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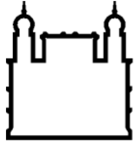
HISTÓRIA EVOLUTIVA, CARACTERIZAÇÃO E VIGILÂNCIA MOLECULAR DAS DIFERENTES LINHAGENS DO VÍRUS DENGUE TIPO 1 NO BRASIL

FERNANDA DE BRUYCKER NOGUEIRA

Rio de Janeiro
Junho de 2018



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Programa de Pós-Graduação em Biologia Parasitária

FERNANDA DE BRUYCKER NOGUEIRA

História evolutiva, caracterização e vigilância molecular das diferentes linhagens do vírus dengue tipo 1 no Brasil

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências

Orientadora: Profª Drª Flavia Barreto dos Santos

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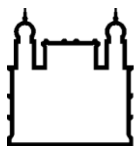

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Programa de Pós-Graduação em Biologia Parasitária

AUTOR: FERNANDA DE BRUYCKER NOGUEIRA

HISTÓRIA EVOLUTIVA, CARACTERIZAÇÃO E VIGILÂNCIA MOLECULAR DAS DIFERENTES LINHAGENS DO VÍRUS DENGUE TIPO 1 NO BRASIL

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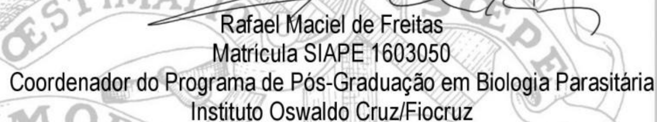

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Este trabalho é dedicado:

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em especial à minha orientadora,
Dra Flavia Barreto dos Santos,
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e relatar a história do DENV-1no Brasil.

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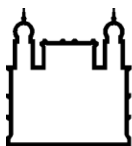
"Tudo tem o seu tempo determinado,
e há tempo para todo o propósito debaixo do céu."

Eclesiastes 3:1
Bíblia Sagrada

"Tudo posso naquele que me fortalece."

Filipenses 4:13
Bíblia Sagrada



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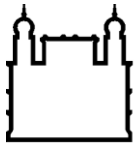
HISTÓRIA EVOLUTIVA, CARACTERIZAÇÃO E VIGILÂNCIA MOLECULAR DAS DIFERENTES LINHAGENS DO VÍRUS DENGUE TIPO 1 NO BRASIL

RESUMO

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Fernanda de Bruycker Nogueira

Este trabalho apresenta a análise filogenética, filogeográfica e caracterização molecular de amostras do vírus dengue tipo 1 (DENV-1) no Brasil no período de 30 anos (1986 - 2016). O estudo foi conduzido, inicialmente, com a análise filogenética, caracterização molecular e análise de recombinação do gene E de 48 amostras e da região codificante completa de 6 amostras do período de 1986 a 2011. Foi demonstrado que todos os vírus analisados pertenciam ao genótipo V, porém agrupando-se em diferentes linhagens no Brasil. Posteriormente, foi realizada a reconstrução da história evolutiva do genótipo V nas Américas com mais de 3 mil sequências disponíveis no Genbank, incluindo as brasileiras e observou-se que a diversidade do genótipo V do DENV-1 no continente resultou da evolução local a partir de duas cepas introduzidas da Índia por volta do início dos anos 70 e 80, independentemente. A primeira cepa sendo responsável pela maioria das infecções pelo genótipo V nas Américas, enquanto a segunda parecendo estar restrita principalmente ao Brasil. As Pequenas Antilhas foram a principal origem das linhagens de DENV-1 disseminadas nas Américas até meados da década de 1980; e Venezuela e Nicarágua se tornaram posteriormente os países mais importantes de manutenção e disseminação viral no continente. Embora várias linhagens de DENV-1 tenham estabelecido surtos de sucesso em diferentes países da América durante os anos 1980, elas foram posteriormente disseminadas e extintas com dinâmicas distintas. Com o intuito de demonstrar a circulação continuada do DENV-1 no Brasil até 2016, conduzimos o estudo sobre a caracterização molecular e o padrão de dispersão dos DENV-1 brasileiros entre os anos de 2012 a 2016, período após a reemergência deste sorotipo no país. Nossos achados demonstraram que as diferentes linhagens do genótipo V continuaram cocirculando no Brasil até o ano de 2016. No entanto, duas novas substituições de aminoácidos localizadas nos domínios II e III do gene E foram identificadas nas amostras do Brasil e Argentina. Foram também demonstrado um evento de dispersão do Brasil para China, provavelmente relacionado a um caso pontual de viagem e um caso de coinfecção por DENV-1/DENV-4 em um paciente do estado do Rio de Janeiro no ano de 2012, nunca identificado até então. Os dados desta tese ressaltam a importância da vigilância molecular constante dos sorotipos, genótipos e linhagens no território brasileiro.



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EVOLUTIONARY HISTORY, CHARACTERIZATION AND MOLECULAR SURVEILLANCE OF THE DIFFERENTS LINEAGES OF DENGUE VIRUS TYPE 1 IN BRAZIL

ABSTRACT

DOCTORAL THESIS IN BIOLOGY PARASITARY

Fernanda de Bruycker Nogueira

This thesis presents the phylogenetic, phylogeographic and molecular characterization of dengue virus type 1 (DENV-1) in Brazil in the period of 30 years (1986 - 2016). The study was conducted initially with phylogenetic analysis, molecular characterization and recombination analysis of the E gene of 48 samples and the complete coding region of 6 samples from the period 1986 to 2011. It was demonstrated that all viruses analyzed belonged to genotype V , but grouping in different strains in Brazil. Subsequently, the reconstruction of the evolutionary history of genotype V in the Americas was performed with more than 3,000 sequences available at Genbank, including Brazilian ones, and it was observed that the diversity of DENV-1 genotype V on the continent resulted from local evolution from two strains introduced from India around the early 70's and 80's, regardless. The first strain was responsible for most genotype V infections in the Americas, while the second strain appeared to be restricted mainly to Brazil. The Lesser Antilles were the main origin of the DENV-1 lineages disseminated in the Americas up to the mid-1980s; and Venezuela and Nicaragua later became the most important countries for viral maintenance and dissemination on the continent. Although several DENV-1 strains have established successful outbreaks in different American countries during the 1980s, they were later disseminated and extinguished with distinct dynamics. In order to demonstrate the continued circulation of DENV-1 in Brazil until 2016, we conducted the study on the molecular characterization and dispersion pattern of Brazilian DENV-1 between the years 2012 to 2016, after the re-emergence of this serotype in the country . Our findings demonstrated that the different strains of genotype V continued to co-circulate in Brazil until the year 2016. However, two new amino acid substitutions located in domains II and III of the E gene were identified in the samples from Brazil and Argentina. We also demonstrated a Brazilian dispersion event for China, probably related to a one-time travel case and a case of co-infection by DENV-1/DENV-4 in a patient from the state of Rio de Janeiro in the year 2012, never identified until so. The data of this thesis emphasize the importance of the constant molecular surveillance of the serotypes, genotypes and lineages in the Brazilian territory.

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LISTA DE SIGLAS E ABREVIATURAS

%	sinal de porcentagem
~	aproximadamente
µL	microlitro
aa	aminoácido
Ae.	*Aedes*
AL	Alagoas
AP	Amapá
BR	Brasil
C	proteína do capsídeo
C6/36	cultura celular de *Ae. albopictus* clone 6 passagem 36
cDNA	DNA complementar
CE	Ceará
CR	complexo de replicação
d.C.	depois de Cristo
D1	iniciador sense D1
D2	iniciador sense D2
DC	dengue clássica
DCC	dengue com complicação
DCSA	dengue com sinais de alerta
DENV	Vírus dengue
DENV-1	Vírus dengue sorotipo 1
DENV-2	Vírus dengue sorotipo 2
DENV-3	Vírus dengue sorotipo 3
DENV-4	Vírus dengue sorotipo 4
DG	dengue grave
DNA	ácido desoxirribonucleico
DSSA	dengue sem sinais de alerta
E	proteína do envelope
ECP	efeito citopático
ELISA	ensaio imunoenzimático indireto
ES	Espírito Santo
EUA	Estados Unidos da América

FD	febre da dengue
FHD	febre hemorrágica da dengue
FIOCRUZ	Fundação Oswaldo Cruz
FR	forma replicativa
h	hora
HI	Inibição da hemaglutinação
IgG	imunoglobulina da classe G
IgM	imunoglobulina da classe M
IOC	Instituto Oswaldo Cruz
IR	intermediário replicativo
Kb	quilobase
kDa	quilodalton
L-15	Leibovitz-15
LABFLA	Laboratório de Flavivírus
M	proteína de membrana
MAC ELISA	ensaio imunoenzimático indireto de captura de anticorpo M (do inglês: *IgM antibody capture enzyme-linked immunosorbent assay*)
mg	miligrama
MG	Minas Gerais
mL	mililitro
MS	Mato Grosso do Sul
MS	Ministério da Saúde
MV	Máxima Verossimilhança
NC	não codificante
NIH	*National Institutes of Health*
nm	nanômetro
NS	proteína não-estrutural
NS1	proteína não-estrutural 1
NS2A	proteína não-estrutural 2A
NS2B	proteína não-estrutural 2B
NS3	proteína não-estrutural 3
NS4A	proteína não-estrutural 4A
NS4B	proteína não-estrutural 4B
NS5	proteína não-estrutural 5

ºC	Grau Celsius
OMS	Organização Mundial de Saúde
OPAS	Organização PanAmericana de Saúde
PAHO	PanAmerican Health Organization
pb	pares de base
pH	potencial hidrogeniônico
PI	Piauí
PM	peso molecular
Poli-A	poli-adenilada
prM	proteína da pré-membrana
RE	retículo endoplasmático
RJ	Rio de Janeiro
RN	Rio Grande do Norte
RNA	ácido ribonucleico
RNA(-)	RNA polaridade negativa
RNA(+)	RNA polaridade positiva
RNAdf	RNA dupla fita
RNAfs	RNA fita simples
RNAm	RNA mensageiro
RT-PCR	transcrição reversa seguida da reação em cadeia pela polimerase
SCD	síndrome do choque por dengue
SFB	soro fetal bovino
SVS	Secretaria de Vigilância em Saúde
TS1	iniciador tipo-específico para DENV-1
TS2	iniciador tipo-específico para DENV-2
TS3	iniciador tipo-específico para DENV-3
TS4	iniciador tipo-específico para DENV-4
TTO	transmissão transovariana
UTR	*untranslated region*
UV	luz ultravioleta
V	Volts
WHO	*World Health Organization*

1 INTRODUÇÃO

1.1 Os vírus dengue (DENV)

Os vírus dengue (DENV) pertencem à família *Flaviviridae*, gênero *Flavivirus,* e devido às suas propriedades antigênicas distintas, apresentam quatro sorotipos denominados DENV-1, DENV-2, DENV-3 e DENV-4 (Sabin, 1952; Hammon *et al.*, 1960; Westaway *et al.*, 1985). A infecção por um sorotipo induz a produção de anticorpos específicos e imunidade duradoura contra o mesmo, podendo um indivíduo ser infectado pelos quatro sorotipos ao longo da vida (Sabin, 1952). São considerados arbovírus, pois a sua manutenção na natureza ocorre através de um ciclo de transmissão envolvendo hospedeiros vertebrados e artrópodos hematófagos (Gubler, 1998), tendo como o principal vetor o mosquito da espécie *Aedes* (*Ae.*) *aegypti* (Linnaeus, 1762 *apud* (Knight e Stone, 1977), mais prevalente em áreas tropicais e subtropicais do mundo (Vasilakis e Weaver, 2017).

A existência de um novo sorotipo de transmissão silvestre em Sarawak, Malásia, detectado em humanos foi descrito há aproximadamente cinco anos (Vasilakis, 2013). Porém, até o momento não houve novas informações sobre o fato, embora tenha sido alvo de discussão como um novo dilema para o controle da dengue (Mustafa *et al.*, 2015).

A morfologia da partícula viral é esférica, envelopada e com cerca de 40 a 50 nanômetros (nm) de diâmetro. O vírion (partícula infecciosa) consiste de RNA de fita simples (RNAfs) de polaridade positiva de aproximadamente 11 quilobase (Kb), envolto por um nucleocapsídeo de simetria icosaédrica (Kuhn *et al.*, 2002) (Figura 1.1).

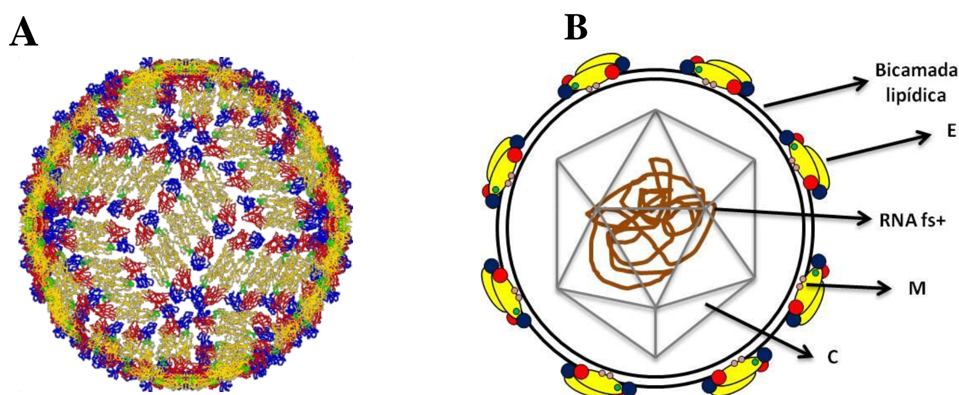

Figura 1.1 Vírus dengue. (A) Estrutura da partícula viral inteira (Kuhn *et al.*, 2002). (B) Esquema das estruturas da partícula viral, apresentando o capsídeo icosaédrico "C", proteína de membrana "M" após maturação da partícula viral, RNA fita simples polaridade positiva "RNAfs+", proteína do envelope "E" e bicamada lipídica.

O RNAfs funciona como o próprio RNA mensageiro (RNAm) e é traduzido a partir da maquinaria da célula hospedeira em uma poliproteína única e posteriormente clivada em três proteínas estruturais (capsídeo [C], pré-membrana [prM] e envelope [E]) e sete não-estruturais (NS1, NS2A, NS2B, NS3 , NS4A , NS4B e NS5) (Chambers *et al.*, 1990; Miller *et al.*, 2010) (Figura 1.2).

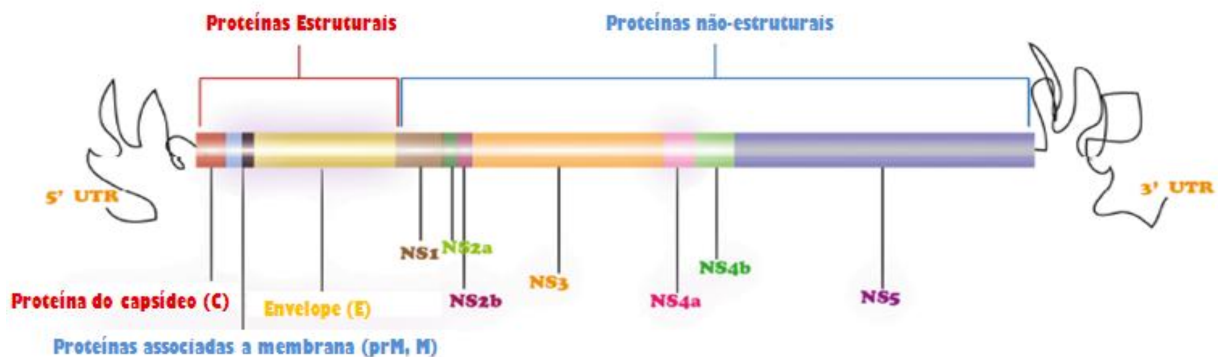

Figura 1.2 Organização do genoma dos vírus dengue (DENV). RNAfs+ com ~10,7Kb, apresentando a região codificante para a poliproteína e as duas extremidades não codificantes (5' UTR e 3'UTR - do inglês *untranslated region*). Adaptado de (Idrees e Ashfaq, 2012)**.**

Entre as proteínas estruturais, a proteína C é a primeira a ser sintetizada, possuindo ~11 quilodalton (kDa). Ela é carregada positivamente e forma o componente estrutural do nucleocapsídeo, que consiste na proteína C e RNA genômico. Em condições de alta salinidade o nucleocapsídeo torna-se instável, tornando as proteínas C em dímeros (Lindenbach *et al.*, 2007).

O precursor prM é uma proteína de 22 kDa, que ao sofrer uma clivagem proteolítica específica durante a maturação viral, origina a proteína M com cerca de 8 kDa, envolvida no aumento da infectividade do vírus e na organização da estrutura viral (Randolph *et al.*, 1990).

O envelope (E) é o maior constituinte da superfície dos DENV (53 kDa), sendo formado por proteínas diméricas que medeiam a ligação e fusão do vírus com a membrana celular do hospedeiro. É responsável por atividades biológicas do ciclo viral, além de ser o principal indutor de anticorpos neutralizantes e apresentar atividade hemaglutinante (Chambers *et al.*, 1990; Heinz e Allison, 2001; Weaver e Vasilakis, 2009). Possui 495 aminoácidos (aa), distribuídos em três domínios (I, II, III). Esses domínios são regiões distintas presentes nas duas subunidades idênticas do complexo dimérico da estrutura

tridimensional da proteína E. O domínio I, região central da molécula, contendo o radical amino terminal, é responsável pela organização estrutural da partícula viral; domínio II contém a maior parte dos contatos do dímero, está relacionado com a fusão da partícula viral, tem a estrutura composta por um par de alças descontínuas, sendo uma altamente conservada entre todos os flavivírus, funcionando como um peptídeo interno de fusão, estabilizado por três pontes dissulfeto; e o domínio III, localizado na superfície lateral externa do dímero, inclui o C terminal e tem relação com a virulência de determinadas cepas virais, sendo um componente altamente imunogênico. Acredita-se que o domínio III dos flavivírus interajam com receptores celulares para a entrada dos vírus (Figueiredo, 1999; Lindenbach *et al.*, 2007; Nayak *et al.*, 2009; Weaver e Vasilakis, 2009) (Figura 1.3).

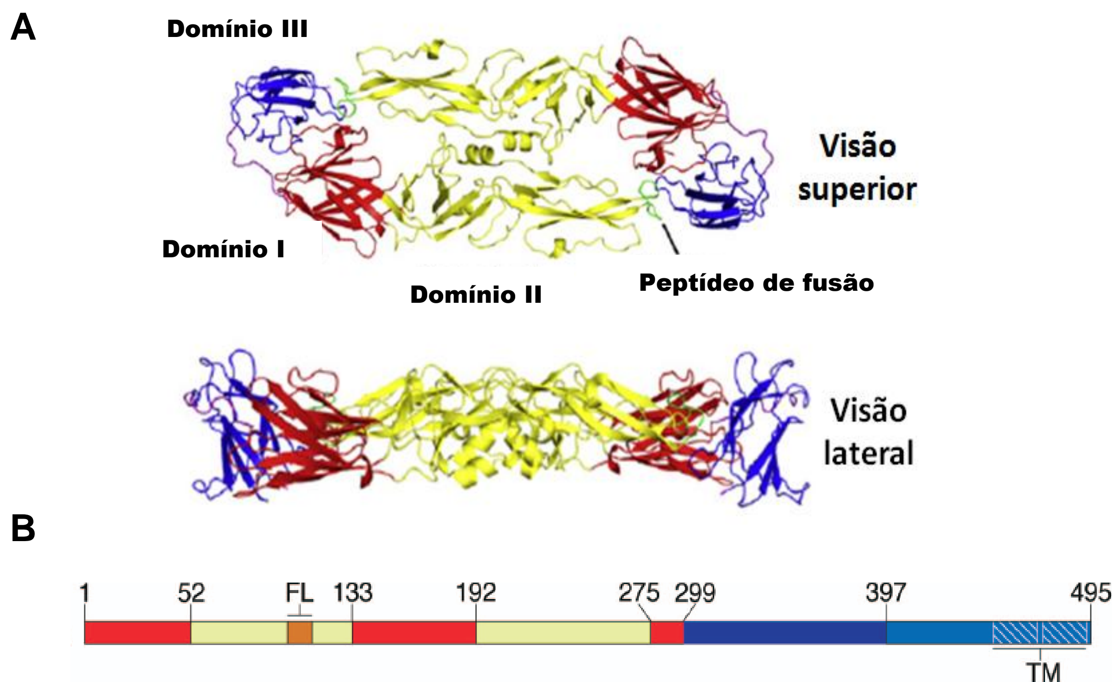

Figura 1.3 Estrutura da proteína do envelope (E). (A) Diagrama em fita da estrutura cristalina da proteína do envelope (E). Domínio I (vermelho), domínio II (amarelo), domínio III (azul) e o peptídeo de fusão (verde). Adaptado de (Heinz e Stiasny, 2012). (B) Estrutura da proteína E. Segmentos dos domínios I (vermelho), II (amarelo), III (azul), peptídeo de fusão (FL – *fusion loop*) (laranja). Região "stem" (turquesa), liga o ectodomínio a uma âncora transmembrana C-terminal dupla-hélice (TM). Adaptado de Nayak *et al.*, 2009.

As partículas virais, no curso do ciclo replicativo, passam de uma conformação imatura para o maduro, obtendo assim a capacidade de infectar novas células, se tornando um vírion. O arranjo das glicoproteínas nas partículas virais maduras e imaturas é diferente. As partículas maduras dos DENV exibem uma superfície exterior relativamente lisa, com os dímeros da proteína E ligada paralelamente à bicamada lipídica da membrana. O envelope apresenta 90 dímeros da proteína E bem ajustados em padrão de espinha de peixe incomum de simetria icosaédrica, sugerindo que tais dímeros podem sofrer rearranjos de rotação em torno do eixo de simetria 3 e 5 formando complexos triméricos fusogênicos. Esta mudança ocorre em ambiente com baixo pH. Já as partículas virais imaturas são maiores e exibem 60 picos, cada um composto por três monômeros da proteína E em torno de um trímero de prM. A proteína M, produzida durante a maturação das partículas dentro da via de secreção, é um pequeno fragmento proteolítico da proteína precursora prM. A prM liga-se ao peptídeo de fusão localizado do domínio II da proteína E, e após clivagem ocorre a liberação do peptídeo pr e os 60 trímeros são dissociados girando e rearranjando em 90 dímeros antiparalelos, configurando a superfície da glicoproteína do envelope em partículas virais maduras (Lindenbach *et al.*, 2013) (Figura 1.4).

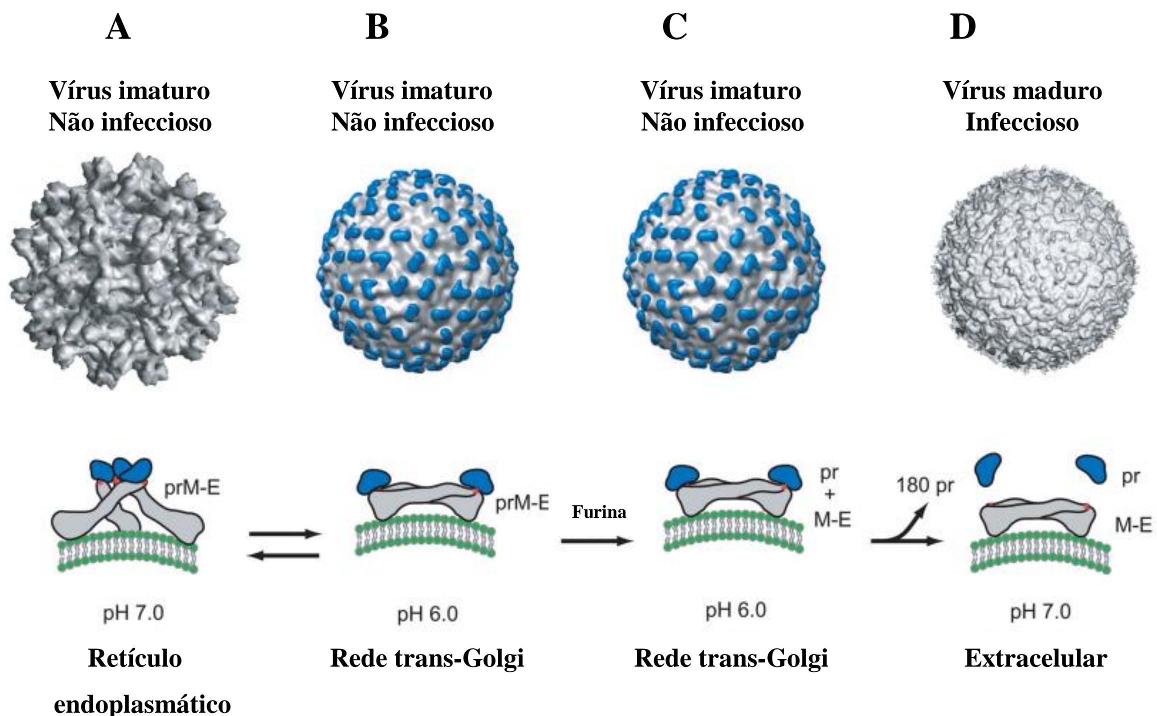

Figura 1.4 Conformações da proteína de envelope (E) durante o processo de maturação do vírus dengue (DENV). (A) Heterodímeros da proteína E (cinza) com prM (azul) formando trímeros em formato de espícula. (B) Dímero das proteínas prM-E em pH ácido. (C) Clivagem do peptído "pr" pela furina da célula hospedeira. (D) Liberação do peptído "pr". Adaptado de (Perera e Kuhn, 2008).

