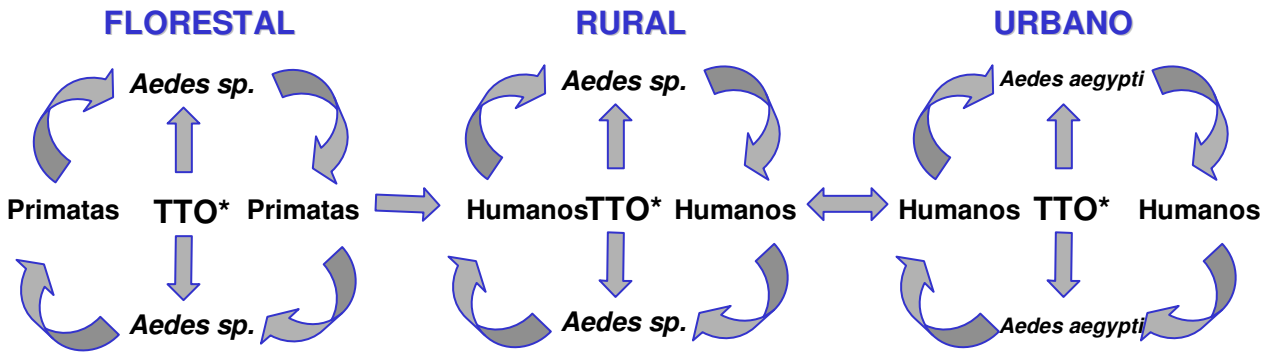
1.9 Epidemiologia

O dengue é a arbovirose humana de maior importância médica no mundo em termos de morbidade e mortalidade, com aproximadamente 3 bilhões de pessoas expostas ao risco de infecção possuindo caráter endêmico em cerca de 100 países de clima tropical e subtropical (Gubler, 1998).

A prevalência global dessa doença cresceu exponencialmente nas últimas décadas. As estimativas da Organização Mundial de Saúde (OMS) apontam que podem ocorrer de 50-100 milhões de casos de infecções causadas pelos DENV em todo o mundo, resultando em 250.000 a 500.000 casos de Dengue Hemorrágico (DHF) e 24.000 mortes por ano (WHO, 1997; Gibbons & Vaughn, 2002).

Existem três grupos de hospedeiros naturais para os DENV: o homem, primatas inferiores e mosquitos do gênero *Aedes*. O homem é o único hospedeiro conhecido que desenvolve a expressão clínica da infecção. Já os primatas como Chimpanzés ou outros primatas não humanos, quando infectados experimentalmente, desenvolvem viremia, mas não apresentam outros sinais da doença (Gubler, 1998).

Os principais vetores do gênero *Aedes* incluem espécies dos sub-gêneros *Stegomyia*, *Finlaya* e *Diceromyia* que estão envolvidos em diferentes ciclos de transmissão rural e florestal enzoótico dos vírus (Gubler, 1998) (Figura 1.3).



* Transmissão Transovariana (TTO)

África (*Ae. diceromyia* e *Ae. stegomyia*) *Ae. aegypti* e *Ae. albopictus* *Ae. aegypti*
Ásia (*Ae. finlaya* e *Ae. stegomyia*) *Ae. polynesiensis* e *Ae. mediovittatus*
Américas (*Aedes sp.*)

Figura 1.3: Ciclos de transmissão e manutenção dos vírus dengue (adaptado de Gubler, 1998).

O *Ae. aegypti* devido a seus hábitos domésticos, é a espécie mais importante na transmissão dos DENV ao homem, estando associado a epidemias explosivas de DC e FHD. Este mosquito é altamente antropofílico e se alimenta repetidamente durante toda sua vida, funcionando como um excelente vetor (Knight & Stone, 1977).

A transmissão dos DENV se dá através da fêmea, durante o repasto sanguíneo. A fêmea pica o homem preferencialmente durante o dia e faz posturas parciais, podendo produzir vários focos (Halstead, 1984).

Os recipientes artificiais abundantemente proporcionados pela moderna sociedade industrial são os mais importantes criadouros de *Ae. aegypti*, sendo essenciais para o desenvolvimento e manutenção de grandes populações deste mosquito.

Identificado no Brasil em 1986 no estado do Rio de Janeiro, o *Ae. albopictus* pela sua capacidade de sobreviver tanto no ambiente silvestre quanto no urbano e periurbano, torna-se uma preocupação embora não tenha até o momento sido comprovado como vetor de dengue no Brasil.

1.9.1 Dengue nas Américas

No continente americano, as primeiras epidemias de dengue foram registradas a partir de 1896, embora os sorotipos envolvidos nestas epidemias só fossem conhecidos em 1953, quando foram isoladas as primeiras amostras de DENV-2 em Trinidad (Anderson *et al*., 1956). Dez anos depois, foram isoladas as primeiras amostras de DENV-3 em Porto Rico, sendo estes dois sorotipos responsáveis pelas epidemias ocorridas no continente durante a década de 60 (Gubler, 1992).

Em 1977, o DENV-1 foi introduzido na Jamaica causando uma pandemia que se expandiu por todas as Ilhas do Caribe, países das Américas Central e do Sul. No período entre 1977 e 1980, foram notificados mais de 700.000 casos da doença, praticamente todos estes causados pelo DENV-1 (PAHO, 1989).

No mesmo período, o DENV-3 apresentou pouca atividade na região com o último isolamento ocorrido em 1978 em Porto Rico (Anonymus, 1995).

Em 1981, o DENV-4 foi introduzido no continente americano na ilha de São Bartolomeu, causando surtos no Caribe, México, países da América Central e América do Sul, tornando-se endêmico na região (Pinheiro, 1989).

Neste mesmo ano, uma nova variante do DENV-2 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 116.000 internações e 158 óbitos (Kouri *et al*., 1986).

Nos anos 80 ocorreu uma expansão da área de transmissão, aumento de casos notificados e neste período circularam nas Américas os sorotipos DENV-1, DENV-2 e DENV-4. No Brasil, Bolívia, Equador, Paraguai e Peru foram notificados epidemias explosivas, depois de várias décadas sem a presença desta doença (Gubler, 1993; PAHO, 1997).

No período de 1989 a 1990, ocorreu na Venezuela a segunda maior epidemia de FHD/SCD das Américas, com cerca de 22 mil casos notificados com a co-circulação dos DENV-1, DENV-2 e DENV-4 (PAHO, 1997; Pinheiro & Chuit, 1998).

A década de 90 foi caracterizada por epidemias de DC e/ou FHD/SCD, que afetaram cerca de 25 países do continente americano (Pinheiro & Chuit, 1998).

No ano de 1994, foi comprovada a re-introdução do DENV-3 na Nicarágua e Panamá e, em 1995, no México (PAHO, 1997). Esta variante de DENV-3 (genótipo III) mostrou-se geneticamente distinta daquela que anteriormente circulava nas Américas (genótipo IV) (Lanciotti *et al*., 1994; Anonymus, 1995).

Este genótipo foi associado à ocorrência de epidemias de FHD/SCD no Sri Lanka e India e casos de FHD no México e países da América Central (Gubler 1997; Gubler & Meltzer, 1999). Nos anos seguintes, o DENV-3 se espalhou para outros países do continente, chegando à América do Sul inclusive ao Brasil (Pinheiro & Corber, 1997; Rigau-Perez *et al*., 2002).

1.9.2 Dengue no Brasil

Os primeiros relatos de dengue no Brasil datam de 1846 e descrevem surtos ocorridos simultaneamente nos estados do Rio de Janeiro, Bahia, Pernambuco e em localidades do norte do país (Mariano, 1917). Posteriormente, novos casos foram notificados no Paraná em 1890 (Reis, 1896), em São Paulo em 1916, no Rio Grande do Sul em 1917 (Mariano, 1917) e no Rio de Janeiro em 1923 (Pedro, 1923).

Em 1904, Oswaldo Cruz iniciou a Campanha Brasileira de erradicação do vetor *Ae. aegypti*, sendo que, somente na década de 40, com o apoio da Fundação Rockefeller, a Campanha ganhou impulso nacional e culminou com a erradicação do mosquito no país até a década de 70 (Franco, 1961; Figueiredo, 1996; 2000). Entretanto, o declínio no controle do mosquito vetor, associado à introdução de novos sorotipos virais na América Central, resultou na reintrodução dos DENV no Brasil após mais de 50 anos (Figueiredo, 2000).

A re-infestação do *Ae. aegypti* no país em 1977, a pandemia de DENV-1 e a introdução do DENV-4 no continente americano, resultaram na re-introdução dos DENV no Brasil.

Em 1981 em Boa Vista (RR) ocorreu um surto de dengue, onde foram isoladas as primeiras amostras de DENV-1 e DENV-4, e um total de 7.000 casos da doença foram notificados (Osanai *et al*., 1983).

Após um período de 5 anos sem ser notificado no país, o DENV-1 causou uma epidemia com inicio no mês de abril no município de Nova Iguaçu, (RJ) com 1 milhão de casos estimados (Schatzmayr *et al*., 1986)

O intenso movimento humano permitiu a rápida dispersão do vírus, avançando neste mesmo ano para os estados de Alagoas e Ceará e no ano seguinte, para os estados de São Paulo, Minas Gerais, Pernambuco e Bahia (Figueiredo, 1996).

A situação no país foi agravada pela introdução do DENV-2 em Niterói (RJ) no ano de 1990, quando foram notificados os primeiros casos de FHD/SCD (Nogueira *et al*., 1990; 1991).

A dificuldade de se implantar uma política nacional eficaz de combate ao vetor, resultou em uma rápida dispersão deste sorotipo pelo país que foi evidenciada pela ocorrência de epidemias nos estados de Tocantins e Alagoas (1991) e posteriormente, nos estados da Bahia e

Ceará (1994), Espírito Santo, Minas Gerais, Pernambuco, Rio Grande do Norte, Mato Grosso do Sul (1995) e São Paulo (1996) (Vasconcelos *et al*., 1993; Nogueira *et al*., 1995; Souza *et al*., 1995; Figueiredo, 1996; Cunha *et al*., 1999).

Em 1998, o DENV-3 foi isolado de um caso importado na cidade de Limeira, estado de São Paulo, (Rocco *et al*., 2001) sem repercussão epidemiológica.

Em dezembro de 2000, este sorotipo foi isolado no município de Nova Iguaçu (RJ) sendo o responsável pela maior e mais grave epidemia de dengue do país até o ano de 2002 (Nogueira *et al*., 2001).

Com epidemias ocorrendo quase que anualmente desde 1986, mais de 5 milhões de casos foram registrados no Brasil, resultantes de epidemias de DENV-1, DENV-2, e mais recentemente, DENV-3 (Figueiredo, 1996; Da Silva Jr *et al.,* 2002, Nogueira et al., 2007).

No ano de 2002 o Brasil notificou 813.104 casos de dengue, e desse total 288.245 correspondem aos casos no estado de Rio de Janeiro. Naquele ano, 1831 casos de DH e 91 óbitos foram confirmados. Este total excedeu o número de casos notificados de DH e óbitos em um período de 20 anos desde a introdução do dengue no país (Nogueira *et al*., 2005). O DENV-3 modificou sobremaneira a epidemiologia do dengue no país, com características de hiperendemicidade. Um fato novo observado durante a epidemia de 2002 foi a apresentação de formas graves e óbitos em menores de 15 anos, bem como casos fatais em infecções primárias (Nogueira *et al*., 2005; Araújo *et al.,* 2009b; 2009c).

No Estado do Rio de Janeiro, o número de casos notificados de dengue diminuiu nos anos 2003 (9.242), 2004 (2.694) e 2005 (2.580), sendo os anos 2004 e 2005 considerados como interepidêmicos (Araújo *et al.,* 2006).

Entretanto, o número de casos de dengue se elevou alcançando em 2006 (31.054) em 2007 (66.553) e 2008 (259.392). O DENV-3 foi predominante nos anos 2006 e 2007, porém em abril de 2007, observou-se a re-emergência do DENV-2 vindo este sorotipo a causar uma grave epidemia no ano 2008 (SVS, 2009).

Um fato novo para o estado do Rio de Janeiro foi a observação da notificação na faixa etária de menores de 15 anos, que até o ano de 2006 situava-se em torno de 20% do total das notificações. Posteriormente, no ano de 2007 e 2008 este percentual se elevou para 30% e com parâmetros de gravidade (SMS/RJ). Quarenta e dois por cento (42%) dos óbitos ocorreram em crianças em idade escolar na faixa de 0-15 anos. Do número total de internações 8620 casos, quarenta e oito por cento (48%) ocorreram na faixa etária de 15 anos (SES/RJ).

Em relação a gestantes foram notificados 732 casos suspeitos de dengue. Três casos de morte materna e fetal foram confirmados.

A epidemia de 2008 é considerada a mais grave para o estado do Rio de Janeiro até o momento, onde foram confirmados 1.776 casos de FHD e 240 óbitos até mês de janeiro de 2009 (SVS, 2009b). Trinta e três por cento dos óbitos ocorreram na faixa etária de 0 a 15 anos (SVS, 2009b).

Um aspecto importante observado durante a epidemia de 2008 foi a ocorrência de 1.140 casos suspeitos de dengue em gestantes. Dos casos investigados, quatro evoluíram para óbito materno e fetal; e outros quatro casos maternos foram confirmados (SVS, 2009b).

Atualmente, 26 das 27 Unidades Federativas já registraram epidemias de dengue. Apenas o Estado de Santa Catarina (SC) não possui registro de casos autóctones sendo considerados até o momento como casos importados (SVS, 2008).

O monitoramento contínuo dos sorotipos isolados no Brasil tem confirmado apenas um genótipo dos DENV-1 e DENV-2 e dois genótipos do DENV-3 (Nogueira *et al*., 2005; Araújo *et al*., 2009d).

1.10 Diagnóstico Laboratorial

Os métodos de diagnóstico laboratorial mais amplamente utilizados em dengue envolvem o isolamento viral em cultura de células, detecção do ácido nucléico viral pelo método de RT-PCR, técnicas sorológicas para pesquisa de anticorpos específicos (IgM/IgG), detecção de antígenos virais em tecidos através da técnica de imunohistoquímica e, mais recentemente, a pesquisa do antígeno não-estrutural 1 (NS1).

1.10.1 Isolamento Viral e Técnica de Imunofluorescência Indireta (IFI)

O isolamento viral é um método definitivo e ainda permanece como o "Padrão Ouro". O sistema para o isolamento mais utilizado consiste na inoculação de espécimes em culturas de células de mosquitos *Ae. albopictus* clone C6/36 (Igarashi, 1978). Esta linhagem celular é de fácil manutenção, podendo ser mantida à temperatura ambiente, além de ser mais sensível do que linhagens celulares de vertebrados (Tesh, 1979).

O isolamento viral pode ser observado pela presença de efeito citopático (ECP) ou pela detecção de antígenos pelo teste de imunofluorescência. A tipagem se realiza utilizando anticorpos monoclonais (Gubler *et al.,* 1984).

1.10.2 Testes Imunoenzimáticos e de Inibição de Hemaglutinação (HI)

O diagnóstico sorológico das infecções por DENV baseia-se na pesquisa de anticorpos específicos em duas amostras de sangue coletadas com intervalos de 14 a 21 dias após o inicio dos sintomas. As provas pareadas são de grande valor possibilitando observar soroconversões.

A técnica da inibição da hemaglutinação (IH) (Clarke & Casais, 1958), recomendada pela OMS possibilita ainda a caracterização da resposta imune se primária ou secundária.

O teste imunoenzimático de captura de anticorpos da classe IgM (MAC-ELISA) (Kuno *et al.,* 1987), tem se mostrado extremamente útil no diagnóstico de casos suspeitos. A presença de anticorpos da classe IgM em uma única amostra de soro indica infecção ativa ou recente, contornando as dificuldades da obtenção de uma segunda coleta de sangue.

O teste imunoenzimático tem sido igualmente utilizado para detecção de anticorpos da classe IgG (IgG-ELISA). Esta técnica é rápida, de fácil execução e pode ser utilizada em larga escala, permitindo a caracterização do tipo de infecção (primária ou secundária), considerando-se os títulos de anticorpos e o tempo de coleta do sangue em relação ao inicio do quadro clínico (Miagostovich *et al.,* 1999).

Recentemente testes comerciais têm sido disponibilizados para o diagnóstico de dengue, inclusive a pesquisa do NS1 (Wu *et al.,* 1997; Lam & Devine, 1998; Lam *et al.,* 2000; Kumarasamy *et al.,* 2007).

Atualmente o MS tem recomendado a pesquisa de NS1 no monitoramento de casos suspeitos de dengue, sendo os casos positivos encaminhados para identificação do sorotipo através do isolamento ou RT-PCR.

1.10.3 Teste de Imunohistoquímica

A detecção de antígenos virais pode ser realizada em amostras de tecidos fixados em formalina em casos de óbito (Miagostovich *et al.,* 1997).

1.10.4 RT-PCR (Sistema "semi-nested")

Diferentes 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 DENV.

Estes protocolos detectam e caracterizam o sorotipo infectante e podem confirmar o diagnóstico em situações onde o material disponível não é adequado para o isolamento viral (Morita *et al.,* 1991; Lanciotti *et al.,* 1992).

O protocolo desenvolvido por Lanciotti *et al.* (1992) permite detectar os quatro sorotipos de DENV simultaneamente em um procedimento "semi-nested" e é recomendado pela OPAS.

1.10.5 RT-PCR em tempo real

Para a realização da técnica de PCR em tempo real, pode-se utilizar o sistema SYBR Green ou o sistema TaqMan (Forster, 1948; Holland *et al.,* 1991; Heid *et al.,* 1996).

O SYBR Green possui função semelhante ao Brometo de Etídio, ligando-se a qualquer DNA de dupla fita.

No sistema TaqMan, uma sonda duplamente marcada com corantes distintos, um reporter (ex: FAM) e outro quencher (ex: TAMRA), hibridiza na região amplificada flanqueada pelos iniciadores.

Quando os dois corantes estão próximos, e a sonda ainda está intacta, o corante (TAMRA [N,N,N´, N´- tetramethyl-6-carboxyrhodamine]) absorve energia do corante FAM (5-carboxyfluorescein) evitando a emissão de fluorescência. A atividade 5´ exonuclease da enzima taq polimerase degrada a sonda durante o curso do PCR. A degradação da sonda conduz a separação dos dois corantes em solução, com um aumento subseqüente do nível de fluorescência. A intensidade de fluorescência é detectada a cada ciclo de amplificação. A quantidade de fluorescência medida em uma amostra, que é proporcional a quantidade de produto de PCR gerado, é comparada com a fluorescência de uma amostra padrão cujo número de cópias de RNA ou PFU (Unidade Formadora de Placa) é conhecido.

Dessa forma, pode-se detectar e quantificar o DNA/RNA viral de uma determinada amostra de forma sensível e específica (Holland *et al.,* 1991; Laue *et al.,* 1999).

O sistema é totalmente automatizado, e o material amplificado é normalmente descartado sem que seja preciso abrir os tubos. Dessa forma, a contaminação das amostras por DNA pode ser completamente evitada.

Essa técnica permite acompanhar a amplificação em tempo real de até 96 amostras simultaneamente.

1.11 Prevenção e Controle

Ainda não existe nenhuma vacina licenciada contra a dengue, entretanto, existem diversas vacinas candidatas em desenvolvimento. Diversas estratégias foram desenvolvidas para uma vacina contra a dengue: vacinas de vírus vivos (atenuação em cultura, atenuação através de engenharia genética, construção de vacinas quiméricas e vacinas de DNA), vacinas de vírus

mortos (vacinas contendo proteínas recombinantes do envelope dos vírus, com ou sem adjuvantes), vacinas de subunidades (proteínas e/ou peptídeos) e estratégias mistas.

Os estudos clínicos mais avançados foram realizados com uma vacina quimérica tetravalente que utiliza como base genética o vírus vacinal da febre amarela (17D). A maioria dos adultos e crianças vacinados com essa vacina desenvolveu anticorpos neutralizantes, que persistem por, pelo menos, um ano após vacinação. Apesar de se detectar viremia na primeira semana após a vacinação, os títulos virais foram baixos e a taxa de eventos adversos foi similar à da vacina contra febre amarela. Os vírus vacinais não replicam no trato gastrintestinal dos vetores e são bastante estáveis. Até o presente, os estudos de fase I e II, realizados na Ásia, nos Estados Unidos e na América Latina, com vacina quimérica tetravalente atenderam a todas as exigências para a realização de estudos de fase III, que deverão ser iniciados em breve (Pugachev *et al.,* 2005; Monath, 2007; Durbin *et al.,* 2006a; 2006b; Mateu *et al.,* 2007; Deauvieau *et al.,* 2007).

A Universidade do Mahidol (Bangkok, Tailândia) e o Instituto de Pesquisa Walter Reed, do Exército Norte-Americano (WRAIR), têm usado métodos convencionais para desenvolver vacinas vivas atenuadas para os DENV, através da passagem destes vírus em cultura de células.

As vacinas candidatas da Universidade do Mahidol não alcançaram resposta imune equilibrada para cada um dos quatro sorotipos, bem como na formulação tetravalente (Kanesa-thasan *et al.,* 2001; Sabchareon *et al.,* 2004; Kitchener *et al.,* 2006).

As vacinas candidatas do WRAIR foram passadas inicialmente em cultura de células primárias de rim de cão (PDK) e posteriormente em cultura de células fetais de pulmão de Macaco Rhesus. As candidatas monovalentes foram avaliadas em Macaco Rhesus em ensaios clínicos da Fase I, com vistas a avaliar cada passagem em células PDK e selecionar os vírus que possam fornecer um equilíbrio adequado entre atenuação e imunogenicidade.

Em estudos humanos, as vacinas de DENV-2, DENV-3 e DENV-4 foram levemente reativas, mas a vacina para os DENV-1, a reação foi moderada, onde 40% dos vacinados desenvolveram febre e exantema generalizado (Sun *et al.,* 2003). Na sequência de uma única administração de vacinas monovalentes em voluntários adultos soronegativos, as taxas de soroconversão variaram de 46 a 100% para cada sorotipo dos DENV, com a taxa mais elevada alcançada pelo DENV-1. A alta reatividade do DENV-1 também ficou evidente no início dos ensaios para a formulação tetravalente, que também indicou baixa reatividade do DENV-4 (Sun *et al.,* 2003; Edelman *et al.,* 2003).

Para abordar estas questões, a dosagem dos níveis de cada componente foi ajustada. O componente do DENV-1 foi substituído por uma passagem maior (PDK-27, em vez de PDK-20)

e o componente do DENV-4 foi substituído por uma passagem menor (PDK-6, em vez de PDK-20), com o objetivo de melhorar a imunogenicidade. As atuais formulações tetravalentes estão em testes na Fase II na América do Norte e Sudeste Asiático.

Com a exceção do candidato DENV-2 (PDK-53) (Butrapet *et al.,* 2000), as mutações que contribuem para a atenuação dos fenótipos da Universidade do Mahidol ou do WRAIR, ainda não foram identificadas, em parte devido ao fato de nunca terem sido clonadas biologicamente, o que tem dificultado sua análise genética (Sanchez *et al.,* 2006).

Uma outra estratégia foi utilizada no Laboratório de Doenças Infecciosas do Instituto Nacional de Alergias e Doenças Infecciosas (EUA). Nessa abordagem, técnicas de genética reversa foram utilizadas para inserir mutações ou deleções definidadas na região 3' não-codificante (3'NC), utilizando o comprimento total de clones cDNA dos DENV-1 e DENV-4 (Men *et al.,* 1996; Whitehead *et al.,* 2003a). A deleção da posição 172-143 da 3'NC, mais tarde denominada Δ30, especificou um desejável equilíbrio entre o nível de atenuação e imunogenicidade para ambos DENV-1 e DENV-4 em macacos e seres humanos, mas não para o DENV-2 e DENV-3 (Blaney et al., 2004a; 2004b). As vacinas candidatas para os DENV-1 e DENV-4 contendo a deleção Δ30 foram seguras, assintomáticas e imunogênicas na dose de 10^3 unidades formadoras de placa (PFU), com leve exantema observado em cerca da metade dos voluntários, leucopenia em 7 a 40%, e aumento transitório dos níveis de alanina aminotransferase (ALT) em alguns voluntários, especialmente, em uma dose maior que 10^5 PFU para DEN4Δ30 (Durbin *et al.,* 2001; 2005; 2006b).

Este vírus não foi transmitido dos vacinados para os mosquitos, provavelmente devido a baixa viremia (10^1 PFU/ml) e pelo fato da mutação Δ30 ser atenuante e não replicar em mosquitos (Troyer *et al.,* 2001). A mutação Δ30 permanece estável geneticamente em humanos. Pelo fato do DEN2Δ30 e DEN3Δ30 não terem tido sucesso, uma estratégia quimérica baseada na vacina candidata DEN4Δ30 foi usada para criar novos candidatos para DENV-2 e DENV-3.

Os DEN2/4Δ30 e DEN3/4Δ30 gerados continham as proteínas prM e E dos DENV-2 e DENV-3 em um "background" genético do DEN4Δ30, respectivamente. Estes vírus quiméricos foram altamente atenuados para macacos (Blaney *et al.,* 2004b; Whitehead *et al.,* 2003b). Tanto o DEN2/4Δ30 quanto o DEN3/4Δ30 possuiram baixa infectividade quando inoculados em mosquitos *Ae. aegypti* (Whitehead *et al.,* 2003b; Blaney *et al.,* 2004b).

A atenuação dos DEN2/4Δ30 e DEN3/4Δ30 foi resultado da quimerização e da mutação Δ30. As vacinas quiméricas candidatas DEN2/4Δ30 e DEN3/4Δ30 foram combinadas com DEN1Δ30 e DEN4Δ30 para criar uma formulação tetravalente, e têm demonstrado ser atenuada

(com títulos de 10^2 PFU/ml), amplamente imunogênica e protetora em Macacos Rhesus (Blaney *et al.,* 2005). Testes na Fase I com a candidata DEN2/4Δ30 demonstraram ser segura e imunogênica com dose única de 10^3 PFU (Durbin *et al.,* 2006b). Testes clínicos com DEN3/4Δ30 estão atualmente em curso. A adequação desta formulação tetravalente para o ser humano ainda não foi estudada.

A plataforma ChimeriVax foi utilizada pela Acambis (Cambridge, E.U.A.) para criar uma vacina quimérica a partir da substituição dos genes que codificam as proteínas prM e E de cada um dos sorotipos dos DENV utilizando a vacina viva atenuada YF17D como base genética. Supõe-se que a atenuação destas candidatas à vacina é proveniente das mutações presentes dos antecedentes genéticos da YF17D, assim como da própria quimerização, embora a contribuição de cada um destes componentes não tenha sido formalmente demonstrado (Guirakhoo *et al.,* 2001). A candidata à vacina monovalente demonstrou baixas taxas de infecção em *Ae. albopictus*, e são essencialmente de natureza não-infecciosa em *Ae. aegypti* (Higgs *et al.,* 2006). Em macacos, a administração de uma formulação tetravalente em doses de 10^3 a $10^{4,5}$ PFU de cada componente, induziu um alto nível de anticorpos neutralizantes, que foi eficaz contra uma ampla coleção de sorotipos de DENV. No entanto, o pico de viremia em macacos (principalmente do DENV-4) foi de 10^2 a 10^3 PFU/ml, superior ao observado com outros sorotipos candidatos a vacina (Guirakhoo *et al.,* 2002). A vacina monovalente ChimeriVax-DEN2 foi avaliada com sucesso em seres humanos e demonstrou ser segura e imunogenica (Guirakhoo *et al.,* 2006). Os relatórios dos ensaios de Fase I da vacina tetravalente produzida pela ChimeriVax, indicam ser uma candidata segura, sem efeitos adversos graves, mas que provavelmente terão de ser administradas em doses superiores a 10^4 PFU/ml (Whitehead *et al.,* 2007).

Usando a vacina atenuada DENV-2 PDK-53 desenvolvida pela Universidade Mahidol, pesquisadores do CDC (EUA) desenvolveram um conjunto de vacinas quiméricas com base nas três mutações atenuantes do DENV-2 PDK-53 existentes fora dos genes estruturais. Esta formulação demonstrou ser imunogênica e protetora em camundongos (Huang *et al.,* 2003), e a formulação tetravalente aplicada em macacos foi recentemente concluída. Estudos prevêem ensaios de Fase I em seres humanos.

No contexto das vacinas inativadas, uma candidata de DENV-2 foi fabricada pelo WRAIR e uma candidata equivalente de DENV-1 entrará em breve nos ensaios clínicos (Putnak *et al.,* 1996a; 2006b). Na preparação destas vacinas, os vírus foram propagados em cultura de células Vero, concentrados por ultrafiltração e purificados em gradiente de sacarose. A alta

titulação dos vírus purificados (aproximadamente 10^9 PFU/ml) foi, então, inativado com formalina. A vacina de DENV-2 com alúmen e outros adjuvantes induziram altos níveis de anticorpos utilizando o modelo de primatas (Putnak *et al.,* 2005).

As vacinas de subunidades ainda não foram testadas em seres humanos. No entanto, dois estudos realizados em Macacos Rhesus foram recentemente concluídos usando a proteína E em formulação monovalente de DENV-2 e DENV-4. Guzman *et al.* imunizaram macacos com quatro doses de 100g da proteína E de DENV-4, utilizando alúmen como adjuvante, e conseguiram apenas uma protecção parcial desafiando com o DENV-2 selvagem (Guzman *et al.,* 2003). Em colaboração com Havaí Biotech, Putnak *et al.* imunizaram macacos com duas doses de DENV-2. Estes autores utilizaram à proteína E produzida em células de Drosophila e testaram cinco diferentes combinações de adjuvantes (Putnak *et al.,* 2005). Apesar dos títulos de anticorpos neutralizantes variarem muito, um grupo de macacos que receberam uma dose maior do antígeno com dois adjuvantes foi completamente protegido. Havaí Biotech atualmente está fabricando este modelo para cada um dos sorotipos e irá em breve iniciar ensaios clínicos de Fase I.

No contexto das vacinas de ácido nucléico, os últimos experimentos usando genes que codificam as proteínas prM e E para DENV-1, demonstraram que três doses desta vacina protegem macacos *Aotus,* com posterior desafio com o vírus selvagem (Raviprakash *et al.,* 2003). Vacinas de DNA convencionais oferecem algumas vantagens, incluindo a facilidade de produção, transporte e estabilidade em temperatura ambiente, diminuição da probabilidade de replicação e interferências na vacinação conjunta com outros patógenos. No entanto, a experiência recente com as vacinas de DNA revelou a necessidade de múltiplas doses em conjunto com adjuvantes adequados, motivos imunoestimulatórios e equipamentos de inoculação especializados. A primeira estratégia utilizando a proteína E expressada em vetores de DNA foi realizada. Entretanto, a formulação tetravalente parece muito complicada (Whitehead *et al.,* 2007).

Finalmente, enquanto uma vacina eficaz não está disponível para aplicação em larga escala, as medidas de controle ao vetor e o diagnóstico precoce de casos suspeitos consistem nos principais instrumentos para a prevenção do dengue, aliado a melhoria do saneamento básico.

2. OBJETIVOS

2.1 Objetivo Geral:

1) Estudar os aspectos relacionados a patogenia, sítios de replicação, filogenia e evolução molecular dos vírus dengue tipo 3 (DENV-3).

2.2 Objetivos específicos:

1) Determinar a viremia em casos fatais e não fatais de DENV-3, avaliando sua importância na patogenia dos casos graves da doença;

2) Pesquisar DENV em diferentes tecidos provenientes de casos fatais considerando-se que ainda não se conhece em profundidade os sítios de replicação dos DENV;

3) Estudar a evolução genética global dos DENV-3;

4) Determinar os genótipos do DENV-3 circulantes no Brasil.

3. RESULTADOS

Este trabalho é apresentado no formato de artigos publicados, obedecendo as Normas e Recomendações do Programa de Pós-Graduação em Biologia Celular e Molecular do Instituto Oswaldo Cruz. Nesse contexto, os artigos científicos substituem as seções de Material e Métodos e Resultados. Cada artigo representa um "capítulo", precedido de uma página de rosto com sua apresentação.

Artigo 1: Determinação da viremia em casos fatais e não fatais de DENV-3.

Referência bibliográfica: Araújo JMG, Filippis AMB, Schatzmayr HG, Araújo ESM, Britto C, Cardoso MA, Camacho LAB, Nogueira RMR. Quantification of dengue virus type 3 RNA in fatal and nonfatal cases in Brazil, 2002. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2009. [Epub ahead of print].

Situação do manuscrito: Aceito para publicação no periódico "Transactions of the Royal Society of Tropical Medicine and Hygiene". **Este artigo atende ao objetivo 1.**

Apresentação: A patogênese da Febre Hemorrágica do Dengue (FHD) e da Síndrome do Choque por Dengue (SCD) tem sido uma das grandes questões na pesquisa da dengue. A ausência de um modelo animal capaz de reproduzir clinicamente a infecção pelos vírus dengue (DENV) tem dificultado a compreensão deste aspecto. Dois principais fatores têm sido atribuídos para a ocorrência da FHD e SCD: a virulência da cepa e a resposta imune do hospedeiro (Halstead, 1988; Gubler, 2002; Guzman & Kouri, 2002; Rothman, 2004; Green & Rothman, 2006). Neste artigo, correspondente ao objetivo 1, apresentamos os resultados da aplicação dos métodos de RT-PCR em tempo real e IgG-ELISA para determinar a carga viral e o tipo de infecção (primária ou secundária) em 23 casos fatais e 19 casos de dengue clássico, ocorridos durante a epidemia de 2002 no estado do Rio de Janeiro.

Title: Quantification of dengue virus type 3 RNA in fatal and nonfatal cases in Brazil, 2002.

Running title: Quantification of DENV-3 RNA.

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Abstract

We examined levels of dengue virus type 3 RNA in association with the type of infection (primary or secondary) in patients with fatal and nonfatal outcomes in Rio de Janeiro, 2002. Subjects with fatal outcomes had mean virus titers significantly higher than those who survived. Because primary infections were confirmed among the fatal cases (52.1%), antibody-dependent enhancement alone did not explain all the cases of severe disease in this study population. In conclusion, these findings suggest that high levels of DENV-3 may have contributed to the severe form of dengue in Rio de Janeiro, 2002.

Keywords: Dengue virus type 3; Real-time RT-PCR; Viremia; Pathogenesis; Brazil.

1. Introduction

The pathogenesis of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) has been one of the major issues in dengue virus research. Among the risk factors reported for DHF and DSS, the viral strain and the host immune status have been identified as key (Gubler, 1978; Halstead, 1988; Guzman and Kouri, 2002). Based on the observation that individuals experiencing secondary infection had a higher probability of developing DHF and/or DSS, the immune hypothesis states that cross-reactive, nonneutralizing antibodies from previous infection may enhance dengue virus infection (Halstead, 1988). Since different dengue viral strains have been associated to outbreaks of very mild or very severe disease, the viral hypothesis contends that severe dengue disease is the result of infection with a more virulent strain (Rosen, 1977; Gubler et al., 1978). For these reasons, we examined levels of dengue virus RNA and the type of infection (primary or secondary) in patients with fatal and nonfatal outcomes in 2002 during a severe DENV-3 epidemic in the State of Rio de Janeiro, Brazil.

2. Materials and methods

The human serum specimens examined in this study were obtained from 42 patients with confirmed DENV-3 infection, who presented acute febrile illness with two or more of the following clinical manifestations: headache, retrobulbar pain, myalgia, arthralgia, rash and hemorrhagic manifestations. All cases were classified according to definitions of the World Health Organization (WHO). Nineteen cases were outpatients categorized as "nonfatal cases" (dengue fever "DF") and the remaining twenty-three were "fatal cases" (19 DHF and 4 DSS).

Viral RNA for real-time RT-PCR assays 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. For the quantitative TaqMan assay, a 10-fold-dilution series containing a known amount of target viral RNA (10^7 RNA copies/ml) was used for RNA extraction. Real-time RT-PCR assays were performed as described previously by Houng et al. (2001). The number of viral RNA copies detected was calculated by generating a standard curve from 10-fold-dilutions of DENV-3 RNA, isolated from a known amount of local virus propagated in *Aedes albopictus* C6/36 cells (Nogueira et al., 2005), the titer of which was determine by plaque assay.

In order to classify the type of infection in fatal and nonfatal cases as either primary or secondary dengue, IgG-ELISA was conducted as described previously by Miagostovich et al. (1999). According to the IgG-ELISA criteria, the immune response is defined as primary when acute-phase serum samples obtained before day 5 of illness have IgG antibody titers <1:160 and convalescent-phase sera have titers <1:40,960. Infections are considered secondary when IgG titers are >1:160 in the acute-phase serum and >1:163,840 in convalescent-phase samples. Parametric (t-test) and nonparametric (Mann-Whitney test) statistical methods within the *SPSS for Windows 8* software were adopted to compare levels of dengue virus RNA in fatal and nonfatal cases, $P < 0.05$ considered significant.

3. Results and discussion

Adopting quantitative real-time RT-PCR, we examined levels of dengue virus RNA in patients with fatal (n=23) and nonfatal (n=19) outcomes. Subjects with fatal outcomes had mean virus titers significantly higher than those who survived, 12.5 $\log_{10}$ RNA copies/ml and 7.9 $\log_{10}$ RNA copies/ml, respectively (t-test, P=0.001; Mann-Whitney test; P=0.002) (Figure 1). These results suggest that high level of DENV is an important factor in the pathogenesis of severe dengue, corroborating several earlier reports (Gubler et al., 1978; Nogueira et al., 2005).

Among the fatal cases, 52.1% (12/23) were classified as primary infections, and 30.4% (7/23) as secondary infections. Four cases could not be characterized due to lack of information. Among the nonfatal cases, 47.3% (9/19) were determined as primary infections and 52.6% (10/19) as secondary infections. Because primary infections were confirmed among the fatal cases, antibody-dependent enhancement alone did not explain all the cases of severe disease in this study population.