A proteína NS1 (PM ~ 48 kDa) está envolvida na morfogênese da partícula viral (Mason *et al.*, 1987; Muylaert *et al.*, 1997). Apresenta-se em três formas: retidas no interior das células infectadas, localizadas na superfície celular e secretada. Ela é translocada para o retículo endoplasmático (RE) e clivada na extremidade C-terminal da proteína E por uma peptidade sinal do hospedeiro e na junção NS1/NS2A por enzimas presentes no retículo endoplasmático (RE) (Lindenbach *et al.*, 2013). A NS1 atua como um cofator no processo de replicação viral e tem sido demonstrado que se colocaliza com RNA dupla fita e outros componentes dos complexos de replicação (CR) (Muller e Young, 2013).

A NS2A é uma proteína hidrofóbica, relativamente pequena (~ 22 kDa). Sua extremidade N-terminal é gerada pela clivagem na junção NS1/NS2A por enzimas do RE. A clivagem na junção NS2A/NS2B é realizada por uma serina protease NS2B-NS3. A NS2A participa do processo de montagem da partícula viral e do complexo de replicação do RNA, interagindo com as proteínas NS3 e NS5. A NS2B também é uma pequena proteína (~ 14 kDa), associada à membrana. Forma um complexo estável com a NS3 e atua como um co-fator para a serina protease NS2B-NS3 (Lindenbach *et al.*, 2013).

A proteína NS3 é altamente conservada entre os flavivírus, apresentando atividade de protease e helicase (Henchal e Putnak, 1990). É uma grande proteína multifuncional (70 kDa), contendo diversas atividades necessárias para o processamento da poliproteína e replicação do RNA. A extremidade N-terminal da proteína é o domínio catalítico do complexo de serina protease da NS2B-NS3. Além da clivagem da NS2A/NS2B, NS2B/NS3, NS3/NS4A e NS4B/NS5, a protease gera as extremidades C-terminal da proteína C madura e NS4A, podendo decompor a NS2A e NS3 internamente (Lindenbach *et al.*, 2013).

A NS4A e NS4B são pequenas proteínas hidrofóbicas (16 kDa e 27 kDa, respectivamente). A NS4A apresenta interação genética com NS1, estando envolvida na replicação do RNA, como um dos componentes do CR. A NS4B está localizada com a NS3 nas estruturas membranosas derivadas do RE, possivelmente o sítio de replicação do RNA (Lindenbach e Rice, 1999; Lindenbach *et al.*, 2013).

A NS5 é uma proteína grande com ~ 103 kDa, e assim como a NS3, é muito conservada entre os flavivírus e multifuncional com função metiltransferase e RNA polimerase RNA-dependente (RpRd) (Henchal e Putnak, 1990). Junto com a NS3 participa do processo de replicação viral, sendo um dos componentes do CR. Forma um complexo com NS3 e pode estimular tanto atividade NTPase e RTPase (Lindenbach *et al.*, 2013).

Nas extremidades do genoma existem as regiões não codificantes (NC) identificadas como 5'NC e 3'NC, com aproximadamente 100 e 400 nucleotídeos, respectivamente. Estas

regiões possuem sequências conservadas e estruturas secundárias de RNA que direcionam os processos de amplificação genômica, tradução e empacotamento (Mandl *et al.*, 1998; Shurtleff *et al.*, 2001; Mutebi *et al.*, 2004).

1.2 Ciclo replicativo dos DENV

Os DENV, assim como os demais vírus, necessitam obrigatoriamente de uma célula hospedeira para realizarem seu processo de replicação do genoma viral e formação de novas partículas virais. O ciclo replicativo envolve as seguintes etapas: adsorção (1), penetração (2), desnudamento (3), transcrição/tradução/replicação (4), montagem (5), maturação e liberação da partícula viral (6) (Lindenbach *et al.*, 2013) (Figura 1.5).

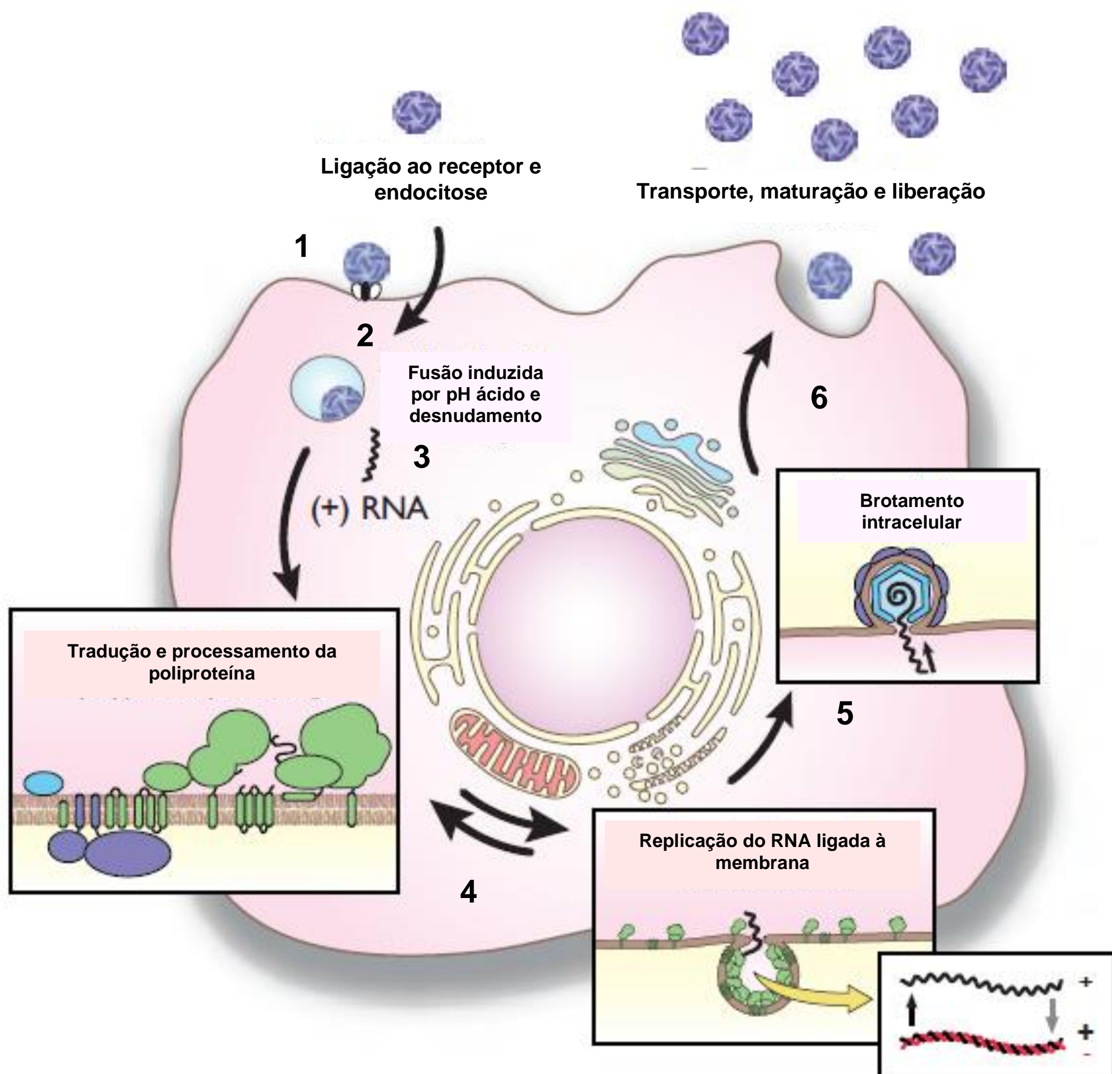

Figura 1.5 Ciclo de replicação da família *Flaviviridae*. Adaptado de Lindenbach *et al.*, 2013.

A primeira etapa do ciclo replicativo é a adsorção da partícula viral infecciosa às células hospedeiras por meio da ligação das proteínas do envelope viral à receptores celulares específicos, seguido do processo de endocitose. No endossomo, o vírion tem o envelope viral fusionado com as membranas intracelulares, induzido pelo baixo pH (potencial hidrogeniônico). Assim, o nucleocapsídeo é desencapsulado permitindo a liberação do RNA genômico no citoplasma (Lindenbach *et al.*, 2013).

A replicação dos DENV ocorre inteiramente no citoplasma das células infectadas, nas estruturas de membranas intracelulares rearranjadas e induzidas pelos vírus chamadas de complexo de replicação (CR). O CR contém proteínas específicas do vírus, RNA viral, e alguns fatores da célula hospedeira envolvidos na produção do RNA progênico. Este ambiente permite uma eficiente síntese de RNA e morfogênese viral e atua como uma estratégia para minimizar a detecção da presença do vírus pelos sensores de defesa do hospedeiro. Aparentemente, após a infecção pelo DENV, o RNA genômico se associa aos ribossomos no retículo endoplasmático (RE) durante a tradução viral Um pré-requisito para a replicação do genoma viral é a expressão e processamento da poliproteína viral, uma vez que a célula hospedeira não codifica todos os fatores necessários para a replicação dos DENV (Welsch *et al.*, 2009; Alcaraz-Estrada *et al.*, 2010; Miller *et al.*, 2010; Lindenbach *et al.*, 2013)..

Durante a replicação viral o RNA(+) serve como molde para a produção da cadeia de RNA(-) que é usado para a geração das novas fitas de RNA(+) (progênie). Além do RNA(+) mais dois tipos de RNA são encontrados dentro da célula infectada. Os RNAs dupla fita (RNAdf) RNase-resistentes são os chamados RNA da forma replicativa (FR) e os RNAs parcialmente RNase-resistente são os intermediários replicativos (IR), ambos precursores do RNA(+) genômico. No atual modelo de replicação dos DENV assume-se que, durante a replicação, o novo RNA(-) sintetizado faz o pareamento de bases com o RNA(+) molde formando a FR. Os FR atuam como molde para gerações de múltiplas fitas de RNA+ via IR, que contém regiões duplas assim como fita livre para a ação da replicase viral. O RNA viral é sintetizado de forma semi-conservativa e assimétrica, com fitas positivas acumulando aproximadamente 10 vezes mais do que as fitas negativas. A nova fita de RNA(+) produzido é liberada do IR e segue para os ribossomos iniciando o ciclo de tradução ou é empacotado na partícula viral recém-gerada (Miller *et al.*, 2010; Lindenbach *et al.*, 2013).

A montagem do CR necessita inicialmente da proliferação e invaginações das membranas do RE. Presumivelmente, este processo é induzido pela NS4A e NS3, em conjunto com outras proteínas celular e viral. Acredita-se que oligômeros da proteína NS4A se intercalem na membrana do RE, formando as invaginações. Essas invaginações dão origem

a vesículas membranosas que tem sido descritas como o local para a replicação viral, uma vez que as proteínas NS e RNA fita dupla são localizados nessas estruturas membranosas. A proteína C se acumula em torno de gotículas lipídicas derivadas do RE que contém um núcleo lipídico neutro delimitado por uma monocamada de fosfolipídeos, sendo direcionada para o RE durante a morfogênese viral. Após a formação do nucleocapsídeo, o envelope viral é adquirido por brotamento no lúmen do RE. Os vírus são depois liberados através da via secretora do hospedeiro, via complexo de Golgi. (Welsch *et al.*, 2009; Alcaraz-Estrada *et al.*, 2010; Lindenbach *et al.*, 2013).

1.3 Ciclo de transmissão dos vírus da dengue

Os quatro sorotipos dos DENV são mantidos na natureza por dois ciclos distintos: silvestre e urbano (Figura 1.6). O ciclo silvestre é ecologicamente e evolutivamente distinto do ciclo de transmissão urbano. Neste ciclo, a transmissão é mediada por mosquitos arborícolas do gênero *Aedes* e primatas não-humanos, parecendo este ser o único hospedeiro amplificador. Já no ciclo urbano, a transmissão é realizada por mosquitos do gênero *Aedes* com comportamento urbano, que habitam no domicílio/peridomicílio, e humanos (Figura 1.6) (Vasilakis e Weaver, 2017).

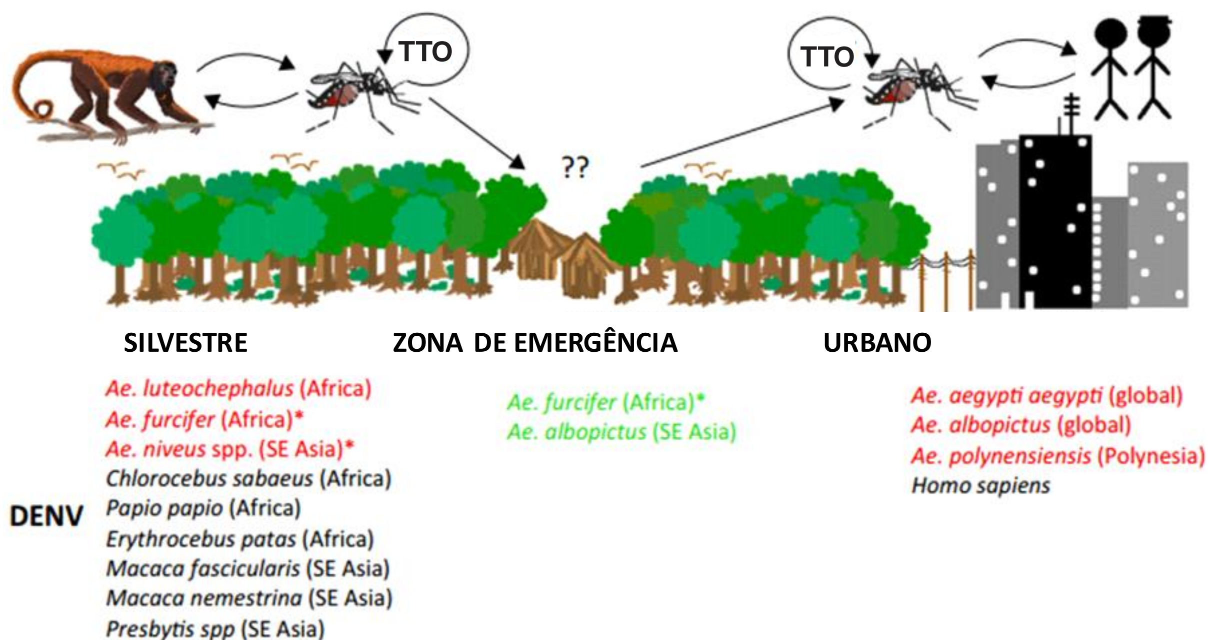

Figura 1.6 Ciclo de transmissão do vírus da dengue. TTO = transmissão transovariana. Vetores envolvidos com os ciclos silvestre e urbano em vermelho. Vetores envolvidos com a zona de emergência em verde. Hospedeiros vertebrados em preto. *Principais vetores. Adaptado de Vasilakis e Weaver, 2017.

Na África os principais vetores são o *Ae.* (Stegomyia) *luteocephalus*, *Ae.* (Diceromyia) *furcifer* e *Ae.* (Diceromyia) *taylori.* Apesar de *Ae. furcifer* serem mosquitos enzoóticos arborícolas, eles são conhecidos por descerem ao nível do solo para se alimentarem em seres humanos. O mosquito ancestral arbóreo, *Ae. aegypti formosus*, principal vetor doméstico de transmissão do DENV é imune à infecção pelo DENV silvestre (Kyle e Harris, 2008; Weaver e Vasilakis, 2009; Chen e Vasilakis, 2011). Os hospedeiros primatas na África incluem o macaco verde africano (*Chlorocebus sabaeus*), babuínos guiné (*Papio papio*) e macaco patas (*Erythrocebus pernas*), e espécies possivelmente relacionadas como: *Papio anubis*, *Papio ursinus* e *Papio cynocephalus*. Na Ásia, os principais vetores incluem os mosquitos primatofílicos do complexo *Ae.* (Finlaya) *niveus* sl., um grupo que inclui *Ae. pseudoniveus*, *Ae. subniveus*, *Ae. vanus*, *Ae. albolateralis*, *Ae. niveoides* e *Ae. novoniveus*. Estas espécies são também conhecidas por descerem das árvores até o solo para se alimentar em humanos. Os hospedeiros primatas asiáticos incluem: macacos cinomolgos (*Macaca fascicularis*), macacos de rabo de porco do sul (*Macaca nemestrina*) e macacos folhas prateadas (*Presbytis cristata*) e, possivelmente, macacos folha verde - mitrados (*Presbytis melaphos*) (Chen e Vasilakis, 2011).

Este comportamento alimentar oportunista dos mosquitos vetores arbóreos poderia facilitar a transferência de DENV silvestre das florestas para ambientes peridomiciliares. Por certo, em áreas rurais da África e Ásia, onde os vetores atingem altas densidades, muitas vezes, o DENV é detectado entre primatas não-humanos e humanos. As savanas úmidas que circundam ambientes silvestres em áreas rurais da África e Ásia são definidas como "zona de emergência" (Chen e Vasilakis, 2011).

Atualmente, quase todas as infecções humanas são devidas a circulação do DENV exclusivamente em ambientes domésticos e peridomiciliares ao longo dos trópicos, onde o homem serve como único reservatório de amplificação. Neste ciclo urbano, *Ae. aegypti* é o principal vetor que transmite o DENV, enquanto outros *Aedes* spp. (por exemplo, *Ae. albopictus*, *Ae. polynesiensis*) servem como vetores secundários. Embora o *Ae. albopictus* possa ter sido o vetor original para transmissão humana, o DENV têm explorado o elevado hábito antropofílico do *Ae. aegypti* para sustentar a transmissão em humanos (Chen e Vasilakis, 2011).

A transmissão transovariana (TTO) também tem sido sugerida como um mecanismo de manutenção dos DENV em ambos os ciclos de transmissão (Figura 1.6), especialmente durante períodos interepidêmicos ou períodos prolongados de seca. O envolvimento da TTO na manutenção dos DENV na natureza foi demonstrado com o isolamento do DENV-2

presumidamente silvestre a partir de um *pool* de *Ae. taylori* em 1980 na Côte d' Ivoire, e um ano depois no Senegal a partir de um *pool* de mosquitos *Ae. furcifer*. Em *Ae. aegypti* coletados em diversas localizações geográficas e estágios de desenvolvimento também foi demonstrada a TTO, bem como em *Ae. albopictus*, *Ae. mediovittatus*, e vários membros do grupo *Ae.* (Stegomyia) *scutellaris*, que desempenham um papel importante na transmissão do DENV no Arquipélago da Indonésia e na Polinésia (Chen e Vasilakis, 2011). Evidências demonstram que o *Ae. albopictus* é mais eficiente na TTO que o *Ae. aegypti*, podendo ser um candidato para manutenção do DENV durante períodos interepidêmicos (Castro *et al.*, 2004). Dado o elevado número de casos assintomáticos de dengue, também é possível que a transmissão silenciosa em humanos por um número reduzido de vetores mantenha a transmissão do DENV entre epidemias (Kyle e Harris, 2008).

Até o momento não há nenhuma evidência concreta da existência da transmissão do ciclo silvestre nas Américas (Chen e Vasilakis, 2011). Entretanto, alguns vertebrados silvestres vêm sendo apontados como possíveis hospedeiros temporários ou finais em alguns países do continente americano. Pesquisas envolvendo morcegos, roedores e marsupiais têm detectado anticorpos e/ou RNA viral dos diferentes sorotipos do DENV nestes mamíferos (De Thoisy *et al.*, 2004; Aguilar-Setién *et al.*, 2008; De Thoisy *et al.*, 2009; Vicente-Santos *et al.*, 2017).

Há evidências de que o DENV silvestre pode facilmente entrar em contato com os seres humanos na Ásia e na África, assim como em outras regiões do mundo, podendo desencadear uma doença mais grave, uma vez que *Ae. aegypti* e *Ae. albopictus* possuem capacidade de transmitirem o vírus silvestre sem necessariamente adaptação do vírus ao vetor (Vasilakis, Durbin*, et al.*, 2008; Vasilakis, Tesh*, et al.*, 2008). Porém, o ciclo silvestre do DENV não tem recebido a atenção necessária, uma vez que estudos recentes apontam que a possível emergência do DENV silvestre representa uma ameaça para a população e para pesquisas envolvendo vacinas, por exemplo (Mayer et al. 2017).

1.4 Breve histórico da dengue

O relato mais antigo de doença com sintomatologia compatível com a dengue ocorreu na China entre 265 a 420 d.C., durante a Dinastia Chin, quando foi descrita em uma Enciclopédia de sintomas e remédios. A mesma foi formalmente editada em 610 d.C. (Dinastia Tang), e posteriormente em 992 d.C. (Dinastia Norte Sung), quando a doença foi denominada de “veneno da água” devido a associação dos insetos voadores com a água. Os

primeiros relatos de grandes epidemias de doença febril compatível com os sintomas da dengue ocorreram em 1779 e 1780, nos continentes Asiático, Africano e Norte Americano. A doença foi caracterizada por *rash*, febre, dor nos olhos, artralgia, mialgia e manifestações hemorrágicas, incluindo sangramento de orofaringe, gengiva, intestinal e vaginal. Surtos de doenças nas Antilhas Francesas em 1635 e no Panamá em 1699 também poderiam ter sido por dengue (Gubler, 1997; Gubler, 1998).

Em 1789, Benjamin Rush relatou uma descrição clínica detalhada da dengue ao descrever uma epidemia de “febre remitente biliar" para descrever a epidemia de 1780 na Filadélfia (Rush, 1789 *apud* (Vasilakis *et al.*, 2010). Este termo já havia sido usado em San Juan, Porto Rico em 1763, sugerindo que a dengue já era bem conhecida no Caribe (Vasilakis *et al.*, 2010). Porém, James Christie, em 1882, menciona que os relatos das epidemias ocorridas de 1779-1784 podem não ter sido realmente sobre "epidemias de dengue", pois são fragmentados. Mas, afirma que a epidemia de 1779 descrita por Gaberts no Cairo e por Brylon, em Batavia, se referem a epidemias similares a dengue, chamada de "*Knockelkoorts"* (febre dos ossos). E que em 1780, um missionário indiano, Mr. Persin, menciona uma epidemia de doença parecida com dengue, prevalente na costa de Coromandel, África, Arábia, Pérsia e Thibet. Além deste relato, aponta que em 1784-85, Fernandez de Castilla e Nieto de Piña descreveram um surto de uma doença semelhante chamada *"la piadosa"*, no Cádiz e Sevilha (Christie, 1882).

A origem do nome dengue é controversa. Sugere-se que o termo “dengue” tenha sido usado pela primeira vez em Cuba. Porém, em 1801, a rainha da Espanha, Maria Luisa, se referiu a uma doença que teve como dengue. Em cartas ao Palácio Real datada de 12 de junho de 1801, ela disse: “Eu estava doente com uma doença chamada “dengue” e, desde ontem, tinha sangramento” (Soler et al., 1949 *apud* (Gubler, 1997). Entretanto, a origem mais provável da palavra dengue é de Suaheli. Em ambas as epidemias relacionadas com doença semelhante à dengue, em Zanzibar (1823) e na Costa Leste Africana (1870), a enfermidade já era chamada de "*Kidinga Pepo"*, descrita por Christie como “uma doença caracterizada por uma cãibra, como um ataque súbito, causado por um espírito maligno” (Christie, 1872). Durante a epidemia em Cuba no ano de 1828, a doença foi inicialmente chamada de Dunga, mas depois mudou para dengue, termo pelo qual tem sido conhecida desde então (Christie, 1882; Gubler, 1997). No Brasil, entre 1846-48 a doença era conhecida como “*Polka fever*” (Christie, 1882).

O termo dengue foi introduzido na literatura médica inglesa durante a epidemia do Caribe em 1827-1828. Em 1869, foi estabelecido pelo “London Royal College of Physicians”

a denominação desta enfermidade, até então referida na literatura como “febre articular”, “febre quebra ossos”, “dinga”, “polka”, entre outros (Siler, 1926; Halstead, 1974; 1980).

A dengue ou doença muito semelhante teve uma ampla distribuição geográfica antes do século XVIII, quando foram descritas as primeiras pandemias (Gubler, 1998). Contudo, ainda hoje existe a dificuldade em confirmar a doença considerando apenas dados clínicos devido aos sinais e sintomas compartilhados com outras doenças tropicais (Vasilakis *et al.*, 2010).