The correlation between levels of DENV-3 RNA and the type of infection (primary or secondary) was examined. In fatal cases, the mean virus titer was higher in patients with primary infections (12.48 $\log_{10}$ RNA copies/ml) than in patients with secondary infections (11.86 $\log_{10}$ RNA copies/ml), however, this difference was not significant (t-test, P=0.10; Mann-Whitney test, P=0.21). In nonfatal cases, the mean virus titer was significantly higher in patients with primary infections (9.74 $\log_{10}$ RNA copies/ml) than in patients with secondary infections (6.23 $\log_{10}$ RNA copies/ml) (t-test, P=0.05; Mann-Whitney test, P=0.02). These results suggest rapid immune response activation in patients classified as secondary infection and consequent reduction of viremia.

In conclusion, these findings suggest that high levels of DENV-3 may have contributed to the severe form of dengue in Rio de Janeiro, 2002. These results are of great importance for understanding the viral pathogenesis of dengue viruses.

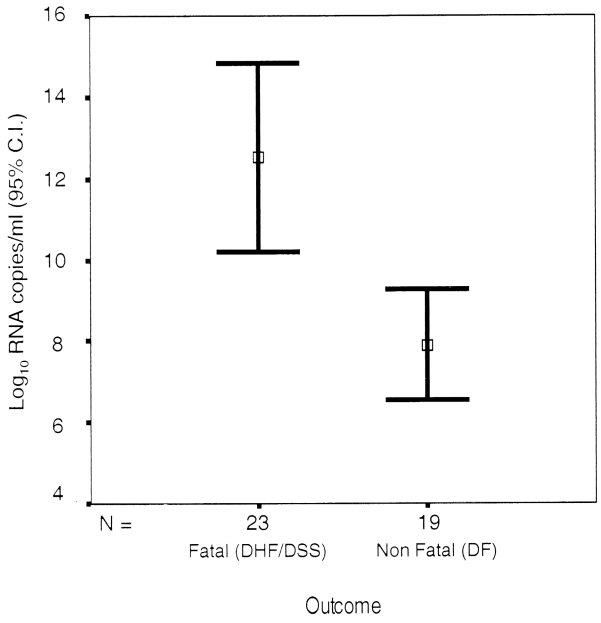

Figure 1. Mean log titer (95% confidence interval) in fatal (n=23) and nonfatal (n=19) cases. Subjects with fatal outcomes had mean virus titers that were significantly higher than those who survived, 12.5 $\log_{10}$ RNA copies/ml and 7.9 $\log_{10}$ RNA copies/ml, respectively (t-test, *P*=0.001; Mann-Whitney test; *P*=0.002).

Authos' contributions: JMGA, HGS, AMBF and RMRN designed the study protocol; JMGA, CB, MAC, LABC and RMRN analyzed the data; ESMA carried out the IgG-ELISA; JMGA, CB and RMRN drafted the manuscript. All authors contributed to preparing the manuscript and read and approved the final version. JMGA and RMRN are guarantors of the paper.

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Artigo 2: Pesquisa dos vírus dengue (DENV) em diferentes tecidos provenientes de casos fatais.

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Apresentação: Os DENV podem infectar muitos tipos de células, incluindo tecidos do sistema vascular, muscular e hematológico, causando diversos sinais clínicos e patológicos (Seneviratne et al., 2006). Estudos com pacientes que desenvolveram FHD/SCD revelaram a presença do antígeno (Ag) e do RNA viral em tecidos como o fígado, baço, cérebro, linfonodos, timo, rins, pulmões, coração, pele e medula óssea (Bhamarapravati *et al.,* 1967; Boonpucknavig *et al.,* 1979; Yoskan *et al.,* 1983; Hall *et al.,* 1991; Bhoopat *et al.,* 1996; Miagostovich *et al.,* 1997; Jessie *et al.,* 2004). Com base nestes estudos, pesquisamos a presença do Ag e do RNA viral em diferentes tecidos provenientes de 29 casos fatais suspeitos de dengue, ocorridos durante a epidemia de 2002 no estado do Rio de Janeiro, com o objetivo de investigar os sítios de replicação viral.

Title: A retrospective survey of dengue virus infection in fatal cases from an epidemic in Brazil.

Running title: A retrospective survey of dengue virus infection in Brazil.

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Abstract

Dengue virus can infect many cell types from the vascular, muscular and hematological systems causing diverse clinical and pathological signs. The purpose of the present study was to investigate by different diagnostic methods dengue virus in human tissue specimens obtained from fatal cases (n=29) during a large-scale dengue fever epidemic in 2002 in the State of Rio de Janeiro, Brazil. The combination of four procedures provided diagnostic confirmation of DENV-3 infection in 26 (89.6%) out of the 29 suspected fatal cases. Dengue virus (DENV) was isolated from 2/74 (2.7%) tissue samples, inoculated into C6/36 cells and identified as DENV-3, nested RT-PCR accusing 22/72 (30.5%) samples as DENV-3. Real-time RT-PCR yielded the highest positivity rate, detecting viral RNA in 45/77 (58.4%) clinical specimens, including the liver (n=18), lung (n=8), spleen (n=8), brain (n=6), kidney (n=3), bone marrow (n=1) and heart (n=1). Immunohistochemical tests recognized the DENV antigen in 26/59 (44%) specimens. Given the accuracy and effectiveness of real-time RT-PCR in this investigation, this approach may play an important role for rapid diagnosis of dengue infections.

Keywords: Dengue virus; Diagnosis; Surveillance; Tissues; Real-Time RT-PCR.

1. Introduction

Dengue infection is the most prevalent arthropod-borne viral disease in subtropical and tropical regions of the world (Halstead, 1980). All four of the dengue virus serotypes (genus *Flavivirus,* family *Flaviviridae*) consist of a single positive-strand RNA surrounded by an icosahedral nucleocapsid (Hammon et al., 1960). Dengue virus infection causes either a relatively mild disease, known as classic dengue fever (DF) or a more severe form, dengue hemorrhagic fever (DHF), a fulminating illness characterized by hemorrhagic manifestations and plasma leakage, which may progress to dengue shock syndrome (DSS) and death (Halstead, 1988). The virus can infect many cell types from the vascular, muscular and hematological systems causing diverse clinical and pathological signs (Seneviratne et al., 2006).

Studies of specimens from patients presenting DHF/DSS revealed the presence of viral antigens or RNA in diverse tissues including liver, spleen, brain, lymph node, thymus, kidney, lung, heart, bone marrow and skin and mainly in mononuclear phagocytic cells (Bhamarapravati et al., 1967; Bhoopat et al., 1996; Boonpucknavig et al., 1979; Hall et al., 1991; Miagostovich et al., 1997; Yoskan et al., 1983). The purpose of the present study was to investigate by different diagnostic approaches dengue virus in human tissue specimens from fatal cases during a large-scale dengue fever epidemic in 2002 in the State of Rio de Janeiro, Brazil.

2. Materials and methods

2.1. Study Population

The human tissue specimens examined included liver (n=28), lung (n=14), spleen (n=16), brain (n=11), kidney (n=5), bone marrow (n=1) and heart (n=2) obtained from 29 patients presenting with acute febrile illness with two or more of the following clinical manifestations: 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 -70ºC until tested. Epidemiological aspects and the description of disease evolution resulting in death were reported previously by Nogueira et al. (2005). All samples were collected between January and March 2002 from patients ranging in age from 11 to 64 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.

2.2. Tissue treatment

Fragments of tissue (1-2 grams) were ground in 1.5mL of Leibovitz-15 culture medium (Sigma) and centrifuged (10,000 rpm at 4ºC, for 15 min). The supernatant was inoculated in a C6/36 cell culture and used for RNA extraction.

2.3. Virus isolation

A total of 74 tissue samples were prepared for virus isolation by inoculation into monolayers of a C6/36 *Aedes albopictus* cell line (Igarashi, 1978). Dengue virus isolates were identified by an indirect fluorescent antibody test (IFAT) with serotype-specific monoclonal antibodies, as described previously by Gubler et al. (1984).

2.4. RNA extraction

Viral RNA for the nested RT-PCR and real-time RT-PCR assays was extracted from 140 µL of tissue specimens (supernatant) 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.

2.5. Nested Reverse Transcriptase PCR assay

The nested RT-PCR protocol for DENV detection and typing was performed on 72 tissue samples, as described previously by Lanciotti et al. (1992).

2.6. Real-time Reverse Transcriptase PCR (TaqMan) assay

One-step real-time RT-PCR assays were performed in the ABI Prism® 7000 Sequence Detection System (SDS) (Applied Biosystems, Foster City, CA). Samples were assayed in a 30 µL reaction mixture containing 8.5 µL of extracted RNA, 0.63 µL of 40X Multiscribe enzyme plus RNAse inhibitor, 12.5 µL TaqMan 2X Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 300 nM of each specific primer and fluorogenic probe. Primer sequences (**DV3.U:** 5´-AGC ACT GAG GGA AGC TGT ACC TCC-3'; **DV.L1:** 5'-CAT TCC ATT TTC TGG CGT TCT-3') and probe (**DV.P1:** 5'-CTG TCT CCT CAG CAT CAT TCC AGG CA-3') were obtained from Houng et al. (2001) and designed for the 3' noncoding sequences (3'NC). The TaqMan probe was labeled at the 5' end with 5-carboxyfluorescein (FAM) reporter dye and at the 3' end with 6-carboxy-*N,N,N',N'*-tetramethylrhodamine (TAMRA) quencher fluorophore.

The 5′ nuclease TaqMan assay relies on the 5′ exonuclease activity of the Taq polymerase to free the reporter dye in the quenched probe. The accumulation of the amplified PCR product is proportional to an exponential increase in the fluorescence emitted from the freed reporter dyes (ΔRn). The threshold cycle (Ct) represents the PCR cycle at which the SDS software first detects a noticeable increase in reporter fluorescence above a baseline signal. Positive and negative controls were included in every assay.

Amplification and real-time detection consisted of the following cycling profile: reverse transcription at 45°C for 30 min followed by one step at 95°C for 10 min and 45 cycles at 95°C for 15 sec and 60°C for 1 min. The ABI Prism® 7000 SDS (version 1.1; PE Applied Biosystems) was adopted to examine the fluorescence emitted during amplification. A single fluorescence reading for each sample was taken at the annealing-elongation step.

2.7. Immunohistochemical procedure

The immunohistochemical procedure was undertaken on 59 tissue samples, as described previously by Miagostovich et al. (1997). Briefly, sections of formalin-fixed, paraffin-embedded tissues were processed by the avidin biotin complex (ABC) method according to the manufacturer's protocol (Vectastain AEC Kit, Vector Laboratories, Inc. Burlingame, CA, USA). Monoclonal antibodies for DENV-1, -2, and -3 were directed against the E protein, positive and negative controls were included.

3. Results

The combination of four methods provided diagnostic confirmation of DENV-3 infection in 26 (89.6%) out of the 29 suspected fatal cases. As demonstrated in Table 1, the use of only one diagnostic tool provided positive results in 9/26 cases (34.6%), only one case determined by immunohistochemistry (3.8%), and in 8/26 patients diagnosis was confirmed by real-time RT-PCR (30.8%). In the other 17/26 cases (65.4%), infection was detected by at least two different diagnostic approaches.

Table 1. Investigation of suspected fatal dengue cases (n=29) by virus isolation, nested RT-PCR, immunohistochemistry and real-time RT-PCR.

Case number	Virus isolation	Nested RT-PCR	Immunohistochemistry	Real-time RT-PCR
1	-	+	-	+
2	-	-	-	+
3	-	-	+	+
4	-	-	-	+
5	+	+	NP	+
6	-	-	-	-
7	-	-	NP	+
8	-	-	NP	-
9	-	-	NP	+
10	-	-	+	+
11	-	-	-	+
12	-	-	NP	+
13	-	+	+	+
14	-	+	+	+
15	-	-	-	+
16	-	+	+	+
17	+	+	+	+
18	-	+	+	+
19	-	-	+	-
20	-	-	NP	-
21	-	-	+	+
22	-	-	+	+
23	-	-	+	+
24	-	-	NP	+
25	-	-	+	+
26	-	+	+	+
27	-	+	-	+
28	-	-	+	+
29	-	+	-	+
Total*	2/29 (6.8)	10/29 (34.4)	14/22 (63.6)	25/29 (86.2)

*Positive/total analyzed (%). Positive sample (+), negative sample (-), and not performed (NP).

Table 2. Dengue diagnosis of 77 clinical samples (tissue specimens from liver, lung, spleen, brain, kidney, bone marrow and heart), collected from the 29 patients with fatal outcome, by virus isolation, nested RT-PCR, immunohistochemistry and real-time RT-PCR.

Clinical specimen	Virus isolation positive/total analyzed (%)	Nested RT-PCR positive/total analyzed (%)	Immunohistochemistry positive/total analyzed (%)	Real-time RT-PCR positive/total analyzed (%)
Liver	2/26	8/27	14/22	18/28
Lung	0/14	5/12	3/11	8/14
Spleen	0/15	3/15	5/13	8/16
Brain	0/11	3/11	2/7	6/11
Kidney	0/5	2/5	2/4	3/5
Bone Marrow	0/1	NP	NP	1/1
Heart	0/2	1/2	0/2	1/2
Total	2/74 (2.7)	22/72 (30.5)	26/59 (44.0)	45/77 (58.4)

NP: not performed

A total of 77 clinical samples (tissue specimens from the liver, lung, spleen, brain, kidney, bone marrow and heart) were collected from the 29 patients with fatal outcome. The diagnostic results yielded by the four distinct approaches are shown in Table 2. DENV was isolated from two liver specimens (2.7%) out of 74 after inoculation into C6/36 cells and identified as DENV-3. Nested RT-PCR detected 22 (30.5%) out of 72 tissue samples as infected by the same serotype and real-time RT-PCR confirmed the infection in 45 (58.4%) out of the 77 tissue specimens investigated. The immunohistochemical procedures detected viral antigens in 26 (44.0%) out of 59 tissue samples. In all tissues, the immunoreactivity pattern was positive for cytoplasmic granular staining. DENV was observed mainly in hepatocytes and Kupfer cells (liver), the neuronal cell body (brain) and lymphoid cells - especially macrophages (spleen). The cell type could not be identified in lung, kidney, heart and bone marrow tissues. Only the qualitative analysis was applied for Real-time RT-PCR and the immunohistochemical procedure. Among all the specimens tested, the liver was the most frequent site for the recovery of DENV isolates, nested RT-PCR, immunohistochemistry and real-time RT-PCR yielding positive results in 24 (92.3%) out of the 26 confirmed DENV-3 cases (Table 3). In this investigation, only one single bone marrow specimen was available (patient 11, Table 3), from which the presence of viral RNA was confirmed by real-time RT-PCR. The same method also determined a positive result in a liver sample from the same patient, reinforcing DENV-3 infection in this case.

Table 3. Dengue virus detection regarding tissue samples from 29 suspected fatal cases.

Case number	Liver	Lung	Spleen	Brain	Kidney	Bone Marrow	Heart
1	+	+	+	+	+		
2	-	+	-	+			
3	+						
4	+	+					
5	+		-				
6	-	-	-	-	-		
7	+	+		+			
8	-						
9	+	-	-	-			
10	+	+	+	+			
11	+	-				+	
12	+		-	+			
13	+	+	+				
14	+		+		+		+
15	-	-	+	-			
16	+						
17	+	+	+	+			
18	+	+	+	+			
19	+						
20	-						
21	+						
22	+		+				
23	+						
24	+						
25	+						
26	+	+	+		+		+
27	+						
28	+		+				
29	+	+	-	+	+		
Total*	24/29 (82.7)	10/14 (71.4)	10/16 (62.5)	8/11 (72.7)	4/5 (80)	1/1 (100)	2/2 (100)

* Positive/total analyzed (%). Positive sample (+), negative sample (-) and unavailable sample (in white).

4. Discussion

During 2002, a total of 813,104 dengue cases were notified in Brazil, mainly in the southeastern and northeastern regions (Nogueira et al., 2007). This number corresponded to 80% of all the reported dengue cases in the Americas (Nogueira et al., 2007). The introduction of DENV-3 into Rio de Janeiro in 2000 placed the region at high risk of a new epidemic involving this serotype, since the emergence of a new serotype into a susceptible population with high mosquito densities may produce a large-scale epidemic after a lag period (Rigau-Perez et al., 2002). Indeed one year after the appearance DENV-3, this serotype was responsible for the most severe epidemic in the State's history in terms of the number of reported cases, severity of clinical manifestations and the number of confirmed deaths. In this DENV-3 epidemic, the number of DHF/dengue shock syndrome (DSS) cases (1,831) and deaths (91) exceeded the total number of

DHF/DSS cases (1,621) and deaths (76) in the entire country from 1986 to 2001 (Barbosa da Silva et al., 2002). Furthermore, an increase in unusual clinical features was observed during this epidemic characterized by the incidence of Central Nervous System (CNS) involvement and hepatitis (Nogueira et al., 2005).

In this study, the liver was the most important organ for virus detection, according to all four diagnostic methods. Recently, the liver was recognized as a major target organ in the pathogenesis of DENV infection, its active hepatocyte replication perhaps accounting for these findings (Couvelard et al., 1999; Lin et al., 2000). In this study, examination of liver specimens confirmed virus infection in 24 out of 26 fatal cases (Table 3), similar to that described previously in Indonesia (Sumarmo et al., 1983).

Heart involvement has been reported during dengue fever (Basílio-de-Oliveira et al., 2005; Horta Veloso et al., 2003), and in the present study heart involvement was confirmed in two cases by the identification of DENV-3 RNA by nested or real-time RT-PCR. Cardiac manifestations of dengue infection are uncommon, nevertheless cardiac rhythm disorders, such as atrioventricular block (Donegani and Briceño, 1986; Khongphatthallayothin et al., 2000) and ectopic ventricular beats (Chuah, 1987), have been described during episodes of dengue hemorrhagic fever, most of them presenting a benign course with spontaneous resolution. These clinical features have been attributed to viral myocarditis, however the exact mechanism has yet to be elucidated definitively.

Although CNS involvement has been reported previously during dengue epidemics, including those in Brazil (Leao et al., 2002; Nogueira et al., 2002), it increased expressively of this clinical features during the 2002 outbreak when many patients complained of dizziness. In 8 fatal cases of this investigation, CNS involvement was clarified further by the detection of viral antigens or DENV-3 RNA in brain tissue samples (Table 3). Neurological disorders associated with dengue cases have been referred to as dengue encephalopathy, attributed to immunopathological responses and not to CNS viral infection. However, the isolation of DENV-3 and detection of DENV-2 by RT-PCR in cerebrospinal fluid (CSF) provides evidence that DENV possesses neurotropism and can lead to encephalitis in both primary and secondary infections (Lum et al., 1996). The breakdown of the blood-brain barrier has been shown previously in fatal dengue cases (Miagostovich et al., 1997).

The clinical syndrome of dengue-associated bone marrow suppression has been well documented (La Russa & Innis, 1995), and a review of experimental dengue infections of volunteers together with histopathological studies of bone marrow from patients with severe

dengue virus infection suggest that marrow suppression evolves rapidly (La Russa & Innis, 1995). The strong link of dengue with neutropenia and thrombocytopenia suggests that bone marrow cells may be potential targets for dengue viral infections. Rothwell et al. (1996) infected long-term marrow cultures with DENV-2 and characterized the viral antigen-positive cells. These investigations demonstrated two types of stromal cells that were positive for DENV-2 antigens by immunofluorescence microscopy and immunohistochemical staining. Taking this into account, dengue virus infection in a bone marrow specimen from a suspected fatal case was investigated by virus isolation and real-time RT-PCR. Positivity in this sample was only confirmed by the molecular diagnostic procedure.

In this study population, examination of available tissue samples of the spleen, kidney and lung gave positive results in 10, 4 and 10 fatal cases, respectively, using either immunohistochemistry or nested and real-time RT-PCR (Table 3). These data confirmed the positivity observed in a retrospective study of tissue specimens from patients with DHF/DSS, by the detection of viral antigens or RNA (Bhamarapravati et al., 1967; Miagostovich et al., 1997).

The application of four distinct assays (virus isolation, nested RT-PCR, immunohistochemistry and real-time RT-PCR) provide a better understanding of viral tropism in fatal cases of dengue virus infection. Real-time RT-PCR provided the best performance and was responsible for 58.4% positivity in the 77 histological samples, while nested RT-PCR gave positive results in 30.5% (22/72). Furthermore, real-time RT-PCR alone was able to establish DENV-3 diagnosis in 8 out of 26 confirmed cases (30.8%). Analysis of the current data is in agreement with the results of another Brazilian study by Poersch et al. (2005), where the investigations compared both dengue diagnostic methods.

In Brazil, dengue virus infections continue to be a formidable public health problem. The greater accuracy, sensitivity and speed of the real-time RT-PCR makes it suitable for effective dengue surveillance and indicates its use as a potential complement for the diagnosis of fatal cases.

CONFLICTS OF INTEREST STATEMENT

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

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Artigo 3: Estudo sobre os aspectos evolutivos global do DENV-3.

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Apresentação: As primeiras estimativas para calcular a taxa de evolução e ano de origem ou tempo do mais recente ancestral comum (Tmrca) para os DENV foram investigadas por Twiddy *et al.* (2003). Estes autores estimaram que o Tmrca dos DENV circulantes atualmente foi de aproximadamente 100 anos (Tmrca ~ 1900). Os autores também sugerem que os quatro sorotipos da dengue evoluem de acordo com um relógio molecular, mas, por razões ainda desconhecidas, os DENV-2 e DENV-3 (genótipos Americano e Asiático) possuem uma taxa de evolução significativamente mais elevada quando comparado aos DENV-1 e DENV-4. Estas diferenças entre taxas de evolução podem ser causadas por fatores epidemiológicos ou virológicos. Twiddy *et al.* (2003) sugerem que durante períodos epidêmicos, a taxa de evolução viral é maior quando comparado a períodos endêmicos. Uma maior taxa evolutiva também pode ser conseqüência do surgimento de novas variantes com propriedades biológicas particulares, tais como transmissibilidade e virulência (Cologna & Rico-Hesse, 2003; Messer *et al,* 2003; Rico-Hesse, 2003; Twiddy & Holmes, 2003; Rico-Hesse *et al,* 1997). Assim, a compreensão da possível correlação entre epidemiologia, evolução, dinâmica populacional e diversidade viral, em última análise, pode ajudar a identificar novas variantes virais capazes de causar novas epidemias. Neste capítulo, apresentamos os resultados das estimativas da taxa de evolução e Tmrca dos DENV-3 e seus principais genótipos (G) (GI, GII e GIII) e a determinação dos seus padrões de migração no Globo.

Title: Phylogeography and evolutionary history of Dengue Virus Type 3.

Running title: DENV-3 Evolution.

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Abstract

In this study, we revisited the phylogeography of the three of major DENV-3 genotypes and estimated its rate of evolution, based on the analysis of the envelope (E) gene of 200 strains isolated from 31 different countries around the world over a time period of 50 years (1956 to 2006). Our phylogenetic analysis revealed a geographical subdivision of DENV-3 population in several country-specific clades. Migration patterns of the main DENV-3 genotypes showed that genotype I was mainly circumspect to the maritime portion of Southeast-Asia and South Pacific, genotype II stayed within continental areas in South-East Asia, while genotype III spread across Asia, East Africa and into the Americas. No evidence for rampant co-circulation of distinct genotypes in a single locality was found, suggesting that some factors, other than geographic proximity, may limit the continual dispersion and reintroduction of new DENV-3 variants. Estimates of the evolutionary rate revealed no significant differences among major DENV-3 genotypes. The mean evolutionary rate of DENV-3 in areas with long-term endemic transmissions (*i.e.*, Indonesia and Thailand) was similar to that observed in the Americas, which have been experiencing a more recent dengue spread. We estimated the origin of DENV-3 virus around 1890, and the emergence of current diversity of main DENV-3 genotypes between the middle 1960s and the middle 1970s, coinciding with human population growth, urbanization, and massive human movement, and with the description of the first cases of DENV-3 hemorrhagic fever in Asia.

Keywords: Dengue Virus Type 3; Phylogeography; Evolutionary History.

1. Introduction

Dengue virus (DENV) (family *Flaviviridae*) has a single-stranded positive-sense RNA genome of approximately 11 kb and is transmitted by *Aedes aegypti* mosquitoes among humans. Infection with DENV may cause an acute "influenza-like" febrile disease called classic dengue fever (DF), or the potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The four known distinct antigenic groups (*i.e.*, serotypes) of DENV (DENV-1 to DENV-4) contain well-defined phylogenetic clusters (*i.e.*, genotypes) that are causing human pandemics. The incidence, geographic distribution and severity of DENV epidemics have dramatically increased since the mid-1950s, probably due to the rapid increase in human population size, uncontrolled urbanization, and the advent of massive human movement which facilitates the spread and proliferation of mosquitoes and infected people (Gubler, 1998; Gubler, 2002; Gubler, 2004; Zanotto et al., 1996). It has been estimated that 50-100 million infections occur annually, and more than 2.5 billion people live in areas of risk for DENV infection (Gubler, 1998; Gubler, 2002; Gubler, 2004).

Using a maximum likelihood framework, Twiddy et al. (2003) extended and re-evaluated the first comprehensive estimates of the rate and time frame of DENV evolution (Zanotto et al., 1996). It was inferred that the current global genetic diversity in the four serotypes of DENV appeared around the last 100 years. Moreover, Twiddy et al. (2003) suggested that all serotypes may be evolving according to a molecular clock; but, for reasons that remain unclear, DENV-3 and the DENV-2 American/Asian genotype had significantly higher substitutions rates when compared to other DENV strains. It has been suggested that under epidemic conditions (*i.e.*, when a new variant is introduced into a susceptible population) the viral transmission rate is higher than under endemic conditions, thus increasing the overall diversity and evolutionary rate of the new variant in the population (Twiddy et al., 2003). A higher evolutionary rate could also be a consequence of the emergence of DENV variants with particular biological properties, such as increased transmissibility, infectiousness, and/or virulence (Cologna and Rico-Hesse, 2003; Holmes and Twiddy, 2003; Messer et al., 2003; Rico-Hesse, 2003; Rico-Hesse et al., 1997). Alternatively, the lineage-specific rate differences in DENV evolution described by Twiddy et al. could be also caused by the low number of sequences used, particularly for DENV-1 (n = 9), DENV-3 (n = 21), and DENV-4 (n = 20) serotypes.

DENV-3 was isolated for the first time during an epidemic outbreak in Philippines in 1956 (Hammon et al., 1960), and since then several DF/DHF outbreaks caused by this serotype have been described world-wide. Five distinct genotypes of DENV-3 have been identified to date

(Lanciotti et al., 1994; Wittke et al., 2002). Genotypes I to III (GI to GIII) are responsible for most DENV-3 infections and have been associated with DF/DHF epidemics in Southeast Asia, Indian subcontinent, South Pacific, East Africa, and the Americas. Genotypes IV and V (GIV and GV) were not associated with DHF epidemics and are only represented by a few early sequences from the Americas, South Pacific, and Asia. Many phylogenetic studies on DENV-3 have documented the viral spread within individual countries (Aquino et al., 2006; Chungue et al., 1993; Diaz et al., 2006; Islam et al., 2006; Kobayashi et al., 1999; Peyrefitte et al., 2003; Peyrefitte et al., 2005; Podder et al., 2006; Raekiansyah et al., 2005; Rodriguez-Roche et al., 2005; Usuku et al., 2001; Uzcategui et al., 2003; Wittke et al., 2002; Zhang et al., 2005) or specific regions (Messer et al., 2003), but we still have an incomplete understanding of the global dispersion and evolutionary history of the distinct DENV-3 genotypes.

The objective of the present study was to revise the global phylogeography and evolutionary history of the main DENV-3 genotypes based on the analysis of a large number (n = 200) of envelope (E) gene sequences of DENV-3 strains isolated from 31 different countries around the world over a time period of 50 years (1956 to 2006).

2. Materials and Methods

2.1 Sequence datasets

Complete E gene sequences (1479 bp in length) with known date of isolation and representing the full extent of genetic diversity in DENV-3 were collected from GenBank (www.ncbi.nlm.nih.gov). Sequences were excluded from the analysis if they were previously identified as recombinant (Worobey et al., 1999), or were 100% similar to any other strain in the data set. For those genotypes where there were more than 75 sequences available, such as for GII and GIII, a maximum of six sequences randomly chosen from a particular country in a given year were included. This resulted in a final data set of 200 DENV-3 E sequences from 31 countries spanning a 50-year period. This primary dataset was further divided into six subsets comprising sequences from distinct genotypes and geographical origin. Table 1 shows the number, date of isolation range, and origin of the DENV-3 E sequences included in each dataset. Nucleotide sequences were aligned using CLUSTAL X program (Thompson et al., 1997) and later hand edited. All alignments are available from the authors upon request.

Table 1. Sequence datasets.

Dataset	Number of sequences	Date range	Geographic origin
DENV-3	200	1956 – 2006	Asia, America, South Pacific, and Africa
GI	43	1973 – 2005	Asia and South Pacific
GI-ID	24	1973 – 2005	Indonesia
GII	75	1973 – 2005	Asia
GII-TH	56	1973 – 2002	Thailand
GIII	75	1981 – 2006	Asia, America, and Africa
GIII-AM	63	1994 – 2005	America

2.2 Phylogenetic analysis

A Bayesian phylogenetic tree for the complete data set of 200 DENV-3 E sequences was inferred with MrBayes program (Ronquist and Huelsenbeck, 2003), under a General Time Reversible model of nucleotide substitution (Rodriguez et al., 1990) with gamma-distributed rate variation and a proportion of invariable sites (GTR+ Γ +I). Two runs of 4 chains each (one cold and tree heated, temp = 0.20) were run for 10 x 10^6 generations, with a burn-in of 2.5 x 10^6 generations. Convergence was assessed using the average standard deviation in partition frequency values across independent analyses, using a threshold value of 0.01. Convergence of parameters was also confirmed by calculating the Effective Sample Size (ESS) using TRACER v1.4 program (Rambaut and Drummond, 2007), excluding an initial 10% for each run. All parameters estimates showed ESS values >200.

2.3 Migration analysis

In order to investigate the migratory patterns of DENV-3 we examined each of the three main genotypes separately, since the isolation of viruses from different genotypes at the same locality would sum splits due to cladogenetic and migratory events, causing inferential errors. Geographical origin of each sample was coded as a set of terminal unordered character states for each dengue time-stamped, geo-referenced sample, represented as a single capital letter. The most parsimonious reconstructions (MPRs) sets of changes at each internal state in the viral

phylogeny was calculated with PAUP v4.0b10 (Swofford, 2002) and MacClade v4.07 (Maddison and Maddison, 2005) and taken as surrogate for migration events. In order to help resolve among equally parsimonious reconstructions that leads to ambiguities in character tracing, we used assignments that delay (DELTRAN) or accelerate (ACCTRAN) character transformations (Swofford and Maddison, 1987). The phylogenies used for each genotype came from the global maximum posterior probability (MAP) tree obtained with Bayesian inference (BI) with BEAST v1.4.7 (Drummond et al., 2002; Drummond and Rambaut, 2006) for the entire dataset of 200 DENV-3 E sequences from which, subtrees for each genotype were analyzed in separate. For comparison, phylogenetic trees for each genotype were also inferred with the maximum likelihood (ML) criterion as implemented in the program GARLI v0.95 (Zwickl, 2006) that estimates simultaneously the best topology, branch lengths and the best values for the parameters for the GTR+ Γ +I model of nucleotide evolution. Independent random runs were conducted with GARLI and the tree with highest likelihood was subsequently used as input for further optimization in both GARLI and PAUP, since both programs calculate the same likelihood score for a tree under the same model. The topology used for calculating the MPRs was the fully resolved consensus of 100 bootstrap replicates with GARLI.

2.4 Estimation of evolutionary rates and dates

Seven different datasets were used to estimate the evolutionary rate (μ, units are nucleotide substitutions per site per year; subs/site/year), and the time of the most recent common ancestor (T_{mrca}, years) of the viruses sampled. First, we used the 200 E gene sequences to estimates the overall rate and *T*mrca for DENV-3. The age of relevant internal nodes, such as those corresponding to the MRCA of distinct genotypes, were also estimated by setting up specific taxon subsets within the global DENV-3 dataset. Second, we analyzed the GI, GII, and GIII datasets to obtain rates and divergence times for individual genotypes separately. Third, separate analyses were also performed on the viruses collected from Indonesia (GI-ID dataset), Thailand (GII-TH dataset), and the Americas (GIII-AM dataset) to estimate rates and divergence times for DENV-3 in these specific localities. Evolutionary parameters were estimated by using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST v1.4.7 (Drummond et al., 2002; Drummond and Rambaut, 2006). Analyses were carried out with a Bayesian Skyline coalescent tree prior (Drummond et al., 2005) under the GTR+ Γ+I model, and using both a strict and a relaxed (uncorrelated Lognormal) (Drummond et al., 2006) molecular clock. MCMC chains were run for 1-3 x 10^7 generations for each data set, with a burn-in of 1-3 x

10^6. BEAST outputs were inspected with TRACER v1.4, with uncertainty in parameter estimates reflected by their 95% Highest Posterior Density (HPD) values. All parameters estimates showed ESS values >100. Molecular clock models were compared by calculating the Bayes Factor (BF) (Suchard et al., 2001) from the posterior output of each of the models using TRACER v1.4. as explained in BEAST website (http://beast.bio.ed.ac.uk/Model_comparison). A log BF (natural log units) >2.3 indicates strong evidence against the null model.

3. Results

3.1 Phylogeography of DENV-3

Our phylogenetic analysis of 200 DENV-3 E gene sequences recovered the five genotypes previously described for this serotype (Lanciotti et al., 1994; Wittke et al., 2002). It also suggested the existence of a strong geographical subdivision of DENV-3 population with no evidences of significant co-circulation of distinct genotypes in a single locality (Fig. 1). Genotype I contained most of the Indonesian strains from 1973 to 2004, along with two early Malaysian isolates (1974 and 1981), one Thai isolate (1988), and three well-supported monophyletic groups of strains isolated in South Pacific (1989-1994), Philippines (1997-2005), and East Timor (2005). Genotype II included almost all DENV-3 strains isolated in Thailand between 1973 and 2002, along with two Myanmar strains (1988 and 2005), one isolate from Singapore (1995), one isolate from Indonesia (1998), and three strongly-supported groups of strains from Malaysia (1992-1994), Bangladesh (2000-2002), and Vietnam (1996-2005). Genotype III was composed by Sri Lankan isolates from 1981 to 2000, along with single isolates from India (1984), Samoa (1986), Somalia (1993), Japan (2000), Singapore (2004), and Taiwan (2006). Moreover, all American strains sampled after 1994 were monophyletic, suggestive of a single introduction of this genotype into the continent, consistent with previous studies (Aquino et al., 2006; Diaz et al., 2006; Messer et al., 2002; Peyrefitte et al., 2005; Rodriguez-Roche et al., 2005). The American cluster was further subdivided into four well-supported lineages containing isolates from: 1) Mexico, 2) Venezuela, 3) Cuba/Martinique/Brazil/Paraguay/Bolivia, and 4) Cuba/Peru/Ecuador. Genotype IV was the most divergent group and included three early Puerto Rican strains from 1960s and 1970s. Genotype V was represented by the oldest prototype strain Philippines/1956, and two Asian isolates from 1973 and 1980. Finally, one isolate sampled from Thailand in 1962 did not fall into any established genotype and was located toward the common ancestral node of GII and GIII.

3.2 Migration patterns of DENV-3 genotypes

Before reconstructing the MPRs along the trees for each genotype we tested for panmixis by calculating with MacClade the expected number of changes along 25000 equiprobable trees and again over other 25000 trees obtained by random partition. The observed number of character state changes for each DENV-3 genotype was much lower than expected by chance (P <0.00004 for all analyses). By increasing the number of random trees we could never sample the observed values in the random distribution (data not shown). Therefore, the DENV-3 genotypes are under high constraints for geographical gene flow and the reconstruction of the observed character state changes along the trees was justified.

Because the viral-based tree did not use geographical information at the inference step, it was used for the reconstruction of the migratory history of samples. Sub-trees for each of the three main DENV-3 genotypes, derived from the global MAP tree obtained with BI, had congruent topologies and supported the same sets of MPRs for each genoptype. All the most parsimonious states at each node are shown in Figure 2. Nine steps were required to trace the MPRs for GI (Fig. 2a). The character tracings were unequivocal and had a single MPR for all nodes, irrespective of the resolving option. The node connecting it to the global MAP tree had Indonesia as point of radiation, since it was the ancestral state at the root. From there, independent lineages of the virus spread to the French Polynesia, reaching Fiji and Tahiti; to the Philippines, also reaching Taiwan; and more recently to the East Timor. Three distinct lineages related to Indonesia were also found in Malaysia and in Thailand.

Seven steps were required to trace the MPRs for GII (Fig. 2b). The rooting position in the MAP tree and the ancestral state for GII unambiguously suggested an origin in Thailand (T). From there, the virus appeared to have gone into Myanmar (M) and Bangladesh (B). The ancestral state at that node in the tree is ambiguous, including T, B, or M. Using DELTRAN, the state would resolve into T indicating that the virus went into the two places at different occasions. However, ACCTRAN would result on either B or M, indicating that the virus may have moved serially following either T>B>M, T>M>B (*i.e.*, stepping-stone model) or alternatively, B<T>B. Likewise, the independent movement of the GII into Singapore (S) and Malaysia (Y) from Thailand (T) had an ambiguous set of MPRs, including T, S, or Y. DELTRAN suggested Thailand broadcasting to both places and ACCTRAN indicated a stepping-stone process T>Y>S, T>S>Y, or simultaneously from Thailand Y<T>S.

GIII required the highest number of reconstruction steps (23 on average, Fig. 2c). Moreover, its rooting and internal MPRs were problematic. The MAP tree obtained with BI indicated a strain from American Samoa isolated in 1986 at the root of the genotype, but older sequences from 1981-1985 were sampled in Sri Lanka. Both rooting options did not alter either the cost or the character transformations (and therefore implied migration events) across the tree, but rooting at American Samoa (as shown in Fig. 2c) leaves the state at the root of the tree undefined. In any case, the virus apparently spread from Sri Lanka into nearby India and more recently into Japan, Singapore and Taiwan. An interesting event, which epitomized the cosmopolitan nature of GIII, was its sampling in Somalia in 1993 making the elucidation of the entry into the Americas problematic. DELTRAN suggested that the virus was broadcasted into Africa and the Americas from Sri Lanka. ACCTRAN pointed to a scenario in which the virus may have gone from Sri Lanka into Africa, and then into the Americas.

The spread of GIII into the Americas appears to have had Mexico as hub, since the data at hand allowed the unequivocal reconstruction of the ancestral state into the Americas there. Moreover the data resolved well the movement of the virus from México into Venezuela and certainly, two more independent entries into South America that had several possible routes. DELTRAN resolved additional entries into South America via Mexico passing the virus via two routes. The first route went into the Pacific side of the Andes hitting Ecuador and Peru, and the second one went via the Caribbean, with Martinique passing the virus into Brazil and from there into Bolivia and Paraguay. ACCTRAN did not resolve if Mexico or Martinique, was the origin of the virus that went into the Pacific side of South America. However, there was no issue with the reconstruction of the entry into Brazil from Martinique. Moreover, both DELTRAN and ACCTRAN were unanimous in that a virus coming from Pacific side of the Andes moved back from South America into Cuba.

3.3 Estimation of evolutionary rates and dates

The phylogenetic tree of globally sampled DENV-3 isolates was characterized by a clear temporal structure, with the oldest sampled viruses tending to fall closest to the root of the tree, while those sampled more recently were located at the most distal tips (Fig. 1). This temporal structure allowed us to estimates the rate of molecular evolution and the T_{mrca} for different DENV-3 datasets. The substitution rate was first estimated for the complete DENV3 data set of 200 sequences, using both strict and relaxed molecular clock models. The BF analysis clearly favored a relaxed molecular clock model over a strict clock model (Table 2), indicating

detectable variation in evolutionary rates among branches. The coefficient of rate variation was estimated at 0.28 (95% HPD, 0.15-0.41). Despite this variation, the median evolutionary rate (E gene) and T_{mrca} of DENV-3 estimated under both strict (μ = 8.7 x 10^{-4} subs./site/yr; T_{mrca} = 1891) and relaxed (μ = 8.9 x 10^{-4} subs./site/yr; T_{mrca} = 1893) molecular clock models were very close (Table 3). This result was also similar to that previously obtained by Twiddy et al. (μ = 9.0 x 10^{-4} subs./site/yr, T_{mrca} ~1900) using a much smaller data set of DENV-3 sequences (n = 21) (Twiddy et al., 2003). By setting up specific taxon sub-groups within the global DENV-3 phylogeny, we also estimated the age of the major genotypes resulting in a median T_{mrca} estimate of 1967 for GI and GII, and 1975 for GIII, under either strict or relaxed molecular clock models (Table 3).

Table 2. Bayes Factors between different molecular clock models for DENV-3.

Dataset	Model comparison	log BF[1]	Evidence against H_0[2]
DENV-3	Strict (H_0) vs Relaxed (H_1) clock	22.8 (0.4)	Decisive
GI	Strict (H_0) vs Relaxed (H_1) clock	-0.8 (0.4)	Negative
GII	Strict (H_0) vs Relaxed (H_1) clock	5.0 (0.5)	Decisive
GIII	Strict (H_0) vs Relaxed (H_1) clock	10.8 (0.5)	Decisive
GI-ID	Strict (H_0) vs Relaxed (H_1) clock	-0.4 (0.3)	Negative
GII-TH	Strict (H_0) vs Relaxed (H_1) clock	1.6 (0.4)	Weak
GIII-AM	Strict (H_0) vs Relaxed (H_1) clock	12.8 (0.4)	Decisive

[1]log BF (Bayes Factor) is the difference (in natural log units) of the marginal likelihood of null (H_0) and alternative (H_1) model. The standard error of the estimates is given in parenthesis. [2] Evidence against H_0 is assessed in the following way: ln BF <0 indicates no evidence against the null model; ln BF between 0-2.3 indicates weak evidence against the null model, ln BF between 2.3-3.4 indicates strong evidence against the null model; ln BF between 3.4-4.6 indicates very strong evidence against the null model; ln BF >4.6 indicates decisive evidence against the null model.

The detected variation in evolutionary rates among DENV-3 lineages could reflect rate differences among distinct genotypes (Twiddy et al., 2003), and/or rate heterogeneity within a

single genotype. To test these hypotheses, we analyzed each genotype separately. The BF analysis showed that the relaxed clock method was favored over the strict clock method in the GII and GIII data sets (Table 2), indicating significant rate heterogeneity within these genotypes. The coefficient of rate variation for GII and GIII was estimated at 0.25 (95% HPD, 0.01-0.44) and 0.37 (95% HPD, 0.13-0.61), respectively. On the other hand, the median evolutionary rate estimates of the distinct genotypes were very similar and displayed a considerable overlap of HPD intervals (Table 3), clearly suggesting that there are no major differences in evolution rate among main DENV-3 genotypes. Accordingly, the median T_{mrca} estimated for each genotype was almost equal to that previously obtained using the complete DENV-3 data set (Table 3).

Table 3. Estimated substitutions rates and dates for DENV-3 genotypes.

Dataset	Molecular clock	μ	*T*mrca DENV-3	*T*mrca GI	*T*mrca GII	*T*mrca GIII
DENV-3	Strict	$8.7x10^{-4}$ ($7.7x10^{-4}$-$9.7x10^{-4}$)	1891 (1876-1904)	1967 (1963-1970)	1967 (1965-1970)	1975 (1972-1978)
	Relaxed	$8.9x10^{-4}$ ($7.9x10^{-4}$-$10.0x10^{-4}$)	1893 (1865-1918)	1967 (1963-1970)	1967 (1964-1970)	1975 (1972-1978)
GI	Strict	$8.3x10^{-4}$ ($6.8x10^{-4}$-$9.8x10^{-4}$)	-	1966 (1961-1970)	-	-
	Relaxed	$8.4x10^{-4}$ ($6.9x10^{-4}$-$10.1x10^{-4}$)	-	1966 (1961-1970)	-	-
GII	Strict	$10.1x10^{-4}$ ($8.7x10^{-4}$-$11.7x10^{-4}$)	-	-	1969 (1966-1971)	-
	Relaxed	$10.3x10^{-4}$ ($8.7x10^{-4}$-$12.0x10^{-4}$)	-	-	1969 (1966-1971)	-
GIII	Strict	$8.1x10^{-4}$ ($6.6x10^{-4}$-$9.7x10^{-4}$)	-	-	-	1974 (1969-1978)
	Relaxed	$8.2x10^{-4}$ ($6.6x10^{-4}$-$9.9x10^{-4}$)	-	-	-	1974 (1967-1979)

Estimates of the median evolutionary rate (μ, substitutions $site^{-1}$ $year^{-1}$) and, median time for the most recent common ancestor (*T*mrca, year). The age estimated for some relevant internal nodes within the global DENV-3 tree (corresponding to the *T*mrca of major genotypes) are also shown. 95% HPD intervals are shown between parentheses.

It has been suggested that under epidemic conditions, such as when a new variant is introduced into a susceptible population, the mean viral evolutionary rate could be higher than under endemic conditions (Twiddy et al., 2003). To test this hypothesis, we compared the

DENV-3 in Indonesia and Thailand, where GI and GII have been evolving since the early 1970s, with DENV-3 lineages from the Americas, where GIII only emerged in the early 1990s. Significant rate heterogeneity was detected within GIII American lineages (Table 2). The median evolutionary rate of DENV-3 lineages circulating in different regions, however, was very similar and displayed a considerable overlap of HPD intervals (Table 4), indicating no major differences in rates of DENV-3 among regions with endemic or epidemic patterns of dengue transmission. The median T_{mrca} estimated for GI-ID and GII-TH datasets were close to that previously estimated using all GI and GII sequences (Table 4), supporting the notion that Indonesia and Thailand are the epicenters for these DENV-3 lineages; whereas the median T_{mrca} for the GIII-American clade was estimated around 1991 (Table 4).

Table 4. Estimated substitutions rates and dates for DENV-3 in Indonesia, Thailand and the Americas.

DENV	Genotype	Region	μ	*T*mrca
3	I	Indonesia	$8.4x10^{-4}$ ($6.2x10^{-4}$-$10.7x10^{-4}$)	1970 (1966-1973)
	II	Thailand	$10.0x10^{-4}$ ($8.2x10^{-4}$-$11.8x10^{-4}$)	1969 (1966-1971)
	III	America	$9.2x10^{-4}$ ($6.9x10^{-4}$-$11.5x10^{-4}$)	1991 (1987-1993)
2	Asian I[a]	Thailand	$8.5x10^{-4}$ ($7.2x10^{-4}$-$9.9x10^{-4}$)	-
	Asian-American[b]	America	$8.0x10^{-4}$ ($6.6x10^{-4}$-$9.5x10^{-4}$)	-
4	I[c]	Thailand	$10.7x10^{-4}$ ($8.4x10^{-4}$-$13.1x10^{-4}$)	-
	II[b]	America	$8.3x10^{-4}$ ($6.8x10^{-4}$-$10.0x10^{-4}$)	-

Estimates of the median evolutionary rate (μ, substitutions site^{-1} year^{-1}), and median time of the most recent common ancestor (*T*mrca, year) for the GI-ID (strict molecular clock), GII-TH (relaxed molecular clock), and GIII-AM (relaxed molecular clock) datasets. 95% HPD intervals are shown between parentheses. [a] Data taken from Zhang et al. (2006). [b] Data taken from Carrington et al. (2005). [c] Data taken from Klungthon et al. (2004).

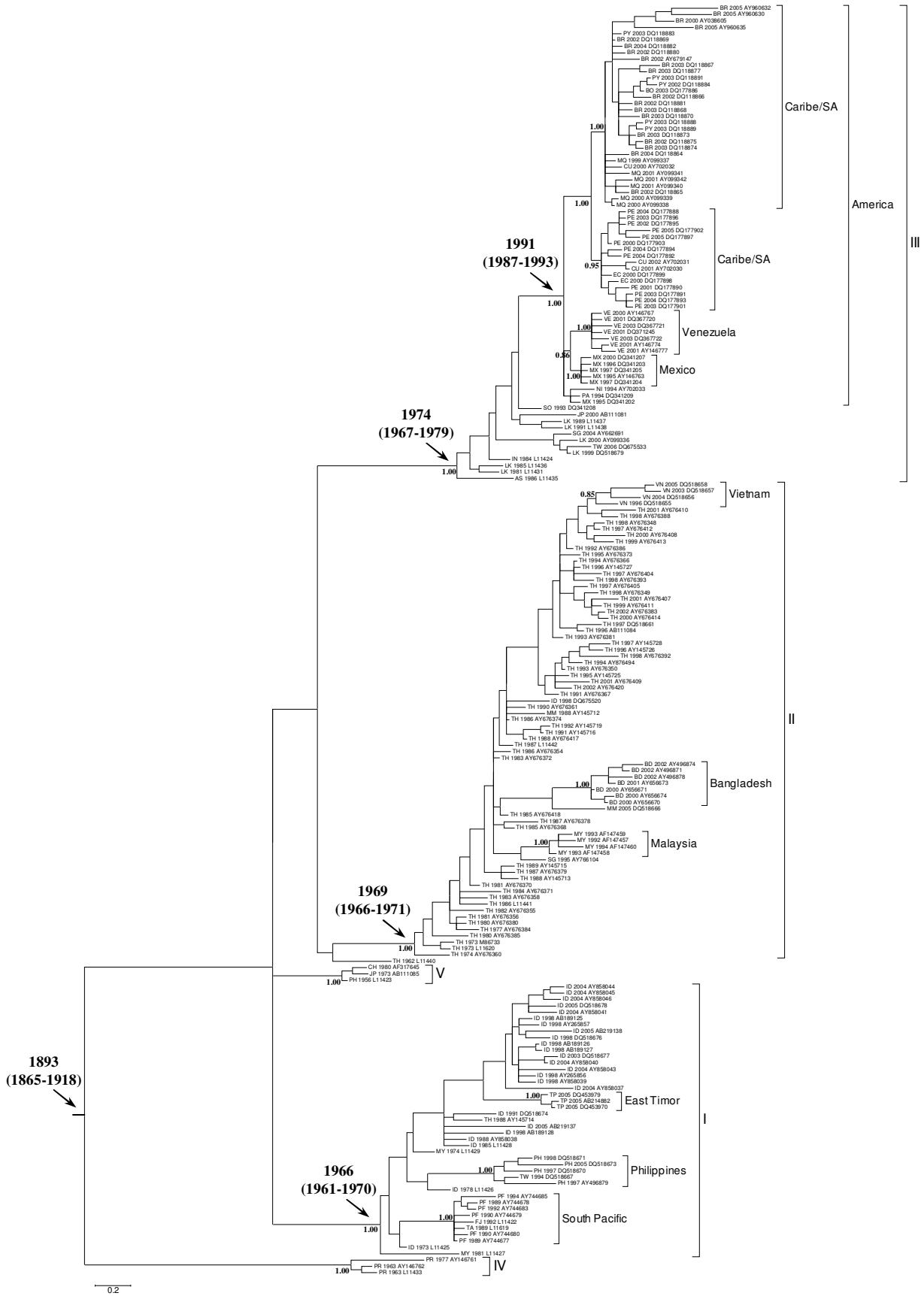
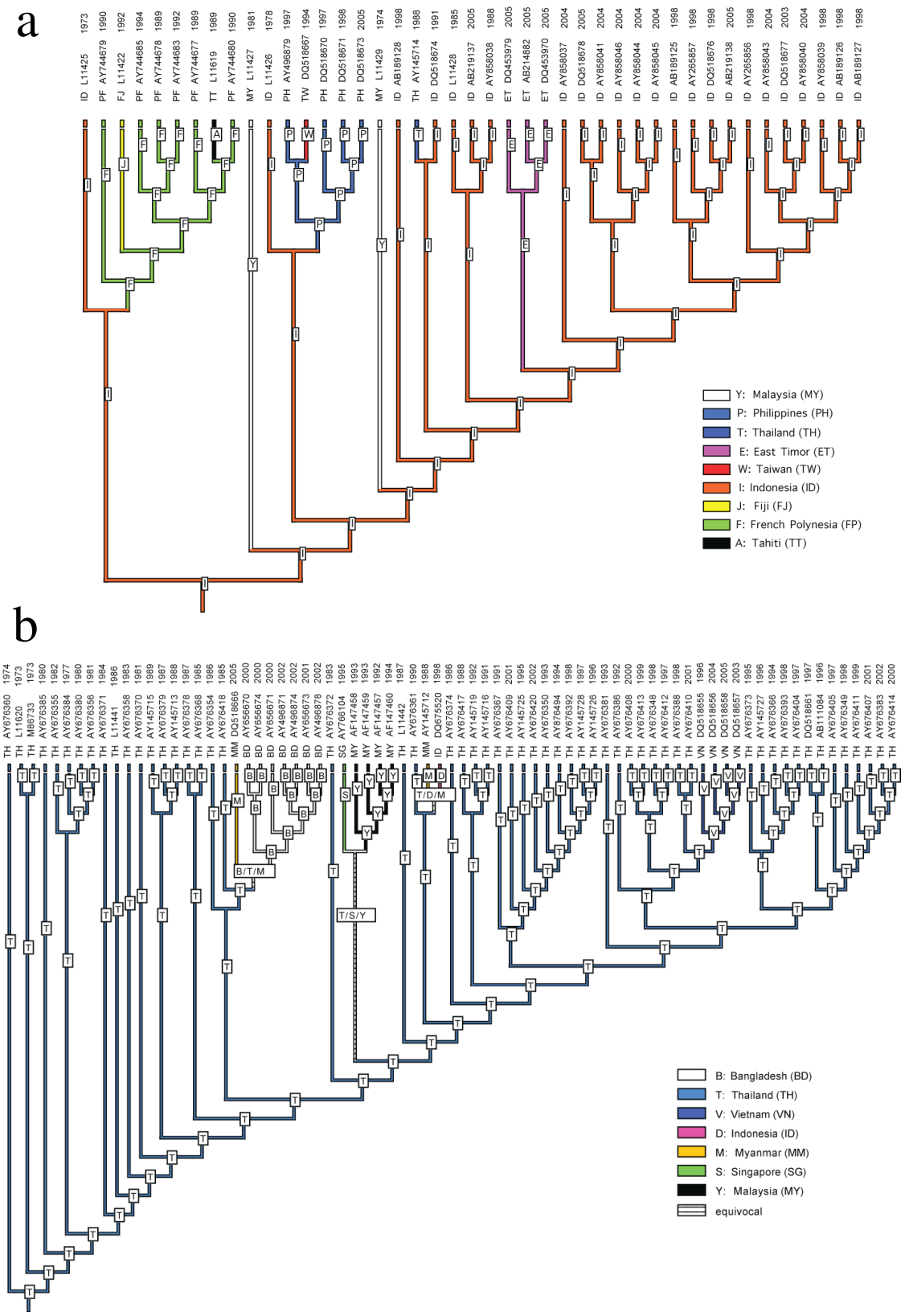

1893
(1865-1918)
1966
(1961-1970)
1969
(1966-1971)
1974
(1967-1979)
1991
(1987-1993)
Caribe/SA
Caribe/SA
America
III
Venezuela
Mexico
Vietnam
II
Bangladesh
Malaysia
V
East Timor
I
Philippines
South Pacific
IV
0.2

Figure 1. Majority-rule Bayesian consensus tree of 200 E gene sequences representing the global diversity of DENV-3. Genotypes (roman numerals) and country-specific clades are indicated. Estimates for the age of some relevant nodes on the tree (point to by arrows) are also highlighted. Posterior probabilities are shown for key nodes. The names of DENV-3 isolates include reference to country origin, year of isolation, and GenBank accession number. Country represented are American Samoa (AS), Bangladesh (BD), Bolivia (BO), Brazil (BR), China (CH), Cuba (CU), Ecuador (EC), Fiji (FJ), Indonesia (ID), India (IN), Japan (JP), Sri Lanka (LK), Myanmar (MM), Martinique (MQ), Mexico (MX), Malaysia (MY), Nicaragua (NI), Panama (PA), Peru (PE), French Polynesia (PF), Philippines (PH), Puerto Rico (PR), Paraguay (PY), Singapore (SG), Somalia (SO), Tahiti (TA), Thailand (TH), East Timor (TP), Taiwan (TW), Venezuela (VE), Vietnam (VN). The tree is rooted using GIV, which have been shown to represent an appropriate outgroup. Horizontal branch lengths are drawn to scale with the bar at the bottom indicating 0.2 nucleotide substitutions per site.


a
Y: Malaysia (MY)
P: Philippines (PH)
T: Thailand (TH)
E: East Timor (ET)
W: Taiwan (TW)
I: Indonesia (ID)
J: Fiji (FJ)
F: French Polynesia (FP)
A: Tahiti (TT)
b
B/T/M
T/S/Y
T/D/M
B: Bangladesh (BD)
T: Thailand (TH)
V: Vietnam (VN)
D: Indonesia (ID)
M: Myanmar (MM)
S: Singapore (SG)
Y: Malaysia (MY)
equivocal

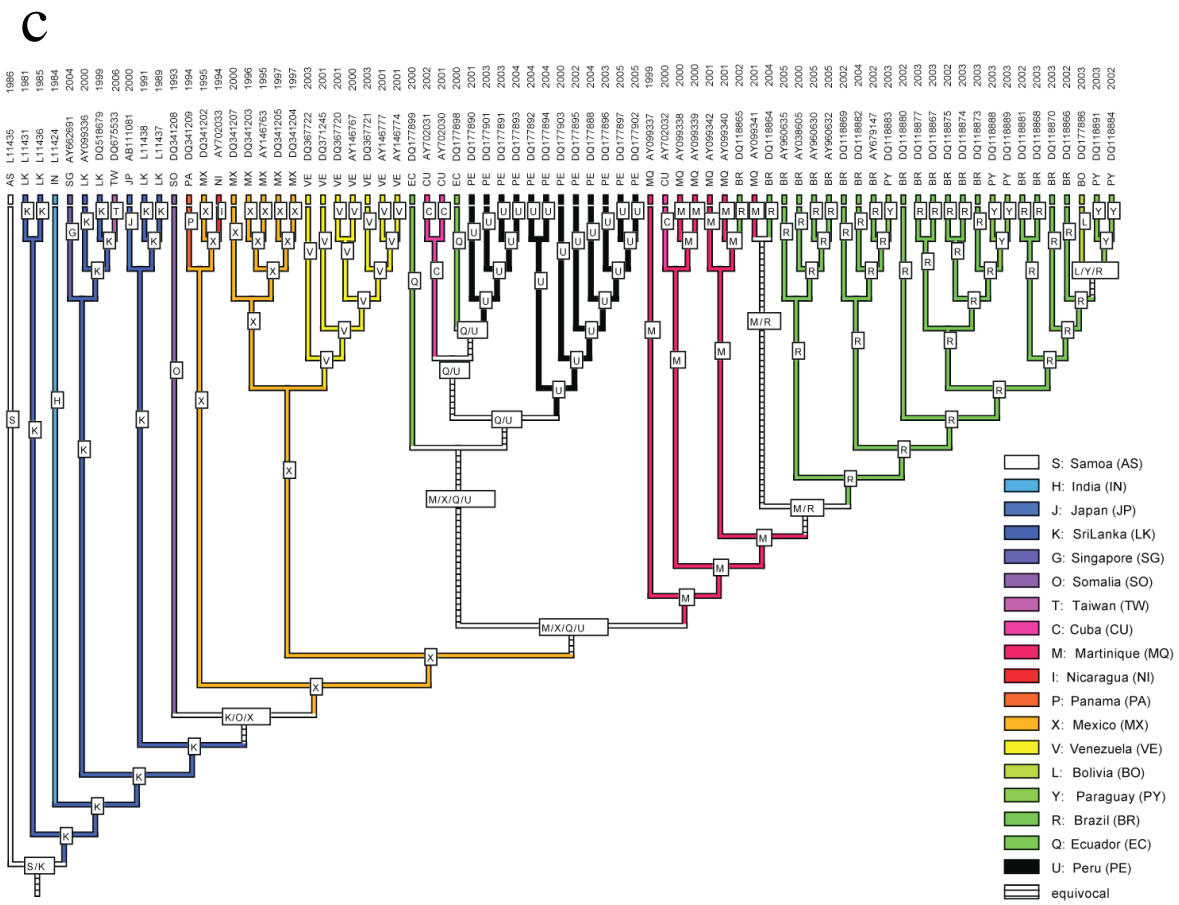


Figure 2. Migration patterns of genotypes I (a), II (b), and III (c). The names of DENV-3 isolates include reference to country origin, GenBank accession number, and year of isolation. The color of each branch represents the country of origin of the sequence corresponding to that branch, according to the figure legend.

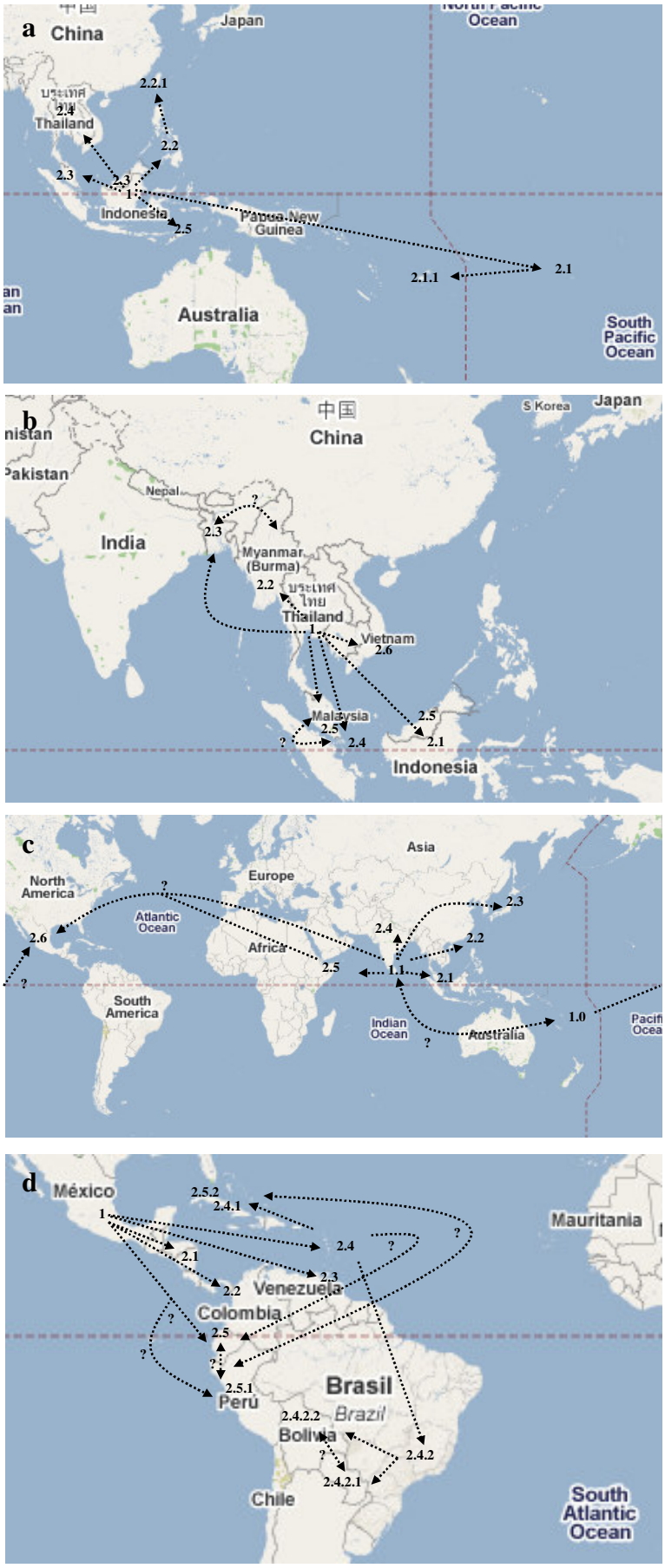

a
China
Japan
North Pacific Ocean
Thailand
2.2.1
2.4
2.2
2.3
2.3
1
Indonesia
2.5
Papua New Guinea
2.1.1
2.1
Australia
South Pacific Ocean
b
中国
China
S Korea
Japan
Pakistan
Nepal
?
2.3
India
Myanmar (Burma)
2.2
Thailand
1
Vietnam
2.6
Malaysia
2.5
2.5
?
2.4
2.1
Indonesia
c
Asia
North America
?
Europe
Atlantic Ocean
2.3
2.4
2.6
Africa
2.2
2.5
1.1
2.1
?
South America
Indian Ocean
Australia
1.0
?
d
México
2.5.2
2.4.1
1
Mauritania
?
2.4
?
2.1
2.3
2.2
Venezuela
Colombia
?
2.5
?
?
2.5.1
Peru
Brasil
Brazil
2.4.2.2
Bolivia
?
2.4.2
2.4.2.1
Chile
South Atlantic Ocean

Figure 3. Plausible sites of origin and migration routes of DENV-3 genotypes I (a), II (b), III (c) and III-AM (d). (a): 1) Indonesia, 2.1) French Polynesia/Tahiti, 2.1.1) Fiji, 2.2) Philippines, 2.2.1) Taiwan, 2.3) Malaysia, 2.4) Thailand, 2.5) East Timor. (b): 1) Thailand, 2.1) Indonesia, 2.2) Myanmar, 2.3) Bangladesh, 2.4) Singapore, 2.5) Malaysia, 2.6) Vietnam. (c): 1.0) Samoa, 1.1) Sri Lanka, 2.1) Singapore, 2.2) Taiwan, 2.3) Japan, 2.4) India, 2.5) Somalia, 2.6) Mexico. (d): 1) Mexico, 2.1) Nicaragua, 2.2) Panama, 2.3) Venezuela, 2.4) Martinica, 2.4.1) Cuba (2000), 2.4.2) Brazil, 2.4.2.1) Paraguay, 2.4.2.2) Bolivia, 2.5) Ecuador, 2.5.1) Peru, 2.5.2) Cuba (2001/2002). Inconclusive routes of DENV-3 were identified with the signal "?".

4. Discussion

This study represents the largest phylogeographic and evolutionary analysis reported for DENV-3 to date. Our phylogenetic analysis of 200 DENV-3 E sequences with world-wide distribution revealed a clear geographical subdivision of viral strains. Genotypes I, II, and III have been evolving independently in Indonesia, Thailand, and Sri Lanka, respectively, over the last 30-40 years. Our data supports the notion that these countries not only sustain the oldest DENV-3 epidemics but also were sources for dengue lineages that have subsequently spread over the world. Strains more recently isolated in South Pacific (1989-1994), Philippines (1997-2005), East Timor (2005), Malaysia (1992-1994), Bangladesh (2000-2002), Vietnam (1996-2005), and the Americas (1994-2006) segregated into distinct monophyletic clusters within the main genotypes; indicating that each country formed a geographically distinct mostly self-contained region with regard to DENV-3 viruses, with few instances of repeated gene flow among regions.

The plausible routes of DENV-3 migration are described in Fig. 3. According to our analyses the spread of GI was mainly circumspect to the maritime portion of Southeast-Asia (East Timor, Malaysia, and Philippines) and South Pacific, where most migrant strains appeared to have been broadcasted from Indonesia. By contrast, most GII strains appeared to have been broadcasted from Thailand and stayed within continental areas in South-East Asia (Bangladesh, Myanmar, Singapore, and Vietnam), with the exception of Malaysia. GIII was the most widely spread of all DENV-3 genotypes, and most GIII strains found in Asia, East Africa and the Americas appeared to have been transmitted from or near from Sri Lanka. It is unclear if the American GIII lineage came from Africa or Asia. The oldest GIII sequences in the Americas were identified in Panama and Nicaragua in 1994 (CDC, 1995; Guzman et al., 1996), but our migration data suggested that the GIII was introduced into the Americas through Mexico where the first GIII strains were identified in 1995 (Briseno-Garcia et al., 1996). More sampling in those

countries will be necessary to elucidate the precise point of introduction of GIII to the Americas. In any case, GIII viruses rapidly spread to others countries in the region (Nogueira et al., 2001; Peyrefitte et al., 2003; Rigau-Perez et al., 2002; Usuku et al., 2001; Uzcategui et al., 2003), using several independent routes from Central America to the Caribbean and South America.

Our phylogeographic analysis also revealed that the co-circulation of different DENV-3 genotypes in a single location is a rare event. This could result from a limited gene flow among distantly geographic regions. However, genotype differences were observed even between neighboring countries like Indonesia and Thailand, where DENV-3 epidemics have been consistently dominated by GI and GII, respectively, since the 1970s. Notwithstanding, there is evidence of incursions into Indonesia by "Thai-like" GII strains in 1988 (represented by strain DQ675520) (Raekiansyah et al., 2005), as well as into Thailand by "Indonesian-like" GI strains in 1998 (represented by strain AY145714) (Wittke et al., 2002). Nevertheless, these incursions seem to have failed to become established, since none of the later Indonesian and Thai isolates grouped within GII and GI, respectively. These facts suggested that several factors, other than geographic proximity, could have had a significant impact on the observed spatial dispersion patterns of DENV-3.

It is hard to envisage a general vicariance mechanism that would explain a lack of genotype overlapping at the same geographic locality. Possibly competition among genotypes, and/or regional differences in mosquito vector competence for each genotype (Anderson and Rico-Hesse, 2006; Armstrong and Rico-Hesse, 2001; Cologna et al., 2005) may be involved. Another possible explanation for the observed patterns could involve viral neutralization by cross-immunity among closely related strains caused by a pre-exposed human population. This would allow for distinct serotype co-circulation but make it difficult for intra-serotype (*i.e.*, genotype) co-circulation, due to a reduction in numbers of the available susceptible human hosts to levels below that necessary to sustain significant epidemics (Adams et al., 2006). This would help explain why the evolution of DENV-3 is characterized by phylogenetic trees with a strong temporal structure as previously noted for this and other DENV serotypes (Goncalvez et al., 2002; Klungthong et al., 2004; Twiddy et al., 2003; Wittke et al., 2002; Zhang et al., 2005; Zhang et al., 2006), which may indicate the strong pruning effect of DENV lineages by host immunity. Finally, we can't also exclude the possibility that GI and GII are circulating as minor variants in Thailand and Indonesia, respectively, but have remained undetectable because the low number of sequences analyzed (sampling bias).

Our analyses of rates of nucleotide substitution in DENV-3 revealed that the relaxed clock model outperforms the strict clock model, indicating detectable variation in evolutionary rates among DENV-3 lineages. Significant rate variation among genotypes was described previously for DENV-2 (Twiddy et al., 2003), suggesting that rate variation detected in DENV-3 could be also consequence of differences among genotypes. However, the analysis of each DENV-3 genotype separately revealed that rate heterogeneity in DENV-3 can be mainly explained by rate differences within genotypes, particularly within GII and GIII, rather than among genotypes. The median evolutionary rate of the distinct DENV-3 genotypes analyzed was very similar (ranging from 8.3×10^{-4} subs./site/yr to 10.3×10^{-4} subs./site/yr) and displayed a considerable overlap of the HPD intervals, clearly suggesting no major differences in evolution rate among genotypes. It is also interesting to note that although the relaxed clock model outperforms the strict clock model in most DENV-3 datasets analyzed, the median value of estimates obtained under both relaxed and strict clock models were very close in all analyses. This is fully consistent with the concept that the substitution rate estimated from large data sets are reliable indicators of the average rate of evolution, even if rate heterogeneity is present (Jenkins et al., 2002).

A previous study suggested that DENV-3 is evolving at a rate ($\mu = 9.0 \times 10^{-4}$ [7.3×10^{-4} – 10.8×10^{-4}] subs./site/yr) significantly faster than other DENV serotypes; and proposed similar rates of substitutions for each DENV-3 genotype (GI = 7.5×10^{-4} [4.5×10^{-4} – 10.7×10^{-4}] subs./site/yr; GIII = 11.6×10^{-4} [7.8×10^{-4} – 15.9×10^{-4}] subs./site/yr) (Twiddy et al., 2003). Those estimates, however, were based on the analysis of very small DENV-3 datasets (total = 21, GI = 8, GIII = 8), and no estimations of the substitution rate for GII were provided. We re-estimated these evolutionary rates using much larger data sets of DENV-3 sequences (total = 200, GI = 43, GII = 75, GIII = 75). Our median rate estimates for DENV-3 (8.9×10^{-4} subs./site/yr) and GI (8.3×10^{-4} subs./site/yr) were similar to those previously reported by Twiddy et al. whereas the median rate estimated for GIII (8.2×10^{-4} subs./site/yr) was considerably lower, although within the large confidence interval of the previous estimate. The median evolutionary rate estimated for GII was 10.3×10^{-4} subs./site/yr. Overall, the confidence intervals of our estimates (6.6×10^{-4} – 12×10^{-4}) were significantly narrower than those described by Twiddy et al. (4.5×10^{-4} – 15.9×10^{-4}), probably due to the higher number of sequences used in the present study.

It has been suggested that the ecological conditions for DENV dissemination may alter the viral evolutionary rate, which could explain some rate differences among dengue lineages previously described (Twiddy et al., 2003). Our analyses revealed, however, that the median

evolutionary rate of GI in Indonesia and GII in Thailand (areas with long-term endemic infections), was similar to that observed for GIII in the Americas (a region with a recent epidemic pattern of DENV-3 transmission). Our DENV-3 rate estimates were also comparable to those estimated for DENV-2 (genotype Asian I) and DENV-4 (genotype I) circulating in Thailand (Klungthong et al., 2004; Zhang et al., 2006), and for DENV-2 (genotype Asian-American) and DENV-4 (genotype II) circulating in the Americas (Carrington et al., 2005) (Table 4). These studies confirmed a lack of association between dengue substitution rate and ecological pattern of virus spread, and revealed no major lineage-specific rate differences among DENV-2, DENV-3, and DENV-4. Whether lineage-specific rate differences in DENV evolution previously described (Twiddy et al., 2003) really exist, or simply reflects a previous use of much smaller datasets needs further investigation.

We estimated the T_{mrca} of DENV-3 at around 1890, fully consistent with previous estimation (T_{mrca} ~ 1900) (Twiddy et al., 2003). Our analysis also suggested that the current global genetic diversity of genotypes I, II, and III arose almost simultaneously within a short time period between the middle 1960s and the middle 1970s, coinciding with the description of the first cases of DHF by DENV-3 in Asia (Gubler et al., 1979; Nisalak et al., 2003; Sumarmo, 1987; Wallace et al., 1980) and the rapid increase in human population size, urbanization, and human movement. According with our estimations, GIII strains were probably introduced into Latin America around 1991, few years earlier than the initial detection of this genotype in the continent in 1994 (CDC, 1995; Guzman et al., 1996). Similar time intervals of few years between the estimated introduction and initial detection were also described for DENV-2 and DENV-4 in the Americas (Carrington et al., 2005).

In conclusion, this study proposes that global DENV-3 evolution could be well characterized as a collection of discrete, country-specific viral population bursts, with limited co-circulation of distinct genotypes in a single region. Despite this strong spatial subdivision, DENV-3 strains of distinct genotypes and from different localities have been evolving at roughly the same rate over time. Whether such similar evolutionary rate estimates translate into comparable biological properties (such as transmissibility, infectiousness, and/or virulence) across distinct DENV-3 lineages is still unclear. Our data also suggested that the current diversity of the three main DENV-3 genotypes arose within the last 30-40 years, coinciding with the emergence of large-scale DHF/DSS epidemics in Asia.

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Artigo 4: Estudo filogenético dos DENV-3 circulantes no Brasil.