Durante os séculos XVIII e XIX a febre do dengue dispersou na América do Norte, América do Sul, Bacia do Caribe, Ásia e Austrália. Nas Américas, a grande propagação foi devido às repetidas introduções do vetor *Stegomyia (St.) aegypti* (*Aedes aegypti*), que foram introduzidos pelos navios negreiros e de outros navios comerciais que atravessaram o oceano Atlântico partindo da África (Rodriguez-Roche e Gould, 2013).

A constatação de que mosquitos eram os vetores de transmissão da dengue ocorreu no início da década de 1900, a partir de estudos conduzidos por Graham (1903) e Bancroft (1906), sendo o último a apresentar conclusões de que o *Ae. aegypti* poderia transmitir a doença. Os estudos realizados em Taiwan, Filipinas e no Pacífico demonstraram que *Ae. albopictus*, *Ae. polynesiensis* e *Ae. scuttellaris*, poderiam ser eficientes vetores dos DENV (Gubler, 1997).

Segundo os registros históricos, entre 1823 e 1916, houve uma segunda série de pandemias da dengue percorrendo o mundo, da África para a Índia e da Oceania para as Américas, com duração de 3 a 7 anos. Embora não seja possível identificar o sorotipo envolvido, estes surtos foram causados provavelmente pelo mesmo sorotipo e foram transportados entre as regiões geográficas através do tráfico de escravos e do comércio (Gubler, 1997; Weaver e Vasilakis, 2009).

Leichtenstern (1896) foi o primeiro a reconhecer a dengue com uma doença dos portos marítimos das regiões costeiras que poderia se dispersar pelo interior ao longo dos rios, como o Ganges e o Indo na Índia, ou do Mississippi nos Estados Unidos. A invasão dos trópicos pelo mosquito vetor *Ae. aegypti* africano, provavelmente devido à circulação de pessoas e o armazenamento de águas nos veleiros foram fatores que mudaram radicalmente o comportamento da dengue no Sudeste Asiático, Subcontinente Indiano e Filipinas que resultou no súbito aparecimento de epidemias urbanas (Weaver e Vasilakis, 2009).

Durante a Segunda Guerra Mundial, no período de 1944 e 1945, pesquisadores japoneses (Hotta, 1952) e americanos (Sabin, 1952) isolaram as primeiras cepas do vírus

dengue, sendo os sorotipos 1 e 2 identificados. Seguindo na década de 1950 com o isolamento dos sorotipos 3 e 4 na Tailândia e Filipinas (Hammon *et al.*, 1960).

Nas Américas, durante os anos 1960 e 1970, houve a circulação de um único sorotipo num determinado momento dentro de uma dada região. No entanto, após este período, um aumento significativo da doença foi observado em outras regiões tropicais no mundo, com uma série de epidemias associadas a um aumento na gravidade dos sintomas apresentados. Ao final deste período, era evidente a cocirculação dos quatro sorotipos do vírus em todo o Sudeste da Ásia e Subcontinente Indiano (Gubler, 1998).

Na década de 1990 e início do século XXI, a expansão populacional urbana, o aumento da densidade vetorial devido à insustentabilidade dos programas de controle e aumento do transporte comercial aéreo, facilitando a movimentação rápida de pessoas, levaram à distribuição global de todos os sorotipos, favorecendo a reemergência rápida de casos graves da doença ao longo dos trópicos (Weaver e Vasilakis, 2009). Atualmente, os quatro sorotipos do vírus circulam na África, Sul e Sudeste da Ásia, Regiões do Pacífico Ocidental, Bacia do Caribe, e Américas Central e do Sul. Frequentes introduções no sudeste da América do Norte também tem sido reportadas (Rodriguez-Roche e Gould, 2013).

1.5 Origem e evolução dos DENV

A origem geográfica dos DENV tem sido especulada por décadas. Sugere-se que os DENV endêmicos evoluíram de cepas silvestres na África ou na Ásia, que circulavam entre primatas não-humanos e vetores do gênero *Aedes*. Entende-se o ciclo silvestre como ancestral, uma vez que para que a transmissão inter-humana eficiente ocorra, necessita-se de população mínima de 10.000 a 1 milhão de habitantes, o que só passou a existir por volta de 4.000 anos atrás com o início da civilização (Gubler, 1997; Weaver e Vasilakis, 2009).

As primeiras hipóteses para a origem destes vírus apontam para a África, principalmente porque muitos dos flavivírus transmitidos por mosquitos circulam exclusivamente no continente africano e por vezes infectam os primatas, o que sugere que este grupo como um todo se originou neste local. Além disso, acredita-se que o *Ae. aegypti* tenha se originado na África, embora esta espécie tenha sido adotada como um vetor para a transmissão em humanos em um passado recente (Gubler, 1997).

Em contrapartida, a presença de todos os quatro sorotipos, tanto em humanos quanto em macacos na Ásia, além da profunda similaridade filogenética das cepas asiáticas silvestres

em particular, sugere que o vírus tenha origem asiática, ao invés de uma origem africana (Holmes e Twiddy, 2003; Weaver e Vasilakis, 2009; Vasilakis *et al.*, 2010).

Até a década de 1940 a informação sobre o causador da dengue era apenas de um agente filtrável. Foi durante a Segunda Guerra Mundial que as primeiras cepas virais foram isoladas. Hotta e Kimura foram os primeiros a isolar o vírus em 1943, através de inoculação de soro de pacientes com doença aguda em camundongos lactentes (Gubler, 1997), mas infelizmente, este trabalho foi publicado em uma revista japonesa pouco conhecida. No verão de 1943, o Dr. Hotta conseguiu o isolamento de uma cepa de vírus da dengue a partir do sangue de um paciente chamado Mochizuki, em Nagasaki, Japão (Hotta, 1952). Naquela época, em um país em guerra, preservar cepas de vírus isolados era difícil, pois não possuíam gelo seco e os freezers eram inúteis por causa das frequentes falhas de energia. Por isso a cepa de DENV-1 foi mantida através de passagens em camundongos. Mas, em um momento em que a oferta de camundongos foi baixa, a manutenção da cepa viral Mochizuki foi até mesmo realizada por inoculação na sua própria mãe (Konishi e Kuno, 2013).

Na mesma época, Sabin e seus colaboradores isolaram o vírus de soldados americanos na Índia, Nova Guiné e Havaí. Utilizando teste de inibição da hemaglutinação, foi possível demonstrar que algumas cepas virais das três regiões geográficas eram antigenicamente similares (Sabin e Schlesinger, 1945; Sabin, 1952). Este vírus foi chamado de dengue tipo 1 (DENV-1), sendo a cepa havaiana designada como protótipo (Haw-DEN-1). Outra cepa viral antigenicamente distinta proveniente da Nova Guiné foi nomeada de dengue tipo 2 (DENV-2), sendo o protótipo chamado de Nova Guiné C (NG"C"-DEN-2) (Sabin, 1952). O vírus de origem japonesa isolado por Hotta e Kimura (1952) foi posteriormente confirmado como DENV-1. Mais dois sorotipos, DENV-3 e DENV-4, foram isolados subsequentemente de pacientes com doença hemorrágica numa epidemia em Manila, nas Filipinas, em 1956 (Hammon *et al.*, 1960; Gubler, 1997).

Embora estudos antigênicos tenham demonstrado que os DENV se classificam como flavivírus, estudos filogenéticos passaram a ser cada vez mais necessários para o entendimento da história evolutiva destes vírus (Zanotto *et al.*, 1996; Kuno *et al.*, 1998; Holmes e Twiddy, 2003). A filogenia baseada na sequências nucleotídica do gene NS5, altamente conservado entre os flavivírus, caracterizou o gênero em três grupos: 1. vírus transmitidos por carrapatos; 2. vírus transmitidos por mosquitos; e 3. vírus com vetor desconhecido (Holmes e Twiddy, 2003; Vasilakis e Weaver, 2008). Os quatro sorotipos do DENV apresentaram forte suporte dentro do clado dos vírus transmitidos por mosquitos, embora os grupos relacionados aos DENV tenham apresentado fraco suporte de *bootstrap*,

não podendo assim ser classificado com certeza (Holmes e Twiddy, 2003). As análises filogenéticas tanto do gene NS5 (Vasilakis e Weaver, 2008), quanto do gene E (Zanotto *et al.*, 1996) demonstraram a mesma ordem de separação dos ramos entre os quatro sorotipos do DENV, com o DENV-4 divergindo primeiramente, seguido do DENV-2 e por último o DENV-1 e DENV-3 (Figura 1.7).

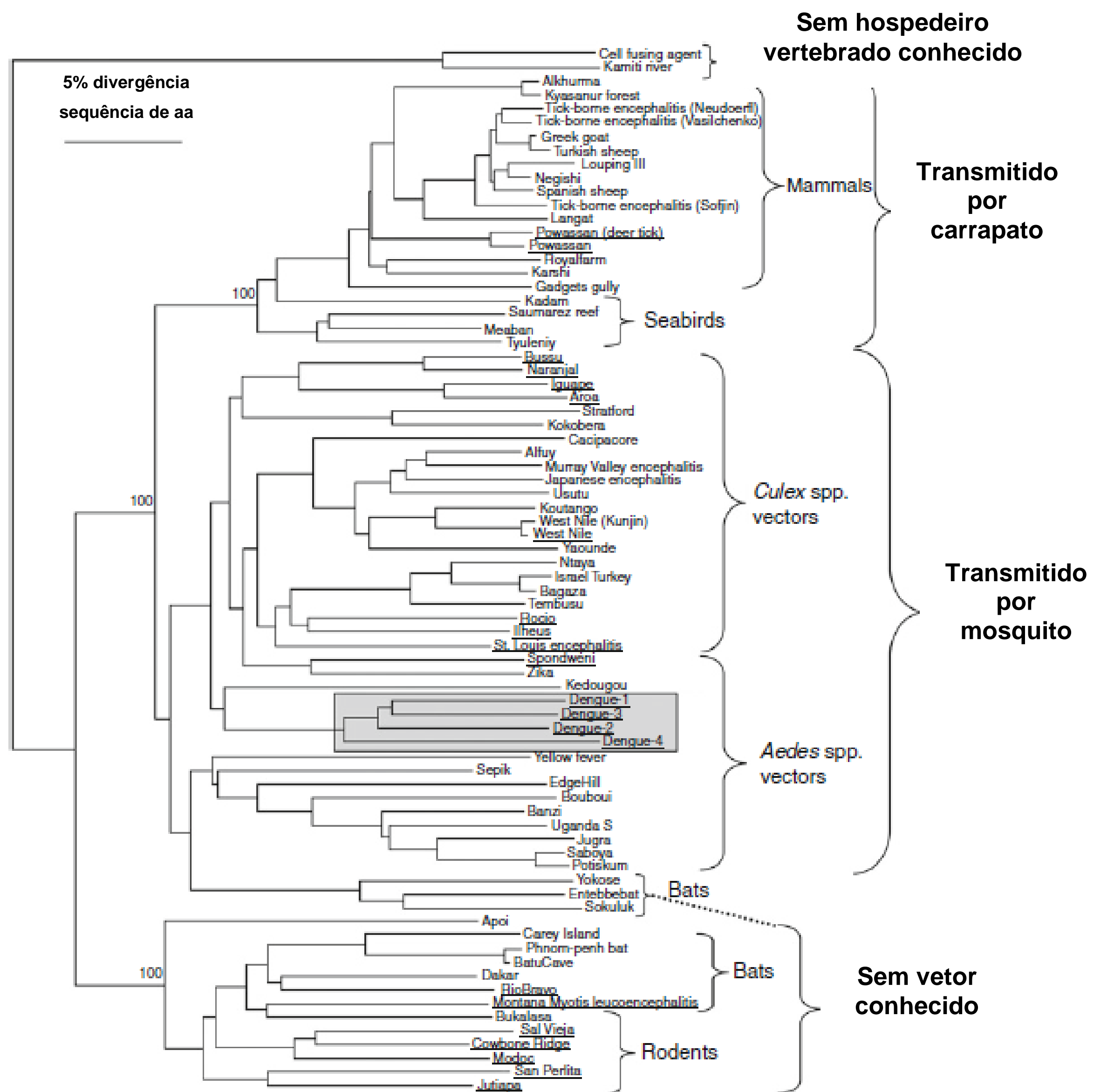

Figura 1.7. Árvore filogenética dos flavivírus baseada no gene não estrutural NS5 representando os grupos virais de acordo com o tipo de vetor. Método de *Neighbor-joining*, *bootstrap* apresentado nos principais clados. Em destaque, os DENV em ordem de divergência (Adaptado de (Vasilakis e Weaver, 2008)

Outra maneira de inferir a história evolutiva é utilizando a reconstrução de uma escala de tempo molecular. Isto foi alcançado pelas taxas de estimativa de substituição de nucleotídeos usando um método de Máxima Verossimilhança (*Maximum Likelihood*) que

analisa a quantidade de mudança evolutiva ocorrida entre os vírus amostrados em momentos diferentes (Rambaut, 2000; Twiddy, Holmes, *et al.*, 2003). Utilizando este método em um grande número de sequências do gene E revelou-se que a evolução dos DENV segue um relógio molecular, embora tenham sido observadas taxas diferentes em linhagens específicas (Holmes e Twiddy, 2003).

A taxa de substituição de nucleotídeo por sítio por ano (subs./sítio/ano) variando de 4.55 x 10^{-4} (DENV-1) a 11.53 x 10^{-4} (DENV-3) vem sendo demonstrada para os quatro sorotipos (Twiddy, Holmes, *et al.*, 2003; Araújo, Nogueira, *et al.*, 2009; Weaver e Vasilakis, 2009; Allicock *et al.*, 2012; Villabona-Arenas e Zanotto, 2013; Mir *et al.*, 2014). Estas taxas são similares às descritas para os vírus RNA transmitidos por vetor, embora ligeiramente inferiores às dos vírus RNA que possuem outros mecanismos de transmissão, possivelmente relacionado com a seletividade inerente ao replicar em hospedeiros tão divergentes como insetos e mamíferos. Usando estas taxas de substituição, a divergência dos quatro sorotipos do DENV foi datada como cerca de 1000 anos atrás, e de acordo com a diversidade genética atualmente reconhecida estima-se que cada sorotipo surgiu quase simultaneamente e apenas durante o século passado (Holmes e Twiddy, 2003; Twiddy, Holmes, *et al.*, 2003; Weaver e Vasilakis, 2009).

Estudos prévios demonstraram que os vírus de origem silvestre e os envolvidos com epidemias apresentaram grande diferença genética, sugerindo que os ciclos silvestres e urbano sejam evolutivamente e ecologicamente distintos. A análise filogenética do sorotipo 2 conduzida por Rico-Hesse foi a primeira a apoiar esta hipótese, apresentando a diferença entre os ciclos silvestres e urbanos, utilizando cepas do Oeste da África, que possuíam alta divergência nucleotífica baseada no fragmento dos genes E/NS1 (Rico-Hesse, 1990). Tal fato foi reforçado por estudo baseado no gene E completo conduzido por Wang, utilizando isolados silvestres de DENV-1, 2 e 4 do Sudeste da Ásia das décadas de 1960 e1970 e de DENV-2 do Oeste da África. Estes vírus apresentara divergência de 7% a 17% entre as cepas silvestres e urbanas. Em relação ao DENV-3, a presença de primatas não-humanos apresentando soroconversão para este sorotipo no Sudeste da Ásia, apoia a possível existência de um ciclo silvestre nesta região (Wang *et al.*, 2000; Vasilakis e Weaver, 2008; Weaver e Vasilakis, 2009).

Estudos mais recentes utilizando o relógio molecular permitiram elucidar um dos questionamentos quanto à ancestralidade das cepas endêmicas/epidêmicas dos DENV, circulantes no ciclo urbano, demonstrando que as cepas silvestres são distintas das urbanas e

parecem datar anteriormente à estas (Vasilakis e Weaver, 2008; Weaver e Vasilakis, 2009) (Figura 1.8).

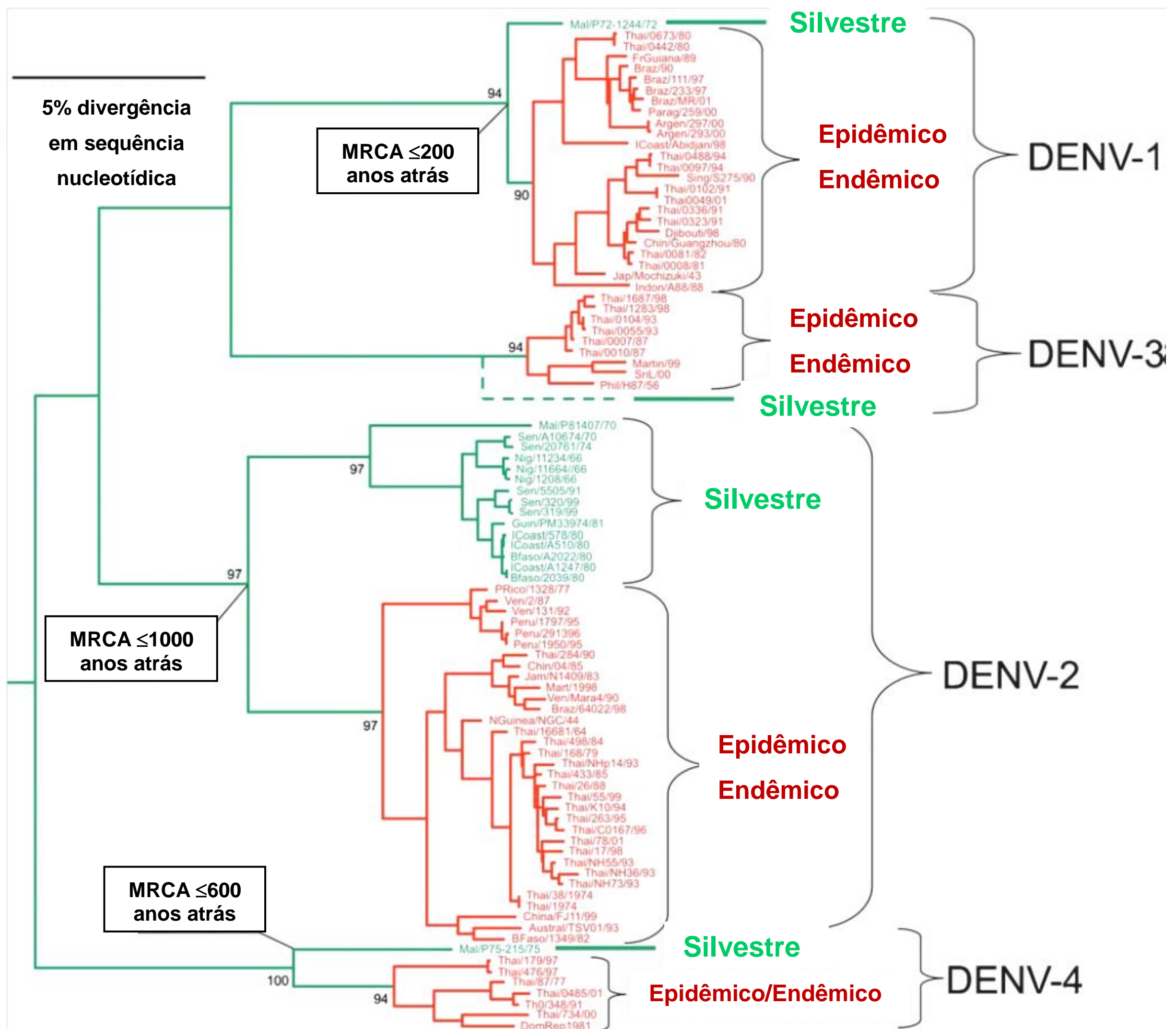

Figura 1.8. Árvore filogenética dos 4 sorotipos dos DENV baseada na sequência nucleotídica codificante completa. Filogenica inferida por análise Bayesiana apresentando o MRCA (ancestral comum mais recente) das cepas silvestres anteriores às epidêmicas/endêmicas. Adaptado de Weaver e Vasilakis, 2009.

A evolução dos DENV tem tido um impacto importante sobre a sua virulência para os seres humanos e epidemiologia da doença em todo o mundo. Embora diferenças antigênicas e genéticas nas cepas virais sejam evidentes, a falta de modelo animal da doença dificulta estudos sobre a virulência dos DENV. Entretanto, estudos filogenéticos permitem a correlação entre estas variantes específicas e gravidade da doença. Atualmente, os DENV

podem ser classificados como sendo de baixo, médio ou alto impacto epidemiológico. Alguns vírus podem permanecer em ciclos silvestres de pequena ou baixa transmissibilidade para humanos, outros causam somente casos brandos, e alguns genótipos têm sido associados como causador de casos graves. Apesar do complexo conjunto de fatores que contribuem para a epidemiologia, estudos têm sugerido que estruturas virais específicas podem contribuir para o aumento da replicação nas células-alvo humanas e intensificar a transmissão pelo mosquito vetor. Entretanto, o estado imunológico e possivelmente características genéticas do hospedeiro também são determinantes de virulência ou manifestação da doença (Rico-Hesse, 2003; Weaver e Vasilakis, 2009).

1.6 Variabilidade genética e filogenia dos DENV

Os DENV, assim como outros vírus de genoma RNA, apresentam maior variabilidade genética devido às suas rápidas taxas de replicação e ao alto grau de mutação associado com a RNA polimerase RNA-dependente, que não possui a capacidade de correção, gerando aproximadamente uma mutação a cada ciclo replicativo do genoma (Steinhauer *et al.*, 1992; Drake, 1993; Tao e Ye, 2010). Esta variabilidade genética resulta na existência dos quatro sorotipos antigenicamente distintos e nas variantes virais dentro de cada sorotipo. Antes mesmo de se conhecer a sequência dos genes já se sabia que variações genéticas existiam dentro de cada sorotipo (Holmes e Twiddy, 2003). A evidência de cepas diferentes entre os DENV foi primeiramente detectada sorologicamente usando anticorpos produzidos por inoculação em animais de laboratório (Sabin, 1952).

As primeiras evidências genéticas para diferenças entre os sorotipos dos DENV foram determinadas pela técnica RNA *fingerprinting*, utilizando enzimas para digestão da fita do RNA, com o número e tamanho dos fragmentos variando de acordo com a sequência nucleotídica do vírus, agrupando-os em topotipos (Vezza *et al.*, 1980; Repik *et al.*, 1983; Rico-Hesse, 2003).

Baseado no gene E, foi mostrado que os quatro sorotipos dos DENV compartilham similaridade de aminoácido de 60 a 70%, e entre cepas de um mesmo sorotipo a similaridade se apresenta como ≥ 90% (Pierson e Diamond, 2013).

O sequenciamento do genoma viral permitiu, com maior eficiência e exatidão, a caracterização das cepas dos DENV e permitiu delinear as relações evolutivas entre sorotipos. O termo "genótipo" foi definido como o agrupamento de DENV com divergência nucleotídica

≥ 6% para uma determinada região do genoma (Rico-Hesse, 1990; Chen e Vasilakis, 2011). Assim, dentro de cada sorotipo são observados diferenças de 6% e 3% em sequências de nucleotídeo e de aminoácido, respectivamente (Pierson e Diamond, 2013).

Estudo envolvendo os quatro sorotipos puderam estabelecer os diferentes genótipos dentro de cada sorotipo viral. Alguns genótipos apresentam forte relação geográfica, enquanto outros apresentam grande dispersão ao longo dos continentes.

Para o DENV-1 análises filogenéticas baseadas nas sequências nucleotídicas da junção E/NS1 ou do gene E completo indicam a divisão do sorotipo em cinco genótipos: (1) genótipo I, representando cepas do Sudeste Asiático, China e Leste da África; (2) genótipo II, representando linhagens da Tailândia coletados na década de 1950 e 1960; (3) genótipo III, representando a cepa selvagem coletada na Malásia; (4) genótipo IV, representando cepas das ilhas do oeste do Pacífico e da Austrália; e o (5) genótipo V, representando todas as cepas derivadas das Américas, África Ocidental, e um parte das cepas asiáticas (Rico-Hesse, 1990; 2003; Weaver e Vasilakis, 2009; Chen e Vasilakis, 2011)

Análises filogenéticas baseadas em sequências nucleotídicas do gene E caracterizou cinco genótipos para o DENV-2: (1) genótipo asiático, consistindo em genótipo asiático 1, representado por cepas da Malásia e Tailândia e genótipo asiático 2, representado por cepas do Vietnam, China, Taiwan, Sri Lanka e Filipinas; (2) genótipo cosmopolita, incluindo cepas de ampla distribuição geográfica, possuindo cepas da Austrália, África Oriental e Ocidental, Ilhas do Oceano Índico e Pacífico, Subcontinente Indiano e do Oriente Médio; (3) genótipo americano, com representantes da América Latina e cepas mais antigas do Caribe, Subcontinente Indiano e Ilhas do Pacífico das décadas de 1950 e 1960; (4) genótipo Sudeste Asiático/Americano, incluindo cepas da Tailândia, do Vietnam e coletadas nas Américas nas últimas três décadas; e (5) genótipo silvestre, representando cepas de humanos, mosquitos silvestres, ou macacos sentinela no Oeste da África e Sudeste da Ásia (Rico-Hesse *et al.*, 1997; Twiddy *et al.*, 2002; Weaver e Vasilakis, 2009; Chen e Vasilakis, 2011).