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Situação do manuscrito: Aceito para publicação no periódico "Memórias do Instituto Oswaldo Cruz". **Este artigo atende ao objetivo 4.**

Apresentação: No Brasil, o DENV-3 foi isolado pela primeira vez em dezembro de 2000 a partir de um caso autóctone de dengue clássico no Município de Nova Iguaçu, Rio de Janeiro, causando grandes epidemias em 2001 e 2002 (Nogueira *et al.,* 2001; 2005). O DENV-3 circulante na América Latina parece ter sido proveniente do Sri Lanka, Índia e Leste da África, por volta de 1991 (1987-1993) (Araújo *et al.,* 2009a). A circulação de um novo genótipo (G) de DENV-3 foi recentemente descrito no Brasil (Figueiredo *et al.,* 2008, Nogueira *et al.,* 2008) e Colômbia (Usme-Ciro *et al.,* 2008). Este novo genótipo foi classificado como GI por Figueiredo *et al.* e Usme-Ciro *et al.*, mas como GV por Nogueira *et al*. Isto claramente indica que uma padronização internacional da nomenclatura de genótipos de DENV-3 é necessária. Por estas razões, nós examinamos a atual classificação filogenética dos DENV-3, com ênfase no novo genótipo descrito no Brasil.

Title: Dengue Virus Type 3 in Brazil: A Phylogenetic Perspective.

Running title: DENV-3 in Brazil.

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Abstract

Circulation of a new DENV-3 genotype was recently described in Brazil and Colombia, but the precise classification has been controversial. Phylogenetic and nucleotide distance analyses of the envelope (E) gene support the subdivision of DENV-3 strains into five distinct genotypes (GI to GV), confirming the classification of this new genotype in South America as GV. The extremely low genetic distances of Brazilian GV strains to the prototype Philippines/L11423 strain isolated in the 1956 GV sample raise important questions regarding the origin of this genotype in South America.

Keywords: Dengue virus type 3; Genotypes; Brazil.

Dengue viruses (DENV) 1 to 4 are members of the *Flavivirus* genus of the *Flaviviridae* family and are responsible for the currently most important arthropod vector transmitted human viral disease worldwide in terms of morbidity and mortality, consequently emerging as a major problem in tropical and subtropical areas (Rosen 1999). The ~11 kb in length genomic RNA is a single strand of positive polarity constituted by a single open reading frame (ORF) flanked by an untranslated region (UTR) in the 5'and 3' termini. The ORF codes for three structural proteins (capsid "C", membrane "prM/M" and envelope "E") and seven non structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), resulting from the cleavage of a single polyprotein of 3386-3433 amino acids (Chambers et al. 1990, Lindebach & Rice 2001).

The phylogenetic analysis of the pre-membrane/membrane (prM/M) and envelope (E) genes of the geographically and temporally distinct dengue virus type 3 (DENV-3) led to the initial identification of four distinct monophylogenetic clusters called genotypes. A subsequent phylogenetic analysis including a larger number of DENV-3 strains demonstrated that genotype I of Lanciotti et al. (1994) encompasses two distinct groups of viruses that were classified separately, constituting a new genotype V. Genotypes I to III (GI to GIII) are responsible for most DENV-3 infections: GI represented by viruses from Indonesia, Malaysia, Philippines and the South Pacific; GII by viruses from Thailand, Bangladesh, Malaysia and Myanmar; and GIII by viruses from Sri Lanka, India, Samoa, Africa and the Americas (sampled after 1994) . In contrast, genotypes IV and V (GIV and GV) are only exemplified by a few older strains: GIV constituted by Puerto Rican strains from the 1960s/1970s and GV represented by the oldest GV strain isolated in the Philippines in 1956 as well as two strains isolated in Japan and China in 1973 and 1980, respectively.

The DENV-3 GIII was detected for the first time in the Americas during dengue fever/ dengue hemorrhagic fever (DF/DHF) outbreaks in Nicaragua and Panama in 1994 (Anonymous 1995). Hitherto, this virus has spread to many countries in Latin America (Briseno-Garcia et al. 1996, Guzman et al. 1998, Balmaseda et al. 1999, Isturiz et al. 2000, Nogueira et al. 2001, Usuku et al. 2001, Uzcategui et al. 2003, Aquino et al. 2006). In Brazil, DENV-3 was isolated for the first time from an autochthonous case of DF in December 2000 from the municipality of Nova Iguaçu, Rio de Janeiro, and soon afterwards, there was a large DENV-3 epidemic in Rio de Janeiro in 2001/2002 (Nogueira et al. 2001). DENV-3 isolates detected in Latin America appeared to have arisen from a single introduction of GIII strains previously circulating in Sri Lanka, India, and East Africa , probably around 1991 (1987-1993) (Araujo et al. 2008). The circulation of a new DENV-3 genotype has recently been reported in Brazil (Figueiredo et al.

2008, Nogueira et al. 2008) and Colombia (Usme-Ciro et al. 2008). This new genotype was classified as GI by Figueiredo et al. and Usme-Ciro et al., but as GV by Nogueira et al. This clearly indicates that an international standardization of DENV-3 genotype nomenclature is needed. For these reasons, we re-examined the current phylogenetic classification of DENV-3 strains, with emphasis on the new genotype described in Brazil.

We analyzed full-length (1,479 bp) and partial (822 bp) E gene sequences of 103 DENV-3 strains from 30 different countries around the world representative of all genotypes retrieved from Genbank (www.ncbi.nlm.nih.gov). Colombian sequences described by Usme-Ciro et al. (2008) were not included in this study, as only a short E gene fragment (224 bp) was available. Tree reconstructions were performed by the Neighbor-Joining (NJ) method (Tamura Nei model) in 1000 bootstrapped data sets as implemented in MEGA 4. Mean nucleotide distances within (intra-genotype) and among (inter-genotype) DENV-3 genotypes, and pairwise genetic distances among sequences were estimated adopting the Tamura Nei model in MEGA 4.

Our phylogenetic analysis of DENV-3 E gene sequences confirmed the five monophyletic groups (genotypes) previously reported for this serotype (Fig. 1). In order to confirm that GI and GV are distinct genotypes, the mean intra-genotype and inter-genotype distances at the full-length E gene were calculated. As displayed in Table 1, the mean nucleotide distance between GV and GI was significantly greater than the mean intra-genotype distances. Most important, the mean distance from GV to GI was not significantly different than the corresponding mean distances from GV to GII or GIII, corroborating the classification of GV as a new genotype distinct from GI.

Table 1. Mean nucleotide distances in E gene within (intra-genotype) and among (inter-genotype) DENV-3 genotypes.

	GI	GII	GIII	GIV	GV
GI	**3.0 ± 0.2**	-	-	-	-
GII	7.1 ± 0.6	**2.4 ± 0.2**	-	-	-
GIII	7.5 ± 0.6	7.6 ± 0.7	**1.8 ± 0.2**	-	-
GIV	11.6 ± 0.9	11.2 ± 0.8	11.7 ± 0.9	**1.2 ± 0.3**	-
GV	5.1 ± 0.5	5.4 ± 0.6	6.5 ± 0.7	10.6 ± 0.9	**0.4 ± 0.1**

Mean intra-genotype (bold) and inter-genotype nucleotide distances and ± standard errors of the mean estimated by the bootstrap method with 100 replicates using *MEGA* 4 program.

Phylogenetic analysis of the new DENV-3 sequences isolated in Brazil confirmed their classification as GV, in agreement with Nogueira et al. (2008) (Fig. 1). The clustering of the GV Brazilian and Colombian isolates with old GV strains (Philippines/1956/L11423, Japan/1973/AB11085, and China/1980/AF317645) is fully consistent with the tree topology previously described by Figueiredo et al. (2008) and Usme-Ciro et al. (2008), but these authors classified the GV strains as GI, based on the initial classification proposed by Lanciotti et al. which merged these two genotypes in a single group (called GI). The identification of GV in DF/DHF South American samples between 2002 and 2004 deserve special attention because GV used to be considered an extinct lineage (Araujo et al. 2008), as only three GV strains (Philippines in 1956, Japan in 1973, and China in 1980) were described in the world before the identification of South American GV strains at the turn of the 21st century.

A detailed analysis of the Genbank database adopting the basic local alignment search tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST) however, allows the identification of one additional DENV-3 E sequence with a high similarity score to the GV strains (accession number: EF110567), that also grouped within this genotype in the phylogenetic tree (Fig. 1). Surprisingly, this unpublished sequence was deposited in the Genbank database in 2006, but seems to corresponds to a virus isolated in Para State, Brazil, in 1989 (Bukin et al. 2006). This finding contrast with the official records that DENV-3 was first isolated in Brazil from an autochthonous case in 2000 (Nogueira et al. 2001).

To further explore the genetic relationships between GV strains, we calculate the pairwise genetic distances among sequences within an overlapping fragment of 822 bp of the E gene. Nucleotide distances between GV Brazilian strains were extremely low ranging from 0% to 0.5%. Of note, two GV Brazilian strains isolated near the turn of this century (accession nos. EF625833 and EF625835) were identical to the Brazilian strain from 1989 in the gene fragment analyzed. This analysis further revealed a surprisingly low genetic distance (0.1% to 0.4%) between the GV Brazilian isolates and the prototype DENV-3 Philippines/1956/L11423 strain isolated in 1956, which corresponds to only 1-3 nucleotide differences throughout the 822 bp fragment. These mean genetic distances were even lower than the corresponding distances between the Philippines/1956/L11423 isolate and early GV Asian strains (Japan/1973/AB111085 [0.6%] and China/1980/AF317645 [0.5%]) and much lower than that expected considering the mean evolutionary rate estimated for the DENV-3 E gene (9×10^{-4} substitutions per site per year) (Araujo et al. 2008). No insertions, deletions, or point mutations that could distinguish GV Brazilian strains from other strains of this genotype were observed.

The results presented here support the classification of DENV-3 sequences from genotypes I and V as distinct genotypes, and confirm that new DENV-3 sequences described in Brazil and Colombia correspond to GV. This study also revealed that a DENV-3 GV sequence, apparently isolated in Brazil in 1989 and deposited in the Genbank database, was identical or very similar to the GV Brazilian strains isolated during the 2000s in the E gene fragment analyzed. These GV Brazilian strains were also unexpectedly similar to the prototype DENV-3 strain identified in the Philippines 48 years ago. These results raise some important questions: Which is the origin of the GV Brazilian strains? How can GV Brazilian strains isolated during the 2000s display a higher similarity with the prototype Philippines/1956/L11423 isolate than GV Asian strains isolated during 1973 and 1980? More GV sequences should be analyzed in Brazil and South America to answer these questions. Increased surveillance, accurate genetic classification of DENV-3 viruses, and molecular epidemiology studies are critical to support new findings and provide an adequate knowledge of dengue virus infections in Brazil.

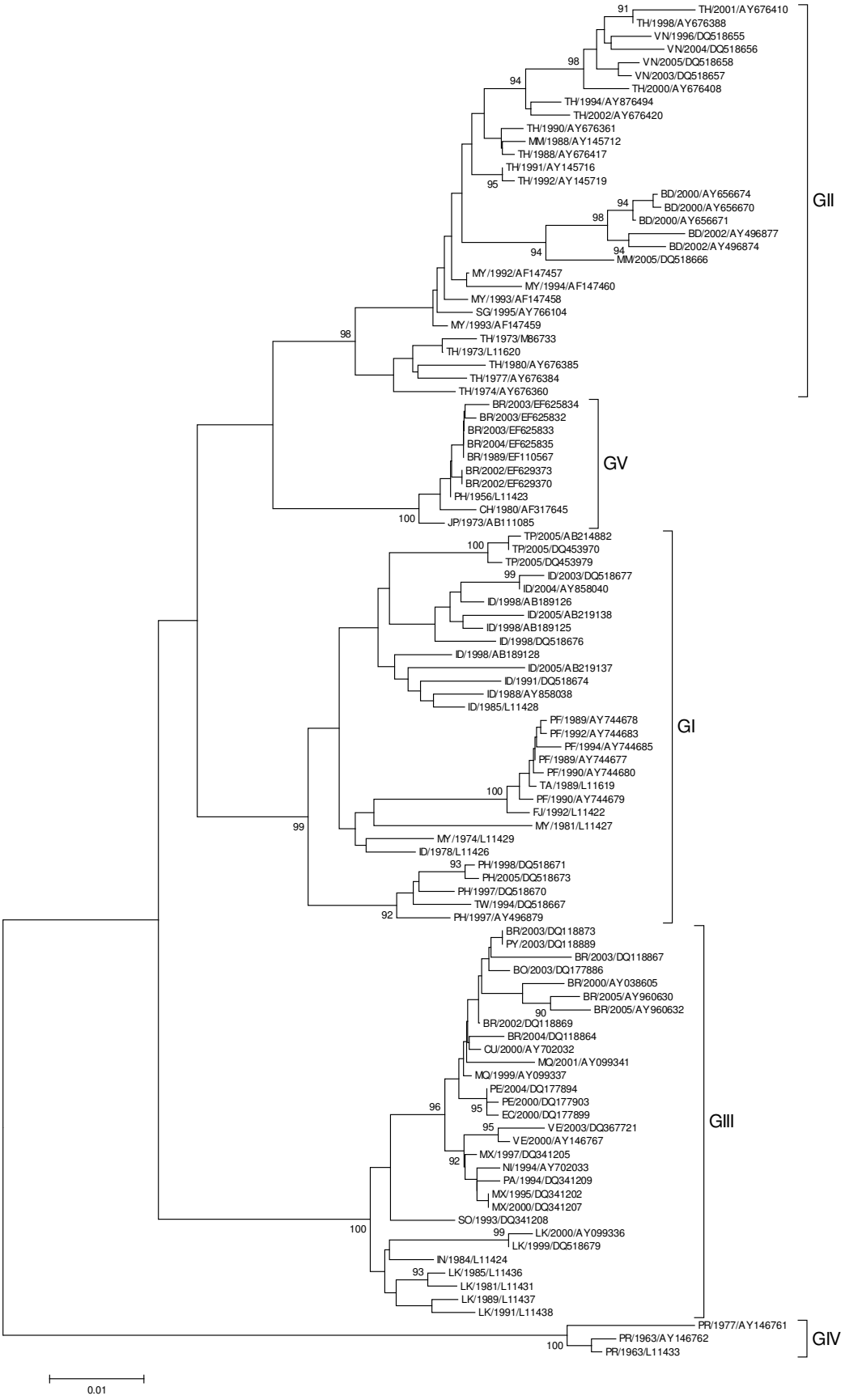
TH/2001/AY676410
TH/1998/AY676388
VN/1996/DQ518655
VN/2004/DQ518656
VN/2005/DQ518658
VN/2003/DQ518657
TH/2000/AY676408
TH/1994/AY876494
TH/2002/AY676420
TH/1990/AY676361
MM/1988/AY145712
TH/1988/AY676417
TH/1991/AY145716
TH/1992/AY145719
BD/2000/AY656674
BD/2000/AY656670
BD/2000/AY656671
BD/2002/AY496877
BD/2002/AY496874
MM/2005/DQ518666
MY/1992/AF147457
MY/1994/AF147460
MY/1993/AF147458
SG/1995/AY766104
MY/1993/AF147459
TH/1973/M86733
TH/1973/L11620
TH/1980/AY676385
TH/1977/AY676384
TH/1974/AY676360
GII
BR/2003/EF625834
BR/2003/EF625832
BR/2003/EF625833
BR/2004/EF625835
BR/1989/EF110567
BR/2002/EF629373
BR/2002/EF629370
PH/1956/L11423
CH/1980/AF317645
JP/1973/AB111085
GV
TP/2005/AB214882
TP/2005/DQ453970
TP/2005/DQ453979
ID/2003/DQ518677
ID/2004/AY858040
ID/1998/AB189126
ID/2005/AB219138
ID/1998/AB189125
ID/1998/DQ518676
ID/1998/AB189128
ID/2005/AB219137
ID/1991/DQ518674
ID/1988/AY858038
ID/1985/L11428
PF/1989/AY744678
PF/1992/AY744683
PF/1994/AY744685
PF/1989/AY744677
PF/1990/AY744680
TA/1989/L11619
PF/1990/AY744679
FJ/1992/L11422
MY/1981/L11427
MY/1974/L11429
ID/1978/L11426
PH/1998/DQ518671
PH/2005/DQ518673
PH/1997/DQ518670
TW/1994/DQ518667
PH/1997/AY496879
GI
BR/2003/DQ118873
PY/2003/DQ118889
BR/2003/DQ118867
BO/2003/DQ177886
BR/2000/AY038605
BR/2005/AY960630
BR/2005/AY960632
BR/2002/DQ118869
BR/2004/DQ118864
CU/2000/AY702032
MQ/2001/AY099341
MQ/1999/AY099337
PE/2004/DQ177894
PE/2000/DQ177903
EC/2000/DQ177899
VE/2003/DQ367721
VE/2000/AY146767
MX/1997/DQ341205
NI/1994/AY702033
PA/1994/DQ341209
MX/1995/DQ341202
MX/2000/DQ341207
SO/1993/DQ341208
LK/2000/AY099336
LK/1999/DQ518679
IN/1984/L11424
LK/1985/L11436
LK/1981/L11431
LK/1989/L11437
LK/1991/L11438
GIII
PR/1977/AY146761
PR/1963/AY146762
PR/1963/L11433
GIV
0.01

Figure 1. NJ phylogenetic tree of 103 partial E gene sequences representing the global diversity of DENV-3. Genotypes are indicated by brackets. Only bootstrap values >90% are shown. The names of DENV-3 isolates include reference to country origin and year of isolation. Countries represented are Bangladesh (BD), Bolivia (BO), Brazil (BR), China (CH), Cuba (CU), Ecuador (EC), Fiji (FJ), Indonesia (ID), India (IN), Japan (JP), Sri Lanka (LK), Myanmar (MM), Martinique (MQ), Mexico (MX), Malaysia (MY), Nicaragua (NI), Panama (PA), Peru (PE), French Polynesia (PF), Philippines (PH), Puerto Rico (PR), Paraguay (PY), Singapore (SG), Somalia (SO), Tahiti (TA), Thailand (TH), East Timor (TP), Taiwan (TW), Venezuela (VE) and Vietnam (VN). Horizontal branch lengths are drawn to scale, and the tree was rooted using GIV which always appears as the most divergent.

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Conflicts of Interest Statement

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

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4. DISCUSSÃO

Várias teorias têm sido propostas para explicar a ocorrência de FHD/SCD, trazendo elementos valiosos para a compreensão da patogenia do dengue. No entanto, em geral, muitos estudos superestimam um aspecto apenas em detrimento de outros e desconhecendo a interação entre eles.

A forma grave do dengue é a expressão de um mecanismo complexo no qual interagem o vírus, o hospedeiro, o vetor e outros fatores relacionados ao ambiente.

A maioria dos estudos atuais demonstra que a infecção secundária é o principal fator de risco para desenvolver a forma grave do dengue, porém outros fatores, como a virulência da cepa viral, as condições do hospedeiro, são também de grande importância.

A hipótese proposta por Halstead (1970), conhecida como "teoria da infecção seqüencial", tem como base estudos epidemiológicos e de experimentação em animais. Dessa forma, casos de FHD/SCD se apresentariam em pacientes que já tivessem anticorpos para dengue, os quais, em presença de uma nova infecção por outro sorotipo, formariam imunocomplexos com o vírus infectante, facilitando a endocitose viral no fagócito mononuclear pelo receptor Fc do monócito/macrófago, seguido da replicação viral, aumento da viremia e disseminação aos tecidos (Morens *et al.,* 1991). Este fenômeno é conhecido como imunoamplificação, ou amplificação dependente de anticorpos (ADA).

Esta teoria tem sido comprovada em diversos países do Sudeste Asiático e em países das Américas. No Brasil, casos de FHD foram confirmados após a introdução do DENV-2 no estado do Rio de Janeiro e em outros estados onde tinham ocorrido previamente epidemias pelo DENV-1 (Nogueira *et al.,* 1990), apoiando a teoria da infecção sequencial.

Outra hipótese, proposta por Rosen (1977, 1982), afirma que a virulência pode variar de uma linhagem para outra, em cada um dos quatro sorotipos, e que as formas graves podem ser explicadas pela virulência destas linhagens.

Rosen em 1977 enfatizou um só fator, ou seja, a patogenicidade do vírus, não correlacionando este fator com os aspectos imunológicos do hospedeiro. Sua hipótese encontra explicação nos casos de FHD/SCD em infecções primárias.

Em Cuba, após estudar a epidemia de 1981, Kourí e colaboradores (Kourí *et al.,* 1989) formularam a hipótese integral, na qual se reconhecia o valor do critério de Halstead quanto à importância dos anticorpos pré-existentes, e o de Rosen quanto à importância da virulência da cepa. Considerou-se, então, que para a ocorrência das formas graves do dengue seriam

necessários a co-existência de três fatores: epidemiológicos, virais e do hospedeiro. Estes fatores isoladamente não explicavam o fenômeno satisfatoriamente.

O Brasil teve o seu perfil epidemiológico agravado pela introdução do DENV-3 em dezembro de 2000 (Nogueira *et al.,* 2001). Este sorotipo se dispersou de forma rápida e contínua, alcançando oito estados brasileiros em apenas três meses. Durante o ano de 2002, o Brasil notificou 813.104 casos de dengue, e desse total 288.245 correspondem aos casos no estado de Rio de Janeiro. Naquele ano, 1831 casos de FHD e 91 óbitos foram confirmados. Este total excedeu o número de casos notificados de FHD e óbitos em um período de 20 anos desde a introdução do dengue no país (Nogueira *et al*., 2005).

Este mesmo sorotipo/genótipo foi responsável por grandes epidemias com formas graves no Sudeste Asiático, América Central e América do Sul (Messer *et al.,* 2003).

Um fato novo observado durante a epidemia de 2002 foi a apresentação de formas graves e óbitos em menores de 15 anos, bem como casos fatais em infecções primárias (Nogueira *et al*., 2005; Araújo *et al.,* 2009b; 2009c). A gravidade da doença pôde ser destacada pelos sinais e sintomas na admissão hospitalar: hipotensão (59,5%), dor abdominal (35,7%), pré-choque (35,7%) e óbito por SCD em 57,8% dos casos (Azevedo *et al.,* 2002).

No contexto do primeiro artigo desta tese intitulado "**Quantification of dengue virus type 3 RNA in fatal and nonfatal cases in Brazil, 2002**", determinamos a viremia em casos fatais e não fatais por DENV-3, ocorridos durante a epidemia de 2002 no estado do Rio de Janeiro, com a finalidade de investigar uma possível correlação entre viremia e gravidade da doença. Este estudo contempla a maior casuística de casos fatais por DENV-3 da literatura, entre os estudos que correlacionam a viremia e gravidade da doença.

Constatamos uma média maior dos níveis de RNA viral em casos fatais (12,5 $\log_{10}$ cópias/ml), em comparação ao grupo de casos não fatais por dengue (7,9 $\log_{10}$ cópias/ml). Esta diferença foi significativa utilizando testes paramétricos e não paramétricos (t-test t, P = 0,001; Mann-Whitney test; P = 0,002). Estes dados sugerem que a viremia é um fator importante na patogênese da forma grave do dengue.

O papel da viremia nas infecções por dengue foi primeiramente investigado nos anos 1975 e 1978 por Gubler e colaboradores (1981). Estes autores analisaram a viremia dos quatro sorotipos de dengue em 153 pacientes em Jakarta, na Indonésia, e detectaram títulos virais que variavam desde níveis muito baixos até $10^8 MID_{50}$/ml. Os autores observaram uma viremia maior, porém não significativa, em casos classificados como infecção primária, em comparação aos casos de infecção secundária. Os títulos virais em pacientes infectados com DENV-4 foram

aproximadamente 100 vezes menor em relação aos demais sorotipos. A análise da viremia nos casos de FHD em Jakarta revelou grandes quantidades de vírus no momento do óbito.

Outro estudo conduzido por Vaughn *et al.* (2000) na Tailândia, entre os anos 1994 e 1996, determinou a viremia em 41 casos de DENV-1 e 46 casos de DENV-2, e demonstrou que a alta viremia estava associada à gravidade da doença em 31 casos, no qual foi detectada uma média do título viral de $10^{7,6}$ MID_{50}/ml para o grupo de DC e $10^{8,5}$ MID_{50}/ml para o grupo de FHD.

Murgue e colaboradores (2000) analisaram a viremia em 49 crianças durante uma epidemia de DENV-2 na Polinésia Francesa, durante os anos 1996 e 1997, e demonstraram uma viremia menor em pacientes com DC do que aqueles com FHD (4,3 ± 0,8 equivalente log_{10} $TCID_{50}$/ml e 5 ± 0,6 equivalente log_{10} $TCID_{50}$/ml, respectivamente).

Durante a epidemia de dengue ocorrida no ano de 1998 em Taiwan, Wang e colaboradores investigaram os níveis de RNA dos DENV-3 utilizando um qRT-PCR convencional, e demonstraram que pacientes com FHD possuíam níveis significativamente mais elevados de RNA quando comparados a pacientes com DC. Durante a defervescencia, o RNA viral foi detectado na maioria dos pacientes com DC e em níveis mais elevados em todos os pacientes com FHD (Wang *et al.,* 2003). Pelo exposto ficou evidente que a viremia é um fator importante nos casos graves de dengue.

A técnica de RT-PCR em tempo real tem sido amplamente utilizada para a quantificação de dengue e outros flavivírus (Guilarde *et al.,* 2008; Linnen *et al.,* 2008, Trindade *et al.,* 2008).

No Brasil, Guilarde *et al.* (2008) determinaram a viremia dos DENV por este método em 185 pacientes durante uma epidemia ocorrida em 2005 na cidade de Goiânia (GO). Entretanto, a diferença entre as médias dos títulos virais não foram significativas entre o grupo de pacientes classificados como FHD e intermédiário DC/FHD (4,45 e 4,78 log_{10} cópias de RNA/ml, respectivamente) e o grupo de pacientes classificados como FHD (5,62 log_{10} cópias de RNA/ml). No trabalho de Goiânia, o kit utilizado pelos autores não permitiu identificar o sorotipo envolvido em cada caso, pela utilização de iniciadores consenso para os quatro sorotipos.

Diversas vantagens têm sido atribuídas à técnica de PCR em tempo real como sensibilidade, especificidade, reprodutibilidade e rapidez (Callahan *et al.,* 2001; Araújo *et al.,* 2006). Uma das características do método consiste em permitir o monitoramento da amplificação em tempo real. Esta técnica, por possuir um sistema automatizado de detecção do ácido nucléico e considerando que os tubos de reação não são abertos durante a prova, reduz a possibilidade de contaminação com produtos amplificados (Araújo *et al.,* 2006).

Os iniciadores e sonda utilizados na técnica de PCR em tempo real foram desenhados por Houng *et al.* (2001) para a região 3´NC do DENV-3. Essa mesma região tem sido utilizada por vários autores como alvo para detecção e quantificação dos DENV, como os trabalhos realizados por Callahan *et al.* (2001), Drosten *et al.* (2002), Parida *et al.* (2005) e Chutinimitkul *et al.* (2005).

Na estratégia para preparação da curva padrão da técnica de RT-PCR em tempo real, aplicamos o modelo descrito por Johnson e colaboradores (2004; 2005), no qual é obtido um controle de RNA (quantificado) com base nos títulos obtidos por PFU, considerando que 1 RNA infeccioso corresponde a 1 PFU. Contudo, é sabido que, na aplicação do RT-PCR em tempo real, além do RNA infeccioso, é quantificado o RNA não-infeccioso, produzido durante o processo de replicação viral e não incorporado aos novos virions. Um estudo de dengue conduzido por Linen et al. (2008), estimou que 1 PFU corresponde a 300 cópias de RNA.

Nesse sentido, tentativas para a obtenção de um controle de ácido nucléico foram realizadas a partir da quantificação do RNA viral pelo espectrofotômetro. Contudo, os níveis de RNA obtidos foram discrepantes, variando de $5{,}14x10^9$ a $8{,}38x10^9$ cópias de RNA por microlitro, partindo de uma amostra com valor de 10^7 PFU por mililitro. Dessa forma mantivemos a metodologia proposta por Johnson e colaboradores.

Após determinar os níveis de RNA pelo método de RT-PCR em tempo real, analisamos os níveis de anticorpos da classe IgG através da técnica de IgG-ELISA com vistas a classificar os pacientes examinados quanto ao tipo de infecção (primária ou secundária). Na nossa casuística 52,1% dos casos fatais foram classificados como infecções primárias por dengue. Diante deste resultado, a teoria da infecção seqüencial por si só não explica todos os casos aqui estudados.

Os testes de inibição da hemaglutinação (HI) e IgM/IgG ELISA são tradicionalmente realizados na classificação de casos primários ou secundários por dengue (Miagostovich *et al.,* 1999; Vaughn *et al.,* 1999). IgG-ELISA é rápido, fácil de executar, apresenta várias vantagens sobre o método convencional de HI e pode ser aplicado no diagnóstico de rotina. Boa correlação entre os resultados obtidos por HI e IgG-ELISA foram obtidos anteriormente (Chungue *et al.,* 1989, Figueiredo *et al.,* 1989).

Ainda no primeiro artigo, fizemos a correlação entre o tipo de infecção (primária ou secundária) e a viremia obtida nos casos estudados, e observamos que a viremia dos casos não fatais foi significativamente maior em casos classificados como infecção primária em comparação aos casos com infecção secundária. Este resultado sugere uma rápida ativação da

resposta imune nos casos classificados como infecção secundária e conseqüente diminuição da viremia.

Em conclusão, os dados obtidos no primeiro artigo sugerem que elevada viremia pode contribuir para a patogênese das formas graves do dengue.

No segundo artigo, intitulado "**A retrospective survey of dengue virus infection in fatal cases from an epidemic in Brazil**", realizamos a pesquisa dos DENV em diferentes tecidos provenientes de casos fatais ocorridos durante a epidemia de DENV-3 no estado do Rio de Janeiro, ano 2002, com o propósito de obter uma melhor compreensão sobre o tropismo desses vírus.

Para este fim, estabelecemos a metodologia do RT-PCR em tempo real para a pesquisa dos DENV em tecidos, e comparamos os resultados obtidos com outras três metodologias: isolamento viral, RT-PCR convencional e imunohistoquímica.

Cabe ressaltar que nossa comparação entre metodologias com alvos diferentes (Ex: imunohistoquímica versus RT-PCR em tempo real) está exclusivamente relacionada a contribuição de cada uma na confimação de casos fatais por dengue.

Este é o primeiro estudo descrito na literatura que envolve a aplicação do RT-PCR em tempo real em amostras de tecidos (n=77) provenientes de casos fatais suspeitos de dengue, contribuindo para o conhecimento dos sítios de replicação desses vírus.

O RT-PCR em tempo real foi o método que apresentou o melhor desempenho, sendo responsável por 58,4% da positividade em 77 amostras histológicas, enquanto o RT-PCR convencional obteve resultados positivos em 30,5% (22/72). Além disso, o RT-PCR em tempo real foi o único método capaz de detectar o DENV-3 em 8 dos 26 casos estudados (30,8%). Estes resultados estão de acordo com os estudos realizados por Poersch et al. (2005), onde os autores observaram maior sensibilidade da técnica de RT-PCR em tempo real quando comparado ao RT-PCR convencional no diagnóstico da dengue em amostras de soro.

Na nossa casuística, o fígado foi o espécime mais importante para detecção dos DENV-3, onde o mesmo foi detectado em 24 (92,3%) dos 26 casos estudados. Este tecido é reconhecido como um dos principais órgãos-alvo na patogênese da infecção por DENV (Couvelard *et al.,* 1999; Lin *et al.,* 2000).

Apesar de a bile ter sido descrita como tendo efeito virucida, inclusive para os DENV, alguns autores admitem que a presença do DENV no fígado seja tão freqüente como no soro (Rosen & Khin, 1989; Itha *et al.,* 2005; Larreal *et al.,* 2005).

Um estudo conduzido por Huerre et al. (2001) no Vietnã, descreve uma investigação dos DENV em amostras de fígado provenientes de cinco casos fatais que foram confirmados por nested RT-PCR e imunohistoquímica. Estes autores observaram locais de recrutamento de células inflamatórias, hepatite difusa com necrose e esteatose em dois casos, áreas focais de necrose em outros dois casos e histologia normal em um caso. Células de Kupffer foram destruídas principalmente em casos com necrose focal ou grave, assumindo que hepatócitos e células de Kupffer podem ser alvos de replicação dos DENV, corroborando com os resultados apresentados neste artigo.

Bhamarapravati propôs que lesões hepáticas são indicativas da própria lesão viral durante certas fases da doença (Bhamarapravati, 1965), e já foi relatada a possível participação do complemento na agressão hepática, a partir de sua ativação por anticorpos contra algumas proteínas virais (Torres, 2005).

O envolvimento do coração em infecções por DENV tem sido investigado em vários estudos, inclusive no Brasil (Horta Veloso *et al.,* 2003; Basílio-de-Oliveira *et al.,* 2005). No nosso estudo, este envolvimento foi confirmado em dois casos a partir da detecção do RNA viral pelos métodos de nested RT-PCR e RT-PCR em tempo real. Manifestações cardíacas durante infecções por DENV são pouco freqüentes, no entanto, alterações no ritmo cardíaco (Donegani e Briceño, 1986; Chuah, 1987; Khongphatthallayothin *et al.,* 2000), tem sido descrito durante a febre hemorrágica da dengue. Na maioria dos casos, esta alteração evolui de forma benigna com cura espontânea. Contudo, pesquisas apontaram a presença de miocardite em algumas crianças que evoluíram ao óbito durante uma epidemia em Cuba, e essa alteração foi muito evidente em pacientes adultos que evoluíram ao óbito durante um surto em Santiago de Cuba em 1997. Nos cortes histológicos pôde-se evidenciar edema intersticial, presença de eosinófilos e alguns linfócitos e monócitos, bem como citólise (Torres, 2005). Os mecanismos exatos destas manifestações ainda não são bem esclarecidos.

O envolvimento do Sistema Nervoso Central (SNC) em casos de dengue tem sido relatado na literatura (Leao *et al.,* 2002; Nogueira *et al.,* 2002). Neste estudo, descrevemos o envolvimento do SNC em 8 casos a partir da detecção do Ag e RNA viral em amostras de cérebro.

Algumas vezes, o paciente poderá ser hospitalizado com o diagnóstico inicial de meningite asséptica e diagnosticado depois como dengue (Torres, 2005).

Em pacientes com manifestações neurológicas e dengue confirmado, têm-se encontrado anticorpos IgM anti-dengue no líquido cefalorraquidiano (LCR) (Chen *et al.,* 1991) e se

conseguido isolar o DENV do LCR (Lum *et al.,* 1996). As manifestações neurológicas nesses casos começaram em fase precoce da doença, antes da instalação do choque.

No Brasil, Miagostovich e colaboradores estudaram cinco casos fatais de dengue utilizando técnicas de imunohistoquímica, e puderam evidenciar antígenos virais em numerosas células do cérebro, em três casos. As células positivas de infecção viral eram macrófagos CD68+ e principalmente dos espaços de Virchow Robin de tamanho médio e de veias pequenas, e infiltravam substância cinzenta e substância branca, comumente situada junto aos neurônios que mostravam mudanças citopáticas (Miagostovich *et al.,* 1997).

Manifestações neurológicas associadas a casos de dengue foram definidas como encefalopatia por dengue, atribuída a respostas imunopatológicas e não pela infecção viral no SNC (Leao *et al.,* 2002). Em algumas situações, o quadro neurológico poderá estar associado a um comprometimento hepático intenso, ao choque prolongado, no qual tem sido considerado que a afecção do SNC foi secundária a anoxia, à isquemia ou a outras alterações metabólicas (Torres, 2005).

A forte ligação de dengue com leucopenia e trombocitopenia sugere que células da medula óssea podem ser alvos potenciais para infecções por DENV. Estudos *in vitro* demonstraram que células da medula óssea são suscetíveis a infecções por esses vírus (Rothwell *et al.,* 1996).

Considerando este aspecto, investigamos a presença dos DENV em uma amostra disponível de medula óssea através das técnicas de isolamento viral e RT-PCR em tempo real. Obtivemos neste caso a positividade de DENV-3 pelo RT-PCR em tempo real. Esta é a primeira descrição da detecção do DENV-3 na medula óssea humana, representando assim uma contribuição original.

Jessie e colaboradores (2004) investigaram a presença e localização dos DENV por imunohistoquímica (IHQ) e hibridização "*in situ*" (ISH), em 5 autópsias, 24 biópsias e 20 amostras de sangue. A técnica de ISH foi aplicada em amostras positivas para IHQ. Antígenos virais foram detectados em células de Kupffer, células endoteliais sinusoidais do fígado; macrófagos, células multinucleadas e células linfóides do baço; macrófagos e endotélio vascular no pulmão; túbulos renais, monócitos e linfócitos no sangue. O RNA viral foi detectado em células do baço e sangue (Jessie et al., 2004).

Estudos anteriores realizados por Bhamarapravati *et al.* (1967) e Miagostovich *et al.* (1997), investigaram a participação do baço, rins e pulmão em casos de FHD/SCD e fatais por DENV, demonstrando o envolvimento destes órgãos.

Neste contexto, aplicamos os métodos de isolamento viral, nested RT-PCR, imunohistoquímica e RT-PCR em tempo real nestes tecidos e observamos a presença do Ag e/ou do RNA viral em 10 casos no baço, 10 casos no pulmão e em 4 casos nos rins. A contribuição por cada metodologia pode ser visualizada na tabela 2 do artigo 2, no qual pode-se observar que a técnica de RT-PCR em tempo real foi a metodologia mais sensível.

Nossos resultados reforçam a importância da incorporação da técnica de RT-PCR em tempo real como mais um método a ser aplicado em casos fatais suspeitos de dengue.

Em algumas oportunidades foi o único método capaz de confirmar a infecção em especial no cérebro e medula óssea, confirmando formas atípicas da doença.