Inicialmente foram caracterizadas quatro genótipos para o DENV-3 (Lanciotti et al., 1994), o que foi corroborado por outras análises baseadas no sequenciamento do genoma parcial ou completo (Chao *et al.*, 2005; King *et al.*, 2008; Amarilla *et al.*, 2009; Araújo *et al.*, 2009; Ramírez *et al.*, 2010; Sharma *et al.*, 2011). Porém, análises utilizando um maior número de sequências do gene E disponíveis no Genbank caracterizaram cinco genótipos para este sorotipo: (1) genótipo I, representando cepas do Sudeste da Ásia, principalmente Indonésia, Singapura, Malásia, Filipinas, Taiwan e isolados das ilhas do Pacífico Sul; (2) genótipo II , representando cepas da Tailândia (de 1962 até recentemente), uma única cepa da Singapura

(1995), uma da Indonésia (1998), Taiwan, Vietnam, Bangladesh, Cambodia, China, Japão e Myanmar; (3) genótipo III, com cepas da Sri Lanka, Índia, Japão, Taiwan, Singapura, Samoa, Lesta da África, Américas Central e Latina, Caribe e cepas importadas da Europa; (4) genótipo IV representando cepas de Porto Rico (das décadas de 1960-70) e Tailândia; e (5) o genótipo V representando pela cepa protótipo das Filipinas (H87), Japão, China (1987-2009) e cepas do Brasil, do início dos anos 2000 (Araújo *et al.*, 2009; Chen e Vasilakis, 2011). Embora cepas silvestres de DENV-3 não tenham sido isoladas, acredita-se que estas existam na Malásia, com base na soroconversão de macacos sentinelas, como já citado anteriormente (Vasilakis e Weaver, 2008).

For DENV-4, dois genótipos foram inicialmente caracterizados pela análise de sequências do gene E (Lanciotti *et al.*, 1997). Entretanto, análises mais robustas, com maior número de sequências e análises de bioinformáticas mais complexas, reportam a existência de quatro genótipos: (1) genótipo I, representado por cepas das Filipinas (incluindo a cepa protótipo H241), Tailândia, Vietnam, Myanmar, Malásia, Sri Lanka, Índia, Brasil (casos importados nos anos 2000 e a partir de 2011) e casos importados do Japão e China; (2) genótipo II, representado por cepas do Sudeste asiático, China, Ilhas do Leste do Oceano Pacífico, Austrália, Caribe, Américas e Ilha de Páscoa; (3) genótipo III, representados por cepas do Tailândia (1997-2001); e (4) genótipo V, também chamado de genótipo silvestre, contendo a cepa silvestre isolada de macacos sentinelas na Malásia na década de 1970 (Klungthong *et al.*, 2004; Chen e Vasilakis, 2011; Villabona-Arenas e Zanotto, 2011; Nunes *et al.*, 2012; Pinho *et al.*, 2015).

Os avanços nas técnicas moleculares, reagentes e programas de bioinformática permitiram análises de fragmentos maiores do genoma, utilizando maiores quantidades de sequências. Cada vez mais sequências genômicas originárias de diversos países estão sendo disponibilizadas em bancos de genes públicos on-line, tais como o *GenBank*® (http://www.ncbi.nlm.nih.gov), o *European Nucleotide Archive* (http://www.ebi.ac.uk/ena), DNA *Data Bank* do Japão (http://www.ddbj.nig.ac.jp/) e o *Virus Pathogen Resource* (https://www.viprbrc.org/), permitindo a continuidade de estudos filogenéticos, assim como os filogeográficos, que permitem a análise dos padrões e dinâmicas evolutivas do DENV.

1.7 Recombinação nos DENV

Métodos filogenéticos têm sido utilizados para identificar a recombinação dentre os sorotipos de DENV, principalmente para o sorotipo 1. Evidências de recombinação para o DENV-1 podem ser encontradas nos seguintes estudos: análise de uma amostra asiática e duas africanas, demonstrando que uma isolada na Singapura (S275/90) continha fortes indícios de ser uma amostra que sofreu eventos de recombinação com as duas linhagens africana e asiática (Tolou *et al.*, 2001); uma amostra de um paciente da Nova Caledônia que foi infectado com ambos os genótipos I e II, assim como com uma cepa recombinante (Aaskov *et al.*, 2007); em um cepa originária da China apresentando três regiões recombinantes nas sequências da junção prM/E, NS1 e NS3 (Chen *et al.*, 2008); e em amostras brasileiras (Dos Santos *et al.*, 2002; Carvalho *et al.*, 2010). Porém, a função da recombinação na evolução dos DENV ainda se encontra controverso (Weaver e Vasilakis, 2009).

Ainda não foi possível alcançar experimentalmente a recombinação nos flavivírus, portanto deve-se ter cuidado ao inferir conclusões sobre eventos de recombinação. Para tal, algumas condições são necessárias para confirmar a transmissão de uma cepa recombinante natural: a transmissão do recombinante deve ser demonstrada em um único fragmento amplificado por PCR após a clonagem para assegurar que ocorre em uma única molécula de cDNA, a recombinação deve ser demonstrada repetidamente em populações de clones de vírus viáveis e o recombinante deve ser mantido durante a evolução pós-recombinação (Weaver e Vasilakis, 2009). As condições ecológicas facilitam a recombinação nos DENV, envolvendo a cocirculação de múltiplas populações virais, incluindo distintos genótipos, permitindo ao mosquito vetor ingerir múltiplas variantes por se alimentar em diferentes hospedeiros, ou pelo fato do hospedeiro ter se infectado simultaneamente por vetores infectados com diferentes vírus. Locais onde há presença de múltiplos genótipos são considerados altamente oportunos para a ocorrência de infecções mistas com diferentes cepas do DENV (Aaskov *et al.*, 2007).

1.8 Epidemiologia da dengue

A dengue é um dos principais problemas de saúde pública, sendo a doença viral transmitida por mosquito que se espalha mais rápido no mundo, com relevante impacto econômico e social devido ao aumento da expansão geográfica, número de casos e gravidade

da doença (Guzman e Harris, 2015). Nos últimos 50 anos, a incidência tem elevado 30 vezes com o aumento da expansão geográfica para novos países. Em 2012, a distribuição geográfica da dengue incluiu mais de 125 países. Esse aumento reforça a necessidade da implementação de programas para prevenção sustentável e intervenções de controle. O surgimento e disseminação dos quatro sorotipos na África, Américas, Sudeste da Ásia e Mediterrâneo Oriental representa uma ameaça de pandemia (WHO, 2013) (Figura 1.9).

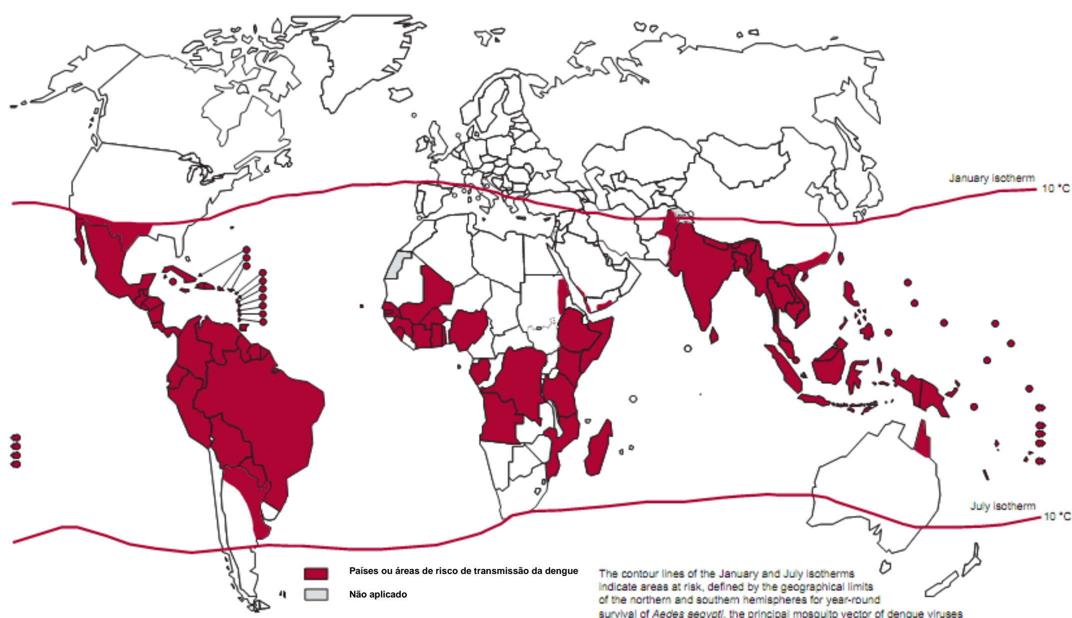

Figura 1.9 Distribuição de países ou áreas de risco de transmissão da dengue no mundo – 2011 (WHO, 2013).

Estima-se que 3 bilhões de pessoas vivam em áreas de risco de contrair a dengue e cerca de 390 milhões de infecções (96 milhões sintomáticos) e 20.000 mortes por dengue ocorram a cada ano (Bhatt *et al.*, 2013). Entre os anos de 1955-2011 houve um aumento constante no número de casos de dengue e dengue grave registrados pela Organização Mundial de Saúde (OMS) (WHO, 2013) (Figura 1.10).

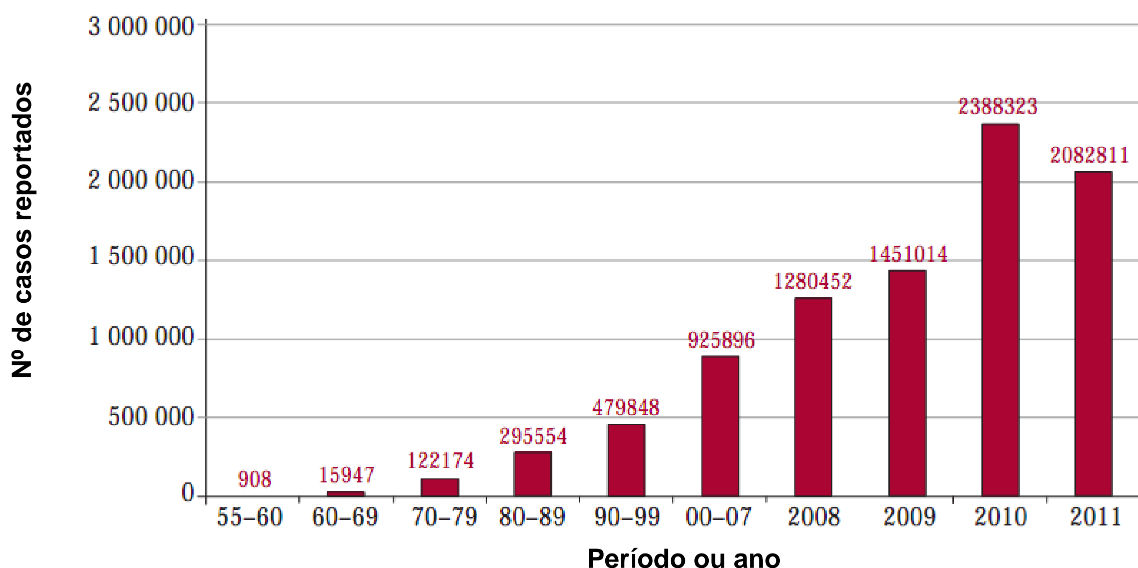

Figura 1.10 Média anual de casos de dengue e dengue grave reportado pela OMS, 1955–2011 (WHO, 2013).

Após o ano de 2010 até 2016 o total de casos de dengue registrado pela OMS, provenientes das Américas, Sudeste Asiático e Oeste do Pacífico, se mantiveram acima de 1 milhão de casos, ultrapassando ou aproximando da faixa dos 3 milhões de casos nos anos de 2013, 2015 e 2016 (Figura 1.11), sendo o continente americano responsável pelo maior número de casos em todos os anos. A figura 1.12 apresenta a média do número de casos no período de 2010 a 2016 referente a cada país destas três regiões reportadas pela OMS.

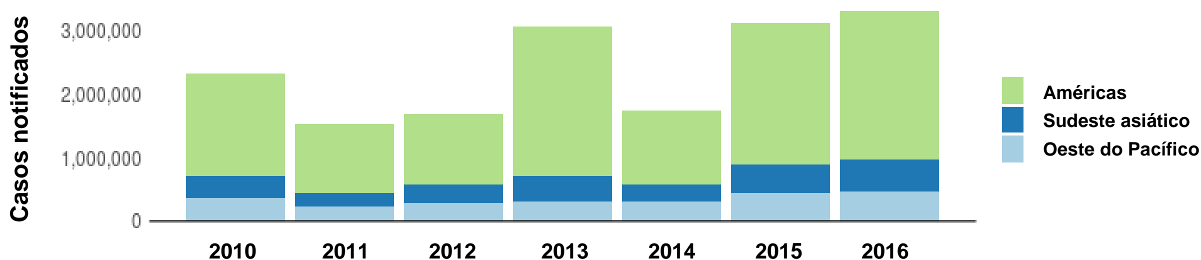

Figura 1.11 Casos de dengue notificados pela OMS, 2010-2016. Adaptado de WHO, 2018b.

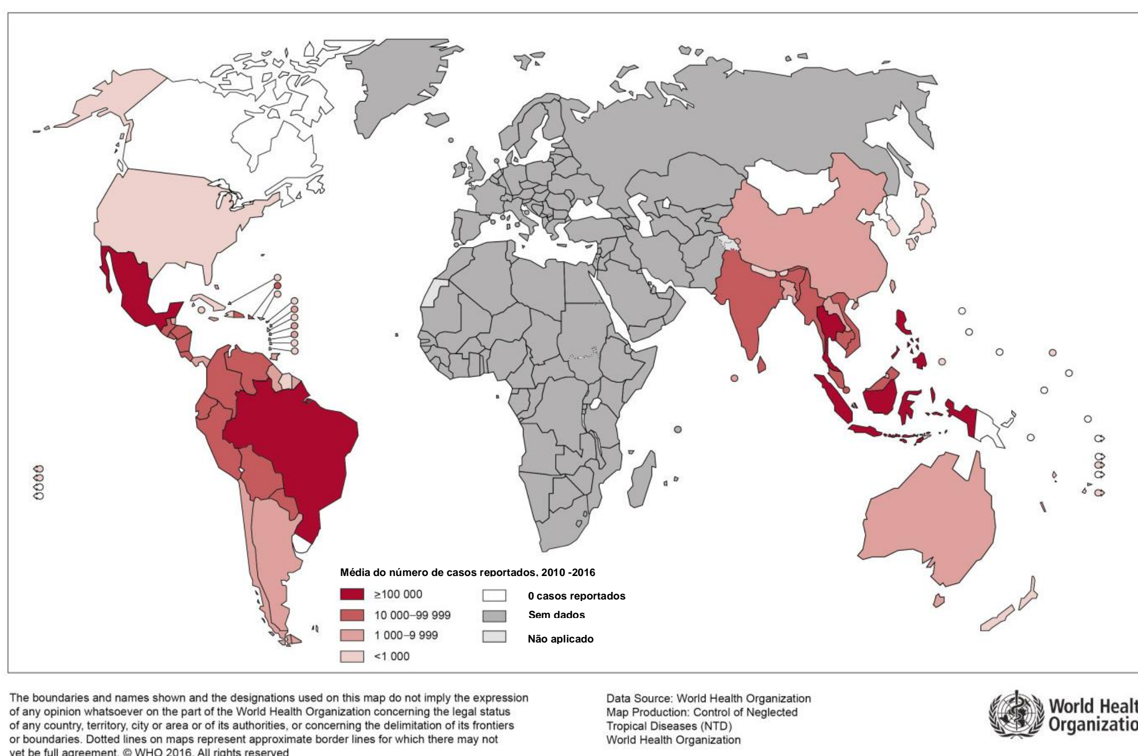

Figura 1.12 Número médio de casos suspeitos e confirmados de dengue reportados a OMS, 2010-2016. (Fonte: http://www.who.int/denguecontrol/epidemiology/en/. Acesso em junho de 2018)**.**

1.8.1 Dengue nas Américas

A dengue provavelmente foi introduzida nas Américas no século XVII, quando surgiram os primeiros relatos de doença similar a dengue em 1635 na Martinica e Guadalupe, e em 1699 no Panamá. No século XIX surtos de dengue foram comuns em cidades portuárias do Caribe, Américas do Norte, Central e Sul, mais relacionadas a atividades comerciais. Em 1818, um surto de uma doença semelhante a dengue no Peru causou aproximadamente 50.000 casos da doença. Entre 1827 e 1828, um surto envolvendo o Caribe e o Golfo do México também foi reportado, se iniciando nas Ilhas Virgens e expandindo para Cuba, Jamaica, Colômbia, Venezuela, algumas cidades portuárias dos Estados Unidos e México. Embora originalmente este surto tenha sido descrito como dengue, as características clínicas indicam que tenha sido causada pelo vírus chikungunya, sugerindo a entrada deste vírus nas Américas a partir dos navios negreiros africanos (Brathwaite Dick *et al.*, 2012).

Algumas evidências de doença semelhante a dengue foram relatadas em surtos ocorridos em Nova Orleans, Cuba e Brasil, entre os anos de 1845 a 1849. Em 1850, uma

epidemia da dengue foi relatada em algumas cidades dos Estados Unidos e Havana, Cuba e em 1851, evidências de casos de dengue foram descritas em Lima, Peru. Nos anos seguintes, surtos esporádicos foram relatados no Golfo e portos do Atlântico nos Estados Unidos, sendo o maior em 1873, em Nova Orleans, com 40 mil pessoas afetadas. Outra grande epidemia em várias cidades portuárias do sul dos Estados Unidos foi registrada entre 1879 e 1880 (Brathwaite Dick *et al.*, 2012).

No final do século XIX e início do século XX, uma ampla distribuição de doença semelhante a dengue foi descrita, incluindo países do norte, como o Estados Unidos até do sul, Chile e Argentina. Entre 1880 e 1912, os surtos foram notificados em Curitiba (Brasil); Iquique, Antofagasta, Tarapaca, Tacna e Arica (Chile); Texas e Flórida (Estados Unidos), Havana (Cuba); Bahamas, Bermuda, e Canal do Panamá. Nos anos seguintes, até 1916, epidemias foram reportadas nas Ilhas Virgens, Porto Rico, Rio Grande do Sul (Brasil) e na Argentina. Já em 1918, um surto foi relatado em Galveston, no Texas, seguido por outro de maior proporção em 1922, com uma estimativa de 30 mil casos de doença "tipo" dengue. No mesmo período um surto foi descrito em Niterói, Brasil. Em 1934, uma grande epidemia iniciou em Miami atingindo aproximadamente 10% da população. Entre 1941 e 1946 a dengue continuou a dispersar com surtos no Texas (1941); na zona do canal do Panamá (1941-42); Havana, Cuba (1944); Porto Rico (1945); Caracas e Venezuela (1945-46); Bermudas, Bahamas e Sonora, no México (Brathwaite Dick *et al.*, 2012).

Um programa de erradicação do *Ae. aegypti* iniciado pela Organização PanAmericana de Saúde (OPAS) nas décadas de 1940 e 1950 para prevenir epidemias urbanas de febre amarela resultou na diminuição significativa das epidemias de dengue nas Américas. O programa foi bem sucedido em vários países, alcançando a erradicação no México, Guatemala, Belize, Honduras, El Salvador, Nicarágua, Costa Rica, Panamá, Colômbia, Equador, Peru, Chile, Bolívia, Paraguai, Argentina, Uruguai, Brasil, Ilhas Caiman e Bermudas. Porém, o mesmo não foi observado no Suriname, Guiana, Guiana Francesa, Venezuela, Ilhas Caribenhas e Estados Unidos. No entanto, a descontinuação do programa no início da década de 1970 e a falta de eliminação do vetor em algumas regiões resultou em reinfestação do mosquito nas décadas subsequentes e em 1995, o vetor já apresentava uma distribuição similar aquela da década de 1940 (Gubler, 1997).

Antes de 1977, somente o DENV-2 e 3 estavam presentes nas Américas, sendo o DENV-1 introduzido neste período, causando epidemias na Jamaica e Cuba e em 1978 em Porto Rico e Venezuela. Nos quatros anos subsequentes, este sorotipo dispersou por todas as Ilhas do Caribe, México, Texas, América Central e norte da América do Sul, causando

grandes epidemias e surtos. Em 1981, o DENV-4 foi introduzido no leste das Ilhas do Caribe e dispersou rapidamente para outras regiões, muitas com recente relato de epidemias pelo DENV-1. Algumas dessas epidemias de DENV-4 foram associadas com casos esporádicos de dengue com manifestações hemorrágicas (Gubler, 1997; Guzman e Kouri, 2003).

Ainda em 1981 uma nova cepa de DENV-2 foi introduzida em Cuba proveniente do Sudeste Asiático, trazidas provavelmente dos soldados cubanos infectados no Vietnã entre os anos de 1977-1980, período de epidemias anual de dengue neste país (Rico-Hesse, 1990). A epidemia de DENV-2 em Cuba foi a primeira epidemia de febre hemorrágica da dengue/síndrome do choque por dengue (FHD/SCD) nas Américas, com uma estimativa de 10.000 casos (Gubler, 1997; Guzman e Kouri, 2003).

A segunda maior epidemia das Américas de FHD/SCD ocorreu na Venezuela em 1989-90 com mais de 6.000 casos e 73 óbitos, não se sabendo ao certo qual sorotipo foi o responsável, uma vez que o DENV-1, DENV-2 e DENV-4 foram isolados (Gubler, 1997; Guzman e Kouri, 2003).

Em 1990-91, os primeiros casos de FHD foram registrados no Rio de Janeiro (Brasil) (Nogueira *et al.*, 1991), causados pelo genótipo Sudeste Asiático do DENV-2 (Rico-Hesse, 2003; Faria *et al.*, 2013), que circulou com o DENV-1 (Nogueira *et al.*, 1988; 1993).

Em 1994, o DENV-3 foi reintroduzido no continente americano causando a maior epidemia de febre do dengue (FD)/FHD na Nicarágua e um pequeno surto associado a dengue clássica (DC) no Panamá. Esse vírus era geneticamente distinto do DENV-3, anteriormente circulante nas Américas, e foi demonstrado pertencer ao mesmo genótipo (III) que causou a epidemia de FHD na Sri Lanka e Índia, indicando a introdução da cepa asiática. Este DENV-3 se dispersou pela América Central e México em 1995 (Gubler, 1997; Guzman e Kouri, 2003; Brathwaite Dick *et al.*, 2012) e em 1998 foi detectado em Porto Rico, e dispersou para outras ilhas caribenhas e América do Sul (Guzman e Kouri, 2003).

Em 1997 foi reportado um pequeno surto de DENV-2 em Santiago de Cuba, não sendo detectada transmissão autóctone para outros munícipios da província ou país (Kourí *et al.*, 1998; Guzmán *et al.*, 2000). No ano seguinte, um aumento considerável dos casos foi registrado no México, Venezuela, Colômbia e Brasil. No mesmo ano, epidemias foram registradas em alguns países como Argentina onde a doença não era detectada há 82 anos (Brathwaite Dick *et al.*, 2012).

Em 2001 ocorreu uma epidemia no Peru, com 23.329 casos notificados, com os quatro sorotipos isolados e casos de FHD. Durante o ano de 2002, um número recorde de 1.015.420 casos foi registrado, incluindo 14.374 casos de FHD e 255 óbitos (Brathwaite Dick

et al., 2012). Naquele ano, o Brasil foi responsável por mais de 75% do número total de casos (Nogueira *et al.*, 2002).

No ano de 2005, na Costa Rica, ocorreu uma epidemia com aproximadamente 38.000 casos de dengue e 45 de FHD. Neste período, o DENV-2 foi reintroduzido no país, onde já estava circulando o DENV-1. Nos anos seguintes (2006-2007) ocorreu uma epidemia no Paraguai, com ~4.200 casos de dengue e circulação do DENV-3. No entanto, em 2007, mais de 28 mil casos de dengue, incluindo 52 casos de FHD e 13 óbitos foram registrados naquele país (Brathwaite Dick *et al.*, 2012). No mesmo ano, mais de 550.000 casos suspeitos de dengue foram registrados no Brasil, com 1.514 casos de FHD e 158 casos fatais por FHD (SVS/MS, 2008a). Em 2008, a reemergência do DENV-2 causou uma epidemia com mais de 700 mil casos suspeitos, mais de 4 mil casos confirmados de FHD e 223 óbitos, até a semana epidemiológica 48 (SVS/MS, 2008b).

Em 2009 várias epidemias na América do Sul foram identificadas. Na Bolívia houve uma epidemia fora do padrão para o país, com mais de 84.000 casos de dengue, com 198 casos FHD, 25 mortes, com a cocirculação do DENV-1, DENV-2 e DENV-3. No mesmo período, a Argentina relatou uma epidemia causada pelo DENV-1 com mais de 26 mil casos confirmados e cinco mortes por FHD e SCD. No México, mais de 250 mil casos foram registrados com a detecção dos quatro sorotipos. Naquele ano, a Nicarágua notificou mais de 17 mil casos de dengue, causados pelo DENV-3, DENV-1 e DENV-2 (Brathwaite Dick *et al.*, 2012).

Em 2010, aproximadamente 1.7 milhão de casos de dengue, 50.235 casos graves, e 1.185 casos fatais foram registrados, com uma incidência >200 casos/100.000 habitantes. Vários países sofreram surtos de dengue, com um total de casos que excederam os dados históricos conhecidos, incluindo a introdução da dengue em Key West, Flórida, EUA. No Brasil, o número de pessoas infectadas excedeu 1 milhão de casos, confirmando 17.489 casos de FHD e 678 casos fatais (Siqueira Jr *et al.*, 2011; Dos Santos *et al.*, 2013). Honduras, Caribe, Guadalupe, Martinica, República Dominicana e Porto Rico também registraram epidemias importantes (Brathwaite Dick *et al.*, 2012).

No caso dos EUA, ambas as espécies de vetores de dengue são amplamente distribuídas nas regiões do sul do país, e um ou outro vetor da dengue está presente em todo o território insular. Viajar, especialmente por via aérea, já tem sido considerado um importante fator de risco para a disseminação rápida de patógenos e seus vetores de forma eficiente. Os EUA possuem centros importantes para viagem aérea, consequentemente, recebem um grande número de indivíduos potencialmente infectado com agentes patogênicos que causam doenças

assintomáticas, incluindo vários arbovírus (como o vírus da dengue). Assim, existe um risco crescente de introdução de patógenos aos conglomerados urbanos onde mosquitos vetores estão presentes ou têm potencial para se estabelecer. Pela primeira vez, após várias décadas de ausência de dengue, um panorama preocupante foi esperado. Esta preocupação foi relatada antes e após a reemergência da dengue na Flórida, em 2009. Tal preocupação é aumentada pelas condições climáticas e ecológicas que favorecem a sustentabilidade do vetor e pela alta ocorrência de viagens com posterior importação de casos. Os casos de dengue decorrentes de viagens para países da América Central, América do Sul e Caribe, destino muito procurados pelos turistas norte americanos, tem contribuído para o aumento da dengue nas últimas décadas (Añez e Rios, 2013).

Em 2011, foram notificados mais de 1 milhão de casos de dengue, dos quais mais de 15.000 apresentavam formas graves necessitando de hospitalização e aproximadamente 700 óbitos. A introdução do DENV-4 foi observado no Panamá e em alguns estados do Brasil onde não havia circulado anteriormente (PAHO, 2011; 2018)

Durante o ano de 2012 houve, assim como o ano anterior, mais de 1 milhão de casos de dengue, 23.925 mortes e 521 casos graves. Todos os quatro sorotipos foram circulantes no continente americano. A maior taxa de incidência foi registrada no Cone Sul (242,54/100.000 habitantes), onde 58,1% do total de mortes por dengue no continente foram concentrados. Neste ano, vários países e territórios da Região relataram a ocorrência de surtos de dengue, incluindo Bolívia, Colômbia, Equador, El Salvador, Guatemala, Honduras, Jamaica, México, Nicarágua, Paraguai, Peru, Porto Rico e República Dominicana. Dentre os surtos notificados, alguns registraram a cocirculação de sorotipos, situação que gerou uma sobrecarga nos serviços de saúde, frente à possibilidade do aumento do risco de dengue grave (PAHO, 2012; 2018).

Em 2013, o número de casos notificados nas Américas dobrou, com mais de 2,3 milhões de casos de dengue, sendo 8.406 casos de dengue grave e 346 casos fatais, com a cocirculação dos quatro sorotipos na região. No primeiro semestre de 2013, epidemias de dengue foram identificados no Brasil, Costa Rica, Colômbia, Paraguai e República Dominicana. No Peru, foram registrados casos em áreas onde não houve casos autóctones de dengue anteriormente. Dado o comportamento habitual da dengue na região, foi esperado nos próximos meses um aumento de casos na América Central, México e Caribe, que coincide com a estação das chuvas nesses países (PAHO, 2013).

No ano seguinte, 2014, a quantidade de casos notificados permaneceu por volta de 1 milhão, com o cone sul apresentando mais de 600 mil casos. O total de casos graves e de

óbitos alcançaram o valor de 16.150 e 683, respectivamente. Destes casos, o Brasil sozinho foi responsável por 591.080 casos notificados, com 689 casos graves e 410 óbitos. Os países mais afetados, além do Brasil, neste ano foram: México, Colômbia, Venezuela, El Salvador, Honduras e Nicarágua. Os casos reportados por estes países variaram de 35.000 a 124.000 casos, aproximadamente (PAHO, 2018).

Em 2015, novamente a América vivenciou uma notificação com mais de 2 milhões de casos, sendo o cone sul representando mais de 1.7 milhões de casos. Mais uma vez o Brasil vem liderando o total de casos notificados no continente, contribuindo com mais de 1.6 milhões de casos de dengue. A sub-região andina foi responsável por mais de 200 mil casos e a América Central e México, mais de 400 mil casos. Mais de 12 mil casos foram graves, e 1.3 mil infectados foram a óbito (PAHO, 2018).

O ano de 2016 foi caracterizado por grandes surtos de dengue em todo o mundo. A Região das Américas registrou mais de 2,1 milhões de casos, onde o Brasil sozinho contribuiu em torno de 1.5 milhão de casos, aproximadamente 3 vezes mais do que em 2014. Um total 912 óbitos por dengue também foram registradas na região (PAHO, 2018; WHO, 2018a).

Em 2017, houve uma redução no número de casos em comparação com os anos anteriores, com aproximadamente 500 mil casos notificados, tendo os principais países afetados o Brasil, México, Peru e Nicarágua, nesta ordem. Juntamente com a redução dos casos de dengue, houve a diminuição dos casos graves (2.062) e de óbitos (308) (PAHO, 2018).

No corrente ano, até a semana epidemiológica 22, um total de 191.524 casos foram notificados, com 557 casos graves e 73 óbitos. Aproximadamente 50% do total de casos notificados e de óbitos são provenientes do Brasil, porém a maior parte dos casos graves são derivados de Honduras e Colômbia, ficando o Brasil em 3º lugar nesta lista (PAHO, 2018).

1.8.2 Dengue no Brasil

Em 1981, na cidade de Boa Vista, Roraima, ocorreu um surto de dengue onde as primeiras amostras de DENV-1 e DENV-4 foram isoladas e um total de sete mil casos da doença foram notificados (Osanai *et al.*, 1983). Porém, a atividade dos DENV têm crescido de modo significativo no Brasil após a detecção DENV-1 no estado do Rio de Janeiro, em Nova Iguaçu no ano de 1986 (Schatzmayr *et al.*, 1986; Dietz *et al.*, 1990; Miagostovich *et al.*, 1993; Figueiredo, 1996). A grande circulação de pessoas nesta região facilitou a rápida dispersão do vírus, causando uma epidemia explosiva, com 92 mil casos reportados durante os anos de

1986-1987 (Nogueira *et al.*, 1999; Nogueira *et al.*, 2002). Após emergir no Rio de Janeiro, o DENV-1 alcançou vários estados da região nordeste, todos com infestação elevada do vetor, em especial Alagoas, Pernambuco e Ceará (Nogueira *et al.*, 1988; Schatzmayr e Cabral, 2012). Estima-se que mais de dois milhões de casos clínicos ocorreram principalmente ao longo da costa leste do Brasil, durante o ano seguinte aos primeiros isolamentos do vírus (Nogueira *et al.*, 1988) e o DENV-1 era o único sorotipo circulante no país até 1990, quando o DENV-2 foi introduzido (Nogueira *et al.*, 1990).

O DENV-2 foi isolado na cidade de Niterói, na Região Metropolitana do estado do Rio de Janeiro no ano de 1990, e a situação do dengue no país se agravou a partir de então, sendo, notificados os primeiros casos de FHD/SCD (Nogueira *et al.*, 1990; Nogueira *et al.*, 1991; Nogueira *et al.*, 1993).

Em 1995-1996, uma nova epidemia foi reconhecida no estado do Rio de Janeiro sendo os sorotipos isolados DENV-1 e DENV-2 (Nogueira *et al.*, 1999). E pouco tempo depois, no final do ano 2000 houve a detecção de um novo sorotipo (DENV-3) no município de Nova Iguaçu, estado do Rio de Janeiro (Nogueira *et al.*, 2000), resultando no ano seguinte, na cocirculação dos três sorotipos (DENV-1, DENV-2 e DENV-3), e na maior e, até então, a mais grave epidemia do país, no ano de 2002 (De Simone *et al.*, 2004; Nogueira *et al.*, 2005).

Desde a última epidemia em 2002, até o ano de 2007 o sorotipo predominante até a semana epidemiológica 48 no estado do Rio de Janeiro era o DENV-3 (SVS/MS, 2008b). Porém o DENV-2 ressurgiu em 2007 ocasionando uma extensa epidemia no ano de 2008, com 632.680 casos notificados, um total de 24.571 casos graves e 561 óbitos no Brasil, sendo o estado do Rio de Janeiro o mais afetado com 235.353 casos, 15.730 casos graves e 263 óbitos (Dos Santos, F. B. *et al.*, 2013; Svs/Ms, 2016b). Esta epidemia apresentou um aumento significativo no número de FHD em menores de 15 anos, representando cerca de 50% dos casos de dengue e 86% das mortes ocorrendo em indivíduos nesta faixa etária (Teixeira *et al.*, 2009).

Em 2009 foram notificados 406.269 casos de dengue, destes 10.418 casos foram de dengue grave. Um total de 341 óbitos foi registrado (SVS/MS, 2013). Neste ano houve a reemergência do DENV-1, ocasionando no ano seguinte uma epidemia com o maior número de casos já registrado no país (Dos Santos *et al.*, 2013).

Em 2010, foram registrados 1.011.548 casos de dengue, com 17.474 casos graves e 656 óbitos. A região sudeste foi responsável por 478.003 casos, com os estados de Minas Gerais e São Paulo contribuindo cerca de 90% do total dos casos (SVS/MS, 2013; 2016b). Este ano foi marcado pelo número elevado de casos de dengue, sendo uma das maiores

epidemias ocorrida no país (Dos Santos *et al.*, 2013). O sorotipo predominante foi o DENV-1 que reemergiu no ano de 2009 (Dos Santos *et al.*, 2011).

A epidemia de 2010 apresentou um padrão espacial bastante distinto das epidemias de 2002 e 2008. Observou-se uma grande concentração de municípios com alta transmissão da doença na Região Centro-Oeste e parte da Região Sudeste. Os estados com maior número de casos registrados foram os estados de Minas Gerais (21,1%) e São Paulo (20,3%). O estado do Rio de Janeiro que foi o epicentro das epidemias anteriores representou apenas 2,9% dos casos. A principal alteração na epidemiologia foi a ocorrência de óbitos em pacientes que apresentavam comorbidades e a incidência foi de 538,4 casos por 100 mil habitantes. Destacam-se as taxas de internações de maiores de 60 anos de idade que apresentaram o maior incremento na comparação com o ano de 2008, onde as crianças foram as mais afetadas (Siqueira Jr *et al.*, 2011).

Em julho de 2010, o DENV-4 reemergiu no estado de Roraima (RR), cerca de 30 anos após a primeira detecção deste sorotipo no país ocorrida em Boa Vista (RR) em 1981-1982. Menos de 20 casos de DENV-4 foram confirmados neste estado ao longo do segundo semestre de 2010, e os primeiros casos decorrentes da dispersão do vírus foram detectados somente a partir de janeiro de 2011 no Amazonas e Pará (Região Norte). Em março, o vírus foi subsequentemente detectado na Região Nordeste (nos estados do Piauí, Pernambuco, Bahia e Ceará) e Região Sudeste (Rio de Janeiro e São Paulo). Os primeiros casos de DENV-4 detectados no RJ ocorreram na Região Oceânica do município de Niterói, localizado na região metropolitana, apenas 15 km de distância da capital (Nogueira e Eppinghaus, 2011).

O número de casos de dengue em 2011 continuou elevado, porém menor do que no ano anterior. Um total 764.032 foram notificados no país, com 10.546 casos grave e 482 casos fatais. No ano de 2012 foram notificados 589.591 casos da doença, com 4.425 casos graves e 327 óbitos (SVS/MS, 2016b).

Em 2013, o número elevado de notificações retornou no país, com mais 1.4 milhões de casos registrados, com predominância do DENV-4, que corresponde a 60% dos casos confirmados laboratorialmente. Neste período, mais de 6 mil casos graves e mais de 500 óbitos foram notificados. A região Sudeste, responsável por aproximadamente 63% dos casos com mais de 900 mil registros, tem o maior número de casos, seguida pela região Centro-Oeste (265.456 casos), Nordeste (152.357 casos), Sul (66.903 casos) e Norte (49.247 casos) (SVS/MS, 2016b) .

No ano seguinte, 2014 o total de casos notificados apresentou uma queda, somando 589.107 casos da doença. Porém, nos anos seguintes foi observado uma elevação no número

de casos, com 1.688.688 registrados em 2015 e 1.500.535, em 2016. O total de casos graves notificados para estes anos foram: 768 (2014), 1.714 (2015) e 861 (2016). Os sorotipos 1 e 4 foram cocirculantes neste período, porém o DENV-1 foi prevalente, sendo responsável por aproximadamente 90% dos casos confirmados laboratorialmente (SVS/MS 2015; 2016a).

Em 2017, até a semana epidemiológica 52, o número de casos prováveis de dengue decresceu drasticamente, sendo registrados 252.054 casos ao longo do ano. A região Nordeste apresentou o maior número de casos prováveis (86.386 casos; 34,3%) em relação ao total do país. Em seguida aparecem as regiões Centro-Oeste (78.729 casos; 31,2%), Sudeste (59.601 casos; 23,6%), Norte (22.660 casos; 9,0%) e Sul (4.678 casos; 1,9%). Foram confirmados 271 de dengue grave e 2.590 casos de dengue com sinais de alarme. No mesmo período de 2016, foram confirmados 919 casos de dengue grave e 9.153 casos de dengue com sinais de alarme. O total de óbitos foi de 141 em 2017, enquanto em 2016 701 foram confirmados (SVS/MS, 2018a).

No momento, até a semana epidemiológica 16, foram registrados 101.863 casos prováveis de dengue no país. Destes, 37.598 (36,9%) foram confirmados e outros 54.682 casos suspeitos foram descartados. A região Centro-Oeste apresentou o maior número de casos prováveis (38.082 casos; 37,4%) em relação ao total do país. Em seguida, aparecem as regiões Sudeste (33.828 casos; 33,2%), Nordeste (19.050 casos; 18,7%), Norte (8.401 casos; 8,2%) e Sul (2.502 casos; 2,5%). Os casos com sinais de alarme confirmados já chegam a 917 e graves, 82, sendo o estado de Goiás o detentor de mais de 70% dos casos com sinais de alarme e quase 50% dos casos graves. Do total de 40 óbitos, 16 pertencem ao estado de Goiás. Foram enviadas para investigação do sorotipo viral um total de 7.050 amostras, sendo 216 confirmadas. Os sorotipos identificados foram o DENV-1 e DENV-2 (SVS/MS, 2018b).

Nos últimos 32 anos extensas epidemias de dengue vêm ocorrendo no Brasil, com mais de doze milhões de casos notificados (Silva Jr, 2013; SVS/MS, 2016b; 2018b) (Figura 1.13).

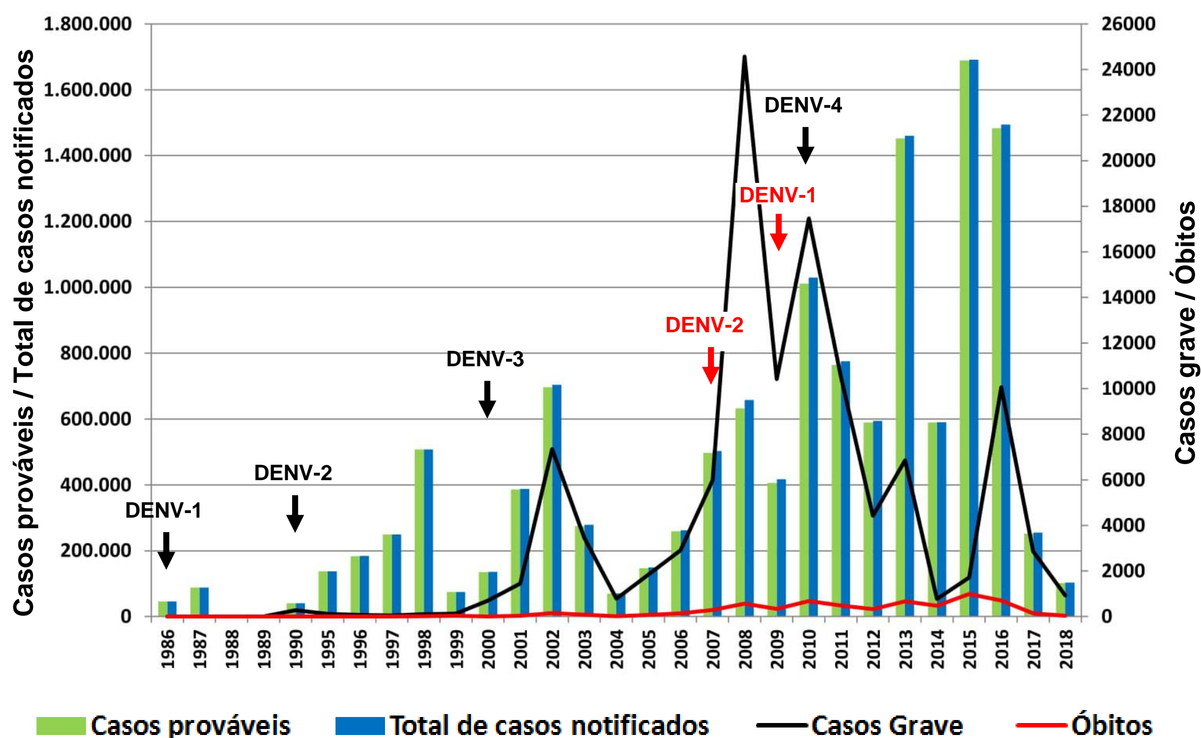

Figura 1.13 Número de casos de dengue no Brasil, 1986-2018. Sorotipos emergentes em preto e reemergentes em vermelho.

A dispersão dos DENV pelo território brasileiro, com aumento nas notificações de casos de dengue, dengue grave e óbitos ressalta a importância da vigilância virológica destes agentes no país. Como a dengue é uma doença viral sem tratamento específico e uma vacina anti-DENV eficaz ainda não se encontra disponível, apesar de inúmeros estudos e esforços para seu desenvolvimento desde a década de 40 (Sabin e Schlesinger, 1945; WHO, 2012), as medidas de controle ao vetor consistem no principal instrumento para a prevenção das infecções por estes vírus. Portanto, a participação da população é essencial para o controle da doença.

Desde a década de 1980, Gubler já salientava que o monitoramento das infecções por DENV através de um programa de vigilância ativa em conjunto com ações envolvendo aspectos clínicos, epidemiológicos, virológicos, sorológicos e entomológicos, principalmente em períodos inter-epidêmicos, são necessários para a detecção da circulação viral em tempo hábil para que sejam evitadas extensas epidemias (Gubler, 1989).

1.9 Manifestações clínica e classificação da dengue

A dengue é uma doença febril aguda, que pode ser de curso benigno ou grave, dependendo da forma como se apresente. As manifestações clínicas da dengue incluem febre, normalmente de início súbito (podendo alcançar 40ºC, diminuindo ao longo dos dias, persistindo em média por 2 a 7 dias), seguida de cefaleia, dor retrorbital, mialgia, artralgia, náuseas, vômitos, prurido cutâneo, exantema e/ou prostração. A doença tem duração de 5 a 7 dias, mas o período de convalescência pode ser acompanhado de grande debilidade física, e prolongar-se por várias semanas (Souza *et al.*, 2008; SVS/MS, 2010).

Manifestações hemorrágicas e/ou evidência de perda de plasma, podendo levar ao choque hipovolêmico também podem ocorrer. A fragilidade capilar é evidenciada pela positividade da prova do laço. Outras manifestações hemorrágicas incluem petéquias, equimoses, epistaxe, gengivorragia, hemorragia em diversos órgãos (gastrointestinais, intracraniana, dentre outras) e hemorragia espontânea pelos locais de punção venosa. Nos casos graves, o choque geralmente ocorre entre o 3º e 7º dia de doença, geralmente precedido por dor abdominal. O choque é decorrente do aumento da permeabilidade vascular, seguida de extravasamento plasmático (evidenciado por hemoconcentração, derrames cavitários e hipoalbuminemia) e falência respiratória. É de curta duração podendo levar ao óbito em 12 a 24 horas ou à recuperação rápida após terapia antichoque apropriada (Svs/Ms, 2010).

Inicialmente, baseada no padrão da dengue de origem asiática, a dengue foi classificada como febre do dengue (FD), febre hemorrágica do dengue/síndrome do choque por dengue (FHD/SCD) e dengue com complicações (DCC) (OMS, 1997). Esse amplo espectro de manifestações clínicas se relacionava a um quadro oligo ou assintomático até formas mais graves, com hemorragia e choque, podendo evoluir pra o óbito. Porém, mudanças na epidemiologia da dengue dificultaram a aplicação da forma da classificação. Neste cenário, em 2009 a OMS preconizou uma nova forma de classificação dos casos de dengue onde seria possível definir os casos de dengue como: dengue sem sinais de alerta (DSSA), dengue com sinais de alerta (DCSA) e dengue grave (DG). Tal proposta foi desenvolvida a partir de um estudo prospectivo multicêntrico em várias regiões endêmicas da doença, utilizando critérios de gravidade (OMS, 2009; SVS/MS, 2010; PAHO, 2016) (Figura 1.14).

Figura 1.14 Classificação atual dos casos de dengue e níveis de gravidade (OMS, 2009).

O período de incubação do vírus no ser humano varia de 3 a 15 dias, após o qual a doença poderá evoluir para uma das formas clinicas. A sintomatologia da doença pode ser semelhante a outras enfermidades, usadas como diagnóstico diferencial, tais como: gripe, rubéola, sarampo e outras infecções virais, bacterianas e exantemáticas, que dificulta em muitos casos o diagnóstico da doença (Souza *et al.*, 2008; SVS/MS, 2010).

1.10 Diagnóstico laboratorial

O diagnóstico laboratorial das infecções pelos DENV é realizado através do isolamento viral e/ou detecção do ácido nucleico, de técnicas sorológicas para detecção de anticorpos específicos (IgM/IgG) e captura de antígeno (NS1), e pela detecção de antígenos virais em tecidos (Igarashi, 1978; Kuno *et al.*, 1987; Chungue *et al.*, 1989; Lanciotti *et al.*, 1992; Miagostovich *et al.*, 1997; Miagostovich *et al.*, 1999).

O estabelecimento da cultura de células de mosquito no sistema de isolamento viral representou um grande avanço aos métodos virológicos utilizados no diagnóstico do dengue

(Gubler e Sather, 1988). O clone C6/36 (Igarashi, 1978) tem sido o mais utilizado nas últimas décadas, pois demonstrou ser altamente sensível à infecção pelos DENV, além de sua fácil manutenção, já que pode ser mantida à temperatura ambiente, aproximadamente a 28ºC (Nogueira *et al.*, 1988; Miagostovich *et al.*, 1993).

A presença do vírus pode ser detectada pelo efeito citopático (ECP) na monocamada celular ou pela técnica de imunofluorescência indireta, com a utilização de soros hiperimunes aos quatro sorotipos dos DENV. Para a identificação dos DENV, utilizam-se anticorpos monoclonais específicos para os quatro sorotipos (Gubler *et al.*, 1984).

Diversos protocolos de amplificação genômica utilizando transcrição reversa seguida da reação em cadeia pela polimerase (RT-PCR), têm sido utilizados no diagnóstico rápido das infecções por dengue (Lanciotti *et al.*, 1992; Brown *et al.*, 1996; Figueiredo *et al.*, 1997; De Paula *et al.*, 2002). Esses protocolos têm sido úteis tanto para a clínica quanto para a vigilância virológica, ao permitirem a identificação do sorotipo infectante (Morita *et al.*, 1991; Lanciotti *et al.*, 1992).