Dessa forma acreditamos que o RT-PCR em tempo real será mais uma ferramenta a integrar os Programas de Vigilância Epidemiológica do Ministério da Saúde.

O terceiro artigo, intitulado "**Phylogeography and evolutionary history of dengue virus type 3**", representa a maior análise sobre os aspectos filogenéticos, migratórios e evolutivos destes vírus até o momento. Nossa análise filogenética envolveu 200 seqüências do gene E dos DENV-3, representando 31 países do globo.

Observamos uma clara subdivisão geográfica dos três principais genótipos deste vírus. Os genótipos I, II e III evoluíram independentemente na Indonésia, Tailândia e Sri Lanka, respectivamente, ao longo dos últimos 30-40 anos. Nossos dados apóiam a idéia de que esses países não apenas foram alvos das mais antigas epidemias de DENV-3, mas também foram epicentros de distribuição de linhagens virais por todo o mundo.

Cepas isoladas no Sul do Pacífico (1989-1994), Filipinas (1997-2005), Timor-Leste (2005), Malásia (1992-1994), Bangladesh (2000-2002), Vietnã (1996-2005) e Américas (1994-2006), formaram diferentes grupos monofiléticos, indicando que cada país possui uma linhagem geograficamente distinta, com poucas evidências de fluxo gênico entre regiões.

As análises dos padrões de migração dos DENV-3 sugerem que a propagação do GI foi direcionada principalmente para a porção marítima do Sudeste Asiático (Timor Leste, Malásia e Filipinas) e Sul do Pacífico, onde a maioria dos vírus parece ter sido originado da Indonésia.

Em contrapartida, a maioria das linhagens do GII parece ter sido proveniente da Tailândia e permaneceu dentro de zonas continentais no Sudeste Asiático (Bangladesh, Mianmar, Singapura, e Vietnã), com exceção da Malásia.

O GIII atingiu o maior território dos DENV-3, com circulação na Ásia, África e nas Américas, parecendo ter sido originado da Ásia (Sri Lanka). Não ficou claro se a linhagem americana teve origem na África, Ásia ou Samoa. Nossos dados de migração do GIII sugerem

que a introdução desta linhagem nas Américas tenha sido pelo México, onde os primeiros isolados foram identificados no ano 1995 (Briseno-Garcia et al, 1996). No entanto, as mais antigas seqüências de GIII foram identificadas no Panamá e Nicarágua no ano de 1994 (CDC, 1995; Guzman *et al,* 1996), dificultando a elucidação desta introdução.

Em qualquer caso, o GIII rapidamente foi disseminado para outros países da região (Nogueira *et al,* 2001; Usuku *et al,* 2001; Rigau-Perez *et al,* 2002; Peyrefitte *et al,* 2003; Uzcategui *et al,* 2003), utilizando diferentes rotas em direção a América Central, Caribe e América do Sul.

Nossa análise filogeográfica revelou que a ocorrência de co-circulação de diferentes genótipos de DENV-3 em uma única localidade é um evento raro. Isto pode ser resultado de um fluxo genético limitado entre regiões geográficas distintas. No entanto, o mesmo genótipo foi observado em países vizinhos como a Indonésia e Tailândia, onde o DENV-3 causa epidemias desde a década de 70, causadas pelos GI e GII, respectivamente. Observamos a co-circulação dos GI e GII na Malásia, Tailândia e Indonésia, GII e GIII em Singapura e GI/GIII em Taiwan. Contudo, a ocorrência destas co-circulações parece não ter se estabelecido.

Nossas estimativas da média da taxa evolutiva foram semelhantes aos descritos anteriormente por Twiddy *et al.* (2003), apesar dos intervalos de confiança de nossos cálculos serem significativamente mais precisos, provavelmente devido a um maior número de seqüências estudadas. A mediana da taxa de evolução estimada para o GII foi ligeiramente mais elevada do que a estimada para GI e GIII. Entretanto, com a sobreposição dos intervalos de máxima densidade a posteriori (95% HPD), não houve grandes diferenças nas taxas entre os principais genótipos.

Twiddy *et al.* (2003) sugerem que durante períodos epidêmicos, a taxa de evolução viral seja maior quando comparada a de períodos endêmicos. Para testar esta hipótese, comparamos a média da taxa evolutiva dos isolados da Indonésia e Tailândia, onde GI e GII circulam desde o início dos anos 1970, com os isolados das Américas, que circulam neste território desde o início dos anos 1990.

As médias da taxa de evolução destas linhagens foram similares, variando de $8{,}4x10^{-4}$ subs/sítio/ano para GI-ID, $9{,}8x10^{-4}$ para GII-TH e $9{,}2x10^{-4}$ subs/sítio/ano para GIII-AM, indicando que não há grandes diferenças nas taxas de evolução entre estas linhagens.

O ano de origem das atuais linhagens de DENV-3 foi estimado para 1890, totalmente coerente com as estimativas anteriores (Tmrca ~ 1900) descritas por Twiddy *et al.* (2003). A

estimativa do ano de origem dos três principais genótipos dos DENV-3 foi cerca do final da década de 1930.

Nossa análise sugere que a atual diversidade genética dos genótipos I, II e III surgiu quase simultaneamente dentro de um curto período de tempo, compreendido entre 1960 e 1970, coincidindo com o aumento do número de casos de FHD descritos na Ásia por DENV-3 (Gubler *et al.,* 1979; Wallace *et al.,* 1980; Sumarmo, 1987; Nisalak *et al.,* 2003).

As linhagens de DENV-3 recentemente introduzidas na América Latina parecem ter começado a se propagar no ano de 1991, três anos mais cedo que a primeira detecção do GIII no continente, em Panamá e Nicarágua no ano de 1994 (CDC, 1995; Guzman *et al.,* 1996).

Finalmente, este estudo revela uma forte subdivisão espacial de linhagens específicas de DENV-3, com pouca evidência de co-circulação de genótipos em uma mesma localidade. Foi observada uma taxa de evolução similar entre os diferentes genótipos de DENV-3. Nossos dados sugerem que a atual diversidade dos três principais genótipos de DENV-3 surgiu nos últimos 30-40 anos, coincidindo com o aumento da população humana, urbanização, e com o aumento do número de casos de FHD/SCD na Ásia.

As mudanças genéticas virais são resultado de mutações acumuladas e recombinações provocadas pelo aumento do contato entre os vírus e as populações humanas cada vez maior, bem como pela mistura intertípica e também intratípica viral que podem ocorrer em sua passagem no vetor (Kuno, 1997).

Um vírus pode ser considerado como um *pool* de variantes pelos quais certos genótipos são dominantes (Nuttal et al., 1991). Tais variações virais poderiam modular a transmissão da infecção, a capacidade de penetrar e replicar-se em células específicas do vetor, dos hospedeiros vertebrados, assim como influir sobre os mecanismos de sobrevivência na natureza, a virulência e a transmissibilidade entre hospedeiros (Kuno, 1997).

Holmes e Burch, da Universidade de Oxford, referiram-se às causas e consequencias da variação genética nos DENV e concluíram: “Os estudos de sua evolução revelam que sua diversidade genética está aumentando. Isso, aliado à evidência de que as linhagens podem diferir quanto a virulência, sugerem que no futuro poderíamos estar expostos a vírus com amplitude expandida de propriedades patogênicas” (Holmes & Burch, 2000).

Aos fatores relacionados a mudanças climáticas, extensão geográfica dos vetores e perigos quanto à propagação e modificações nos vírus, agrega-se o aumento populacional previsto no século XXI, assim como a crescente relação entre urbanização e ecologia do dengue (Tauil, 2001). A cada ano a população mundial aumenta em 80 milhões, pelo que é de seis

bilhões no início deste século e será de oito bilhões pouco depois da metade do mesmo. Mais de 90% dessa população estará localizada nos países chamados em desenvolvimento, na maioria dos quais existe atividade do dengue, estimando que a quantidade de suscetíveis aumente a cada ano.

Tais considerações, aliadas aos estudos de epidemiologia molecular dos DENV, nos dá base para compreender como estes vírus evoluem geneticamente e como são seus padrões de migração na natureza. Além disso, nos permite examinar a origem de linhagens re-emergentes, como foi o caso do genótipo V (GV) abordado no quarto artigo deste trabalho, intitulado "**Dengue Virus Type 3 in Brazil: A Phylogenetic Perspective**", e discutido a seguir.

A análise filogenética dos genes prM/M/E dos DENV-3 conduziu à identificação inicial de quatro grupos monofiléticamente distintos chamados de genótipos (G) (Lanciotti *et al.,* 1994). Após algum tempo, uma análise filogenética incluindo um número maior de seqüências de DENV-3, demonstrou que genótipo I descrito pelo Lanciotti *et al.* (1994) envolve dois genótipos distintos, que foram classificados separadamente como GI e GV (Wittke *et al.,* 2002).

Os genótipos I, II e III (GI, GII e GIII) são responsáveis pela maioria das infecções por DENV-3: GI representado pelos vírus circulantes na Indonésia, Malásia, Filipinas e no Sul do Pacífico; GII por vírus provenientes da Tailândia, Bangladesh, Malásia e Mianmar; e GIII por vírus circulantes no Sri Lanka, Índia, Samoa, África e nas Américas.

Em contraste, os genótipos IV e V (GIV e GV) são provenientes de isolados mais antigos do DENV-3: GIV constituído por isolados de Porto Rico circulantes durante as décadas de 60 e 70; e GV representado pelo protótipo isolado nas Filipinas em 1956 e duas cepas isoladas no Japão e na China em 1973 e 1980, respectivamente.

O DENV-3 GIII foi detectado pela primeira vez nas Américas durante uma epidemia na Nicarágua e no Panamá em 1994 (Anônimo, 1995) e se disseminou para muitos países da América Latina (Briseno-Garcia *et al.,* 1996, Guzman *et al.,* 1998, Balmaseda *et al.,* 1999, Isturiz *et al.,* 2000, Nogueira *et al.,* 2001, Usuku *et al.,* 2001, Uzcategui *et al.,* 2003, Aquino *et al.,* 2006; Araújo *et al.,* 2009a).

No Brasil, o DENV-3 foi isolado pela primeira vez a partir de um caso autóctone de DC em dezembro de 2000, no município de Nova Iguaçu, Rio de Janeiro (Nogueira *et al.,* 2001). Dois anos mais tarde, este sorotipo foi responsável por uma grave epidemia no estado do Rio de Janeiro (Nogueira *et al.,* 2005).

O DENV-3 parece ter sido introduzido na América Latina por uma única rota, provavelmente do Sri Lanka, Índia e Leste da África, por volta do ano de 1991 (1987-1993) (Araujo *et al.,* 2009a).

A circulação de um novo genótipo do DENV-3 foi recentemente descrito no Brasil (Figueiredo *et al.,* 2008, Nogueira *et al.,* 2008) e Colômbia (Usme-Ciro *et al.,* 2008). Este novo genótipo foi classificado como GI por Figueiredo *et al.* e Usme-Ciro *et al.*, mas como GV por Nogueira et al. Isto indica claramente que uma padronização internacional da nomenclatura de genótipos de DENV-3 é necessária. Por estas razões, nós examinamos a atual classificação filogenética dos DENV-3, com destaque para o novo genótipo descrito no Brasil.

A análise filogenética das seqüências de DENV-3 isoladas no Brasil confirmou a circulação dos genótipos III e V, de acordo com Nogueira *et al.* (2008). O agrupamento dos isolados GV brasileiros e colombianos com o protótipo GV (Philippines/1956/L11423, Japan/1973/AB11085, e China/1980/AF317645) é perfeitamente coerente com a topologia descrita anteriormente por Figueiredo *et al.* (2008) e Usme-Ciro *et al.* (2008), mas estes autores classificam estes isolados GV como pertencentes ao GI, com base na classificação inicial proposto por Lanciotti *et al.* (1994).

A identificação do GV em casos de dengue clássico e em casos de FHD em amostras Sul-Americanas entre 2002 e 2004 merece atenção especial. O GV foi considerado uma linhagem extinta (Araújo *et al.,* 2009a), uma vez que apenas três isolados pertencentes ao GV (Filipinas/1956, Japão/1973 e China/1980) foram descritos em todo o mundo antes da identificação dos isolados recentes na América do Sul.

Uma análise detalhada utilizando o banco público de seqüências (GenBank), adotando a ferramenta (BLAST) (www.ncbi.nlm.nih.gov/BLAST), permitiu a identificação de uma seqüência adicional de DENV-3 com alta similaridade com isolados do GV (número de acesso: EF110567).

Surpreendentemente, a inédita seqüência foi depositada no GenBank em 2006, correspondendo a um vírus isolado no estado do Pará (Brasil) em 1989 (Bukin *et al.,* 2006). Este achado contrasta com os registros oficiais de que o DENV-3 foi primeiro isolado no Brasil a partir de um caso autóctone em dezembro de 2000 (Nogueira *et al.,* 2001).

Para aprofundar as relações genéticas entre os isolados do GV, calculamos as distâncias genéticas entre as seqüências, utilizando um fragmento de 822 pb do gene E. As distâncias genéticas do GV entre as seqüências brasileiras foram extremamente baixas, variando de 0% a 0,5%. Observamos que duas seqüências brasileiras do GV proveniente de isolados recentes (número de acesso: EF625833 e EF625835) foram idênticas à sequencia brasileira de 1989.

Esta análise revelou uma distância genética surpreendentemente baixa (0,1% para 0,4%) entre os isolados brasileiros GV e o protótipo isolado nas Filipinas em 1956 (número de acesso:

L11423), correspondendo a 1-3 nucleotídeos de diferença. A distância genética entre as GV brasileiras e a amostra protótipo Filipinas/1956 foi mais baixa do que as distâncias entre os isolados Filipinas/1956/L11423 e Ásia (Japão/1973/AB111085 [0,6%] e China/1980/AF317645 [0,5%]). Este resultado é inesperado, considerando a média da taxa de evolução estimada para o gene do envelope dos DENV-3 (9×10^{-4} substituições por sítio por ano) (Araújo *et al.,* 2009a). Não foram observadas inserções, deleções ou mutações pontuais que diferenciem isolados brasileiros do GV de outros deste mesmo genótipo.

Os resultados apresentados neste estudo apoiam que GI e GV são genótipos distintos e confirma que as novas seqüências de DENV-3 descritas no Brasil e na Colômbia pertencem ao GV.

Este estudo revelou que uma seqüência pertencente ao GV, aparentemente isolada no Brasil em 1989 e depositadas no GenBank, foi idêntica ou muito semelhante as seqüências brasileiras do mesmo genótipo isoladas recentemente (2002-2004). Estes isolados brasileiros pertencentes ao GV foram inesperadamente semelhantes ao protótipo do DENV-3 identificado nas Filipinas há 48 anos atrás.

Nesse sentido, estes resultados levantam algumas questões importantes: Qual é a origem dos isolados brasileiros pertencentes ao GV? Como podem as amostras GV brasileiras isoladas em 2002-2004 possuir uma maior similaridade com o protótipo Filipinas/1956/L11423 do que as amostras Asiáticas isoladas durante os anos 1973 e 1980? Especulamos que possa existir um mecanismo ainda desconhecido de manutenção desses vírus na natureza, que atualmente não conseguimos observar. Todavia, um maior número de seqüências do GV deverá ser analisado no Brasil e na América do Sul para responder a estas perguntas.

A precisa classificação genética dos DENV-3 e estudos de epidemiologia molecular são fundamentais para apoiar novos resultados e proporcionar um conhecimento adequado das infecções por dengue no Brasil.

Como considerações finais, observamos que desde os anos 70 do século XX, alguns sorotipos de dengue têm sido correlacionados com a gravidade do dengue (Rosen, 1977; Gubler *et al.,* 1978). Atualmente, com os avanços da biologia molecular e da bioinformática, torna-se possível investigar com maior profundidade os fatores genéticos de diferentes linhagens virais e sua importância na determinação da gravidade da doença.

Os genótipos dos DENV-2 e DENV-3, originários do Sudeste Asiático e do Subcontinente Indiano, respectivamente, tem sido identificados em casos graves de FHD/SCD, provenientes de infecções primárias (Rico-Hesse, 2007; Araújo *et al.,* 2009b). Em alguns países,

como o Peru, estudos epidemiológicos têm demonstrado que, mesmo após a infecção secundária, os pacientes não desenvolveram FHD e/ou SCD, mesmo quando ocorre a circulação de um genótipo menos patogênico (Watts *et al.,* 1999).

Os genótipos mais virulentos foram introduzidos nas Américas, onde foram observadas graves epidemias, incluindo a descrição dos primeiros casos de FHD neste continente (Rico-Hesse, 2003). Os mecanismos que conduzem a uma maior virulência nestes vírus estão sob investigação.

Até o momento não sabemos se existem evidências significativas no aumento da taxa de evolução de novas variantes genéticas para os DENV (Araújo *et al.,* 2009a). Estudos com o genótipo Sudeste Asiático do DENV-2, considerado virulento, demonstraram que esta linhagem tem estado estável geneticamente desde a década de 1940. De fato, o genótipo Sudeste Asiático deslocou variantes menos virulentas (isto é, não associadas à FHD e/ou SCD), como o genótipo Americano, em muitos países (Rico-Hesse, 2007).

No Norte do México, o último relato da circulação do genótipo Americano foi em 1995 e, desde então, predominou o genótipo Sudeste Asiático, que se disseminou para outras regiões do México, bem como para outros países do continente americano. Os últimos isolados do genótipo Americano foram detectados no Peru no ano de 1996, e não há evidências de que o genótipo Sudeste Asiático tenha causado surtos neste país (Rico-Hesse, 2007).

Neste trabalho, demonstramos que os três principais genótipos do DENV-3 (GI-III) apresentaram uma forte estrutura temporal, com os mais antigos isolados situados próximos a raiz da árvore filogenética e os mais recentes localizados na porção distal. Este resultado indica claramente que os DENV-3 estão evoluindo geneticamente ao longo do tempo, provavelmente devido ao processo de adaptação a novas populações suscetíveis, independente da forma clínica da doença. Estudos de evolução molecular com foco em diferentes formas clínicas da doença, como dengue clássico e dengue grave, são fundamentais para esclarecer estas relações.

O fato de dois diferentes genótipos de DENV-3 (GIII e GV) circularem no Brasil requer análise especial, pois não sabemos qual será a repercussão epidemiológica e clínica deste novo genótipo no país. Levando em consideração a alta virulência do genótipo III do DENV-3, é pouco provável que o GV deste sorotipo se dissemine de forma significativa para outras regiões do país, considerando ainda que em muitas localidades exista a co-circulação de diferentes sorotipos (DENV-1-3).

5. CONCLUSÕES

1) O grupo de casos fatais de dengue ocorridos durante a epidemia de 2002 no Rio de Janeiro, apresentou uma viremia por DENV-3 significativamente maior quando comparado ao grupo de casos não fatais (12,5 $\log_{10}$ cópias de RNA/ml e 7,9 $\log_{10}$ cópias de RNA/ml, respectivamente) (t-teste, *P*=0,001; Mann-Whitney test, *P*=0,002). Este resultado sugere uma correlação entre viremia e gravidade da doença;

2) Nos casos fatais, 52,1% foram classificados como infecção primária. Portanto, a teoria da infecção seqüencial por si só não explicou todos os casos aqui estudados. Este resultado sugere que as formas graves por DENV-3 podem ter sido conseqüência da patogenicidade ou virulência deste vírus;

3) No grupo de casos não-fatais, observou-se uma viremia significativamente maior em casos classificados como infecção primária. Este resultado sugere uma rápida resposta imune antiviral em casos de infecção secundária, com conseqüente diminuição da viremia.

4) A pesquisa dos vírus dengue em diferentes tecidos provenientes de casos fatais permitiu a detecção do antígeno e/ou do RNA viral em amostras de fígado, pulmão, baço, cérebro, rim, medula óssea e coração, demonstrando a ampla circulação dos DENV-3 no organismo.

5) A positividade pelo RT-PCR em tempo real alcançou 58,4% (45/77), sendo superior a imunohistoquímica 44% (26/59), RT-PCR convencional 30,5% (22/72) e isolamento viral 2,7% (2/74).

6) No estudo de evolução molecular, a árvore filogenética identificou cinco genótipos para os DENV-3, denominados GI-V.

7) A árvore filogenética dos DENV-3 foi caracterizada por uma forte estrutura temporal, com os antigos isolados situados próximos a raiz da árvore e os mais recentes localizados na porção distal.

8) A árvore filogenética dos DENV-3 sugere uma forte subdivisão geográfica, com poucas evidências de co-circulação de genótipos em uma mesma localidade.

9) Não foram observadas diferenças significativas nas médias das taxas de evolução dos três principais genótipos dos DENV-3 [GI ($8,3x10^{-4}$ substituições/sítio/ano), GII ($10.1x10^{-4}$ substituições/sítio/ano) e GIII ($8.1x10^{-4}$ substituições/sítio/ano)].

10) A taxa de evolução estimada para o DENV-3 foi de $8.7x10^{-4}$ ($7.7x10^{-4}$ – $9.7x10^{-4}$) substituições/sítio/ano.

11) O tempo estimado para o mais recente ancestral comum (Tmrca) dos DENV-3 foi de 1891 (1876 – 1904).

12) A taxa de evolução viral em países que sofreram epidemias de dengue desde a década de 70 como Indonésia ($8.4x10^{-4}$ substituições/sítio/ano) e Thailândia ($9.8x10^{-4}$ substituições/sítio/ano) foi similar a de países que sofreram epidemias de dengue desde a década de 90 [América ($9.2x10^{-4}$ substituições/sítio/ano)].

13) As análises dos padrões de migração dos DENV-3 sugerem que a propagação do GI foi direcionada principalmente para a porção marítima do Sudeste Asiático (Timor Leste, Malásia e Filipinas) e Sul do Pacífico, onde a maioria dos vírus parece ter sido originada da Indonésia.

14) A maioria das linhagens do GII parece ter sido proveniente da Tailândia e permaneceu dentro de zonas continentais no Sudeste Asiático (Bangladesh, Mianmar, Singapura, e Vietnã), com exceção da Malásia.

15) O GIII atingiu o maior território dos DENV-3, com circulação na Ásia, África e nas Américas, parecendo ter sido originado da Ásia (Sri Lanka).

16) Os dados de migração do GIII sugerem que a introdução desta linhagem nas Américas tenha sido pelo México.

17) O GIII rapidamente foi disseminado nas Américas utilizando várias rotas em direção a América Central, Caribe e América do Sul.

18) Nossa análise filogeográfica revelou que a ocorrência da co-circulação de diferentes genótipos de DENV-3 em uma única localidade é um evento raro. Isto pode ser resultado de um fluxo genético limitado entre regiões geográficas distintas.

19) Um mesmo genótipo foi observado em países vizinhos como a Indonésia e Tailândia, onde o DENV-3 causa epidemias desde a década de 70, causadas pelos GI e GII, respectivamente.

20) Foi observada a co-circulação dos GI e GII na Malásia, Tailândia e Indonésia, GII e GIII em Singapura e GI/GIII em Taiwan. No entanto, a ocorrência destas co-circulações parece não ter se estabelecido.

21) A distância genética observada entre as amostras brasileiras classificadas como GV (no período de 2002 a 2004) e o vírus protótipo isolado nas Filipinas em 1956 foi de 0,3%. Esta distância é significativamente baixa considerando a taxa de evolução dos DENV-3 ($8.7x10^{-4}$ substituições/sítio/ano).

6. PERSPECTIVAS

No contexto da aplicação do RT-PCR em tempo real para a quantificação dos vírus dengue, diferentes protocolos precisam ainda ser avaliados e desenvolvidos. Um protocolo que detecte e quantifique os quatro sorotipos com alta sensibilidade, ainda é um desafio.

Como demonstrado neste estudo, esta abordagem é especialmente útil para correlacionar a viremia e a gravidade da doença, o que contribui para as questões relacionadas à compreensão da patogenia desta doença. Nesse sentido, pode-se aplicar este modelo para os DENV-1 e DENV-2 em diferentes epidemias.

Um aspecto interessante será avaliar a viremia dos DENV por dia de doença em diferentes grupos: dengue clássico e dengue grave, a partir da aplicação do RT-PCR em tempo real.

No aprimoramento da técnica de RT-PCR em tempo real, a avaliação dos sistemas TaqMan e Sybr Green para diminuir os custos da reação é outra questão relevante.

Outro estudo importante a ser realizado será compreender a dinâmica de circulação dos DENV no Brasil. Para este fim, torna-se necessário a união de diferentes grupos de pesquisa do país na execução de um projeto multidisciplinar de caracterização genética, inicialmente com o sequenciamento do gene E.

Como demonstrado neste estudo, o gene E é uma região informativa. Durante o desenvolvimento desta tese, foram iniciadas várias atividades nesse sentido, primeiro com o desenho de oligonucleotídeos iniciadores para o sequenciamento completo dos DENV1-3 (Tabelas 6.1, 6.2 e 6.3), no treinamento de pesquisadores de outros Estados nas técnicas de sequenciamento e análise filogenética dos DENV e no estabelecimento de colaborações com vários Estados brasileiros.

Como resultado, esperamos em curto prazo, determinar os padrões de migração dos DENV no Brasil. Em contrapartida, a caracterização genética a partir do sequenciamento completo do genoma dos DENV abre caminhos para a pesquisa de marcadores genéticos de virulência e outros estudos de evolução.

A implantação e o desenvolvimento de métodos para a pesquisa de novos Flavivírus circulantes no país representam um passo importante considerando a circulação do vírus do Nilo Ocidental em países visinhos como a Argentina. O isolamento do vírus São Luiz no estado de São Paulo alerta a necessidade da pesquisa deste agente etiológico no Rio de Janeiro e em outros estados brasileiros. No futuro, a pesquisa de outros vírus do gênero *Alphavirus*, como o Chikungunya, também será de grande importância.

Tabela 6.1: Oligonucleotídeos para o sequenciamento completo do genoma dos DENV-1, de acordo com seqüência obtida a partir de um isolado brasileiro (nº acesso no GenBank AF513110). Os oligos foram desenhados para amplificar ~900pb/região, com ~200pb de sobreposição entre regiões.

Região	Primer sense A (5'- 3')	Primer antisense B (5'- 3')	Posição do genoma (de acordo com AF513110)	Produto (pb)	Tm (ºC) 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	8608 – 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

Tabela 6.2: Oligonucleotídeos para o sequenciamento completo do genoma dos DENV-2, de acordo com seqüência obtida a partir de um isolado brasileiro (nº acesso no GenBank AF489932). Os oligos foram desenhados para amplificar ~900pb/região, com ~200pb de sobreposição entre regiões.

Região	Primer sense A (5'- 3')	Primer antisense B (5'- 3')	Posição do genoma (de acordo com AF489932)	Produto (pb)	Tm (ºC) 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 GTA GCC ATG GTA AC	708 – 1586	878	62/62
3	ATG GCA CTG TCA CGA TGG AG	CAC TAT CAG CCT GCA CCA TAG CT	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 TTC CTA 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

Tabela 6.3: Oligonucleotídeos para o sequenciamento completo do genoma dos DENV-3, de acordo com seqüência obtida a partir de um isolado brasileiro (nº acesso no GenBank AY679147). Os oligos foram desenhados para amplificar ~900pb/região, com ~200pb de sobreposição entre regiões.

Região	Primer sense A (5'- 3')	Primer antisense B (5'- 3')	Posição do genoma (de acordo com AY679147)	Produto (pb)	Tm (ºC) A/B
1	GTT GTT AAT CTA CGT GGA CCG ACA	ACC ACC TTC TGG GTC AAG GAT	2 – 874	872	62/62
2	GAT CAG TGG CGT TAG CTC CC	TCC TTC CTG TTC CAG GTT GG	707 – 1613	907	62/62
3	TGC CTG AAT ATG GAA CCC TTG	CTT GCA CCA CAG CTC CCA G	1452 – 2393	941	62/63
4	TGG TGT TCT GAA CTC ATT AGG CA	GGC CAT GTG CAG GTT TTC AC	2206 – 3071	865	62/64
5	TGA CCA TAG GCT AAT GTC GGC	GAG CGA TTC CAT TTG CCA TT	2950 – 3835	885	62/62
6	GGG AAT GGG CGT TAC CTA CC	CCC ATA GAA CGC CGG ATC TT	3673 – 4510	837	63/63
7	GGA GGA AGA GGC TGA GCA AA	TCT TCC ATC TCA GCT GCA ACC	4305 – 5194	889	62/63
8	GAA TAG CGC AAA CGA ATG CA	TGA CTG GCA TTG GTC CAG C	5004 – 5846	842	62/63
9	GCA TTA AAG CCG GAA ATG ACA	CGC CAT GTT CTG ACG TAT GC	5607 – 6463	856	62/63
10	CCC GCA CTT ATT CAG ATC CC	TTC TGA GCT TCA CGA GTG GCT	6309 – 7204	895	62/63
11	CTA TAG CCA ACC AGG CAG TGG	CTG GTT CTT CGT GTC CTG GAC	7020 – 7884	864	62/62
12	ATG CCG TGT CCA GAG GTA GC	GCT GGC CAA ATG GAG TTG TAT C	7722 – 8594	872	63/63
13	CAA CAT GGC ACT ATG ATG ACG A	TCC ACT GCC TCT TTG GTC TTT C	8430 – 9346	916	62/63
14	CAG CTG GTT GGG ACA CAA GA	TCC ACT GAT GGT GAG CAT GAA	9165 – 9954	789	62/63
15	TGG AGC CTT AGA GAA ACC GC	TTC TGT GCC TGG AAT GAT GCT	9790 - 10647	857	62/63

7. CONSIDERAÇÕES SOBRE OS ASPECTOS ÉTICOS

O presente estudo utiliza a casuística do Centro de Referência de Dengue e aprovado pelo Comitê de Ética em Pesquisa-CEP/FIOCRUZ. Protocolo CEP: 274/05 (anexo).

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7. ANEXOS

Neste item estão incluidos os trabalhos publicados desta tese e os trabalhos publicados como co-autor.

Concurrent infection with dengue virus type-2 and DENV-3 in a patient from Ceará, Brazil

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Dengue outbreaks have occurred in several regions in Brazil and cocirculating dengue virus type 1 (DENV-1), DENV-2, and DENV-3 have been frequently observed. Dual infection by DENV-2 and DENV-3 was identified by type-specific indirect immunofluorescence assay and confirmed by reverse transcription polymerase chain reaction in a patient in Ceará with a mild disease. This is the first documented case of simultaneous infection with DENV-2 and DENV-3 in Brazil. Sequencing confirmed DENV-2 and DENV-3 (South-East/American) genotype III and (SriLanka/India), genotype III respectively.

Key words: dengue - concurrent infection - DEN2 - DENV-3 - Ceará - Brazil

Dengue is one of the most important arboviral infections affecting humans, caused by one of the four serotypes of dengue virus (DENV-1, 2, 3, 4). They belong to the genus *Flavivirus*, family *Flaviviridae* (Gubler 1998). The prevalence of this disease has grown in recent decades and is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and the Western Pacific (Chaturvedi et al. 2004). Since 1986, dengue epidemics have been reported in Brazil, initially involving DENV-1 and after 1990, DENV-2. With the introduction of DENV- 3 in 2000, three serotypes are now circulating in Brazil (Nogueira et al. 2005). With the change in the epidemiological pattern that has occurred in approximately 30 years, the Americas, and more recently Brazil, can be considered a hyperendemic region, with multiple cocirculating serotypes. Since 1986, the state of Ceará began reporting dengue transmission with epidemic peaks in 1987, 1990, 1994, and 2001. Only DENV-1 was in circulation until 1994, when DENV-2 was introduced. This serotype caused a large epidemic with 47,789 notified cases, in which 14 deaths have been reported (Souza et al. 1995). In March 2002, DENV-3 was first isolated in Ceará by the Central Laboratory of Public Heath, thus it became an area of high endemicity with three cocirculating serotypes. Interestingly, in spite of the high or hyperendemicity of dengue viruses in some populations, relatively few cases of double infection have been reported (Gubler et al. 1985, Laille et al. 1991, Maneekarn et al. 1993, Sisouk et al. 1995). More recently two cases were reported in Brazil involving DENV-1 DENV-2 (Santos et al. 2003). Simultaneous infection with DENV-2 and DENV-3 was reported by Kanesa-Thasan et al. (1994), by Wang et al. (2003) during an outbreak in Taiwan in 2000, and more recently by Wenming et al. (2005), who confirmed simultaneous infection with dengue 2 and 3 viruses in a Chinese patient returning from Sri Lanka.

A case of concurrent infection due to DENV-2 and DENV-3 was confirmed during an outbreak of dengue in the state of Ceará in the Northeastern region of Brazil in 2003. The patient was a 36 year-old male who lived in Tauá, a town of approximately 52,000 inhabitants, located 337 km far from Fortaleza city, the state capital. The onset of symptoms started on February 2, with high fever, headache, arthralgia, myalgia, retrobulbar pain, and asthenia. No hemorrhagic manifestation and no severe signs were observed, and the patient was fully recovery after one week. A mild disease, in spite of dual infection, has been observed by other authors and does not support the hypothesis that double infection with dengue viruses leads to more severe hemorrhagic disease (Gubler et al. 1985, Santos et al. 2003). Interestingly, DENV-2 and DENV-3 viruses have been well documented in severe cases in Brazil (Nogueira et al. 2001, 2005). The serum was obtained two days after the onset of symptoms and the procedures for virus isolation and virus identification were performed using the protocol previously described by Nogueira et al. (2001) using monoclonal antibodies. Polymerase chain reaction directly from the serum has been suggested in order to avoid selection of a dengue serotype during attempts of virus isolation in tissue culture. The present results show that both serotypes were identified, by the virus isolation process and by molecular method (Fig. 1).

For RNA extraction the supernatant of C6/36 was extracted using a QIAmp Viral Mini Kit (Qiagen, US) according to the manufacturer's protocol. Reverse transcription (RT-PCR) for detecting and typing DENV in acute sera and tissue culture fluid were carried out according to

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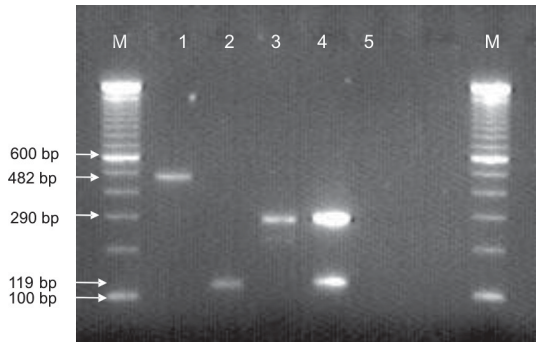

Fig. 1: M: 100-bp, ladder (Gibco). Lanes - 1: DENV-1 positive control; 2: DENV-2 positive control; 3: line DENV-3 positive control; 4: patient serum showing dual infection with DENV-3 and DENV-2; 5: negative control.

Lanciotti et al. (1992). Primers for sequencing DENV-2 and DENV-3 were performed according to Rico-Hesse (1990) and Lanciotti et al. (1994), respectively.

Fig. 1 shows gel exhibiting two bands corresponding DENV-2 and DENV-3 viruses. Figs 2 and 3 show the phylogenetic analysis of isolates corresponding to DENV-2 E/NSI (Rico-Hesse 1990) and DENV-3 prM/M/E gene regions (Lanciotti et al. 1994). The genotypes found corresponded to genotype III (South-East/Asia) and genotype III (Indian Subcontinent) DENV-2 and DENV-3, respectively.

Vertical transmission is of great epidemiological importance and suggests that vector mosquitoes may play an important role in the maintenance (Castro et al. 2004) of virus in the environment and mosquitoes may act as reservoirs of these viruses (Joshi et al. 2006). Laboratory data demonstrated that the mosquito *Aedes aegypti* may be infected with double combinations of different arboviruses and that it is also capable of transmitting these viruses simultaneously. According to Wenming et al. (2005) it is possible to assume that mosquitoes infected with DENV-2 and DENV-3 may transmit them both in areas where two or more serotypes of the virus exists, together with a high prevalence of the vector.

In spite of both serotypes involved in this case can lead to severe form of dengue in Brazil, a dual infection did not caused a dengue severe clinical presentation in this patient. Understanding of the DHF pathogenesis is

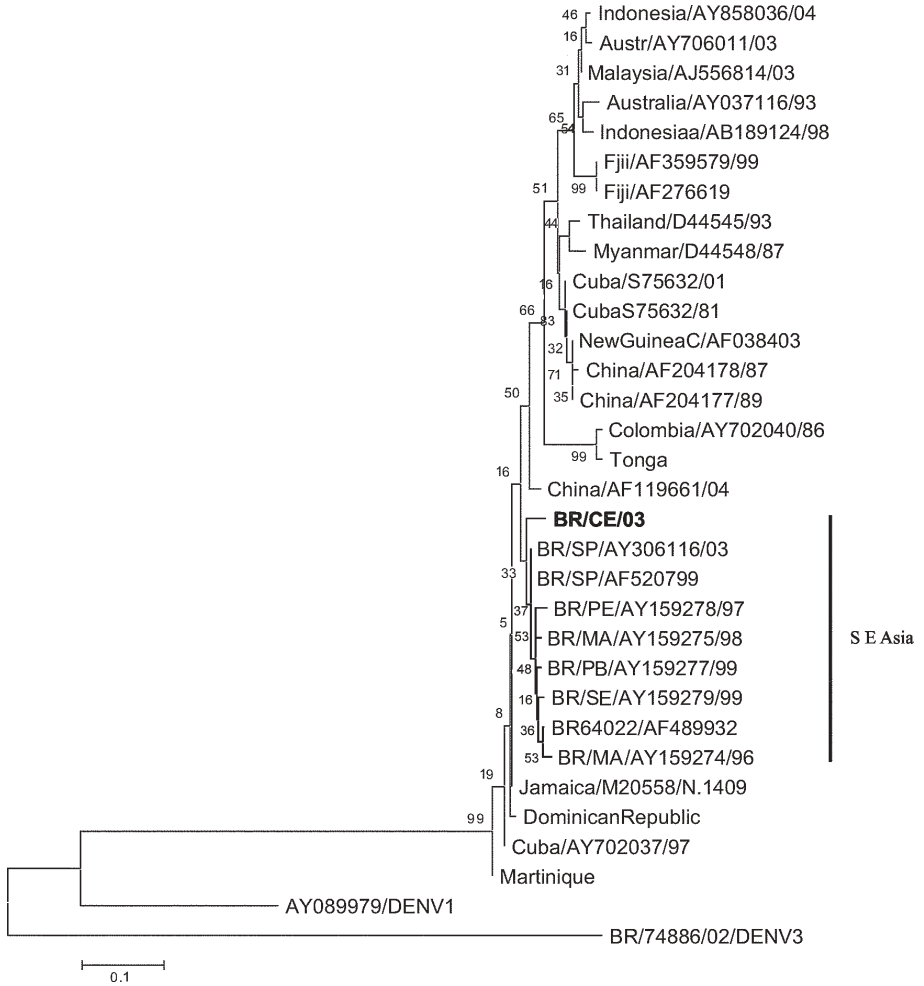

Fig. 2: phylogenetic analysis of 30 dengue viruses type 2 using 240 nucleotides from the E/NS1 junction. Representatives of the other serotypes were used as an out group; 1000 bootstrap replications were used to estimate the reliability of the predicted tree.