O protocolo descrito por Lanciotti e colaboradores (1992) é o indicado pela OPAS e foi estabelecido no Brasil em 1995 como uma ferramenta molecular rápida e específica para a detecção e tipagem dos DENV. Ele permitiu a identificação dos sorotipos 1 e 2 em 41% dos casos de dengue confirmados no Laboratório de Referência Regional do Instituto Oswaldo Cruz (Miagostovich *et al.*, 1997)

A sorologia é o método utilizado para confirmação laboratorial de rotina. Através das técnicas sorológicas é possível detectar infecções atuais ou recentes por meio da captura das imunoglobulinas da classe M (IgM) por ELISA (MAC ELISA). Na maioria dos casos, somente uma amostra de soro coletada é suficiente para a confirmação diagnóstica. Contudo um resultado negativo em amostra coletada em fase precoce (6 a 10 dias após o início dos sintomas) não exclui o diagnóstico de dengue, uma vez que, em alguns casos, os níveis de IgM tornam-se detectáveis pelo teste somente após esse período. O anticorpo IgM anti-dengue desenvolve-se rapidamente, geralmente a partir do quinto dia do início da doença, e tanto em infeções primárias, quanto secundárias, os indivíduos apresentam esses anticorpos detectáveis. A pesquisa de anticorpos IgG ELISA e o teste de inibição de hemaglutinação (HI), que exigem amostras do soro pareadas (fase aguda e convalescente recente) de casos suspeitos também podem ser utilizadas no diagnóstico sorológico (Svs/Ms, 2010). Miagostovich e colaboradores (1999) avaliaram a técnica IgG (ELISA) em substituição ao uso do HI, e concluíram que o teste IgG ELISA pode substituir o teste HI para caracterizar o tipo de infecção, se primária ou secundária (Miagostovich *et al.*, 1999).

A detecção de antígeno NS1 é realizada pelo ELISA que permite a detecção de antígenos virais específicos de dengue do tipo NS1. É um método que, a princípio, é bastante sensível e inicialmente era utilizado em pesquisas e nos casos graves. O Ministério da Saúde disponibiliza kits de teste NS1 ELISA para triagem das amostras para isolamento viral em unidades sentinelas (Svs/Ms, 2010). Pesquisas de avaliação dos kits para detecção do antígeno NS1 disponíveis para o diagnóstico da dengue indicam que os kits comerciais são úteis para o diagnóstico laboratorial da dengue primária e secundária na fase aguda e que detecção do NS1 deve ser usado em combinação com o MAC-ELISA para a detecção de casos e como triagem para complementar o isolamento viral (Lima *et al.*, 2010). Além disso, a técnica é uma alternativa para diagnóstico em casos fatais através da detecção do antígeno NS1 em tecidos (Lima *et al.*, 2011). Neste caso, a imunohistoquímica também pode ser realizada em cortes de tecidos fixados em formalina e emblocados em parafina, permitindo a detecção de antígenos virais. Os cortes são corados pela fosfatase alcalina ou peroxidase marcados com anticorpos específicos. Podem ser utilizados anticorpos monoclonais ou policlonais. Essa técnica é bastante sensível e específica, sendo considerada exame confirmatório e deve ser utilizada após diagnóstico histopatológico presuntivo (Svs/Ms, 2010; PAHO, 2016).

Além dos métodos de diagnóstico utilizados na rotina, métodos moleculares como a PCR seguido a sequenciamento genômico viral são de grande importância para a vigilância dos sorotipos e/ou genótipos de DENV circulantes em uma dada região e a estratégia de sequenciamento vai depender do objetivo proposto. Para a caracterização molecular e genotipagem dos sorotipos circulantes de DENV utiliza-se o sequenciamento parcial (gene E) ou completo, com o uso de oligonucleotídeos iniciadores (*primers*) que amplificam o genoma dos DENV (Miagostovich *et al.*, 2006; Dos Santos *et al.*, 2011; Faria *et al.*, 2013; De Bruycker-Nogueira *et al.*, 2015).

1.11 Vacina

Desenvolver uma vacina contra os DENV tem sido prioridade pela OMS, porém o desenvolvimento tem enfrentado algumas dificuldades, tais como: a necessidade de imunizar contra os quatro sorotipos com alta eficiência para evitar o desencadeamento de uma doença grave em caso de infecção secundária e a ausência de um modelo experimental para estudar a resposta pós-vacinal. Para a vacina ser considerada ideal deve cumprir algumas exigências: promover imunização prolongada contra os quatro sorotipos do DENV; ser de baixo custo e

toxicidade; manter títulos virais em refrigeração ou à temperatura ambiente (Figueiredo, 1999).

Diversas abordagens para o desenvolvimento de uma vacina eficaz, estão sendo utilizadas, como as vacinas de vírus vivo atenuado, de vírus inativados, vacinas recombinantes, quiméricas e de DNA (Clements *et al.*, 2010; Coller e Clements, 2011; Murrell *et al.*, 2011; Schmitz *et al.*, 2011; Kirkpatrick *et al.*, 2015; Osorio *et al.*, 2015; Screaton *et al.*, 2015; Guy *et al.*, 2016; Shukla *et al.*, 2017; Rajpoot *et al.*, 2018).

Atualmente, podemos destacar duas vacinas tetravalentes de vírus atenuado que se encontram em fase III de avaliação. Uma sendo desenvolvida pela empresa farmacêutica Takeda (Sáez-Llorens *et al.*, 2018) e outra pelos Institutos Nacionais de Saúde (NIH) dos Estados Unidos em parceria com o Instituto Butantan (Butantan DV; TetraVax-DV-TV003) (Precioso *et al.*, 2015; Whitehead *et al.*, 2017), com previsão de término de estudo no ano de 2022, de acordo com dados disponíveis em https://clinicaltrials.gov/ct2/show/NCT02406729 do *National Institutes of Health* (NIH).

Uma vacina recombinante quimérica tretavalente atenuada produzida pela empresa francesa Sanofi-Pasteur, de nome Dengvaxia® ou CYD-TDV, foi licenciada em 2015 para uso em indivíduos de 9 a 45 anos, e em 2017 já estava sendo utilizada em 14 países endêmicos do continente americano e asiático (Guy *et al.*, 2017). Porém, indivíduos vacinados passaram a apresentar quadro grave da doença, sendo observada elevada taxa de hospitalização (Hadinegoro *et al.*, 2016). Tal fato desencadeou a suspensão das doses de vacina disponíveis para maiores averiguações quanto a seguridade da mesma. Em 13 de dezembro de 2017 a OMS informou que a vacina Dengvaxia só deveria ser administrada em indivíduos que já tiveram infecção natural prévia por algum dos sorotipos do DENV, como informada em nota pela própria Sanofi (WHO, 2017).

2 JUSTIFICATIVA

A dengue é um dos principais problemas de saúde pública no mundo. No Brasil a atividade dos DENV têm crescido de modo significativo desde a introdução do DENV-1 no Rio de Janeiro (RJ) em meados da década de 1980. Este sorotipo reemergiu em 2009 após aproximadamente oito anos sem intensa circulação, causando em 2010 mais de um milhão de casos prováveis da doença, com a maior taxa de internações registradas no país, principalmente em maiores de 60 anos de idade (Dos Santos *et al.*, 2013).

A evolução dos DENV tem tido um impacto importante sobre a sua virulência para os seres humanos e epidemiologia da doença em todo o mundo. Embora diferenças antigênicas e genéticas nas cepas virais sejam evidentes, a falta de modelo animal da doença dificulta a detecção da virulência dos DENV. Entretanto, estudos filogenéticos permitem a correlação entre genótipos específicos e gravidade da doença. Apesar do complexo conjunto de fatores que contribuem para a epidemiologia, estudos têm sugerido que estruturas virais específicas podem contribuir para o aumento da replicação nas células-alvo humanas e intensificar a transmissão pelo mosquito vetor (Rico-Hesse, 2003).

Um dos genes mais utilizados para o sequenciamento genômico é o gene E. Este gene tem sido alvo de estudos de caracterização molecular, dinâmica populacional, análises filogenética e filogeográficas (Rico-Hesse, 1990; Zanotto *et al.*, 1996; Holmes e Twiddy, 2003; Rico-Hesse, 2003; Twiddy, Pybus, *et al.*, 2003; Weaver e Vasilakis, 2009; Carrillo-Valenzo *et al.*, 2010; Mendez *et al.*, 2010; Dos Santos *et al.*, 2011; Carneiro *et al.*, 2012). A proteína do envelope possui 495 aminoácidos (aa), distribuídos em três domínios (I, II, III). Acredita-se que o domínio III dos flavivírus interaja com receptores celulares para a entrada dos vírus (Nayak *et al.*, 2009; Weaver e Vasilakis, 2009), além disso é o domínio mais variável, apresentando o maior número de alterações nucleotídicas que acarretam em mudanças de aminoácidos (Carneiro *et al.*, 2012).

A análise filogenética dos DENV-1 isolados desde a introdução deste sorotipo no país caracterizou estes vírus como pertencentes ao genótipo V (América/África). Porém, para o DENV-1, além dos genótipos, a existência de linhagens com distintas relações geográficas e temporais foi sugerida no Brasil, demonstrando alta variabilidade genotípica (Dos Santos *et al.*, 2011; Carneiro *et al.*, 2012; Drumond *et al.*, 2012).

Ainda não está claro se a epidemia explosiva de DENV-1 ocorrida no Brasil em 2009 e 2010, foi devido à circulação de linhagens. Neste contexto, o monitoramento do DENV é de

grande relevância para observar a propagação de cepas potencialmente virulentas, assim como para avaliar o seu impacto sobre a população durante um surto. Devido à geografia do Brasil e epidemiologia da dengue, além do fato do país possuir regiões turísticas, o sequenciamento continuado de vírus circulantes se faz necessário para melhor caracterizar esses eventos de substituições e introduções de possíveis genótipos e linhagens no país.

Estudos de caracterização e evolução molecular podem constituir uma importante ferramenta para monitorar a introdução e entender a dispersão dos vírus, bem como prever as possíveis consequências epidemiológicas de tais eventos. A análise evolutiva das linhagens do DENV-1 pode esclarecer se as diferentes linhagens circulantes no país evoluíram localmente a partir de uma única introdução ou foi consequência de introduções independentes, o que é sugerido atualmente. Além disso, ainda há dúvidas quanto a mais provável origem geográfica do genótipo V e há quanto tempo o mesmo alcançou o continente americano.

3 OBJETIVOS

3.1 Objetivo Geral

Reconstruir a história evolutiva, realizar a caracterização e vigilância molecular das diferentes linhagens de DENV-1 detectadas durante 30 anos de circulação no Brasil (1986 - 2016).

3.2 Objetivos Específicos

1. Realizar a caracterização molecular e análise filogenética das diferentes linhagens de DENV-1 circulantes no Brasil de 1986 até o período da reemergência em 2011;

2. Reconstruir a história evolutiva do genótipo V nas Américas;

3. Demonstrar a migração e dispersão dos DENV-1 no Brasil a partir da primeira detecção deste sorotipo no estado do Rio de Janeiro em 1986;

4. Realizar a vigilância molecular das diferentes linhagens de DENV-1 cocirculantes entre 2012-2016, período após a reemergência deste sorotipo no Brasil.

4 METODOLOGIA E RESULTADOS

Esta seção será apresentada sob a forma de artigos científicos publicados ou submetidos em revistas científicas indexadas, de acordo com os critérios estabelecidos pela Pós-Graduação em Biologia Parasitária.

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

4.2 Artigo 2. Evolutionary history and spatiotemporal dynamics of DENV-1 genotype V in the Americas.

4.3 Artigo 3. DENV-1 Genotype V in Brazil: Spatiotemporal dispersion pattern reveals continuous co-circulation of distinct lineages until year of 2016.

4.4 Artigo 4. First detection and molecular characterization of a DENV-1/DENV-4 co-infection during an epidemic in 2012 in Rio de Janeiro, Brazil.

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

Relação do Manuscrito com os Objetivos:

Objetivo específico 1: Realizar a caracterização molecular das diferentes linhagens de DENV-1 circulantes no Brasil de 1986 até o período da reemergência em 2011;

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Resumo: O vírus dengue tipo 1 (DENV-1) foi isolado no Brasil em 1986 no estado do Rio de Janeiro (RJ) e durante 25 anos esse sorotipo emergiu e reemergiu causando epidemias explosivas no país. Aqui, objetivamos apresentar a filogenia e a caracterização molecular dos DENV-1 (n= 48) isolados durante epidemias ocorridas de 1986 a 2011, baseadas no gene do envelope (E). Seis genomas codificantes completos do DENV-1 foram sequenciados e possíveis eventos de recombinação genômica foram analisados. Os resultados mostraram que os isolados brasileiros de DENV-1 analisados pertencem ao Genótipo V (Américas/África), porém se agrupam em clados distintos. Foram identificados três clados, um com sequências datadas de 1986 a 2002 (linhagem 1a), um segundo clado com isolados de 2009 a 2011 e uma cepa de 2002 (linhagem 2), e um terceiro clado composto de cepas isoladas de 2010 a 2011 (linhagem 1b). As linhagens 1a e 1b foram mais relacionadas com os DENV-1 de origem americana, enquanto a linhagem 2 com origem asiática. As substituições de aminoácidos (aa) foram observadas nos domínios I e III da proteína E e foram associadas à diferenciação das linhagens. Uma substituição em E297 permitiu a separação da linhagem 1a das linhagens 1b e 2. Substituições em E338, E394 (domínio III), E428 e E436 (região-*stem*) foram responsáveis por diferenciar as linhagens 1a, 1b e 2. Com exceção do gene C, todos os genes analisados permitiram a classificação genotípica do DENV-1. Porém, somente o segmento correspondente ao domínio III e a região-*stem* do gene E foram capazes de caracterizar as diferentes linhagens, assim como observado pela análise realizada a partir do gene E e da região codificante completa. Nenhum evento de recombinação foi detectado, mas uma cepa pertencente à linhagem 1a apontou estar intimamente relacionada com uma cepa recombinante conhecida (AF513110/BR/2001).

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

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ABSTRACT

Dengue virus type 1 (DENV-1) was first isolated in Brazil in 1986 in the state of Rio de Janeiro (RJ) and during 25 years, this serotype emerged and re-emerged causing explosive epidemics in the country. Here, we aimed to present the phylogeny and molecular characterization based on the envelope gene (E) of DENV-1 ($n = 48$) isolated during epidemics occurred from 1986 to 2011. Six full coding region genomes of DENV-1 were fully sequenced and possible genomic recombination events were analyzed. The results showed that the Brazilian DENV-1 isolates analyzed belong to genotype V (Americas/Africa), but grouping into distinct clades. Three groups were identified, one dating from 1986 to 2002 (lineage 1a), a second group isolated from 2009 to 2011 and a representative strain isolated in 2002 (lineage 2), and a group of strains isolated from 2010 to 2011 (lineage 1b). The lineages 1a and 1b were more closely related to the American strains, while lineage 2 to the Asian strains. Amino acids (aa) substitutions were observed in the domains I and III of the E protein and were associated to the lineages segregation. A substitution on E_{297} differentiated the lineage 1a from the lineages 1b and 2. Substitutions on E_{338}, E_{394} (domain III), E_{428} and E_{436} (stem region) differentiated lineages 1a, 1b and 2. With the exception of the C gene, all the others genes analyzed allowed the DENV-1 classification into the distinct genotypes. Interestingly, the E gene's domain III and stem regions alone were able to characterize the distinct lineages, as observed by the analysis of the entire E gene and the complete coding region. No recombinant events were detected, but a strain belonging to lineage 1a was closely related to a known recombinant strain (AF513110/BR/2001).

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1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Ethical statement

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

2.2. Dengue viral strains

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

2.3. Viral RNA extraction

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

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

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

2.5. Dengue virus genome amplification, sequencing and recombination analysis

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

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

ID/state/year of isolation	Origin of strain	Passage in C6/36 culture	Case Classification (WHO, 2009)	Genes sequenced	Genbank accession number	References
31768/RJ/1986	Isolated (C6/36)	1st	DWWS	C/prM/M/E	HQ026760	dos Santos et al. (2011)
31807/RJ/1986	Isolated (C6/36)	1st	DWWS	C/prM/M/E	JN122280	dos Santos et al. (2011)
36034/RJ/1988	Isolated (C6/36)	2nd	DWWS	(C → NS5)	KF672761	This study
38159/RJ/1989	Isolated (C6/36)	1st	DWWS	(C → NS5)	KF672762	This study
45907/MS/1991	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672791	This study
58485/MG/1997	Isolated (C6/36)	1st	SD	C/prM/M/E	KF672769	This study
58522/MG/1997	Isolated (C6/36)	1st	NI	C/prM/M/E	KF672770	This study
58540/RJ/1997	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF672771	This study
58610/RJ/1997	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672772	This study
58724/RN/1997	Isolated (C6/36)	1st	NI	C/prM/M/E	KF672773	This study
60068/RJ/1998	Isolated (C6/36)	1st	SD	C/prM/M/E	KF672774	This study
60443/CE/1998	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672775	This study
60606/MG/1998	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF672776	This study
60619/MG/1998	Isolated (C6/36)	1st	SD	C/prM/M/E	KF672777	This study
62114/ES/1998	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672778	This study
62189/ES/1998	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF672779	This study
64450/RJ/1999	Isolated (C6/36)	2nd	DWS	C/prM/M/E	KF672780	This study
64451/RJ/1999	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672781	This study
64616/RJ/1999	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672792	This study
66568/RJ/2000	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672782	This study
66694/ES/2000	Isolated (C6/36)	1st	NI	(C → NS5)	KF672763	This study
67993/PI/2000	Isolated (C6/36)	1st	NI	(C → NS5)	KF672787	This study
68826/RJ/2001	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672764	This study
71602/RJ/2001	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF672788	This study
75424/ES/2002	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672783	This study
15_2010/MS/2010	Serum		DWWS	C/prM/M/E	HQ696612	dos Santos et al. (2011)
876_2010/MS/2010	Isolated (C6/36)	1st	NI	C/prM/M/E	KF672768	This study
2676_2010/RJ/2010	Isolated (C6/36)	2nd	Fatal case	C/prM/M/E	KF672786	This study
1141_2011/AL/2010	Isolated (C6/36)	1st	NI	C/prM/M/E	JQ015184	This study
1142_2011/AL/2010	Isolated (C6/36)	1st	NI	C/prM/M/E	JQ015185	This study
122_2011/RJ/2011	Isolated (C6/36)	1st	DWWS	C/prM/M/E	JN122281	dos Santos et al. (2011)
169_2011/RJ/2011	Isolated (C6/36)	1st	Fatal case	C/prM/M/E	KF672765	This study
325_2011/RJ/2011	Isolated (C6/36)	1st	SD	C/prM/M/E	KF672766	This study
1049_2011/RJ/2011	Isolated (C6/36)	1st	DWS	C/prM/M/E	KF672790	This study
1266_2011/RJ/2011	Isolated (C6/36)	1st	Fatal case	(C → NS5)	KF672760	This study
73834/ES/2001	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672789	This study
55_2009/ES/2009	Isolated (C6/36)	1st	NI	C/prM/M/E	HM043709	dos Santos et al. (2011)
1433_2009/RJ/2009	Serum		DWWS	C/prM/M/E	HQ026761	dos Santos et al. (2011)
1435_2009/RJ/2009	Serum		DWWS	C/prM/M/E	HM043710	dos Santos et al. (2011)
19_2010/RJ/2010	Serum		DWWS	C/prM/M/E	HQ026762	dos Santos et al. (2011)
20_2010/RJ/2010	Serum		DWWS	C/prM/M/E	HQ696613	dos Santos et al. (2011)
188_2010/RJ/2010	Isolated (C6/36)	1st	DWS	C/prM/M/E	HQ696614	dos Santos et al. (2011)
242_2010/RJ/2010	Isolated (C6/36)	1st	DWWS	(C → NS5)	KF672759	This study
516_2010/ES/2010	Isolated (C6/36)	1st	Fatal case	C/prM/M/E	KF672785	This study
568_2010/ES/2010	Isolated (C6/36)	1st	Fatal case	C/prM/M/E	KF719187	This study
594_2011/CE/2010	Isolated (C6/36)	1st	NI	C/prM/M/E	JN982362	This study
512_2011/RJ/2011	Isolated (C6/36)	1st	DWWS	C/prM/M/E	KF672767	This study
514_2011/RJ/2011	Isolated (C6/36)	1st	Fatal case	C/prM/M/E	KF672784	This study

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

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

3. Results

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

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

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

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

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

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

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

The E gene sequences alignment demonstrated conserved nts substitutions among strains of the same lineage, and it was responsible for the changes of some aa in domains I and III of the E gene. In the domain I, a substitution in E_{297} (T → M) was observed between lineage 1a and the lineages 1b and 2, respectively. Four substitutions were detected in domain III, at positions E_{338} (S → L), E_{394} (K → R), E_{428} (V → L) and E_{436} (V → I). The substitutions occurred at positions E_{338}, E_{428} and E_{436} were conserved on strains from lineage 2, while strains from lineage 1be, showed conserved modifications in the position E_{394} (Table 3). The domain III and stem region of the E gene presented the highest number of substitutions causing changes in conserved aa among the strains and, the phylogenetic analysis based on this domain was sufficient to segregate the different lineages (Table 3 and Fig. 2). The aa replacements in the ectodomain of the E protein is represented in a three-dimensional model (Fig. 3). The nts sequences identity of the E gene from 48 DENV-1 strains ranged from 95.6% to 97.2% between lineages 1a and 2, from 96.6% to 98.1%, between lineages 1a and 1b and, from 95.2% to 96.2% between lineages 2 and 1b. Comparing those with genotypes I, II, III and IV, the nts divergences observed, ranged from 6.3 to 8.7%.

3.2. Phylogeny and molecular characterization of DENV-1 strains based on the complete genome's coding region (C → NS5)

The phylogenetic analysis based on the sequences of the entire coding region of six DENV-1 strains, corroborated the analysis of the E gene and characterized those strains as belonging to genotype V (Americas/Africa), Fig. 4. In order to determine which DENV-1 genes could be used to differentiate the distinct lineages, phylogenetic trees were constructed based on the C, prM/M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 genes. Except by the C gene analysis, all genes allowed the genotype V characterization. The analysis of the E, NS3 and NS5 genes enabled the DENV-1 grouping into two different lineages (lineages 1 and 2). However, only the analysis of the E gene was able to characterize the sub-lineages 1a and 1b, with bootstrap support ⩾75%.

The comparison of the 3,392 aa corresponding to the 10,176 nts of the six Brazilian DENV-1 to the strain AF226685.2/BR/90, showed 82 aa substitutions along the entire region (Table S1). The strains 36034/RJ/1988 and 38159/RJ/1989 showed six and seven substitutions, respectively. The strains 66694/ES/2000 and 68826/RJ/2001, belonging to the lineage 1a, showed a higher number of substitutions, nineteen and sixteen, respectively. In the strain 1266_2011/RJ/2011, representative of the lineage 1b, a total of 26 aa substitutions were identified and the strain 242_2010/RJ/2010, representative of lineage 2, presented 43 substitutions. Most substitutions were unique to the sequences, but sixteen were conserved among the strains studied. In the strains isolated in 2000 and 2001 (lineage 1a), nine substitutions were detected in NS1, NS4B and NS5 genes. Three substitutions were found in strains representative of lineage 1b and 2 in E_{297} (T → M), $NS2A_{67}$ (K → R) and $NS5_{678}$ (I → T). The nucleotides identity of the six complete coding region sequences ranged from 96.1% to 96.5% between lineages 1a and 2; from 97.3% to 97.9%, between lineages 1a and 1b and 95.4%, between lineages 1b and 2.