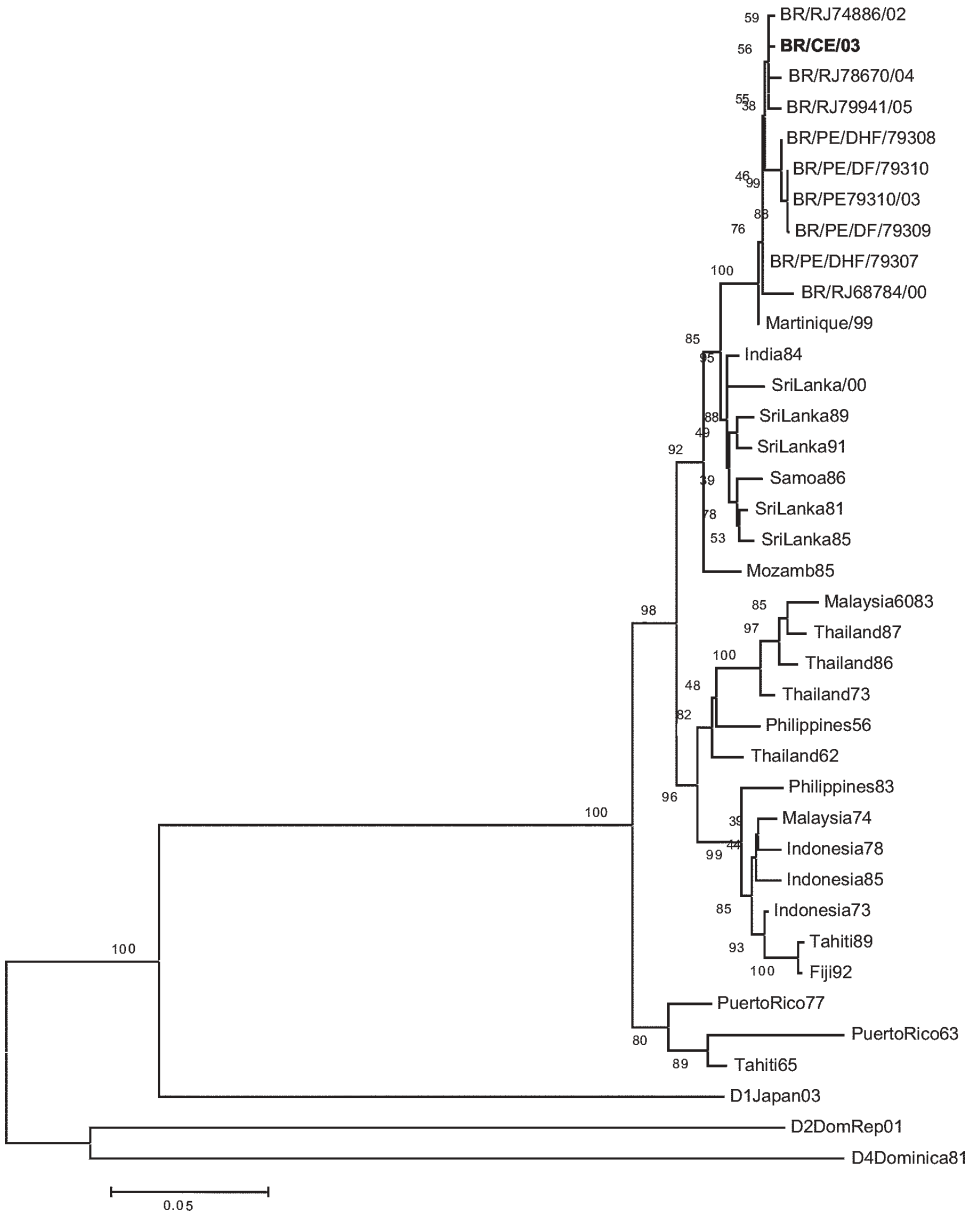

Fig. 3: phylogenetic analysis of 35 dengue viruses type 3 using 1976 nucleotides from the E gene. Representative of the other serotypes were used as an out group; 1000 bootstrap replications were used to estimate the reliability of the predicted tree.

not complete so far and two distinct hypotheses to explain the mechanism of DHF have been suggested. Immune responses and viral virulence have been considered as two major factors responsible for the pathogenesis (Kurane 2001). Serological tests showed a primary infection in this case; therefore it is plausible to admit that this case should be classified as dengue fever, which represents the majority of dengue infection.

This is the first documented case of dual infection with DENV-2 and DENV-3 in Brazil.

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Dengue viruses in Brazil, 1986–2006

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ABSTRACT *A total of 4 243 049 dengue cases have been reported in Brazil between 1981 and 2006, including 5 817 cases of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) and a total of 338 fatal cases. Although all Brazilian regions have been affected, the Northeast and Southeast regions have registered the highest number of notifications. DENV-1 and DENV-4 were isolated for the first time in the Amazon region of Brazil in 1981 and 1982. The disease became a nationwide public health problem following outbreaks of DENV-1 and DENV-2 in the state of Rio de Janeiro in 1986 and 1990, respectively. The introduction of DENV-3 in 2000, also in the state of Rio de Janeiro, led to a severe epidemic with 288 245 reported dengue cases, including 91 deaths. Virus strains that were typed during the 2002 epidemic show that DENV-3 has displaced other dengue virus serotypes and entered new areas, a finding that warrants closer evaluation.*

Unusual clinical symptoms, including central nervous system involvement, have been observed in dengue patients in at least three regions of the country.

Key words Dengue, dengue/epidemiology, dengue virus/classification, diagnosis, genome, Brazil.

The high level of dengue virus activity on the American continent and the reinfestation of Brazil by the *Aedes aegypti* vector in 1977 contributed to the reintroduction of the dengue viruses (DENV) into Brazil in the 1980s (1, 2). Since that time, more than 60% of the reported cases of dengue in the Region of the Americas have occurred in Brazil (Figure 1). In this article, we describe the introduction of dengue viruses in Brazil, the virus strains, and spread of the disease to different regions of the country.

A dengue outbreak caused by the DENV-1 and DENV-4 viruses (1981–1982) occurred in the city of Boa Vista, in the state of Roraima, in the Amazon region close to the Venezuelan border (3). This outbreak was contained by local vector control measures and no dengue activity was reported for the next four years in Brazil. It was only after 1986, with the DENV-1 virus introduction into the state of Rio de Janeiro (4), that dengue infections became a nationwide public health problem. Difficulties implementing effective vector control programs in large urban communities resulted in the rapid spread of the virus and explosive virgin soil epidemics in several states. The situation was aggravated in 1990 by the introduction of DENV-2 virus, also into the state of Rio de Janeiro (5). With its subsequent spread to other regions of the country, there were more severe clinical presentations and the first fatal cases due to secondary infections.

Absent from the Americas for almost 15 years, DENV-3 was reintroduced in 1994 (6), reaching Brazil by 2000 where it caused a widespread and severe dengue epidemic in the summer of 2001–2002 (7–9).

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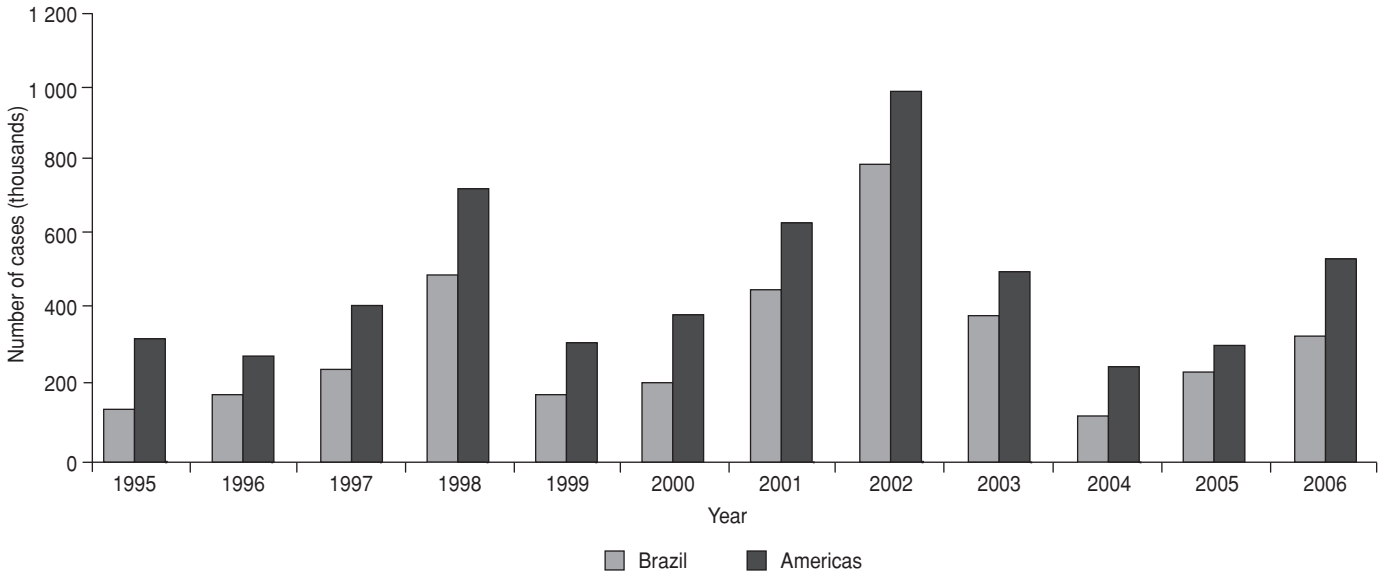
FIGURE 1. Reported dengue cases in Brazil and the Region of the Americas, 1995–2006



Note: Figures were compiled from information provided by the Ministry of Health, Brazil (available at http://portal.saude.gov.br/portal/arquivos/pdf) and the PAHO Health Surveillance and Disease Management Web site (available at: http://www.paho.org/english/ad/dpc/cd/dengue.htm).

The three dengue virus serotypes spread successively throughout the country and by the end of 2006, 25 of the 27 Brazilian states had reported dengue epidemics. A total of 4 243 049 reported cases in the last 20 years in Brazil were caused by DENV-1, DENV-2, and DENV-3 circulation (10).

Dengue in the State of Rio de Janeiro

The state of Rio de Janeiro was the site of the introduction and dissemination of DENV-3, a new serotype in the country, as well as DENV-1 and DENV-2, making it obvious that this area is important for dengue epidemiology in Brazil. As an important tourist center with high levels of *Ae. aegypti* infestation, the area merits close attention in terms of entry of dengue viruses into Brazil.

Dengue infection was first confirmed in the state of Rio de Janeiro by the Flavivirus Laboratory at the Oswaldo Cruz Institute in April 1986. The DENV-1 virus was isolated from patients presenting with exanthematic disease during an explosive epidemic in the municipality of Nova Iguaçu (4). This municipality belongs to the Greater Metropolitan Area of the state, which includes the capital Rio de Janeiro and 20 other municipalities, and has a population of over 11 million out of the state's 14.7 million total. Nova Iguaçu is located about 25 km from downtown Rio de Janeiro, with a large population that commutes daily to surrounding areas. This heavy circulation of people facilitated the rapid spread of dengue virus to susceptible populations, causing an epidemic of approximately 92 000 cases reported during the 1986–1987 period (11). The infections were clinically characterized as classic dengue fever. The only fatality reported as a result of this episode was that of a young patient.

From Rio de Janeiro, the virus spread rapidly along the coast to different states, all previously infested with the vector. Epidemics in these areas presented similar characteristics to that of Rio de Janeiro: only DENV-1 was involved and no severe cases of dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) were reported.

By April 1990, an active surveillance program established by the municipality of Niterói in the Rio de Janeiro Greater Metropolitan Area allowed for early identification of DENV-2 during a period of high DENV-1 virus activity, exactly four years after the first DENV-1 strain isolations. The epidemic in 1990–1991 presented two waves and a significantly higher proportion of patients with thrombocytopenia and clinical presentations requiring hospitalization were seen in the period when DENV-2 was predominant (5).

Both the DENV-1 and DENV-2 viruses were isolated during an epidemic recognized in 1995–1996, with a total of 51 465 reported cases of dengue fever. In January 1998 a new epidemic occurred in the Paraíba river valley, in the western part of the state of Rio de Janeiro and quickly spread to other municipalities, including an important tourist area on the northern coast (11). DENV-2 spread from Rio de Janeiro to different parts of the country, showing more severe clinical presentations in comparison with the previous DENV-1 outbreaks.

Because of the epidemiological importance of the state of Rio de Janeiro, a virological surveillance program was mounted in the city of Nova Iguaçu over the years, including during periods between epidemics. This made it possible to isolate a strain of the DENV-3 virus in 2000 from a case of classic dengue fever as well as from the vector *Ae. aegypti* collected in the field (7, 8, 12).

DENV-3 virus introduction increased the number of reported cases to 69 269 in 2001 and during the summer of 2002, DENV-3 caused the most severe epidemic so far observed in the state of Rio de Janeiro (8, 9). The number of cases in 2001–2002 exceeded the epidemic of 1990–1991, when about 100 000 cases with 462 DHF/DSS cases and 8 deaths were reported. In the DENV-3 epidemic in the summer of 2002, a total of 288 245 cases were reported, with 1 831 cases of DHF/DSS and 91 deaths, corresponding to 1 735 reported cases per 100 000 inhabitants (9). The highest notification of cases was in the Greater Metropolitan Area of the state.

Laboratory studies carried out in 2000/2001 on 1 478 reported dengue cases confirmed a 54.5% infection rate by serology and/or virus isolation and polymerase chain reaction (RT-PCR) (8). Three DENV-1, one DENV-2, and 320 DENV-3 strains were detected, revealing that DENV-3 represented 98.7% of the circulating viruses during the 2002 epidemic in the state of Rio de Janeiro. Forty fatal cases were confirmed for DENV-3 by our institution and at least two different laboratory methodologies were used on 20 of these cases. DENV-3 was the only serotype detected in these fatal cases and virtually all of them were primary infections by DENV-3 (9).

Dengue viruses in other Brazilian states

The southeastern and northeastern regions of Brazil have been the most affected by dengue infections, with epidemics occurring almost yearly. In the southeastern region, besides Rio de Janeiro, the states of Minas Gerais and Espírito Santo have reported epidemics in both the state capitals and inland municipalities. In the state of São Paulo, dengue virus activity was mainly observed in inland municipalities, with some severe epidemics and sporadic activity in coastal locations.

The northeastern region, an important tourist area comprising seven states along the coast, has suffered successive dengue epidemics and was responsible for the highest number of dengue notifications during the late 1990s. In 1994, the state of Ceará reported an epidemic with 47 889 cases, including 25 cases of DHF and 12 deaths. An increase in DHF was observed in 2003, when 23 796 dengue cases were reported, including 291 DHF cases and 20 deaths. This scenario was also observed in 2005 with 22 817 cases reported, including 195 DHF cases and 20 deaths (13–16).

The midwestern region, which includes the Federal District and the nation's capital Brasília, confirmed DENV-1 circulation in 1990. In 1995 DENV-2 was isolated and one case of dual infection was reported (17).

Dengue epidemics caused by DENV-2 occurred in the state of Tocantins in 1991 (18) and in the state of Pará in 1995 (19), both in Brazil's northern region. The state of Roraima confirmed dengue activity in 1996, 14 years after the first outbreak occurred in that area. In 1998, the state of Amazonas reported a dengue epidemic with 23 910 cases. In 2001, all the states in the Amazonas region had epidemics of different magnitudes (10), including Acre and Amapá, confirming the expansion of *Ae. aegypti* in the Amazon basin.

In the southern region, the state of Paraná is the only one that has reported dengue since 1995. No autochthonous cases were reported in 2006 in the states of Santa Catarina or Rio Grande do Sul (10).

It should be emphasized that, according to available epidemiological data, dengue infections in the country are found in all age groups in the period studied, with no predominance in children.

Genetic characterization of dengue viruses

The co-circulation of DENV-1 and DENV-2 in Brazil began in 1990 with the subsequent appearance of DHF/DSS and fatal cases (Figure 2). This occurred initially in Rio de Janeiro and later in other states (5, 20–22). An increase in the number of more severe cases in Brazil, similar to other countries of the Americas, coincided with the introduction of the DENV-2 Southeast Asian genotype into the continent (23).

Analysis by genome sequencing performed on DENV-1 and DENV-2 isolated in Brazil identified genotypes from the Americas and Southeast Asia of DENV-1 and DENV-2, respectively (24–26). The complete nucleotide sequence analysis in our laboratory of one Brazilian DENV-2 isolated in 1998 confirmed that the Southeast Asian genotype III is circulating in Brazil (27).

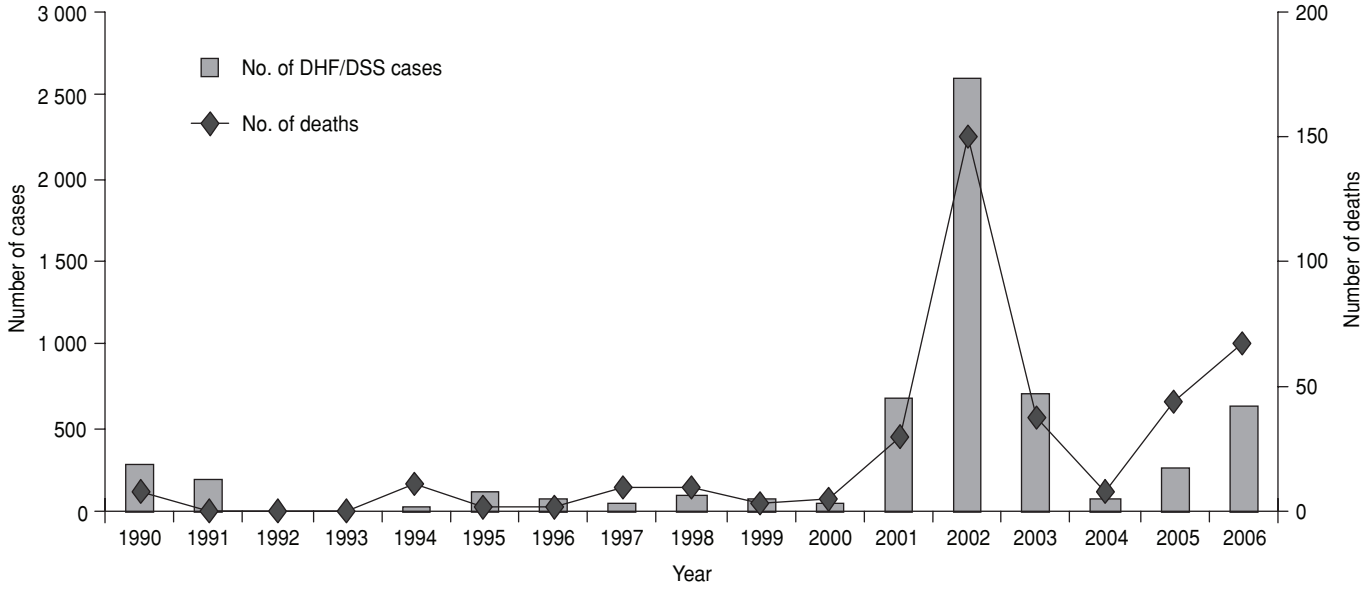
The DENV-3 genotype introduced into the continent has been associated with major DHF/DSS epidemics in Sri Lanka and India and with DHF/DSS cases and deaths in Mexico and Central American countries (28, 29). DENV-3 strains isolated in Rio de Janeiro in 2000, 2001, 2002, and 2003 have been sequenced by our group and in one strain the complete genome was detailed (GenBank accession no. AY679147), confirming that this strain corresponds to genotype III of the Indian subcontinent (30–32).

The data on dengue strain typing after introduction of DENV-3 show that this genotype dislodged other serotypes when first introduced into the area, showing its high infection capacity in both humans and vectors. These data however, need more careful evaluation and comparison with epidemiological data from other countries in the Region of the Americas.

Clinical symptoms

Besides the usual symptoms of dengue observed in the cases of DHF/DSS, unusual manifestations involving the central nervous system were

FIGURE 2: Number of DHF/DSS cases and deaths resulting from dengue infections reported in Brazil, 1990–2006



Source: Ministry of Health, Brazil, http://portal.saude.gov.br/portal/arquivos/pdf

reported during the 1986–1987 epidemic in Rio de Janeiro and later in different states, including one case in which the dengue antigen was detected in neuronal cells by immunohistochemistry (33–36). Neurological manifestations in 41 patients were reported in the state of Pernambuco between March and July 1997 and February and May 2002. The brain was involved in 5 of 7 of these cases in 1997 and 20 of 34 cases in 2002; the spinal cord was involved in 2 of 34 cases in 2002; peripheral nerves were involved in 2 of 7 cases in 1997 and 12 of 34 cases in 2002. Cerebral hemorrhage and acute disseminated encephalomyelitis were also diagnosed (16, 37).

In the state of Rondonia, acute encephalomyelitis was observed in 51 patients presenting signs and symptoms of dengue from November 2004 to March 2005. Anti-dengue IgM antibodies were detected in sera, and one patient, in whom the RT-PCR test detected DENV-3, presented IgM in cerebrospinal fluid (38, 39).

Dengue cases with high levels of serum aminotransferases have also been observed. Yellow fever infections were occasionally suspected but not confirmed by epidemiologic investigations and laboratory results.

During the DENV-3 epidemic in Rio de Janeiro in 2002, viral RNA was detected in the cerebrospinal fluid, liver, brain, lung, spleen, and kidneys of fatal cases by RT-PCR (9).

CONCLUSIONS

Dengue virus activity in Brazil during the past 20 years is demonstrated by the high number of reported cases and states involved in epidemics. The co-circulation of three dengue serotypes is responsible for the increased occurrence of severe forms of the disease, such as DHF/DSS.

The presence of *Ae. aegypti* in about 80% of the country and the difficulties of implementing successful vector control are well-known in Brazil and in many other countries in the region. Barriers to adequate vector control include poor urban planning, demographic pressure, and the shortage of vector surveillance and personnel able to cover huge geographic areas.

While vector control continues to be difficult, progress has been made in notification of dengue outbreaks in Brazil. A network of laboratories that are capable of diagnosing dengue infections has been established in all states of Brazil, under the supervision of Reference Laboratories at the regional level.

Considering the widespread presence of the disease vector and the lack of a vaccine to prevent dengue infections, constant improvement in the rapid identification and correct clinical management of dengue cases by health services is crucial in order to reduce the impact of the disease and the number of fatal cases.

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Virus del dengue en Brasil, 1986–2006

RESUMEN

En Brasil se han notificado 4 243 049 casos de dengue entre 1981 y 2006, de ellos 5 817 casos de dengue hemorrágico/síndrome de choque por dengue (DH/SCD) y un total de 338 casos mortales. A pesar de que la enfermedad ha afectado a todas las regiones brasileñas, el mayor número de casos se ha notificado en las regiones nororiental y suroriental. Los virus del dengue (DENV) 1 y 4 se aislaron por primera vez en la región amazónica de Brasil en 1981 y 1982. La enfermedad se convirtió en un problema nacional de salud pública después de los brotes de DENV-1 y DENV-2 en el Estado de Río de Janeiro en 1986 y 1990, respectivamente. La introducción del DENV-3 en 2000, también en el Estado de Río de Janeiro, llevó a una grave epidemia con 288 245 casos notificados de dengue y 91 muertes. Las cepas del virus identificadas durante la epidemia de 2002 demostraron que el DENV-3 ha desplazado a los otros serotipos y se ha expandido a nuevas zonas, algo que merece una evaluación más profunda. En los pacientes con dengue de al menos tres regiones del país se han observado síntomas clínicos atípicos, entre ellos alteraciones del sistema nervioso central.

Palabras clave Dengue, dengue/epidemiología, virus del dengue, diagnóstico, genoma viral, Brasil.

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ASPECTS OF DENGUE VIRUS INFECTIONS IN BRAZIL 1986-2007

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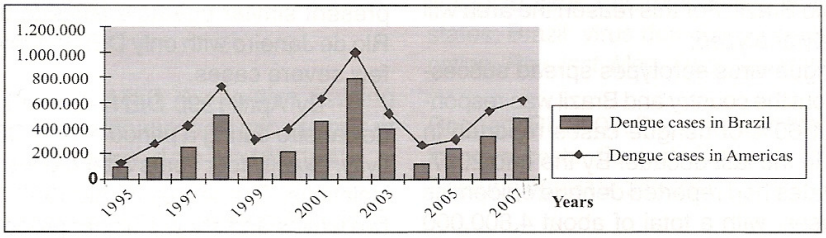
dengue, epidemiology, dengue diagnosis, genome, Brazil

ABSTRACT

Dengue infections were reported and confirmed for the first time at laboratory level, in the northern Region of Brazil in 1981-1982. DENV-1 and DENV-4 were isolated in this outbreak, which was controlled by vector reduction. However following the reinfestation of the American Region by the vector *Aedes aegypti*, the disease spread nationwide after the epidemics that occurred in the state of Rio de Janeiro caused by DENV-1, DENV-2 and DENV-3 in 1986, 1990 and 2000, respectively. The introduction of DENV-3 led to a severe epidemic in 2002, with 288,245 reported dengue cases, including 91 deaths, mostly primary infections. A total of 4,243,049 dengue cases were reported in Brazil up to 2006, including 5,817 cases of DHF/DSS and a total of 338 fatal cases. Provisional data for 2007, collected by December, show about 500,000 reported cases, with 1,076 cases of dengue hemorrhagic fever and 121 deaths. Data on the typing of isolated dengue virus strains after 2002, show that DENV-3 has been able to dislodge the other dengue virus serotypes entering new areas, confirming a high infection capacity in both humans and vectors. DENV-3 predominate until 2005 but later DENV-1 and DENV-2 returned in different parts of the country. Non usual clinical symptoms, like central nervous system involvement have been observed as well as an increasing number of small children among the patients.

INTRODUCTION

Dengue virus activity on the American continent and the reinfestation of Brazil by *Aedes aegypti* around 1977, contributed to the reintroduction of the dengue viruses (DENV) in the 1980's (Pinheiro & Korber 1997, Gubler 1992). Indeed the country has been responsible in last decades for more than 60% of the total reported cases of dengue in the Americas (Figure 1).



Source: http://www.paho.org/english/ad/dpc/cd/dengue.htm and Ministry of Health, Brazil

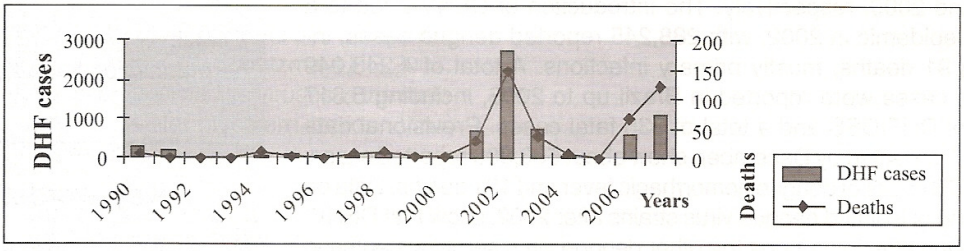
Figure 1: Reported dengue cases in Brazil and Americas

The dengue outbreak due to DENV-1 and DENV-4 viruses (1981-1982) occurred in the city of Boa Vista, in the state of Roraima, close to the Venezuelan border (Osanai et al. 1993).The episode was controlled by local measures of vector control and no dengue activity was reported during the following four years in the country. However after 1986, with the DENV-1 virus introduction into the state of Rio de Janeiro, dengue infections became a nationwide public health problem (Schatzmayr et al. 1986). Poor vector control programs in urban communities resulted in the rapid spread of the virus and consequently the occurrence of explosive epidemics in several states. In 1990 after the introduction of DENV-2 virus, also into the state of Rio de Janeiro (Nogueira et al. 1993a) and its subsequent spread to other regions of the country, more severe clinical presentations and fatal cases were detected (Ministry of Health 2007).

The distribution of the dengue hemorrhagic cases and fatal cases (Figure 2) show a peak in 2002, due to the outbreak in Rio de Janeiro and an increasing number of cases in the last years 2 years, reflecting a tendency of dengue to become an endemic disease in many parts of the country.

Dengue and the state of Rio de Janeiro.

Dengue infections were first confirmed in the area by our laboratory in April 1986, during an explosive epidemic of an exanthematic disease caused only by DENV-1, in the municipality of Nova Iguaçu (Schatzmayr et al. 1986), which belongs to the Greater Metropolitan area of the state, with about 11 mi population, including the city of Rio de Janeiro. Nova Iguaçu is located about 25 km from Rio de Janeiro downtown, with a large population which commutes daily to surrounding areas. In the epidemic DENV-1 spread quickly in the susceptible



Source: Ministry of Health, Brazil.

Figure 2: Number of DHF/DSS cases and deaths reported in Brazil, 1990 / 2007

DENV-3 was reintroduced into the continent in 1994 (Anonimous 1995), reaching Brazil by 2000 when it caused a severe dengue epidemic in Rio de Janeiro city, in summer 2001-2002 (Nogueira et al. 2001, DeSimone et al. 2004, Nogueira et al. 2002).

The Rio de Janeiro area, as an important tourist center with high levels of *Ae. aegypti* infestation, has a crucial importance regarding the entry of dengue viruses into Brazil. For this reason the area will be more closely analyzed.

The dengue virus serotypes spread successively throughout the country and Brazil was responsible for about 60% of dengue cases reported in the Americas in the last decade. By the end 2007, all Brazilian states had reported dengue epidemics or isolated cases, with a total of about 4,800,000 cases in the last 20 years, caused by DENV-1, DENV-2 and DENV-3 (Ministry of Health 2007).

population, causing an epidemic of approximately 92,000 cases, reported during the 1986-1987 period (Miagostovich et al. 1993) but only one fatal case was confirmed.

Causing infections clinically characterized as dengue classical fever, the virus spread rapidly along the Atlantic coast to different states, all infested with the vector. Epidemics in these areas present similar characteristics to the epidemic in Rio de Janeiro with only DENV-1 involved and very few severe cases.

By April 1990 DENV-2 was identified at Rio de Janeiro during a period of high DENV-1 virus activity, exactly four years after the first DENV-1 strain isolations. The new epidemic 1990-1991 presented two waves and show a higher proportion of patients with thrombocytopenia and clinical presentations requiring hospitalization (Nogueira et al. 1993).

DENV-1 and DENV-2 were isolated during a new epidemic recognized in 1995-1996, with a total of 51,465 reported cases of dengue fever. By January 1998 a new epidemic occurred in the Paraíba river valley, in the western part of the state, expanding also to an important tourist area on the northern coast (Nogueira et al. 1993). DENV-2 also spread from Rio de Janeiro to different regions of the country, showing more severe clinical presentations, when compared with previous DENV-1 outbreaks.

A virological surveillance program established by our group in Nova Iguaçu city along the years, resulted in the isolation of a strain of the DENV-3 virus in 2000, from a case of classical dengue fever and also from the vector *Aedes aegypti* collected in the field (Nogueira et al. 2001, Lourenço-de-Oliveira et al. 2002). DENV-3 virus introduction increased the number of reported cases to 69,269 in 2001 and during the summer of 2002 occur the most severe epidemic so far observed in the state of Rio de Janeiro (De Simone et al. 2004). The number of cases exceeded the epidemic of 1990-1991, when about 100,000 cases with 462 DHF/DSS cases and 8 deaths were reported. In the DENV-3 epidemic in summer 2002, a total of 288,245 cases were reported, with 1,831 cases of DHF/DSS and 91 deaths, corresponding to 1,735 reported cases per 100,000 inhabitants (Nogueira et al. 2005).

Three DENV-1, one DENV-2 and 320 DENV-3 strains were detected, revealing that this new serotype represented 98.7% of the circulating viruses during the 2002 epidemic. Forty fatal cases were confirmed by our group and DENV-3 was the only serotype detected in these cases. Virtually all fatal cases were primary infections by DENV-3 (Nogueira et al. 2005).

In the last years, an increase in the numbers of children as dengue patients have observed including severe and fatal cases and these data deserve a closer evaluation.

The dengue situation in other regions of Brazil.

The northeastern region of Brazil, an important tourist area along the Atlantic coast, has suffered successive dengue epidemics and it was responsible for the highest number of dengue notifications during the later 1990´s. The state of Ceará in 1994 reported an epidemic with about 50,000 cases, including 25 cases of DHF and 12 deaths. An increase in DHF was observed in 2003, with 23,796 reported dengue cases, including 291 DHF and 20 deaths. This scenario was also observed in 2005 with 22,817 cases, 195 DHF cases and 20 deaths (Nogueira et al. 1995, Cunha et al. 1998, Cunha et al. 1999, Cordeiro 1997).

In the southeastern region, besides Rio de Janeiro, the states of Minas Gerais São Paulo and Espírito Santo also reported epidemics. In the state of São Paulo, dengue virus activity was mainly observed in inland municipalities, with some severe epidemics and sporadic activity in coastal localities.

The midwestern region, including the Federal District, confirmed DENV-1 circulation in 1990. In 1995 DENV-2 was isolated and one case of dual infection was reported (Rocco et al. 1998).

A dengue epidemic caused by DENV-2 occurred in the state of Tocantins in 1991 (Vasconcellos et al. 1993) and in the state of Pará in 1995 (Travassos da Rosa et al. 2000). Dengue activity was confirmed in 1996 in the state of Roraima, fifteen years after the first outbreak occurred in 1981. In 1998, the state of Amazonas reported a dengue epidemic with 23,910 cases. In 2001 all states in the region were involved in epidemics of variable magnitudes (Ministry of Health 2007), including Acre and Amapá, confirming the expansion of *Ae. aegypti* in the Amazon valley.

In the southern region, the state of Paraná has reported dengue since 1995. No autochthonous cases were reported up to the end of 2006 in the states of Santa Catarina and Rio Grande do Sul but in 2007 dengue epidemics were confirmed in the last state as well as non-epidemic cases in Santa Catarina (Ministry of Health 2007). This new data confirm that dengue infections are now present in all country.

The cocirculation of DENV-1 and DENV-2 in Brazil began in 1990 with the subsequent appearance of DHF/DSS and fatal cases (Figure 2), initially in Rio de Janeiro and later in other states (Zagne et al. 1994, Souza et al. 1995,Vasconcellos et al. 1994). The number of more severe cases in Brazil increases, similarly to other countries of the Americas and was coincident with the introduction of the DENV-2 southeast Asian genotype into the Americas (Rico-Hesse 1997).

Genome sequencing performed on DENV-1 and DENV-2 isolated in Brazil, identified the Caribbean and the Southeast Asian genotypes of DENV-1 and DENV-2, respectively (Rico-Hesse 1990, Deubel et al 1993, Miagostovich et al. 1998). Our laboratory carried on the complete nucleotide sequence analysis in of one Brazilian DENV-2 isolated in 1998 and confirmed that this genotype is the one circulating in Brazil (Santos et al. 2002).

Major DHF/DSS epidemics in Sri Lanka and India and with DHF/DSS cases and deaths in Mexico and Central American countries the DENV-3 has been associated with the DENV-3 genotype introduced into our continent (Lanciotti et al. 1994, Figueroa & Ramos 2000). The DENV-3 strains isolated in Rio de Janeiro in 2000, 2001, 2002 and 2003 have been also sequenced by our group and in one of them the complete genome was detailed (GenBank accession no. AY679147), confirming that this strain corresponds to the genotype III of the Indian subcontinent (Miagostovich et al. 2000, Miagostovich & Nogueira 2004, Miagostovich et al. 2006).

DENV-3, based on the data of dengue strain typing after introduction of this genotype, has been able to dislodge the other serotypes when first introduced into an area. However a more careful evaluation of these data is still needed.

Involvement of the central nervous system in dengue cases were first reported during the 1986-1987 epidemic in Rio de Janeiro and later in different states, including one case when dengue antigen was detected in neuronal cells by immunohistochemistry (Chimelli et al. 1990, Vasconcelos et al. 1998, Miagostovich et al. 1997, Nogueira et al. 2002). In 41 patients neurological manifestations were reported in the state of Pernambuco between March and July 1997 and February and May 2002. The anatomical regions involved were the brain, the spinal cord and peripheral nerves. Cerebral hemorrhage and acute disseminated encephalomyelitis were also diagnosed (Cordeiro 1997, Ferreira et al. 2005).

Acute encephalomyelitis was observed in 51 patients in the state of Rondonia, presenting signs and symptoms of dengue from November 2004 to March 2005. IgM antibodies were detected in sera and one patient presented IgM in cerebrospinal fluid in whom the RT-PCR test detected DENV-3; the same serotype was isolated from the blood (Ministry of Health 2005, Silva Junior & Pimenta Junior 2007).

High levels of serum aminotransferases in dengue cases have also been observed in different areas.