The analysis of the E gene from Brazilian strains belonging to genotype V and other genotypes available in GenBank, showed no evidence of recombination. However, the analysis of the complete coding region identified a possible recombination event in the strain AF513110.1/BR/2001 isolated in the state of Paraná in 2001. The strain 36034/BR/RJ/1988 isolated in RJ in 1988 was more closely related to that possible recombinant, indicated by *P* values

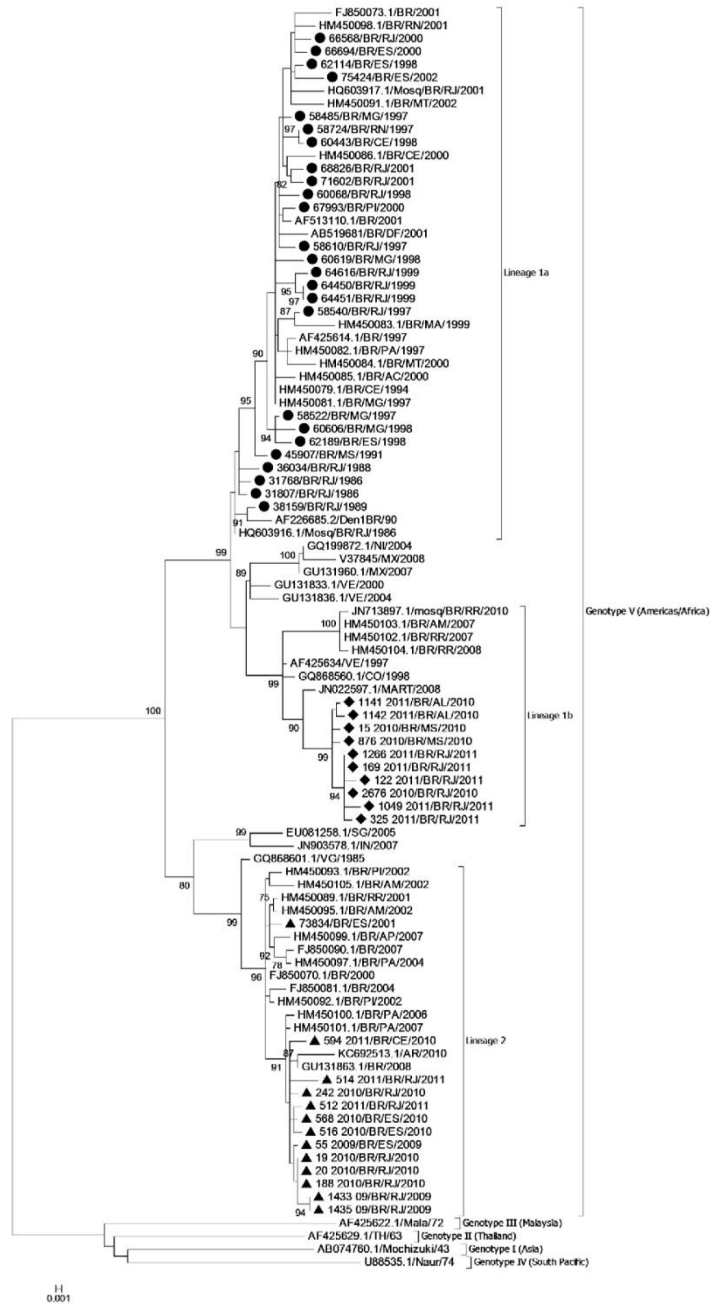

Fig. 1. Phylogenetic analysis based on the E gene of 48 Brazilian DENV-1 isolates during the epidemic periods (1986–2011). Maximum-likelihood method, TN93 + G model with 6 discrete Gamma categories (parameters = 0.2630). The DENV-1 sequences analyzed are represented as: circle (lineage 1a), triangle (lineage 1b) and diamond (lineage 2). DENV strains were named as follows: GenBank accession number/country/year.

of 6.39×10^{-06}, 5.75×10^{-06}, obtained by the RDP and BootScan methods, respectively. The region indicated as recombinant, ranges from nts position 8037 to 8677 (aa 2679–2893) within the NS5 gene, and overlaps the coding region for the FtsJ methyltransferase (aa 2548–2714) and Flavi_NS5 (aa 2743–3386).

4. Discussion

In Brazil, DENV-1 was first detected in 1982 in Roraima during an outbreak that did not spread (Osanai et al., 1983). However, in 1986 the introduction of this serotype in Rio de Janeiro resulted in more than 60,000 cases and the spread of the virus (Schatzmayr et al., 1986) establishing a new national public health concern (Nogueira et al., 2007; Rodriguez-Barraquer et al., 2011). In 2009, DENV-1 reemerged and caused a major DENV-1 epidemic in 2010 and, for the first time, distinct viral lineages were reported (dos Santos et al., 2011).

DENV are highly diverse (Wang et al., 2002; Aaskov et al., 2006; Thai et al., 2012) and this genetic diversity has been described in different genotypes within each serotype (Rico-Hesse, 1990, 2003; Weaver and Vasilakis, 2009). The genotype V of DENV-1 has been previously investigated and the circulation and replacements of lineages have been reported in Brazil and in other American countries (Mendez et al., 2010; dos Santos et al., 2011; Yamanaka et al., 2011; Carneiro et al., 2012; Drumond et al., 2012; Muñoz-Jórdan et al., 2013; Añez and Rios, 2013; Dash et al., 2015). It has been shown that this serotype had three introductions events in Brazil (Drumond et al., 2012).

In this study, the analysis of 48 sequences of DENV-1 representing strains isolated in 25 years in Brazil, characterized distinct lineages and showed that, lineages 1a and 1b are more closely related to DENV-1 of Latin American origin and lineage 2 with viruses of Asian origin. Aa substitutions were observed in all sequences analyzed, mainly in the domain III of the E gene and corroborate

Table 3
Amino acid substitutions predicted to differentiate the distinct Brazilian DENV- lineages based on analysis of the envelope (E) gene.

ID state year of isolation	E gene (position)/substitution of amino acid					Genotype Rico-Hesse (2003), Weaver and Vasilakis (2009)	Lineage
	Domain I	Domain III					
	E_{297}	E_{338}	E_{394}	E_{428}	E_{436}		
31768/RJ/1986	T	S	K	V	V	Americas/Africa (V)	Lineage 1a
31807/RJ/1986	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
36034/RJ/1988	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
38159/RJ/1989	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
45907/MS/1991	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58485/MG/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58522/MG/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58540/RJ/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58610/RJ/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
58724/RN/1997	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
60068/RJ/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
60443/CE/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
60606/MG/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
60619/MG/1998	.	L	.	.	.	Americas/Africa (V)	Lineage 1a
62114/ES/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
62189/ES/1998	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
64450/RJ/1999	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
64451/RJ/1999	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
64616/RJ/1999	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
66568/RJ/2000	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
66694/ES/2000	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
67993/PI/2000	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
68826/RJ/2001	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
71602/RJ/2001	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
75424/ES/2002	.	.	.	.	.	Americas/Africa (V)	Lineage 1a
15_2010/MS/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
876_2010/MS/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
2676_2010/RJ/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
1141_2011/AL/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
1142_2011/AL/2010	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
122_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
169_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
325_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
1049_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
1266_2011/RJ/2011	M	.	R	.	.	Americas/Africa (V)	Lineage 1b
73834/ES/2001	M	L	.	L	I	Americas/Africa (V)	Lineage 2
55_2009/ES/2009	M	L	.	L	I	Americas/Africa (V)	Lineage 2
1433_09/RJ/2009	M	L	.	L	I	Americas/Africa (V)	Lineage 2
1435_09/RJ/2009	M	L	.	L	I	Americas/Africa (V)	Lineage 2
19_2010/RJ/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
20_2010/RJ/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
188_2010/RJ/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
242_2010/RJ/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
516_2010/ES/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
568_2010/ES/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
594_2011/CE/2010	M	L	.	L	I	Americas/Africa (V)	Lineage 2
512_2011/RJ/2011	M	L	.	L	I	Americas/Africa (V)	Lineage 2
514_2011/RJ/2011	M	L	.	L	I	Americas/Africa (V)	Lineage 2

T: threonine; S: serine; K: lysine; V: valine; M: methionine; L: leucine; R: arginine; I: isoleucine.

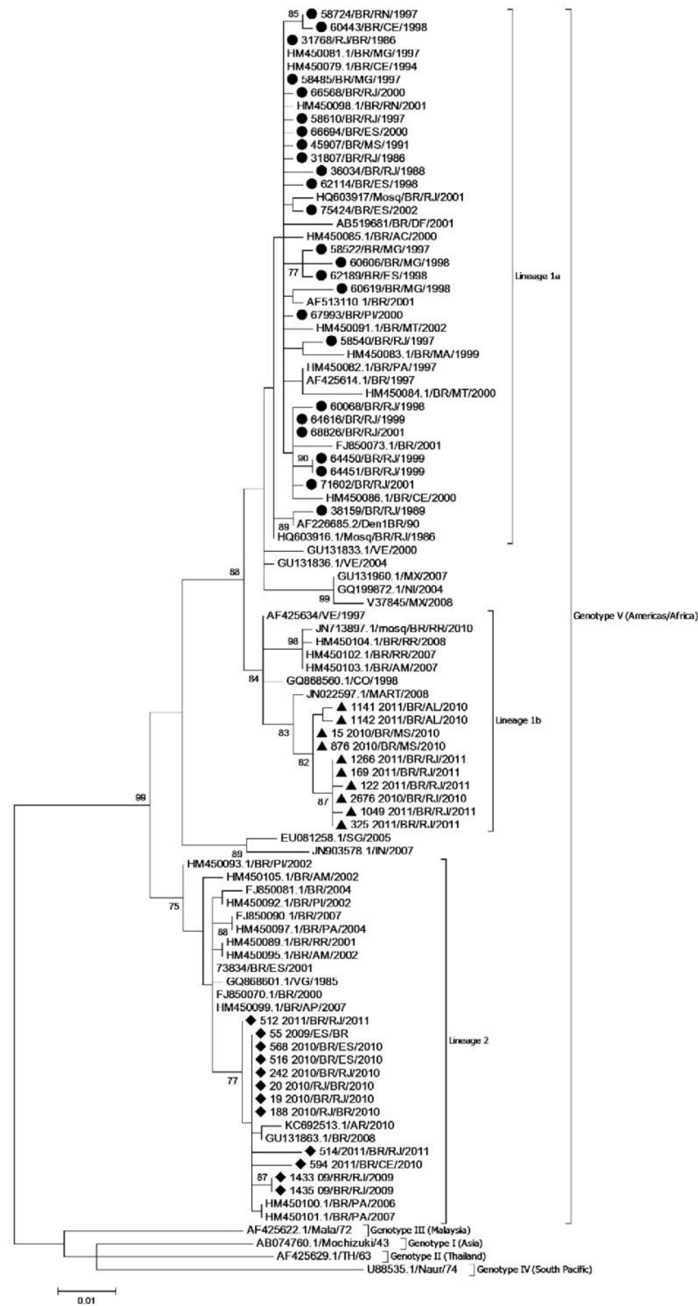

Fig. 2. Phylogenetic analysis based on the coding sequence for the domain III and stem region of the E gene of Brazilian DENV-1 isolates during the epidemic periods (1986–2011). Maximum-likelihood method, TN93 + G model with six discrete Gamma categories (parameters: =0.2941). The DENV-1 sequences analyzed are represented as: circle (lineage 1a), triangle (lineage 1b) and diamond (lineage 2). DENV strains were named as follows: GenBank accession number/country/year.

previous observations (Carneiro et al., 2012; Drumond et al., 2012). Substitutions on domain III may alter the viral particle immunogenicity, may influence pathogenicity and determine the receptor specificity, type of vector, host and cell tropism (Chen et al., 1996; dos Santos et al., 2002; Goncalvez et al., 2002; Drumond et al., 2012; Carneiro et al., 2012). As some regions in domain III are target for neutralizing antibodies in humans, mutations in this region could have important functional consequences (Thai et al., 2012). Carneiro et al. (2012) described aa substitutions at the position E_{338} in four DENV-1 strains from severe dengue patients, however in this study, substitutions at this position were observed in strains isolated from patients with all clinical presentations. Substitution at E_{196} (M → V) and E_{296} (T → P), considered as virulence markers (Bordignon et al., 2008), were not identified in the strains analyzed in this study.

Three substitutions were observed in prM, but at prM_{29}, a single strain representative of the lineage 2, presented a valine instead of an alanine and this observation has been described previously when Indian strains were compared to strains globally (Kukreti et al., 2009). Substitutions at positions $NS1_{84}$ and $NS1_{92}$ identified in two strains from lineage 1a are located immediately by the linear epitope ^{111}HKYSWK. As the NS1 glycoprotein is involved in the early stages of viral replication, it would be important to determine whether those aa differences among the strains studied, would affect the antigenicity and the biological activity of NS1 (dos Santos et al., 2002). Suzuki and collaborators (2007) analyzing a mutant DENV-1 NS1 demonstrated that a tyrosine (Y) residue at position $NS1_{247}$, highly conserved in DENV-1, would be important for NS1 function. In this study, a substitution was observed in a strain from lineage 1b, where the tyrosine (Y) was replaced by a phenylalanine (F). Substitutions at $NS3_{358}$, $NS3_{437}$, $NS3_{465}$ and $NS3_{474}$ were reported on the strains analyzed, and three of them were reported in a strain representative of the lineage 2. At $NS3_{465}$, an asparagine residue was detected in the six complete genomes analyzed, differentiating those, from the reference strain BR/90 (dos Santos et al., 2002). In NS4B, the only conserved substitutions observed were on $NS4B_{34}$ (R → H) and $NS4B_{90}$ (R → L) in two strains isolated in 2000–2001, representative of lineage 1b. This protein has strong interactions with NS1 and is associated with viral replication (Lindenbach et al., 2007). Substitutions at the NS5 protein can directly affect viral replication, since this protein acts as a RNA-dependent RNA polymerase and, along with NS3, can act by stimulating both NTPase activity of NS3 and RTPase (Lindenbach et al., 2007). The substitutions at $NS5_{114}$ (I → V) and $NS5_{784}$ (I → V) observed in this study were prevalent among the strains, and different from BR/90. Two strains isolated in 2000–2001 belonging to lineage 1a, shared aa substitutions at $NS5_{629}$ (L → F) and $NS5_{635}$ (T → S), not detected in the other strains. Although several aa substitutions were observed among the strains studied, no changes in the aa's biochemical character were identified.

The divergence analysis showed that the strains are quite similar among the groups, and that the lineages 1a and 1b are more closely related when compared to lineage 2, as those are phylogenetically separated and supported (Fig. 4). The strains from lineage 2 analyzed here, shared a high similarity with strains of Asian origin.

Phylogeny based on the C gene did not allow genotyping of the strains studied. The lineages separation on 1a, 1b and 2 was only possible by analyzing the E gene or the entire genome coding region. Moreover, the same grouping was observed by analyzing only the domain III and stem region from the E protein, supporting the fact that the substitutions on this region are associated to the lineages separation.

Previous studies describe recombination among DENV-1 strains (Tolou et al., 2001; Aaskov et al., 2007; Carvalho et al., 2010; Shin et al., 2013; Chu et al., 2013), however no evidence of recombination was observed in the analysis of the 48 Brazilian strains in this study.

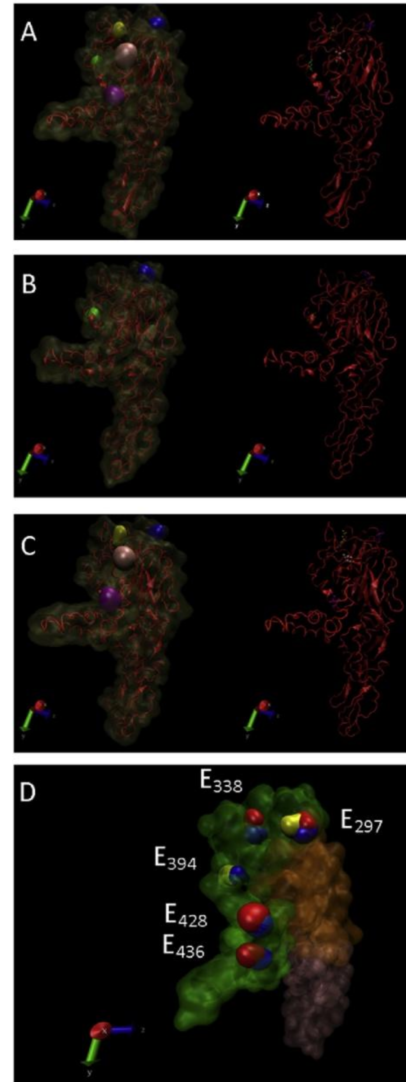

Fig. 3. Three-dimensional structure of DENV-1 E protein. Positions with conserved amino acids in domain I and III in lineage 1a (A), lineage 1b (B) and lineage 2 (C). (D) Representation constructed using as 4CCT template (Kostyuchenko et al., 2013) available from PDB (Protein Data Bank). Residues highlighted represent the amino acids found in the lineages: E_{297} (blue), E_{338} (yellow), E_{394} (green), E_{428} (pink), E_{436} (purple). Protein with a superposition of lineages 1a (blue), 1b (yellow) and 2 (red). Domains I, II and III are in orange, pink and green, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

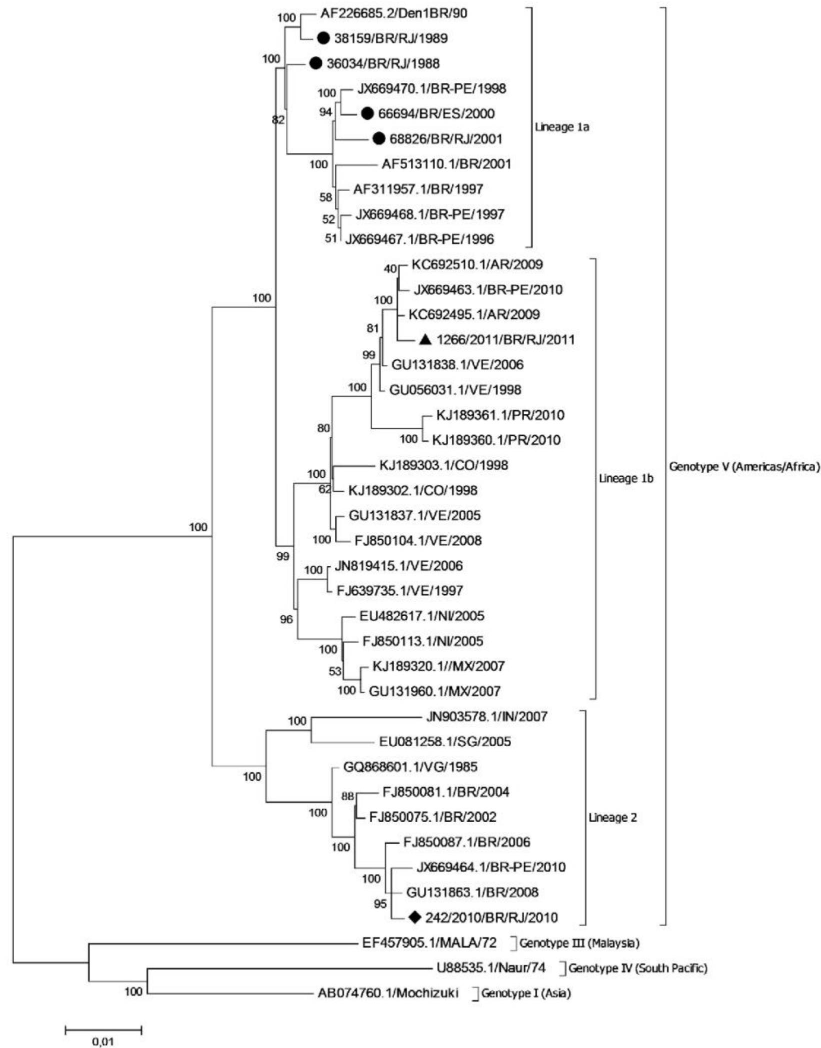

Fig. 4. Phylogenetic analysis based on the complete coding region of 6 Brazilian DENV-1 isolates during the epidemic periods (1986–2011). Maximum-Likelihood method, TN93 + G model with six discrete Gamma categories (parameters: =0.2104). The DENV-1 sequences analyzed are represented as: circle (lineage 1a), triangle (lineage 1b) and diamond (lineage 2). DENV strains were named as follows: GenBank accession number/country/year.

The co-circulation of two distinct lineages in Brazil in recent years has been characterized and, the involvement of both lineages on mild, severe and fatal cases demonstrate that, those are not restrict to a particular disease outcome. It is unknown whether the explosive outbreaks occurred in Brazil in 2009 and 2010, mainly caused by DENV-1, was due to the lineages circulation. The susceptibility of the population to a serotype that did not circulate intensively for nearly a decade, and the occurrence of secondary infections in a hyperendemic country, could have also played a role in the disease epidemiology. Due to Brazil's geography and dengue epidemiology, the surveillance of viral populations, lineages and genotypes is necessary, as the impact of those lineages replacements and co-circulation are yet not fully understood.

5. Conclusions

The phylogenetic analysis of Brazilian DENV-1 confirmed the circulation of the genotype V in the country, and the analysis of the domain III of the E gene and stem region was sufficient to differentiate the distinct lineages identified. A single substitution at

E_{297} was able to differentiate the strains belonging to lineage 1a from lineages 1b and 2. Moreover, substitutions at positions E_{338}, E_{394}, E_{428} and E_{436} were responsible for the segregation of lineages 1b and 2. Lineages 1a and 1b were more closely related to strains of American origin and lineage 2 to the strains of Asian origin. The circulation of multiple lineages in the country in 25 years was reported, with co-circulation and replacement, over time. No recombination events on the strains analyzed in this study was reported.

Author contributions

FBS, RMRN and AMB designed the study. FBN, NRCF, JBSS and PCGN implemented the study. FBN, FBS and RMRN analyzed the data. FBN and FBS wrote the paper. All authors read and approved the final version of the paper.

Competing interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2015.07.007.

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4.1.1 *Dados suplementares relacionados ao artigo 1*

Table S1. Amino acid substitutions in the genes C→NS5 six samples of DENV-1 representing the three distinct lineages compared with the reference sample AF226685.2/BR/90.

ORF	Protein	aa	AF226685.2 BR/90	Strain de DENV-1					
				Lineage 1a				Lineage 2	Lineage 1b
				36034/BR/RJ/1988	38159/ BR/RJ/1989	66694 BR/ES/2000	68826 BR/RJ/2001	242_2010 BR/RJ/2010	1266_2011 BR/RJ/2011
26	C	25	G	*	*	*	*	S	*
95		95	M	*	*	*	*	I	*
106		106	L	F	*	*	*	*	*
143	prM	29	A	*	*	*	*	V	*
199		85	E	G	*	*	*	*	*
203		89	D	*	*	*	*	*	E
222	M	31	K	*	*	*	*	*	R
252		47	L	*	*	*	*	*	F
577	E	297	T	*	*	*	*	M	M
603		329	T	*	I	*	*	*	*
618		338	S	*	*	*	*	L	*
662		382	A	*	*	V	*	*	*
674		394	K	*	*	*	*	*	R
708		428	V	*	*	*	*	L	*
716		436	V	*	*	*	*	I	*
720		440	F	*	L	*	*	*	*
823	NS1	48	K	*	*	R	R	*	*
852		77	H	*	*	*	*	Y	*
859		84	M	*	*	I	I	*	*
867		92	D	*	*	N	N	*	*
868		93	A	*	*	*	*	V	*
869		94	N	*	*	D	*	*	*
873		98	A	*	*	*	T	*	*
921		146	E	*	*	*	*	D	G
937		162	I	*	*	*	*	*	V
950		175	Y	*	*	*	*	H	*
999		224	I	*	*	*	*	*	T
1017		242	I	*	*	*	*	V	*
1021		246	I	*	*	M	M	*	*
1022		247	Y	*	*	*	*	*	F
1068		293	N	*	*	S	S	Y	*
1122		347	R	*	*	*	K	*	*
1144	NS2A	17	I	*	*	*	*	*	M
1145		18	M	*	*	*	*	L	*
1194		67	K	*	*	*	*	R	R
1224		97	A	T	T	T	T	T	T
1225		98	S	*	*	*	*	C	*
1233		106	I	*	*	*	*	V	*
1238		111	V	*	*	M	*	*	*
1240		113	S	*	*	*	*	*	C
1266		139	E	*	*	*	*	D	*
1282		155	I	*	*	*	*	*	V
1283		156	K	*	*	*	*	R	*
1285		158	T	*	*	*	*	S	*
1295		168	T	*	*	*	*	M	*
1298		171	V	*	*	*	*	A	*
1340		213	K	*	*	E	*	*	*
1349	NS2B	4	L	*	*	*	*	I	*
1356		11	I	*	*	*	*	V	*
1408		63	E	*	*	*	*	*	Q
1415		70	T	*	*	*	*	*	A
1487	NS3	12	E	*	*	*	*	K	*
1646		171	S	*	*	*	*	T	*
1830		358	K	*	R	*	*	*	*
1912		437	D	*	*	*	*	E	*
1940		465	S	N	N	N	N	N	N
1949		474	V	*	*	*	*	I	*
2183	NS4A	89	T	*	*	*	*	M	*
2261	NS4B	17	H	*	*	*	*	*	Y
2264		20	A	*	*	*	*	V	*
2268		24	Q	*	*	*	*	H	*
2278		34	R	*	*	H	H	*	H
2334		90	I	*	*	L	L	*	*
2392		148	V	*	*	I	*	*	*
2397		153	A	*	*	*	*	T	*
2523	NS5	30	R	*	*	*	*	K	*
2532		39	S	*	*	*	T	*	*
2607		114	V	I	I	I	I	I	I
2628		135	M	*	*	*	*	T	*
2688		195	Q	*	*	*	*	*	R
2863		370	A	*	*	*	*	*	T
3040		547	Q	*	*	R	*	*	*
3078		585	N	*	*	*	*	S	*
3120		629	L	*	*	F	F	S	*
3128		635	T	*	*	S	S	*	*
3133		640	E	*	*	*	*	K	*
3134		641	R	*	*	*	*	K	*
3135		642	V	*	*	*	*	*	A
3171		678	I	*	*	*	*	T	T
3277		784	I	V	*	V	V	V	V
3282		789	T	*	*	*	*	*	A
3320		827	T	*	A	*	*	*	*

4.2 Artigo 2: Evolutionary history and spatiotemporal dynamics of DENV-1 genotype V in the Americas.

Relação do Manuscrito com os Objetivos:

Objetivo específico 2: Reconstruir a história evolutiva do genótipo V nas Américas;

Objetivo específico 3: Demonstrar a migração e dispersão dos DENV-1 no Brasil, a partir da introdução deste sorotipo no estado do Rio de Janeiro em 1986;

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Resumo: O genótipo V do vírus dengue 1 (DENV-1) tem sido o mais prevalente nas Américas nos últimos 40 anos. Neste estudo, investigamos o padrão espaço-temporal de emergência e disseminação de linhagens de DENV-1 no continente. Foram aplicadas abordagens filogenéticas e filogeográficas a um conjunto abrangente de dados de 836 sequências gênicas do gene do envelope do genótipo V originárias de 46 países diferentes durante um período de 50 anos (1962 a 2014). Nosso estudo revela que a diversidade genética do genótipo V do DENV-1 nas Américas resultou de duas introduções independentes desse genótipo a partir da Índia. A primeira cepa do genótipo V foi provavelmente introduzida nas Pequenas Antilhas por volta do início dos anos 1970 e essa região do Caribe se tornou a fonte de várias linhagens de DENV-1 que se espalharam nas Américas durante as décadas de 1970 e 1980. A maioria dessas linhagens parece extinta durante a década de 1990, exceto uma que persistiu na Venezuela e depois se espalhou para outros países americanos, dominando a epidemia de DENV-1 na região a partir do início dos anos 2000. A segunda linhagem do genótipo V, também de origem indiana, foi provavelmente introduzido nas Pequenas Antilhas por volta do início dos anos de 1980. Esta linhagem permaneceu quase indetectada por quase 15 anos, até ser introduzida na região norte do Brasil em meados da década de 1990, e depois se espalhar para outras regiões do país. Esses resultados demonstram que diferentes regiões geográficas desempenharam um papel na manutenção e na disseminação do genótipo V do DENV-1 nas Américas ao longo do tempo. Linhagens de genótipo V do DENV-1 originaram-se, disseminaram-se e desapareceram nas Américas com dinâmicas muito diferentes e o fenômeno de substituição de linhagem através de sucessivos surtos epidêmicos de DENV-1 tem sido uma característica comum na maioria dos países americanos.