In the DENV-3 epidemic in Rio de Janeiro, viral RNA was detected by RT-PCR in the cerebrospinal fluid, liver, brain, lung, spleen and kidneys of fatal cases (Nogueira et al. 2005).

Dengue virus activity in Brazil during the past 20 years reaches by December 2007, all states of the country. The circulation of three dengue serotypes is certainly responsible for the increased occurrence of more severe forms of the disease. The new data on the increase of children in the dengue patients, at least in the state of Rio de Janeiro, brought the concept that dengue should be already considered an important paediatric disease, which may become of increasing importance in the next future, following the Asian patterns.

The well known difficulties of implementing successful vector control, due to poor urbanization planning, lack of personnel for vector surveillance in huge geographic areas and demographic pressure, have been observed in the country, contributing to the presence of *Ae. aegypti* in about 80% of our municipalities.

It should pointed out that a network of laboratories, capable of diagnosing dengue infections has been implemented in all states, which has been responsible for confirmation of cases in support to the epidemiological surveillance.

The constant improvement of the medical services aiming a rapid and correct handling of dengue cases seems crucial to our country at moment, in order to reduce the impact of the disease and the number of fatal cases.

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SHORT COMMUNICATION

Quantification of dengue virus type 3 RNA in fatal and non-fatal cases in Brazil, 2002

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Summary We examined levels of dengue virus type 3 (DENV-3) RNA in association with the type of infection (primary or secondary) in 42 patients with fatal and non-fatal outcomes in Rio de Janeiro, Brazil, 2002. Subjects with fatal outcomes had mean virus titers significantly higher than those who survived (12.5 vs. 7.9 $\log_{10}$ RNA copies/ml). Because primary infections were confirmed among the fatal cases (52.1%), antibody-dependent enhancement alone did not explain all the cases of severe disease in this study population. These findings suggest that high levels of DENV-3 may have contributed to the severe form of dengue in Rio de Janeiro, 2002.

1. Introduction

The pathogenesis of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) has been one of the major issues in dengue virus research. Among the risk factors reported for DHF and DSS, viral strain and host immune status have been identified as key.[1–3] Based on the observation that individuals experiencing secondary infection had a higher probability of developing DHF and/or DSS, the immune hypothesis states that cross-reactive, non-neutralizing antibodies from previous infection may enhance dengue virus infection.[2] As different dengue viral strains have been associated with outbreaks of very mild or very severe disease, the viral hypothesis contends that severe dengue disease is the result of infection with a more virulent strain.[1,4] For these reasons, we examined levels of dengue virus RNA and the type of infection (primary or secondary) in patients with fatal and non-fatal outcomes in 2002 during a severe DENV-3 epidemic in the State of Rio de Janeiro, Brazil.

2. Materials and methods

The human serum specimens examined in this study were obtained from 42 patients with confirmed dengue virus type

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3 (DENV-3) infection, who presented acute febrile illness with two or more of the following clinical manifestations: headache, retrobulbar pain, myalgia, arthralgia, rash and hemorrhagic manifestations. Informed consent was obtained from patients or from the patients' families. All cases were classified according to WHO definitions.[5] Nineteen cases were outpatients categorized as 'non-fatal cases' [dengue fever (DF)] and the remaining 23 were 'fatal cases' (19 DHF and 4 DSS). Viral RNA for real-time RT-PCR assays was extracted from 140 μl of human serum specimens with the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) in accordance with the manufacturer's suggested protocol. For the quantitative TaqMan assay, a 10-fold-dilution series containing a known amount of target viral RNA (10^7 RNA copies/ml) was used for RNA extraction. Real-time RT-PCR assays were performed as described previously by Houng et al.[6] The number of viral RNA copies detected was calculated by generating a standard curve from 10-fold dilutions of DENV-3 RNA, isolated from a known amount of local virus propagated in *Aedes albopictus* C6/36 cells,[7] the titer of which was determined by plaque assay. In order to classify the type of infection in fatal and non-fatal cases as either primary or secondary dengue, IgG ELISA was conducted as described previously by Miagostovich et al.[8] According to the IgG-ELISA criteria, the immune response is defined as primary when acute-phase serum samples obtained before day 5 of illness have IgG antibody titers <1:160 and convalescent-phase sera have titers <1:40 960. Infections are considered secondary when IgG titers are >1:160 in the acute-phase serum and >1:163 840 in convalescent-phase samples. Parametric (*t* test) and non-parametric (Mann–Whitney test) statistical methods within SPSS for Windows 8 (SPSS Inc., Chicago, IL, USA) were adopted to compare levels of dengue virus RNA in fatal and non-fatal cases; $P < 0.05$ was considered significant.

3. Results and discussion

Adopting quantitative real-time RT-PCR, we examined levels of dengue virus RNA in patients with fatal ($n = 23$) and non-fatal ($n = 19$) outcomes. Subjects with fatal outcomes had mean virus titers significantly higher than those who survived: 12.5 $\log_{10}$ RNA copies/ml and 7.9 $\log_{10}$ RNA copies/ml, respectively (*t* test, $P = 0.001$; Mann–Whitney test; $P = 0.002$) (Figure 1). These results suggest that a high level of DENV is an important factor in the pathogenesis of severe dengue, corroborating several earlier reports.[1,7] Among the fatal cases, 52.2% (12/23) were classified as primary infections, and 30.4% (7/23) as secondary infections. Four cases could not be characterized due to lack of information. Among the non-fatal cases, 47.4% (9/19) were determined as primary infections and 52.6% (10/19) as secondary infections. Because primary infections were confirmed among the fatal cases, antibody-dependent enhancement alone did not explain all the cases of severe disease in this study population.

The correlation between levels of DENV-3 RNA and the type of infection (primary or secondary) was examined. In fatal cases, the mean virus titer was higher in patients with primary infections (12.48 $\log_{10}$ RNA copies/ml) than in patients with secondary infections (11.86 $\log_{10}$ RNA copies/ml); however, this difference was not significant (*t* test, $P = 0.10$; Mann–Whitney test, $P = 0.21$). In non-fatal cases, the mean virus titer was significantly higher in patients with primary infections (9.74 $\log_{10}$ RNA copies/ml) than in patients with secondary infections (6.23 $\log_{10}$ RNA copies/ml) (*t* test, $P = 0.05$; Mann–Whitney test, $P = 0.02$). These results are suggestive of rapid immune response activation in patients classified as secondary infection and consequent reduction of viremia.

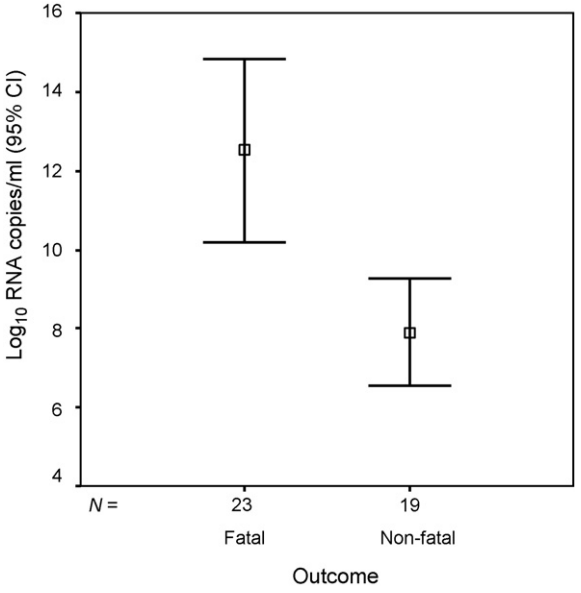

Figure 1 Mean log titer (95% CI) in 23 fatal (dengue hemorrhagic fever and dengue shock syndrome) and 19 non-fatal (dengue fever) cases.

In conclusion, these findings suggest that high levels of DENV-3 may have contributed to the severe form of dengue in Rio de Janeiro, 2002. These results are of great importance for understanding the viral pathogenesis of dengue viruses.

Authors' contributions: JMGA, HGS, AMBF and RMRN designed the study protocol; ESMA carried out the IgG ELISA; JMGA, CB, MAC, LABC and RMRN analyzed the data; JMGA, CB and RMRN drafted the manuscript. All authors contributed to the preparation of the manuscript and read and approved the final version. JMGA and RMRN are guarantors of the paper.

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Conflicts of interest: None declared.

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A retrospective survey of dengue virus infection in fatal cases from an epidemic in Brazil

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A B S T R A C T

Dengue virus can infect many cell types from the vascular, muscular and hematological systems causing diverse clinical and pathological signs. The purpose of the present study was to investigate by different diagnostic methods dengue virus in human tissue specimens obtained from fatal cases ($n = 29$) during a large-scale dengue fever epidemic in 2002 in the State of Rio de Janeiro, Brazil. The combination of four procedures provided diagnostic confirmation of DENV-3 infection in 26 (89.6%) out of the 29 suspected fatal cases. Dengue virus (DENV) was isolated from 2/74 (2.7%) tissue samples, inoculated into C6/36 cells and identified as DENV-3, nested RT-PCR accusing 22/72 (30.5%) samples as DENV-3. Real-time RT-PCR yielded the highest positivity rate, detecting viral RNA in 45/77 (58.4%) clinical specimens, including the liver ($n = 18$), lung ($n = 8$), spleen ($n = 8$), brain ($n = 6$), kidney ($n = 3$), bone marrow ($n = 1$) and heart ($n = 1$). Immunohistochemical tests recognized the DENV antigen in 26/59 (44%) specimens. Given the accuracy and effectiveness of real-time RT-PCR in this investigation, this approach may play an important role for rapid diagnosis of dengue infections.

1. Introduction

Dengue infection is the most prevalent arthropod-borne viral disease in subtropical and tropical regions of the world (Halstead, 1980). All four of the dengue virus serotypes (genus *Flavivirus*, family *Flaviviridae*) consist of a single positive-strand RNA surrounded by an icosahedral nucleocapsid (Hammon et al., 1960). Dengue virus infection causes either a relatively mild disease, known as classic dengue fever (DF) or a more severe form, dengue hemorrhagic fever (DHF), a fulminating illness characterized by hemorrhagic manifestations and plasma leakage, which may progress to dengue shock syndrome (DSS) and death (Halstead, 1988). The virus can infect many cell types from the vascular, muscular and hematological systems causing diverse clinical and pathological signs (Seneviratne et al., 2006).

Studies of specimens from patients presenting DHF/DSS revealed the presence of viral antigens or RNA in diverse tissues including liver, spleen, brain, lymph node, thymus, kidney, lung, heart, bone marrow and skin and mainly in mononuclear phagocytic cells (Bhamarapravati et al., 1967; Bhoopat et al., 1996; Boonpucknavig et al., 1979; Hall et al., 1991; Miagostovich et al., 1997; Yoskan and Bhamarapravati, 1983). The purpose of the present study was to investigate by different diagnostic approaches dengue virus in human tissue specimens from fatal cases during a large-scale dengue fever epidemic in 2002 in the State of Rio de Janeiro, Brazil.

2. Materials and methods

2.1. Study population

The human tissue specimens examined included liver ($n = 28$), lung ($n = 14$), spleen ($n = 16$), brain ($n = 11$), kidney ($n = 5$), bone marrow ($n = 1$) and heart ($n = 2$) obtained from 29 patients presenting with acute febrile illness with two or more of the following clinical manifestations: headache, retrobulbar pain, myalgia, arthralgia, rash and hemorrhage. All samples were received refrigerated and

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separately from private and public hospitals in the metropolitan area of Rio de Janeiro city and stored at −70 °C until tested. Epidemiological aspects and the description of disease evolution resulting in death were reported previously by Nogueira et al. (2005). All samples were collected between January and March 2002 from patients ranging in age from 11 to 64 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.

2.2. Tissue treatment

Fragments of tissue (1–2 g) were ground in 1.5 mL of Leibovitz-15 culture medium (Sigma) and centrifuged (10,000 rpm at 4 °C, for 15 min). The supernatant was inoculated in a C6/36 cell culture and used for RNA extraction.

2.3. Virus isolation

A total of 74 tissue samples were prepared for virus isolation by inoculation into monolayers of a C6/36 *Aedes albopictus* cell line (Igarashi, 1978). Dengue virus isolates were identified by an indirect fluorescent antibody test (IFAT) with serotype-specific monoclonal antibodies, as described previously by Gubler et al. (1984).

2.4. RNA extraction

Viral RNA for the nested RT-PCR and real-time RT-PCR assays was extracted from 140 μL of tissue specimens (supernatant) 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.

2.5. Nested Reverse Transcriptase PCR assay

The nested RT-PCR protocol for DENV detection and typing was performed on 72 tissue samples, as described previously by Lanciotti et al. (1992).

2.6. Real-time Reverse Transcriptase PCR (TaqMan) assay

One-step real-time RT-PCR assays were performed in the ABI Prism® 7000 Sequence Detection System (SDS) (Applied Biosystems, Foster City, CA). Samples were assayed in a 30 μL reaction mixture containing 8.5 μL of extracted RNA, 0.63 μL of 40× Multiscribe enzyme plus RNAse inhibitor, 12.5 μL TaqMan 2× Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and 300 nM of each specific primer and fluorogenic probe. Primer sequences (**DV3.U**: 5′-AGC ACT GAG GGA AGC TGT ACC TCC-3′; **DV.L1**: 5′-CAT TCC ATT TTC TGG CGT TCT-3′) and probe (**DV.P1**: 5′-CTG TCT CCT CAG CAT CAT TCC AGG CA-3′) were obtained from Houng et al. (2001) and designed for the 3′ noncoding sequences (3′NC). The TaqMan probe was labeled at the 5′ end with 5-carboxyfluorescein (FAM) reporter dye and at the 3′ end with 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA) quencher fluorophore. The 5′ nuclease TaqMan assay relies on the 5′ exonuclease activity of the Taq polymerase to free the reporter dye in the quenched probe. The accumulation of the amplified PCR product is proportional to an exponential increase in the fluorescence emitted from the freed reporter dyes (ΔRn). The threshold cycle (Ct) represents the PCR cycle at which the SDS software first detects a noticeable increase in reporter fluorescence above a baseline signal. Positive and negative controls were included in every assay.

Amplification and real-time detection consisted of the following cycling profile: reverse transcription at 45 °C for 30 min followed by one step at 95 °C for 10 min and 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The ABI Prism® 7000 SDS (version 1.1; PE Applied Biosystems) was adopted to examine the fluorescence emitted during amplification. A single fluorescence reading for each sample was taken at the annealing-elongation step.

2.7. Immunohistochemical procedure

The immunohistochemical procedure was undertaken on 59 tissue samples, as described previously by Miagostovich et al. (1997). Briefly, sections of formalin-fixed, paraffin-embedded tissues were processed by the avidin biotin complex (ABC) method according to the manufacturer's protocol (Vectastain AEC Kit, Vector Laboratories, Inc. Burlingame, CA, USA). Monoclonal antibodies for DENV-1, -2, and -3 were directed against the E protein, positive and negative controls were included.

3. Results

The combination of four methods provided diagnostic confirmation of DENV-3 infection in 26 (89.6%) out of the 29 suspected fatal cases. As demonstrated in Table 1, the use of only one diagnostic tool provided positive results in 9/26 cases (34.6%), only one case determined by immunohistochemistry (3.8%), and in 8/26 patients diagnosis was confirmed by real-time RT-PCR (30.8%). In the other 17/26 cases (65.4%), infection was detected by at least two different diagnostic approaches.

A total of 77 clinical samples (tissue specimens from the liver, lung, spleen, brain, kidney, bone marrow and heart) were collected from the 29 patients with fatal outcome. The diagnostic results

Table 1
Investigation of suspected fatal dengue cases ($n = 29$) by virus isolation, nested RT-PCR, immunohistochemistry and real-time RT-PCR.

Case number	Virus isolation	Nested RT-PCR	Immunohistochemistry	Real-time RT-PCR
1	–	+	–	+
2	–	–	–	+
3	–	–	+	+
4	–	–	–	+
5	+	+	NP	+
6	–	–	–	–
7	–	–	NP	+
8	–	–	NP	–
9	–	–	NP	+
10	–	–	+	+
11	–	–	–	+
12	–	–	NP	+
13	–	+	+	+
14	–	+	+	+
15	–	–	–	+
16	–	+	+	+
17	+	+	+	+
18	–	+	+	+
19	–	–	+	–
20	–	–	NP	–
21	–	–	+	+
22	–	–	+	+
23	–	–	+	+
24	–	–	NP	+
25	–	–	+	+
26	–	+	+	+
27	–	+	–	+
28	–	–	+	+
29	–	+	–	+
Total*	2/29 (6.8)	10/29 (34.4)	14/22 (63.6)	25/29 (86.2)

* Positive/total analyzed (%). Positive sample (+), negative sample (–), and not performed (NP).

Table 2
Dengue diagnosis of 77 clinical samples (tissue specimens from liver, lung, spleen, brain, kidney, bone marrow and heart), collected from the 29 patients with fatal outcome, by virus isolation, nested RT-PCR, immunohistochemistry and real-time RT-PCR.

Clinical specimen	Virus isolation positive/total analyzed (%)	Nested RT-PCR positive/total analyzed (%)	Immunohistochemistry positive/total analyzed (%)	Real-time RT-PCR positive/total analyzed (%)
Liver	2/26	8/27	14/22	18/28
Lung	0/14	5/12	3/11	8/14
Spleen	0/15	3/15	5/13	8/16
Brain	0/11	3/11	2/7	6/11
Kidney	0/5	2/5	2/4	3/5
Bone Marrow	0/1	NP	NP	1/1
Heart	0/2	1/2	0/2	1/2
Total	2/74 (2.7)	22/72 (30.5)	26/59 (44.0)	45/77 (58.4)

NP: not performed.

yielded by the four distinct approaches are shown in Table 2. DENV was isolated from two liver specimens (2.7%) out of 74 after inoculation into C6/36 cells and identified as DENV-3. Nested RT-PCR detected 22 (30.5%) out of 72 tissue samples as infected by the same serotype and real-time RT-PCR confirmed the infection in 45 (58.4%) out of the 77 tissue specimens investigated. The immunohistochemical procedures detected viral antigens in 26 (44.0%) out of 59 tissue samples. In all tissues, the immunoreactivity pattern was positive for cytoplasmic granular staining. DENV was observed mainly in hepatocytes and Kupfer cells (liver), the neuronal cell body (brain) and lymphoid cells—especially macrophages (spleen). The cell type could not be identified in lung, kidney, heart and bone marrow tissues. Only the qualitative analysis was applied for real-time RT-PCR and the immunohistochemical procedure. Among all the specimens tested, the liver was the most frequent site for the recovery of DENV isolates, nested RT-PCR, immunohistochemistry and real-time RT-PCR yielding positive results in 24 (92.3%) out of the 26 confirmed DENV-3 cases (Table 3). In this investigation, only one single bone marrow specimen was available (patient 11, Table 3), from which the presence of viral RNA was confirmed by real-time RT-PCR. The same method also determined a positive result in a liver sample from the same patient, reinforcing DENV-3 infection in this case.

4. Discussion

During 2002, a total of 813,104 dengue cases were notified in Brazil, mainly in the southeastern and northeastern regions (Nogueira et al., 2007). This number corresponded to 80% of all the reported dengue cases in the Americas (Nogueira et al., 2007). The introduction of DENV-3 into Rio de Janeiro in 2000 placed the region at high risk of a new epidemic involving this serotype, since the emergence of a new serotype into a susceptible population with high mosquito densities may produce a large-scale epidemic after a lag period (Rigau-Perez et al., 2002). Indeed one year after the appearance DENV-3, this serotype was responsible for the most severe epidemic in the State's history in terms of the number of reported cases, severity of clinical manifestations and the

Table 3
Dengue virus detection regarding tissue samples from 29 suspected fatal cases.

Case number	Liver	Lung	Spleen	Brain	Kidney	Bone marrow	Heart
1	+	+	+	+	+		
2	–	+	–	+			
3	+						
4	+	+					
5	+		–				
6	–	–	–	–	–		
7	+	+		+			
8	–						
9	+	–	–	–			
10	+	+	+	+			
11	+	–				+	
12	+		–	+			
13	+	+	+				
14	+		+		+		+
15	–	–	+	–			
16	+						
17	+	+	+	+			
18	+	+	+	+			
19	+						
20	–						
21	+						
22	+		+				
23	+						
24	+						
25	+						
26	+	+	+		+		+
27	+						
28	+		+				
29	+	+	–	+	+		
Total*	24/29 (82.7)	10/14 (71.4)	10/16 (62.5)	8/11 (72.7)	5/4 (80)	1/1 (100)	2/2 (100)

* Positive/total analyzed (%). Positive sample (+), negative sample (–) and unavailable sample (in white).

number of confirmed deaths. In this DENV-3 epidemic, the number of DHF/dengue shock syndrome cases (1831) and deaths (91) exceeded the total number of DHF/DSS cases (1621) and deaths (76) in the entire country from 1986 to 2001 (Barbosa da Silva et al., 2002). Furthermore, an increase in unusual clinical features was observed during this epidemic characterized by the incidence of Central Nervous System (CNS) involvement and hepatitis (Nogueira et al., 2005).

In this study, the liver was the most important organ for virus detection, according to all four diagnostic methods. Recently, the liver was recognized as a major target organ in the pathogenesis of DENV infection, its active hepatocyte replication perhaps accounting for these findings (Couvelard et al., 1999; Lin et al., 2000). In this study, examination of liver specimens confirmed virus infection in 24 out of 26 fatal cases (Table 3), similar to that described previously in Indonesia (Sumarmo et al., 1983).

Heart involvement has been reported during dengue fever (Basílio-de-Oliveira et al., 2005; Horta Veloso et al., 2003), and in the present study heart involvement was confirmed in two cases by the identification of DENV-3 RNA by nested or real-time RT-PCR. Cardiac manifestations of dengue infection are uncommon, nevertheless cardiac rhythm disorders, such as atrioventricular block (Donegani and Briceño, 1986; Khongphatthallayothin et al., 2000) and ectopic ventricular beats (Chuah, 1987), have been described during episodes of dengue hemorrhagic fever, most of them presenting a benign course with spontaneous resolution. These clinical features have been attributed to viral myocarditis, however the exact mechanism has yet to be elucidated definitively.

Although CNS involvement has been reported previously during dengue epidemics, including those in Brazil (Leao et al., 2002; Nogueira et al., 2002), it increased expressively of this clinical features during the 2002 outbreak when many patients complained of dizziness. In 8 fatal cases of this investigation, CNS involvement was clarified further by the detection of viral antigens or DENV-3 RNA in brain tissue samples (Table 3). Neurological disorders associated with dengue cases have been referred to as dengue encephalopathy, attributed to immunopathological responses and not to CNS viral infection. However, the isolation of DENV-3 and detection of DENV-2 by RT-PCR in cerebrospinal fluid (CSF) provides evidence that DENV possesses neurotropism and can lead to encephalitis in both primary and secondary infections (Lum et al., 1996). The breakdown of the blood–brain barrier has been shown previously in fatal dengue cases (Miagostovich et al., 1997).

The clinical syndrome of dengue-associated bone marrow suppression has been well documented (La Russa and Innis, 1995), and a review of experimental dengue infections of volunteers together with histopathological studies of bone marrow from patients with severe dengue virus infection suggest that marrow suppression evolves rapidly (La Russa and Innis, 1995). The strong link of dengue with neutropenia and thrombocytopenia suggests that bone marrow cells may be potential targets for dengue viral infections. Rothwell et al. (1996) infected long-term marrow cultures with DENV-2 and characterized the viral antigen-positive cells. These investigations demonstrated two types of stromal cells that were positive for DENV-2 antigens by immunofluorescence microscopy and immunohistochemical staining. Taking this into account, dengue virus infection in a bone marrow specimen from a suspected fatal case was investigated by virus isolation and real-time RT-PCR. Positivity in this sample was only confirmed by the molecular diagnostic procedure.

In this study population, examination of available tissue samples of the spleen, kidney and lung gave positive results in 10, 4 and 10 fatal cases, respectively, using either immunohistochemistry or nested and real-time RT-PCR (Table 3). These data confirmed the positivity observed in a retrospective study of tissue specimens from patients with DHF/DSS, by the detection of viral antigens or RNA (Bhamarapravati et al., 1967; Miagostovich et al., 1997).

The application of four distinct assays (virus isolation, nested RT-PCR, immunohistochemistry and real-time RT-PCR) provide a better understanding of viral tropism in fatal cases of dengue virus infection. Real-time RT-PCR provided the best performance and was responsible for 58.4% positivity in the 77 histological samples, while nested RT-PCR gave positive results in 30.5% (22/72). Furthermore, real-time RT-PCR alone was able to establish DENV-3 diagnosis in 8 out of 26 confirmed cases (30.8%). Analysis of the current data is in agreement with the results of another Brazilian study by Poersch et al. (2005), where the investigations compared both dengue diagnostic methods.

In Brazil, dengue virus infections continue to be a formidable public health problem. The greater accuracy, sensitivity and speed of the real-time RT-PCR make it suitable for effective dengue surveillance and indicates its use as a potential complement for the diagnosis of fatal cases.

Conflicts of interest statement

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

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Phylogeography and evolutionary history of dengue virus type 3

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ABSTRACT

In this study, we revisited the phylogeography of the three of major DENV-3 genotypes and estimated its rate of evolution, based on the analysis of the envelope (E) gene of 200 strains isolated from 31 different countries around the world over a time period of 50 years (1956–2006). Our phylogenetic analysis revealed a geographical subdivision of DENV-3 population in several country-specific clades. Migration patterns of the main DENV-3 genotypes showed that genotype I was mainly circumspect to the maritime portion of Southeast-Asia and South Pacific, genotype II stayed within continental areas in South-East Asia, while genotype III spread across Asia, East Africa and into the Americas. No evidence for rampant co-circulation of distinct genotypes in a single locality was found, suggesting that some factors, other than geographic proximity, may limit the continual dispersion and reintroduction of new DENV-3 variants. Estimates of the evolutionary rate revealed no significant differences among major DENV-3 genotypes. The mean evolutionary rate of DENV-3 in areas with long-term endemic transmissions (*i.e.*, Indonesia and Thailand) was similar to that observed in the Americas, which have been experiencing a more recent dengue spread. We estimated the origin of DENV-3 virus around 1890, and the emergence of current diversity of main DENV-3 genotypes between the middle 1960s and the middle 1970s, coinciding with human population growth, urbanization, and massive human movement, and with the description of the first cases of DENV-3 hemorrhagic fever in Asia.

1. Introduction

Dengue virus (DENV) (family *Flaviviridae*) has a single-stranded positive-sense RNA genome of approximately 11 kb and is transmitted by *Aedes aegypti* mosquitoes among humans. Infection with DENV may cause an acute "influenza-like" febrile disease called classic dengue fever (DF), or the potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The four known distinct antigenic groups (*i.e.*, serotypes) of DENV (DENV-1 to DENV-4) contain well-defined phylogenetic clusters (*i.e.*, genotypes) that are causing human pandemics. The incidence, geographic distribution and severity of DENV epidemics have dramatically increased since the mid-1950s, probably due to the rapid increase in human population size, uncontrolled urbanization, and the advent of massive human movement which facilitates the spread and proliferation of mosquitoes and infected people (Gubler, 1998, 2002, 2004; Zanotto et al., 1996). It has been estimated that 50–100 million infections occur annually, and more than 2.5 billion people live in areas of risk for DENV infection (Gubler, 1998, 2002, 2004).

Using a maximum likelihood framework, Twiddy et al. (2003) extended and re-evaluated the first comprehensive estimates of the rate and time frame of DENV evolution (Zanotto et al., 1996). It was inferred that the current global genetic diversity in the four serotypes of DENV appeared around the last 100 years. Moreover, Twiddy et al. (2003) suggested that all serotypes may be evolving according to a molecular clock; but, for reasons that remain unclear, DENV-3 and the DENV-2 American/Asian genotype had significantly higher substitutions rates when compared to other DENV strains. It has been suggested that under epidemic conditions (*i.e.*, when a new variant is introduced into a susceptible population) the viral transmission rate is higher than under endemic conditions, thus increasing the overall diversity and evolutionary rate of the new variant in the population (Twiddy et al., 2003). A higher evolutionary rate could also be a consequence of the emergence of DENV variants with particular

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biological properties, such as increased transmissibility, infectiousness, and/or virulence (Cologna and Rico-Hesse, 2003; Holmes and Twiddy, 2003; Messer et al., 2003; Rico-Hesse, 2003; Rico-Hesse et al., 1997). Alternatively, the lineage-specific rate differences in DENV evolution described by Twiddy et al. could be also caused by the low number of sequences used, particularly for DENV-1 ($n = 9$), DENV-3 ($n = 21$), and DENV-4 ($n = 20$) serotypes.

DENV-3 was isolated for the first time during an epidemic outbreak in Philippines in 1956 (Hammon et al., 1960), and since then several DF/DHF outbreaks caused by this serotype have been described world-wide. Five distinct genotypes of DENV-3 have been identified to date (Lanciotti et al., 1994; Wittke et al., 2002). Genotypes I to III (GI to GIII) are responsible for most DENV-3 infections and have been associated with DF/DHF epidemics in Southeast Asia, Indian subcontinent, South Pacific, East Africa, and the Americas. Genotypes IV and V (GIV and GV) were not associated with DHF epidemics and are only represented by a few early sequences from the Americas, South Pacific, and Asia. Many phylogenetic studies on DENV-3 have documented the viral spread within individual countries (Aquino et al., 2006; Chungue et al., 1993; Diaz et al., 2006; Islam et al., 2006; Kobayashi et al., 1999; Peyrefitte et al., 2003, 2005; Podder et al., 2006; Raekiansyah et al., 2005; Rodriguez-Roche et al., 2005; Usuku et al., 2001; Uzcategui et al., 2003; Wittke et al., 2002; Zhang et al., 2005) or specific regions (Messer et al., 2003), but we still have an incomplete understanding of the global dispersion and evolutionary history of the distinct DENV-3 genotypes.

The objective of the present study was to revise the global phylogeography and evolutionary history of the main DENV-3 genotypes based on the analysis of a large number ($n = 200$) of envelope (E) gene sequences of DENV-3 strains isolated from 31 different countries around the world over a time period of 50 years (1956–2006).

2. Materials and methods

2.1. Sequence datasets

Complete E gene sequences (1479 bp in length) with known date of isolation and representing the full extent of genetic diversity in DENV-3 were collected from GenBank (www.ncbi.nlm.nih.gov). Sequences were excluded from the analysis if they were previously identified as recombinant (Worobey et al., 1999), or were 100% similar to any other strain in the data set. For those genotypes where there were more than 75 sequences available, such as for GII and GIII, a maximum of six sequences randomly chosen from a particular country in a given year were included. This resulted in a final data set of 200 DENV-3 E sequences from 31 countries spanning a 50-year period. This primary dataset was further divided into six subsets comprising sequences from distinct genotypes and geographical origin. Table 1 shows the number, date of isolation range, and origin of the DENV-3 E sequences included in each dataset. Nucleotide sequences were aligned using CLUSTAL X program (Thompson et al., 1997) and later hand edited. All alignments are available from the authors upon request.

Table 1
Sequence datasets.

Dataset	Number of sequences	Date range	Geographic origin
DENV-3	200	1956–2006	Asia, America, South Pacific, and Africa
GI	43	1973–2005	Asia and South Pacific
GI-ID	24	1973–2005	Indonesia
GII	75	1973–2005	Asia
GII-TH	56	1973–2002	Thailand
GIII	75	1981–2006	Asia, America, and Africa
GIII-AM	63	1994–2005	America

2.2. Phylogenetic analysis

A Bayesian phylogenetic tree for the complete data set of 200 DENV-3 E sequences was inferred with MrBayes program (Ronquist and Huelsenbeck, 2003), under a General Time Reversible model of nucleotide substitution (Rodriguez et al., 1990) with gamma-distributed rate variation and a proportion of invariable sites (GTR + Γ + I). Two runs of 4 chains each (one cold and tree heated, temperature = 0.20) were run for 10×10^6 generations, with a burn-in of 2.5×10^6 generations. Convergence was assessed using the average standard deviation in partition frequency values across independent analyses, using a threshold value of 0.01. Convergence of parameters was also confirmed by calculating the Effective Sample Size (ESS) using TRACER v1.4 program (Rambaut and Drummond, 2007), excluding an initial 10% for each run. All parameters estimates showed ESS values >200.

2.3. Migration analysis

To investigate the migratory patterns of DENV-3 we examined each of the three main genotypes separately, since the isolation of viruses from different genotypes at the same locality would sum splits due to cladogenetic and migratory events, causing inferential errors. Geographical origin of each sample was coded as a set of terminal unordered character states for each dengue time-stamped, geo-referenced sample, represented as a single capital letter. The most parsimonious reconstructions (MPRs) sets of changes at each internal state in the viral phylogeny was calculated with PAUP v4.0b10 (Swofford, 2002) and MacClade v4.07 (Maddison and Maddison, 2005) and taken as surrogate for migration events. To help resolve among equally parsimonious reconstructions that leads to ambiguities in character tracing, we used assignments that delay (DELTRAN) or accelerate (ACCTRAN) character transformations (Swofford and Maddison, 1987). The phylogenies used for each genotype came from the global maximum posterior probability (MAP) tree obtained with Bayesian inference (BI) with BEAST v1.4.7 (Drummond et al., 2002; Drummond and Rambaut, 2006) for the entire dataset of 200 DENV-3 E sequences from which, subtrees for each genotype were analyzed in separate. For comparison, phylogenetic trees for each genotype were also inferred with the maximum likelihood (ML) criterion as implemented in the program GARLI v0.95 (Zwickl, 2006) that estimates simultaneously the best topology, branch lengths and the best values for the parameters for the GTR + Γ + I model of nucleotide evolution. Independent random runs were conducted with GARLI and the tree with highest likelihood was subsequently used as input for further optimization in both GARLI and PAUP, since both programs calculate the same likelihood score for a tree under the same model. The topology used for calculating the MPRs was the fully resolved consensus of 100 bootstrap replicates with GARLI.

2.4. Estimation of evolutionary rates and dates

Seven different datasets were used to estimate the evolutionary rate (μ, units are nucleotide substitutions per site per year; subs/site/year), and the time of the most recent common ancestor (T_{mrca}, years) of the viruses sampled. First, we used the

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200 E gene sequences to estimates the overall rate and T_{mrca} for DENV-3. The age of relevant internal nodes, such as those corresponding to the MRCA of distinct genotypes, were also estimated by setting up specific taxon subsets within the global DENV-3 dataset. Second, we analyzed the GI, GII, and GIII datasets to obtain rates and divergence times for individual genotypes separately. Third, separate analyses were also performed on the viruses collected from Indonesia (GI-ID dataset), Thailand (GII-TH dataset), and the Americas (GIII-AM dataset) to estimate rates and divergence times for DENV-3 in these specific localities. Evolutionary parameters were estimated by using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST v1.4.7 (Drummond et al., 2002; Drummond and Rambaut, 2006). Analyses were carried out with a Bayesian Skyline coalescent tree prior (Drummond et al., 2005) under the GTR + Γ + I model, and using both a strict and a relaxed (uncorrelated lognormal) (Drummond et al., 2006) molecular clock. MCMC chains were run for 1–3 × 10^7 generations for each data set, with a burn-in of 1–3 × 10^6. BEAST outputs were inspected with TRACER v1.4, with uncertainty in parameter estimates reflected by their 95% Highest Posterior Density (HPD) values. All parameters estimates showed ESS values >100. Molecular clock models were compared by calculating the Bayes Factor (BF) (Suchard et al., 2001) from the posterior output of each of the models using TRACER v1.4 as explained in BEAST website (http://beast.bio.ed.ac.uk/Model_comparison). A log BF (natural log units) >2.3 indicates strong evidence against the null model.

3. Results

3.1. Phylogeography of DENV-3

Our phylogenetic analysis of 200 DENV-3 E gene sequences recovered the five genotypes previously described for this serotype (Lanciotti et al., 1994; Wittke et al., 2002). It also suggested the existence of a strong geographical subdivision of DENV-3 population with no evidences of significant co-circulation of distinct genotypes in a single locality (Fig. 1). Genotype I contained most of the Indonesian strains from 1973 to 2004, along with two early Malaysian isolates (1974 and 1981), one Thai isolate (1988), and three well-supported monophyletic groups of strains isolated in South Pacific (1989–1994), Philippines (1997–2005), and East Timor (2005). Genotype II included almost all DENV-3 strains isolated in Thailand between 1973 and 2002, along with two Myanmar strains (1988 and 2005), one isolate from Singapore (1995), one isolate from Indonesia (1998), and three strongly supported groups of strains from Malaysia (1992–1994), Bangladesh (2000–2002), and Vietnam (1996–2005). Genotype III was composed by Sri Lankan isolates from 1981 to 2000, along with single isolates from India (1984), Samoa (1986), Somalia (1993), Japan (2000), Singapore (2004), and Taiwan (2006). Moreover, all American strains sampled after 1994 were monophyletic, suggestive of a single introduction of this genotype into the continent, consistent with previous studies (Aquino et al., 2006; Diaz et al., 2006; Messer et al., 2002; Peyrefitte et al., 2005; Rodriguez-Roche et al., 2005). The American cluster was further subdivided into four well-supported lineages containing isolates from: (1) Mexico, (2) Venezuela, (3) Cuba/Martinique/Brazil/Paraguay/Bolivia, and (4) Cuba/Peru/Ecuador. Genotype IV was the most divergent group and included three early Puerto Rican strains from 1960s and 1970s. Genotype V was represented by the oldest prototype strain Philippines/1956, and two Asian isolates from 1973 and 1980. Finally, one isolate sampled from Thailand in 1962 did not fall into any established genotype and was located toward the common ancestral node of GII and GIII.

3.2. Migration patterns of DENV-3 genotypes

Before reconstructing the MPRs along the trees for each genotype we tested for panmixis by calculating with MacClade the expected number of changes along 25,000 equiprobable trees and again over other 25,000 trees obtained by random partition. The observed number of character state changes for each DENV-3 genotype was much lower than expected by chance ($P < 0.00004$ for all analyses). By increasing the number of random trees we could never sample the observed values in the random distribution (data not shown). Therefore, the DENV-3 genotypes are under high constraints for geographical gene flow and the reconstruction of the observed character state changes along the trees was justified.

Because the viral-based tree did not use geographical information at the inference step, it was used for the reconstruction of the migratory history of samples. Sub-trees for each of the three main DENV-3 genotypes, derived from the global MAP tree obtained with BI, had congruent topologies and supported the same sets of MPRs for each genoptype. All the most parsimonious states at each node are shown in Fig. 2. Nine steps were required to trace the MPRs for GI (Fig. 2a). The character tracings were unequivocal and had a single MPR for all nodes, irrespective of the resolving option. The node connecting it to the global MAP tree had Indonesia as point of radiation, since it was the ancestral state at the root. From there, independent lineages of the virus spread to the French Polynesia, reaching Fiji and Tahiti; to the Philippines, also reaching Taiwan; and more recently to the East Timor. Three distinct lineages related to Indonesia were also found in Malaysia and in Thailand.

Seven steps were required to trace the MPRs for GII (Fig. 2b). The rooting position in the MAP tree and the ancestral state for GII unambiguously suggested an origin in Thailand (T). From there, the virus appeared to have gone into Myanmar (M) and Bangladesh (B). The ancestral state at that node in the tree is ambiguous, including T, B, or M. Using DELTRAN, the state would resolve into T indicating that the virus went into the two places at different occasions. However, ACCTRAN would result on either B or M, indicating that the virus may have moved serially following either T > B > M, T > M > B (*i.e.*, stepping-stone model) or alternatively, B < T > B. Likewise, the independent movement of the GII into Singapore (S) and Malaysia (Y) from Thailand (T) had an ambiguous set of MPRs, including T, S, or Y. DELTRAN suggested Thailand broadcasting to both places and ACCTRAN indicated a stepping-stone process T > Y > S, T > S > Y, or simultaneously from Thailand Y < T > S.

GIII required the highest number of reconstruction steps (23 on average, Fig. 2c). Moreover, its rooting and internal MPRs were problematic. The MAP tree obtained with BI indicated a strain from American Samoa isolated in 1986 at the root of the genotype, but older sequences from 1981 to 1985 were sampled in Sri Lanka. Both rooting options did not alter either the cost or the character transformations (and therefore implied migration events) across the tree, but rooting at American Samoa (as shown in Fig. 2c) leaves the state at the root of the tree undefined. In any case, the virus apparently spread from Sri Lanka into nearby India and more recently into Japan, Singapore and Taiwan. An interesting event, which epitomized the cosmopolitan nature of GIII, was its sampling in Somalia in 1993 making the elucidation of the entry into the Americas problematic. DELTRAN suggested that the virus was broadcasted into Africa and the Americas from Sri Lanka. ACCTRAN pointed to a scenario in which the virus may have gone from Sri Lanka into Africa, and then into the Americas.

The spread of GIII into the Americas appears to have had Mexico as hub, since the data at hand allowed the unequivocal reconstruction of the ancestral state into the Americas there. Moreover the data resolved well the movement of the virus from

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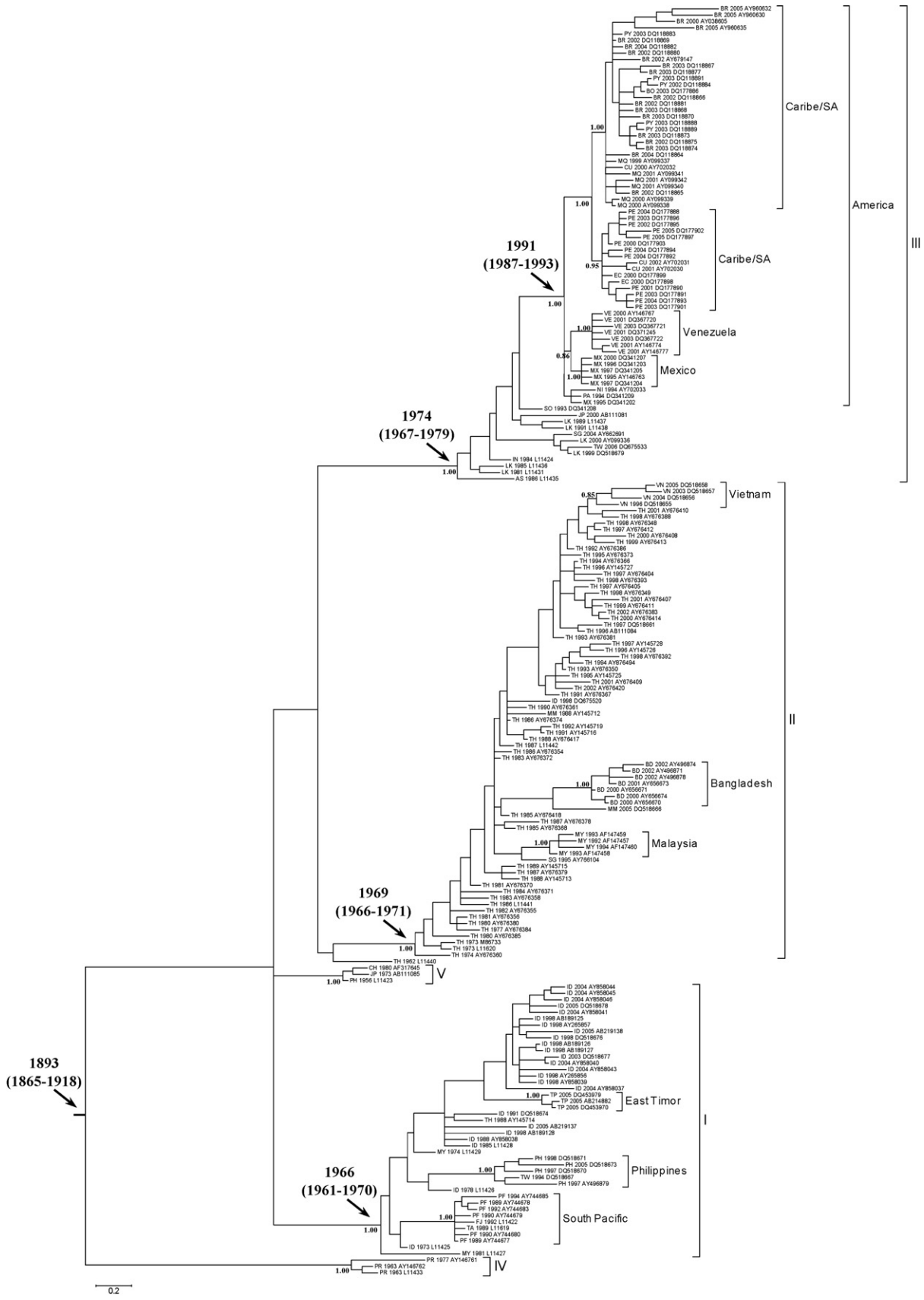

Fig. 1. Majority-rule Bayesian consensus tree of 200 E gene sequences representing the global diversity of DENV-3. Genotypes (roman numerals) and country-specific clades are indicated. Estimates for the age of some relevant nodes on the tree (point to by arrows) are also highlighted. Posterior probabilities are shown for key nodes. The names of DENV-3 isolates include reference to country origin, year of isolation, and GenBank accession number. Country represented are American Samoa (AS), Bangladesh (BD),

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Mexico into Venezuela and certainly, two more independent entries into South America that had several possible routes. DELTRAN resolved additional entries into South America via Mexico passing the virus via two routes. The first route went into the Pacific side of the Andes hitting Ecuador and Peru, and the second one went via the Caribbean, with Martinique passing the virus into Brazil and from there into Bolivia and Paraguay. ACCTRAN did not resolve if Mexico or Martinique, was the origin of the virus that went into the Pacific side of South America. However, there was no issue with the reconstruction of the entry into Brazil from Martinique. Moreover, both DELTRAN and ACCTRAN were unanimous in that a virus coming from Pacific side of the Andes moved back from South America into Cuba.

3.3. Estimation of evolutionary rates and dates

The phylogenetic tree of globally sampled DENV-3 isolates was characterized by a clear temporal structure, with the oldest sampled viruses tending to fall closest to the root of the tree, while those sampled more recently were located at the most distal tips (Fig. 1). This temporal structure allowed us to estimates the rate of molecular evolution and the T_{mrca} for different DENV-3 datasets. The substitution rate was first estimated for the complete DENV3 data set of 200 sequences, using both strict and relaxed molecular clock models. The BF analysis clearly favored a relaxed molecular clock model over a strict clock model (Table 2), indicating detectable variation in evolutionary rates among branches. The coefficient of rate variation was estimated at 0.28 (95% HPD, 0.15–0.41). Despite this variation, the median evolutionary rate (E gene) and T_{mrca} of DENV-3 estimated under both strict ($\mu = 8.7 \times 10^{-4}$ subs./site/yr; $T_{mrca} = 1891$) and relaxed ($\mu = 8.9 \times 10^{-4}$ subs./site/yr; $T_{mrca} = 1893$) molecular clock models were very close (Table 3). This result was also similar to that previously obtained by Twiddy et al. ($\mu = 9.0 \times 10^{-4}$ subs./site/yr, $T_{mrca} \sim 1900$) using a much smaller data set of DENV-3 sequences ($n = 21$) (Twiddy et al., 2003). By setting up specific taxon sub-groups within the global DENV-3 phylogeny, we also estimated the age of the major genotypes resulting in a median T_{mrca} estimate of 1967 for GI and GII, and 1975 for GIII, under either strict or relaxed molecular clock models (Table 3).

The detected variation in evolutionary rates among DENV-3 lineages could reflect rate differences among distinct genotypes (Twiddy et al., 2003), and/or rate heterogeneity within a single genotype. To test these hypotheses, we analyzed each genotype separately. The BF analysis showed that the relaxed clock method was favored over the strict clock method in the GII and GIII data sets (Table 2), indicating significant rate heterogeneity within these genotypes. The coefficient of rate variation for GII and GIII was estimated at 0.25 (95% HPD, 0.01–0.44) and 0.37 (95% HPD, 0.13–0.61), respectively. On the other hand, the median evolutionary rate estimates of the distinct genotypes were very similar and displayed a considerable overlap of HPD intervals (Table 3), clearly suggesting that there are no major differences in evolution rate among main DENV-3 genotypes. Accordingly, the median T_{mrca} estimated for each genotype was almost equal to that previously obtained using the complete DENV-3 data set (Table 3).

It has been suggested that under epidemic conditions, such as when a new variant is introduced into a susceptible population, the mean viral evolutionary rate could be higher than under endemic conditions (Twiddy et al., 2003). To test this hypothesis, we compared the DENV-3 in Indonesia and Thailand, where GI and GII have been evolving since the early 1970s, with DENV-3 lineages from the Americas, where GIII only emerged in the early 1990s. Significant rate heterogeneity was detected within GIII American lineages (Table 2). The median evolutionary rate of DENV-3 lineages circulating in different regions, however, was very similar and displayed a considerable overlap of HPD intervals (Table 4), indicating no major differences in rates of DENV-3 among regions with endemic or epidemic patterns of dengue transmission. The median T_{mrca} estimated for GI-ID and GII-TH datasets were close to that previously estimated using all GI and GII sequences (Table 4), supporting the notion that Indonesia and Thailand are the epicenters for these DENV-3 lineages; whereas the median T_{mrca} for the GIII-American clade was estimated around 1991 (Table 4).

4. Discussion

This study represents the largest phylogeographic and evolutionary analysis reported for DENV-3 to date. Our phylogenetic analysis of 200 DENV-3 E sequences with world-wide distribution revealed a clear geographical subdivision of viral strains. Genotypes I, II, and III have been evolving independently in Indonesia, Thailand, and Sri Lanka, respectively, over the last 30–40 years. Our data supports the notion that these countries not only sustain the oldest DENV-3 epidemics but also were sources for dengue lineages that have subsequently spread over the world. Strains more recently isolated in South Pacific (1989–1994), Philippines (1997–2005), East Timor (2005), Malaysia (1992–1994), Bangladesh (2000–2002), Vietnam (1996–2005), and the Americas (1994–2006) segregated into distinct monophyletic clusters within the main genotypes; indicating that each country formed a geographically distinct mostly self-contained region with regard to DENV-3 viruses, with few instances of repeated gene flow among regions.

The plausible routes of DENV-3 migration are described in Fig. 3. According to our analyses the spread of GI was mainly circumspect to the maritime portion of Southeast-Asia (East Timor, Malaysia, and Philippines) and South Pacific, where most migrant strains appeared to have been broadcasted from Indonesia. By contrast, most GII strains appeared to have been broadcasted from Thailand and stayed within continental areas in South-East Asia (Bangladesh, Myanmar, Singapore, and Vietnam), with the exception of Malaysia. GIII was the most widely spread of all DENV-3 genotypes, and most GIII strains found in Asia, East Africa and the Americas appeared to have been transmitted from or near from Sri Lanka. It is unclear if the American GIII lineage came from Africa or Asia. The oldest GIII sequences in the Americas were identified in Panama and Nicaragua in 1994 (CDC, 1995; Guzman et al., 1996), but our migration data suggested that the GIII was introduced into the Americas through Mexico where the first GIII strains were identified in 1995 (Briseno-Garcia et al., 1996). More sampling in those countries will be necessary to elucidate the precise point of introduction of GIII to the Americas. In any case, GIII viruses rapidly spread to others countries in the region (Nogueira et al., 2001; Peyrefitte et al., 2003; Rigau-Perez et al., 2002; Usuku et al., 2001; Uzcategui et al., 2003), using several independent routes from Central America to the Caribbean and South America.

Our phylogeographic analysis also revealed that the co-circulation of different DENV-3 genotypes in a single location is

Bolivia (BO), Brazil (BR), China (CH), Cuba (CU), Ecuador (EC), Fiji (FJ), Indonesia (ID), India (IN), Japan (JP), Sri Lanka (LK), Myanmar (MM), Martinique (MQ), Mexico (MX), Malaysia (MY), Nicaragua (NI), Panama (PA), Peru (PE), French Polynesia (PF), Philippines (PH), Puerto Rico (PR), Paraguay (PY), Singapore (SG), Somalia (SO), Tahiti (TA), Thailand (TH), East Timor (TP), Taiwan (TW), Venezuela (VE), Vietnam (VN). The tree is rooted using GIV, which have been shown to represent an appropriate outgroup. Horizontal branch lengths are drawn to scale with the bar at the bottom indicating 0.2 nucleotide substitutions per site.

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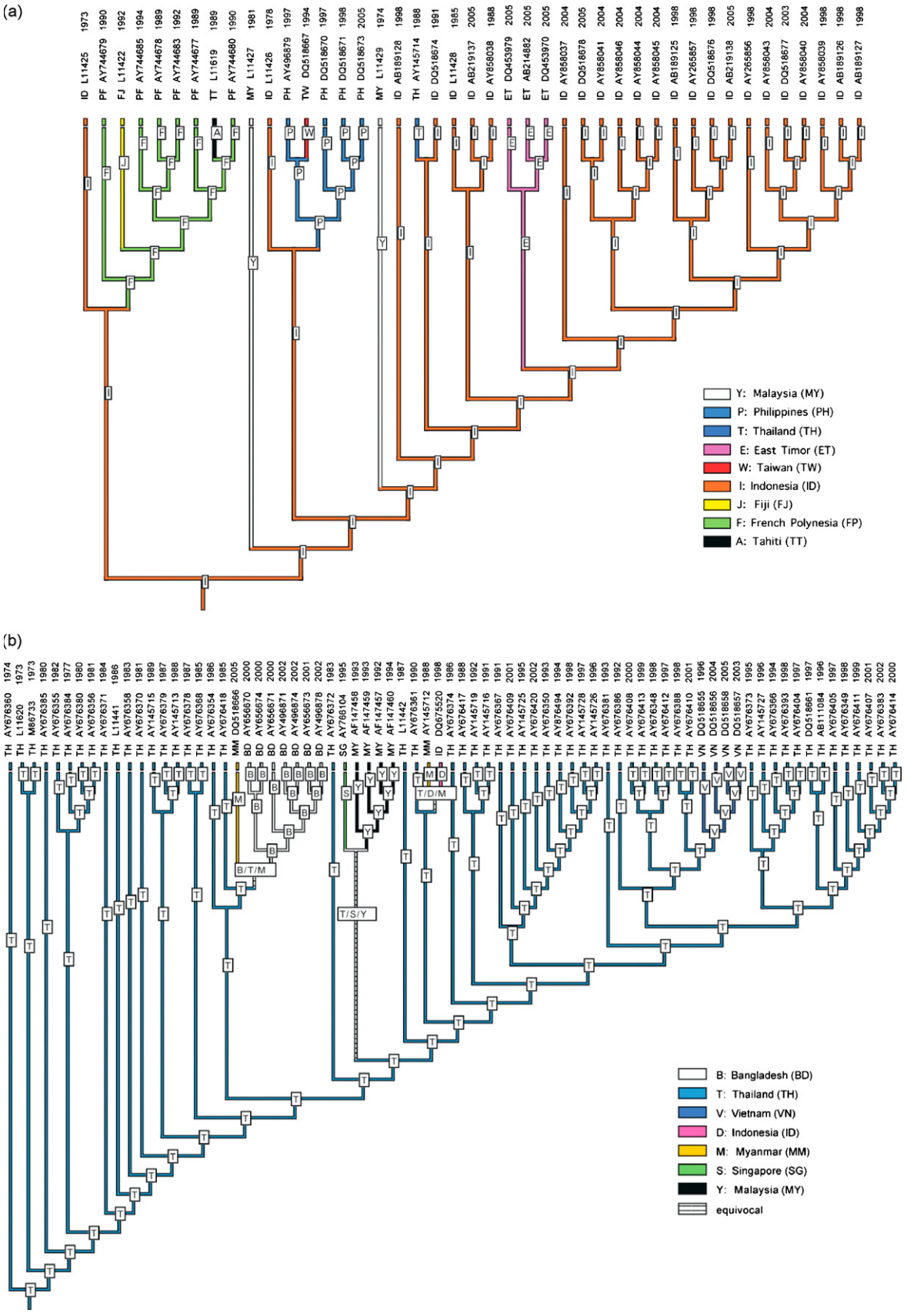

Fig. 2. Migration patterns of genotypes I (a), II (b), and III (c). The names of DENV-3 isolates include reference to country origin, GenBank accession number, and year of isolation. The color of each branch represents the country of origin of the sequence corresponding to that branch, according to the figure legend.

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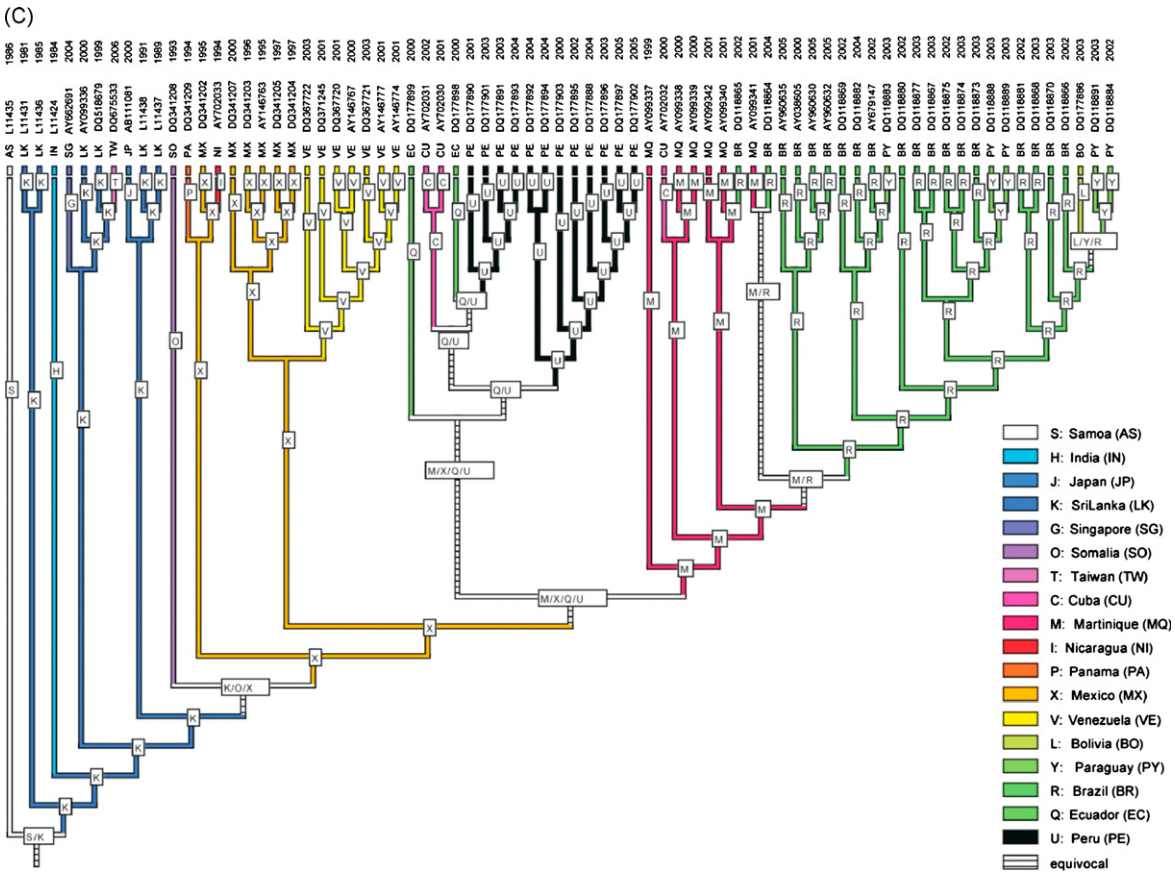

Fig. 2. (*Continued*).

a rare event. This could result from a limited gene flow among distantly geographic regions. However, genotype differences were observed even between neighboring countries like Indonesia and Thailand, where DENV-3 epidemics have been consistently dominated by GI and GII, respectively, since the 1970s. Notwithstanding, there is evidence of incursions into Indonesia by "Thai-like" GII strains in 1988 (represented by strain DQ675520) (Raekiansyah et al., 2005), as well as into Thailand by "Indonesian-like" GI strains in 1998 (represented by strain AY145714) (Wittke et al., 2002). Nevertheless, these incursions seem to have failed to become established, since none of the later Indonesian and Thai isolates grouped within GII and GI, respectively. These facts suggested that several factors, other than geographic proximity, could have had a significant impact on the observed spatial dispersion patterns of DENV-3.

It is hard to envisage a general vicariance mechanism that would explain a lack of genotype overlapping at the same geographic locality. Possibly competition among genotypes, and/or regional differences in mosquito vector competence for each genotype (Anderson and Rico-Hesse, 2006; Armstrong and Rico-Hesse, 2001; Cologna et al., 2005) may be involved. Another possible explanation for the observed patterns could involve viral neutralization by cross-immunity among closely related strains caused by a pre-exposed human population. This would allow for distinct serotype co-circulation but make it difficult for intra-serotype (*i.e.*, genotype) co-circulation, due to a reduction in numbers of the available susceptible human hosts to levels below that necessary to sustain significant epidemics (Adams et al., 2006). This would help explain why the evolution of DENV-3 is characterized by phylogenetic trees with a strong temporal

Table 2
Bayes factors between different molecular clock models for DENV-3.

Dataset	Model comparison	log BF[a]	Evidence against H_0[b]
DENV-3	Strict (H_0) vs. relaxed (H_1) clock	22.8 (0.4)	Decisive
GI	Strict (H_0) vs. relaxed (H_1) clock	−0.8 (0.4)	Negative
GII	Strict (H_0) vs. relaxed (H_1) clock	5.0 (0.5)	Decisive
GIII	Strict (H_0) vs. relaxed (H_1) clock	10.8 (0.5)	Decisive
GI-ID	Strict (H_0) vs. relaxed (H_1) clock	−0.4 (0.3)	Negative
GII-TH	Strict (H_0) vs. relaxed (H_1) clock	1.6 (0.4)	Weak
GIII-AM	Strict (H_0) vs. relaxed (H_1) clock	12.8 (0.4)	Decisive

[a] log BF (Bayes factor) is the difference (in natural log units) of the marginal likelihood of null (H_0) and alternative (H_1) model. The standard error of the estimates is given in parenthesis.

[b] Evidence against H_0 is assessed in the following way: ln BF <0 indicates no evidence against the null model; ln BF between 0 and 2.3 indicates weak evidence against the null model, ln BF between 2.3 and 3.4 indicates strong evidence against the null model; ln BF between 3.4 and 4.6 indicates very strong evidence against the null model; ln BF >4.6 indicates decisive evidence against the null model.

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Table 3
Estimated substitutions rates and dates for DENV-3 genotypes.

Dataset	Molecular clock	μ	T_{mrca} DENV-3	T_{mrca} GI	T_{mrca} GII	T_{mrca} GIII
DENV-3	Strict	8.7×10^{-4} (7.7×10^{-4} to 9.7×10^{-4})	1891 (1876–1904)	1967 (1963–1970)	1967 (1965–1970)	1975 (1972–1978)
	Relaxed	8.9×10^{-4} (7.9×10^{-4} to 10.0×10^{-4})	1893 (1865–1918)	1967 (1963–1970)	1967 (1964–1970)	1975 (1972–1978)
GI	Strict	8.3×10^{-4} (6.8×10^{-4} to 9.8×10^{-4})	–	1966 (1961–1970)	–	–
	Relaxed	8.4×10^{-4} (6.9×10^{-4} to 10.1×10^{-4})	–	1966 (1961–1970)	–	–
GII	Strict	10.1×10^{-4} (8.7×10^{-4} to 11.7×10^{-4})	–	–	1969 (1966–1971)	–
	Relaxed	10.3×10^{-4} (8.7×10^{-4} to 12.0×10^{-4})	–	–	1969 (1966–1971)	–
GIII	Strict	8.1×10^{-4} (6.6×10^{-4} to 9.7×10^{-4})	–	–	–	1974 (1969–1978)
	Relaxed	8.2×10^{-4} (6.6×10^{-4} to 9.9×10^{-4})	–	–	–	1974 (1967–1979)

Estimates of the median evolutionary rate (μ, substitutions site^{-1} year^{-1}) and, median time for the most recent common ancestor (T_{mrca}, year). The age estimated for some relevant internal nodes within the global DENV-3 tree (corresponding to the T_{mrca} of major genotypes) are also shown. 95% HPD intervals are shown between parentheses.

Table 4
Estimated substitutions rates and dates for DENV-3 in Indonesia, Thailand and the Americas.

DENV	Genotype	Region	μ	T_{mrca}
3	I	Indonesia	8.4×10^{-4} (6.2×10^{-4} to 10.7×10^{-4})	1970 (1966–1973)
	II	Thailand	10.0×10^{-4} (8.2×10^{-4} to 11.8×10^{-4})	1969 (1966–1971)
	III	America	9.2×10^{-4} (6.9×10^{-4} to 11.5×10^{-4})	1991 (1987–1993)
2	Asian I[a]	Thailand	8.5×10^{-4} (7.2×10^{-4} to 9.9×10^{-4})	–
	Asian-American[b]	America	8.0×10^{-4} (6.6×10^{-4} to 9.5×10^{-4})	–
4	I[c]	Thailand	10.7×10^{-4} (8.4×10^{-4} to 13.1×10^{-4})	–
	II[b]	America	8.3×10^{-4} (6.8×10^{-4} to 10.0×10^{-4})	–

Estimates of the median evolutionary rate (μ, substitutions site^{-1} year^{-1}), and median time of the most recent common ancestor (T_{mrca}, year) for the GI-ID (strict molecular clock), GII-TH (relaxed molecular clock), and GIII-AM (relaxed molecular clock) datasets. 95% HPD intervals are shown between parentheses.
[a] Data taken from Zhang et al. (2006).
[b] Data taken from Carrington et al. (2005).
[c] Data taken from Klungthong et al. (2004).

structure as previously noted for this and other DENV serotypes (Goncalvez et al., 2002; Klungthong et al., 2004; Twiddy et al., 2003; Wittke et al., 2002; Zhang et al., 2005, 2006), which may indicate the strong pruning effect of DENV lineages by host immunity. Finally, we cannot also exclude the possibility that GI and GII are circulating as minor variants in Thailand and Indonesia, respectively, but have remained undetectable because the low number of sequences analyzed (sampling bias).

Our analyses of rates of nucleotide substitution in DENV-3 revealed that the relaxed clock model outperforms the strict clock model, indicating detectable variation in evolutionary rates among DENV-3 lineages. Significant rate variation among genotypes was described previously for DENV-2 (Twiddy et al., 2003), suggesting that rate variation detected in DENV-3 could be also consequence of differences among genotypes. However, the analysis of each DENV-3 genotype separately revealed that rate heterogeneity in DENV-3 can be mainly explained by rate differences within genotypes, particularly within GII and GIII, rather than among genotypes. The median evolutionary rate of the distinct DENV-3 genotypes analyzed was very similar (ranging from 8.3×10^{-4} subs./site/yr to 10.3×10^{-4} subs./site/yr) and displayed a considerable overlap of the HPD intervals, clearly suggesting no major differences in evolution rate among genotypes. It is also interesting to note that although the relaxed clock model outperforms the strict clock model in most DENV-3 datasets analyzed, the median value of estimates obtained under both relaxed and strict clock models were very close in all analyses. This is fully consistent with the concept that the substitution rate estimated from large data sets are reliable indicators of the average rate of evolution, even if rate heterogeneity is present (Jenkins et al., 2002).

A previous study suggested that DENV-3 is evolving at a rate ($\mu = 9.0 \times 10^{-4}$ [7.3×10^{-4} to 10.8×10^{-4}] subs./site/yr) significantly faster than other DENV serotypes; and proposed similar rates of substitutions for each DENV-3 genotype (GI = 7.5×10^{-4} [4.5×10^{-4} to 10.7×10^{-4}] subs./site/yr; GIII = 11.6×10^{-4} [7.8×10^{-4} to 15.9×10^{-4}] subs./site/yr) (Twiddy et al., 2003). Those estimates, however, were based on the analysis of very small DENV-3 datasets (total = 21, GI = 8, GIII = 8), and no estimations of the substitution rate for GII were provided. We re-estimated these evolutionary rates using much larger data sets of DENV-3 sequences (total = 200, GI = 43, GII = 75, GIII = 75). Our median rate estimates for DENV-3 (8.9×10^{-4} subs./site/yr) and GI (8.3×10^{-4} subs./site/yr) were similar to those previously reported by Twiddy et al. whereas the median rate estimated for GIII (8.2×10^{-4} subs./site/yr) was considerably lower, although within the large confidence interval of the previous estimate. The median evolutionary rate estimated for GII was 10.3×10^{-4} subs./site/yr. Overall, the confidence intervals of our estimates (6.6×10^{-4} to 12×10^{-4}) were significantly narrower than those described by Twiddy et al. (4.5×10^{-4} to 15.9×10^{-4}), probably due to the higher number of sequences used in the present study.

It has been suggested that the ecological conditions for DENV dissemination may alter the viral evolutionary rate, which could explain some rate differences among dengue lineages previously described (Twiddy et al., 2003). Our analyses revealed, however, that the median evolutionary rate of GI in Indonesia and GII in Thailand (areas with long-term endemic infections), was similar to that observed for GIII in the Americas (a region with a recent epidemic pattern of DENV-3 transmission). Our DENV-3 rate estimates were also comparable to those estimated for DENV-2 (genotype Asian I) and DENV-4 (genotype I) circulating in Thailand (Klungthong et al., 2004; Zhang et al., 2006), and for DENV-2 (genotype Asian-American) and DENV-4 (genotype II) circulating in the Americas (Carrington et al., 2005) (Table 4). These studies confirmed a lack of association between dengue substitution rate and ecological pattern of virus spread, and revealed no major

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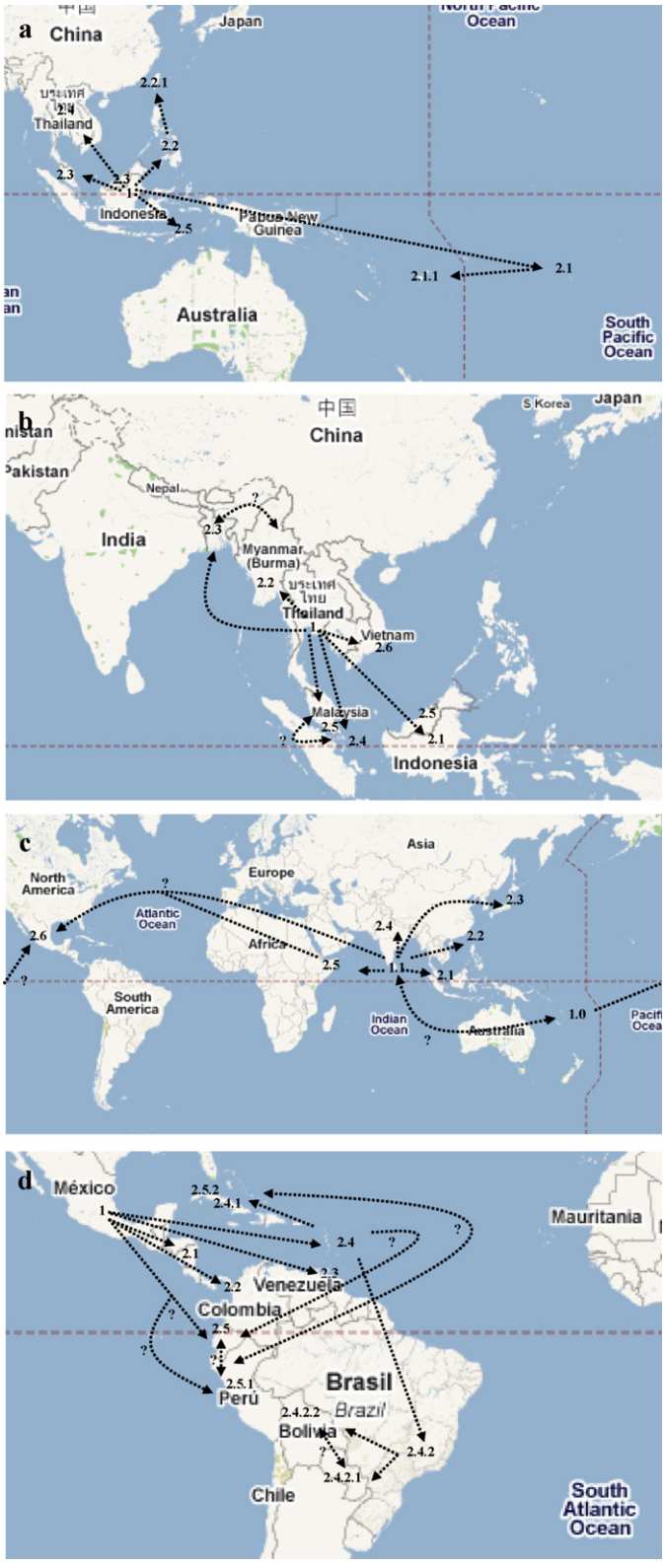

Fig. 3. Plausible sites of origin and migration routes of DENV-3 genotypes I (a), II (b), III (c) and III-AM (d). (a) (1) Indonesia, (2.1) French Polynesia/Tahiti, (2.1.1) Fiji, (2.2) Philippines, (2.2.1) Taiwan, (2.3) Malaysia, (2.4) Thailand, (2.5) East Timor. (b) (1) Thailand, (2.1) Indonesia, (2.2) Myanmar, (2.3) Bangladesh, (2.4) Singapore, (2.5) Malaysia, (2.6) Vietnam. (c) (1.0) Samoa, (1.1) Sri Lanka, (2.1) Singapore, (2.2) Taiwan, (2.3) Japan, (2.4) India, (2.5) Somalia, (2.6) Mexico. (d) (1) Mexico, (2.1) Nicaragua, (2.2) Panama, (2.3) Venezuela, (2.4) Martinica, (2.4.1) Cuba (2000), (2.4.2) Brazil, (2.4.2.1) Paraguay, (2.4.2.2) Bolivia, (2.5) Ecuador, (2.5.1) Peru, (2.5.2) Cuba (2001/2002). Inconclusive routes of DENV-3 were identified with the signal "?".

lineage-specific rate differences among DENV-2, DENV-3, and DENV-4. Whether lineage-specific rate differences in DENV evolution previously described (Twiddy et al., 2003) really exist, or simply reflects a previous use of much smaller datasets needs further investigation.

We estimated the T_{mrca} of DENV-3 at around 1890, fully consistent with previous estimation ($T_{mrca} \sim 1900$) (Twiddy et al., 2003). Our analysis also suggested that the current global genetic diversity of genotypes I, II, and III arose almost simultaneously within a short time period between the middle 1960s and the middle 1970s, coinciding with the description of the first cases of DHF by DENV-3 in Asia (Gubler et al., 1979; Nisalak et al., 2003; Sumarmo, 1987; Wallace et al., 1980) and the rapid increase in human population size, urbanization, and human movement. According with our estimations, GIII strains were probably introduced into Latin America around 1991, few years earlier than the initial detection of this genotype in the continent in 1994 (CDC, 1995; Guzman et al., 1996). Similar time intervals of few years between the estimated introduction and initial detection were also described for DENV-2 and DENV-4 in the Americas (Carrington et al., 2005).

In conclusion, this study proposes that global DENV-3 evolution could be well characterized as a collection of discrete, country-specific viral population bursts, with limited co-circulation of distinct genotypes in a single region. Despite this strong spatial subdivision, DENV-3 strains of distinct genotypes and from different localities have been evolving at roughly the same rate over time. Whether such similar evolutionary rate estimates translate into comparable biological properties (such as transmissibility, infectiousness, and/or virulence) across distinct DENV-3 lineages is still unclear. Our data also suggested that the current diversity of the three main DENV-3 genotypes arose within the last 30–40 years, coinciding with the emergence of large-scale DHF/DSS epidemics in Asia.

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