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Research paper

Evolutionary history and spatiotemporal dynamics of DENV-1 genotype V in the Americas

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ABSTRACT

The genotype V has been the most prevalent dengue virus type 1 (DENV-1) clade circulating in the Americas over the last 40 years. In this study, we investigate the spatiotemporal pattern of emergence and dissemination of DENV-1 lineages in the continent. We applied phylogenetic and phylogeographic approaches to a comprehensive data set of 836 DENV-1 E gene sequences of the genotype V isolated from 46 different countries around the world over a period of 50 years (1962 to 2014). Our study reveals that genetic diversity of DENV-1 genotype V in the Americas resulted from two independent introductions of this genotype from India. The first genotype V strain was most probably introduced into the Lesser Antilles at around the early 1970s and this Caribbean region becomes the source population of several DENV-1 lineages that spread in the Americas during the 1970s and 1980s. Most of those lineages appear to become extinct during the 1990s, except one that persisted in Venezuela and later spread to other American countries, dominating the DENV-1 epidemics in the region from the early 2000s onwards. The second genotype V strain of Indian origin was also most probably introduced into the Lesser Antilles at around the early 1980s. This lineage remained almost undetected for nearly 15 years, until it was introduced in Northern Brazil around the middle 1990s and later spread to other country regions. These results demonstrate that different geographic regions have played a role in maintaining and spreading the DENV-1 genotype V in the Americas over time. DENV-1 genotype V lineages have originated, spread and died out in the Americas with very different dynamics and the phenomenon of lineage replacement across successive DENV-1 epidemic outbreaks was a common characteristic in most American countries.

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1. Introduction

The dengue virus (DENV) belongs to the family Flaviviridae, genus *Flavivirus*, and presents four serotypes antigenically distinct, DENV-1 to DENV-4 (Pierson and Diamond, 2013). They are classified as arboviruses, since its maintenance in nature occurs through a transmission cycle of vertebrate hosts and hematophagous arthropods (Gubler, 1998). In the last 50 years, the incidence of dengue has increased 30-fold with geographic expansion to new countries. A recent estimate suggests that 390 million dengue infections occur per year and 96 million manifest clinically the disease (Bhatt et al., 2013).

DENV-1 was isolated for the first time during an epidemic outbreak in Hawaii in 1944 (Gubler, 1997) and since then several outbreaks of classic dengue fever (DF) and dengue hemorrhagic fever (DHF) caused by this serotype have been described world-wide. Phylogenetic analyzes support the existence of five distinct genotypes of DENV-1 (Weaver and Vasilakis, 2009). Genotypes I, IV and V are responsible for most DENV-1 infections detected up to date and have been associated with DF/DHF epidemics in Asia, the Indian subcontinent, South Pacific, Africa, the Arabian Peninsula and the Americas. Genotypes II and III, by contrast, are only represented by a few early sequences from Thailand and Malaysia, respectively. A previous phylogeographic study indicates that Indochina displayed a pivotal role in the global diffusion of DENV-1 and that Thailand and Indonesia represented the main sources of strains for neighboring countries (Villabona-Arenas and Zanotto, 2013).

The first major epidemics of DENV-1 in the Americas occurred in Jamaica and Cuba in 1977 (Gubler, 1997; Guzman and Kouri, 2003) and since then, DENV-1 has been continuously circulating in the Americas, leading to several waves of DF/DHF epidemics in the region (San Martín et al., 2010). Most DENV-1 infections in the Americas were caused by genotype V probably introduced from Southeast Asia (most probable Singapore or Thailand) (Villabona-Arenas and Zanotto, 2013) or from India (Walimbe et al., 2014). Previous studies documented two independent introductions of genotype V into the Americas, being the first one dated to around the early 1970s and the second one to the early 1980s (Villabona-Arenas and Zanotto, 2013; Walimbe et al.,

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2014). The Caribbean region was pointed as the major epicenter of genotype V diffusion in the Americas until the middle 1980s, after which movement was also observed from Brazil, Venezuela, Argentina, and Colombia to nearby countries (Allicock et al., 2012).

The continuous evolution and dissemination of DENV-1 genotype V in the Americas over the last 45 years resulted in the emergence of different local viral lineages. Phylogenetic analyses of DENV-1 genotype V in the Americas have identified the circulation of one major lineage in Central/North America (2005–2008) (Muñoz-Jordán et al., 2013), two different lineages in Colombia (1978–2005) (Mendez et al., 2010), and up to three distinct lineages in Argentina (2000 – 2010) (Tittarelli et al., 2014), Brazil (1982–2013) (dos Santos et al., 2011; Drumond et al., 2012; Carneiro et al., 2012; de Bruycker-Nogueira et al., 2015; Cunha et al., 2016), Mexico (1980–2007) (Carrillo-Valenzo et al., 2010) and Puerto Rico (1986–2010) (Añez et al., 2012). The geographical and temporal dispersion pattern of the distinct DENV-1 genotype V lineages detected in the Americas and the countries responsible for their dispersion and maintenance is poorly understood.

In this study we reconstruct the origin and spatiotemporal dissemination dynamics of the major DENV-1 genotype V lineages circulating in the Americas by using a comprehensive data set of 836 DENV-1 genotype V envelope (E) gene sequences isolated from 46 different countries around the world over a period of 50 years (1962 to 2014).

2. Materials and methods

2.1. Sequence dataset

All complete E gene (1485 bp) sequences of DENV-1 with known sampling date and location available at GenBank by May 2016 were downloaded and a final data set of 3575 DENV-1 sequences covering a total of 77 countries and spanning a period of 70 years (1944 to 2014) was analyzed. GenBank accession numbers, countries of origin, and year of isolation of all included sequences are shown in Table S1. Nucleotide sequences were aligned using MAFFT v6.902b program (Katoh et al., 2002) and the alignments may be available from the authors upon request.

2.2. DENV-1 phylogenetic analysis

In order to classify the complete data set of 3575 DENV-1 E gene sequences into its five distinct genotypes (I–V), a Neighbor Joining (NJ) phylogenetic tree was inferred with the PhyML program (Guindon et al., 2010), under the GTR + I + Γ4 model of nucleotide substitution as determined by jModelTest program (Posada, 2008). Phylogenetic relationships among genotype V sequences were further resolved using a Maximum Likelihood (ML) tree inferred with PhyML under the GTR + I + Γ4 model of nucleotide substitution and the SPR branch-swapping heuristic tree search algorithm. The reliability of the phylogenies was estimated with the approximate likelihood-ratio (aLRT) SH-like test (Anisimova and Gascuel, 2006) and trees were visualized with FigTree v1.4.2 program (Rambaut, 2012).

2.3. Spatiotemporal dispersion pattern of DENV-1 genotype V

The rate of nucleotide substitution per site per year (subs./site/year), the time to the most recent common ancestor (T_{MRCA}) and the global spatial diffusion were jointly estimated for the DENV-1 genotype V lineages using the Bayesian Markov Chain Monte Carlo (MCMC) statistical framework implemented in the BEAST v1.8 package (Drummond and Rambaut, 2007) with BEAGLE (Suchard and Rambaut, 2009) to improve run performance. Since this methodology is computationally prohibitive on large datasets, the DENV-1 genotype V sequences were subdivided into three subsets and sequences from the mostly densely sampled American countries (n ≥ 200 sequences) were clustered with the CD-HIT program (Li and Godzik, 2006) and only one sequence per cluster was selected (Table S3). The spatiotemporal scale of evolutionary process for all analyses was directly estimated from the sampling dates of the sequences using the GTR + I + Γ_4 nucleotide substitution model, a relaxed uncorrelated lognormal molecular clock model (Drummond et al., 2006), a Bayesian Skyline coalescent tree prior (Drummond et al., 2005) and a non-reversible discrete phylogeography model (Lemey et al., 2009). MCMC was run sufficiently long to ensure stationarity and convergence of parameters was assessed by calculating the Effective Sample Size (ESS) using TRACER v1.6 (http://tree.bio.ed.ac.uk/software/tracer/). Maximum clade credibility (MCC) trees were summarized using TreeAnnotator v1.8 and visualized with FigTree v1.4.2.

3. Results

3.1. Global prevalence and distribution of DENV-1 genotype V

The NJ phylogenetic analysis of 3575 DENV-1 E gene sequences sampled worldwide showed that 836 (23.4%) sequences branched within genotype V, whereas the remaining sequences were distributed within genotypes I (n = 2359; 66.0%), IV (n = 373; 10.4%), II (n = 5; <0.5%) and III (n = 2; <0.5%) (Fig. S1). According to this analysis, genotype V is the most prevalent DENV-1 clade circulating in India/Nepal/Bangladesh (100%), the Americas (99%) and Africa (43%) (Fig. 1). This genotype also accounts for a significant fraction of DENV-1 sequences from Saudi Arabia (33%) and Singapore/Malaysia (17%), while a very low proportion (<10%) of DENV-1 sequences was detected in other geographic region (Fig. 1).

3.2. Origin and dissemination of DENV-1 genotype V in the Americas

The ML phylogenetic tree showed that DENV-1 genotype V sequences split into two sub-clusters, one mostly composed by sequences of Asian origin (Asian clade, n = 119) and the other one composed by sequences from Asia, Africa and the Americas (Cosmopolitan clade, n = 717) (Fig. 2). Within the Cosmopolitan clade, the American sequences were distributed into two major lineages: 1) clade I that comprises 91.5% (n = 628) of the American sequences sampled between 1977 and 2014; and 2) clade II that comprises 8.5% (n = 58) of the American sequences sampled between 1985 and 2013. The earliest clade I sequences (sampled before 1995) were then combined with all clade II sequences and all genotype V sequences detected outside the American continent and subjected to Bayesian phylogeographic analysis (Table S2, subset genotype V). The phylogeographic reconstruction placed the origin of genotype V in the Indian subcontinent (posterior state probability [*PSP*] = 0.56) at around the early 1930s and further supports two independent introductions of this DENV-1 genotype from India (*PSP* = 0.94) into the Caribbean region (*PSP* ≥ 0.98) around the early 1970s and the early 1980s, originating the clades I and II, respectively (Fig. 3 and Table 1).

3.3. Dispersion of DENV-1 genotype V clade I in the Americas

To reduce potential biases in the phylogeographic reconstructions due to sampling heterogeneity between locations, we generate a "non-redundant" subset of the American clade I by removing very closely related sequences from the most densely sampled countries (n ≥ 200 sequences). The American DENV-1 clade I sequences were then distributed across 23 locations, comprising between 1 and 149 sequences each (Table S3, subset clade I), and subjected to Bayesian phylogeographic analysis. This analysis showed that clade I probably arose in the Lesser Antilles (LA) (*PSP* = 0.88) around the early 1970s (Table 1) and this Caribbean region was the main epicenter of genotype V dissemination in the Americas until the middle 1980s (Fig. 4). By this time, the clade I was already disseminated at multiple times from the LA to the Greater Antilles (GA), South America, Central America and Mexico. While

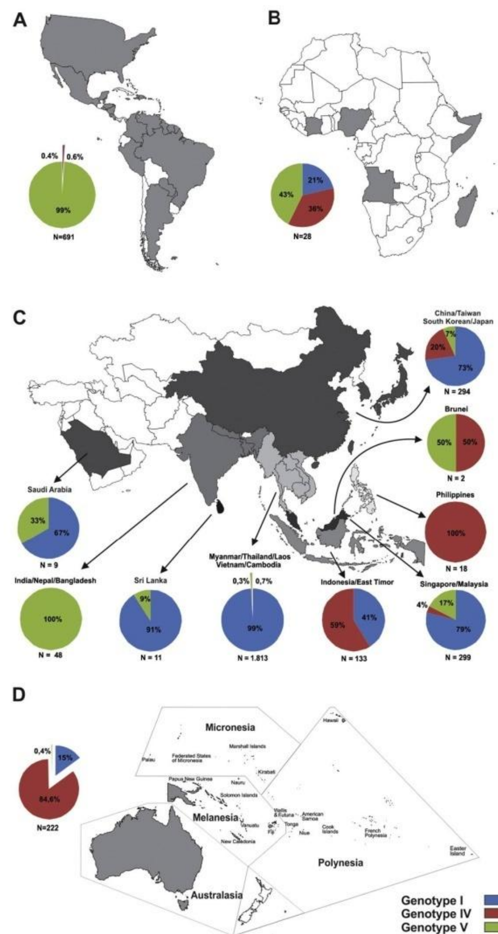

Fig. 1. Estimated proportion of the genotypes I (blue), IV (red) and V (green) among DENV-1 infections in the American (A), African (B), Asian (C) and Oceania (D) continents. The total number of sequences analyzed in each locality is indicated below each pie graph. Countries colored in gray correspond to locations that have DENV-1 sequences. Due to the small size of many islands, only those countries from Oceania represented in our study are named. Maps are not to scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

most clade I strains disseminated outside the LA resulted in secondary outbreaks of small size with no further dissemination after the middle 1990s; three strains established secondary outbreaks of large size, originating the sub-clades I-PR, I-BR and I-VE (Fig. 4).

The sub-clade I-PR probably arose in Puerto Rico (*PSP* = 0.75) around the early 1980s (Table 1) and continues to be the dominant clade in this country up to the late 1990s. This sub-clade I-PR seems to have remained mostly restricted to Puerto Rico, although a few strains belonging to this lineage were also detected in South America (Argentina, Paraguay and Peru) and LA (Barbados and Martinique) between 1989 and 2003. The sub-clade I-BR probably arose in the Southeastern Brazilian region (*PSP* = 0.63) around the early 1980s (Table 1) and was later disseminated to the Northeastern and Central-Western Brazilian regions, circulating in the country up to the early 2000s. We detected only one dissemination event of the sub-clade I-BR outside Brazil, from the Central-Western region (*PSP* = 0.95) to Paraguay and then to Argentina.

The sub-clade I-VE probably arose in Venezuela (*PSP* = 0.96) around the late 1980s (Table 1) and represents the most successfully disseminated viral lineage in the American continent. This sub-clade continues to circulate in Venezuela up to the most recent sampling period (2007–2008) and from Venezuela (*PSP* ≥ 0.88) migrates: 1) to Colombia and the LA around the late 1990s, 2) to the GA around the early 2000s, 3) to Argentina, Northern Brazil and Nicaragua around the middle 2000s, and 4) to the US around the late 2000s. During the 2000s, the sub-clade I-VE was spread from Nicaragua (*PSP* = 1) to other Central American countries, Mexico and the US, establishing the previously recognized Central/North American lineage. At around the late 2000s, the sub-clade I-VE was also disseminated from Argentina (*PSP* = 1) to the Brazilian Northeastern region, from the GA (*PSP* = ≥0.85) to the LA and the US, from Mexico to the US (*PSP* ≥ 0.99) and from the Northeastern (*PSP* = ≥0.53) to the Southeastern and Central-Western Brazilian regions.

3.4. Dispersion of DENV-1 genotype V clade II in the Americas

All American DENV-1 sequences that branched within clade II (n = 58) were distributed across seven locations (Table S3, subset clade II) and subjected to Bayesian phylogeographic analysis. The results obtained suggest that clade II was also introduced to the Americas by the LA (*PSP* = 1) around the early 1980s (Table 1) and then remained undetected until 2000, when it was detected in Northern Brazil. The clade II entered Brazil by the Northern region (*PSP* = 1) around mid-1996 (Table 1) and rapidly moved to the Northeastern and Southeastern Brazilian regions. After the middle 2000s, clade II was disseminated from the Southeastern (*PSP* ≥ 0.97) to the Central-Western region and Argentina, and from Argentina (*PSP* = 0.43) to the Central-Western region (Fig. 5).

4. Discussion and conclusion

Our phylogeographic analysis supports that current diversity of DENV-1 in the Americas resulted from two independent introductions of genotype V from India at around the early 1970s and the early 1980s, thus corroborating the study of Walimbe et al. (2014). Both genotype V strains were probably introduced in the American continent through the Caribbean region and later disseminated within the continent, confirming the central role of Caribbean islands in the initial dispersion of DENV from Asia to the Americas (Allicock et al., 2012; Villabona-Arenas and Zanotto, 2011; Villabona-Arenas and Zanotto, 2013; Mir et al., 2014).

The first DENV-1 genotype V strain introduced in the Americas at around the early 1970s was first identified in the Bahamas in 1977 and rapidly spread to other countries of South, Central and North America, originating the lineage here designated as clade I. According to the results presented here, the LA acted as the primary source population of clade I strains disseminated in the Americas until the middle 1980s, consistent with a previous study (Allicock et al., 2012). Most of clade I strains disseminated during the 1970s and 1980s from the LA, resulted in secondary outbreaks of small sizes and become extinct by the middle 1990s. Two clade I strains were able to ignite secondary outbreaks of large size in Puerto Rico (lineage I-PR) and Brazil (lineage I-BR) and persisted as the prevalent DENV-1 lineages in those countries during the 1980s and 1990s. Those lineages, however, do not appear to have caused large outbreaks outside their countries of origin and eventually also become extinct by the early 2000s.

Another clade I strain from the LA entered Venezuela around the late 1980s and was successfully disseminated within this country, establishing

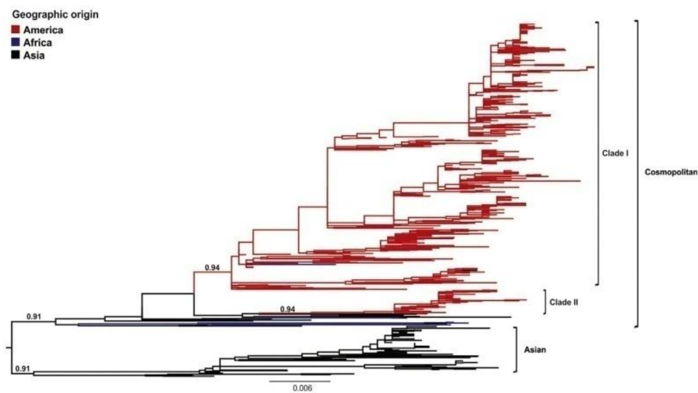

Fig. 2. ML tree of 836 DENV-1 genotype V E gene sequences sampled worldwide. Branches are colored according to the geographic origin of the tips (legend shown on the left side). Brackets indicate the position of major clades. Only the aLRT support values of major clades are shown. The tree was rooted on the midpoint. All horizontal branch lengths are drawn to a scale of nucleotide substitutions per site as shown in the bar at the bottom. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

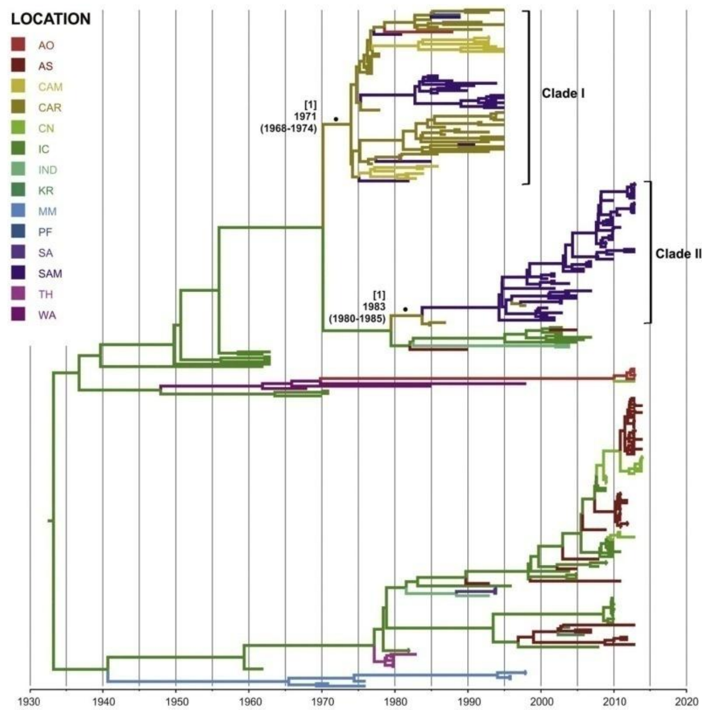

Fig. 3. Time-scalvalue ed Bayesian MCC tree of 278 DENV-1 genotype V E gene sequences belonging to the Cosmopolitan clade. Brackets indicate the position of major American clades. Branches are colored according to the most probable location (legend shown on the left side) of their parental node inferred by discrete phylogeographical analysis. The Posterior Probability (*PP*) support (value in [ ]) and the T_{MRCA} (95% HPD) are only represented for those nodes corresponding to the ancestors of major American clades. All horizontal branch lengths are drawn to a scale of years. The tree is automatically rooted under the assumption of a relaxed molecular clock. (AO: Angola, AS: Brunei, Malaysia, Singapore, CAM: Costa Rica, Mexico, El Salvador, CAR: Bahamas, Greater Antilles, Lesser Antilles, CN: China, IC: Bangladesh, India, Sri Lanka, Nepal, IND: Comoro Island, Reunion Island, KR: South Korea, MM: Myanmar, PF: French Polynesia, SA: Saudi Arabia, SAM: Argentina, Brazil, Colombia, French Guiana, Peru, Suriname, Venezuela, TH: Thailand, WA: Ivory Coast, Nigeria.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Median time of the most recent common ancestor (T_{MRCA}) of major DENV-1 genotype V clades and sub-clades detected in America estimated using different datasets.

Dataset	T_{MRCA}				
	Clade I	Sub-clade I-PR	Sub-clade I-BR	Sub-clade I-VE	Clade II
Genotype V	1971 (1968–1974)	–	–	–	1983 (1980–1985)
Clade I	1974 (1971–1976)	1982 (1979–1984)	1983 (1981–1985)	1989 (1985–1991)	–
Clade II	–	–	–	–	1985 (1983–1985)

the lineage I-VE. The lineage I-VE did not remain restricted to Venezuela, but was successfully dispersed to other American countries and was responsible for nearly all DENV-1 infections detected in the region after 2003. Between the middle 1990s and the middle 2000s, it is possible to identify different entries of the lineage I-VE from Venezuela into other South American countries (Argentina, Brazil and Colombia), Central America (Nicaragua), the Caribbean and also the US. Since the middle 2000s, Nicaragua has become an important secondary hub of dissemination of lineage I-VE to other countries from Central and North America, leading to the establishment of a regional sub-lineage, while Argentina and the GA acted as minor hubs of dissemination of lineage I-VE to nearby countries.

The second DENV-1 genotype V strain introduced in the Americas around the early 1980s was first identified in the Virgin Islands in 1985 and after a decade of cryptic circulation, entered Brazil, originating the lineage here designated as clade II. Probably, the circulation of this minor genotype V clade in the Americas remained unrecognized for such a long period due to the paucity of DENV-1 sequences available from the Caribbean, particularly from the Lesser Antilles islands, during the 1980s and 1990s. According to our estimations, the clade II was introduced from the Caribbean into the Northern Brazilian region at around the middle 1990s and later disseminated to the Southeastern, Northeastern and Central-Western regions, and from the Southeastern region to Argentina.

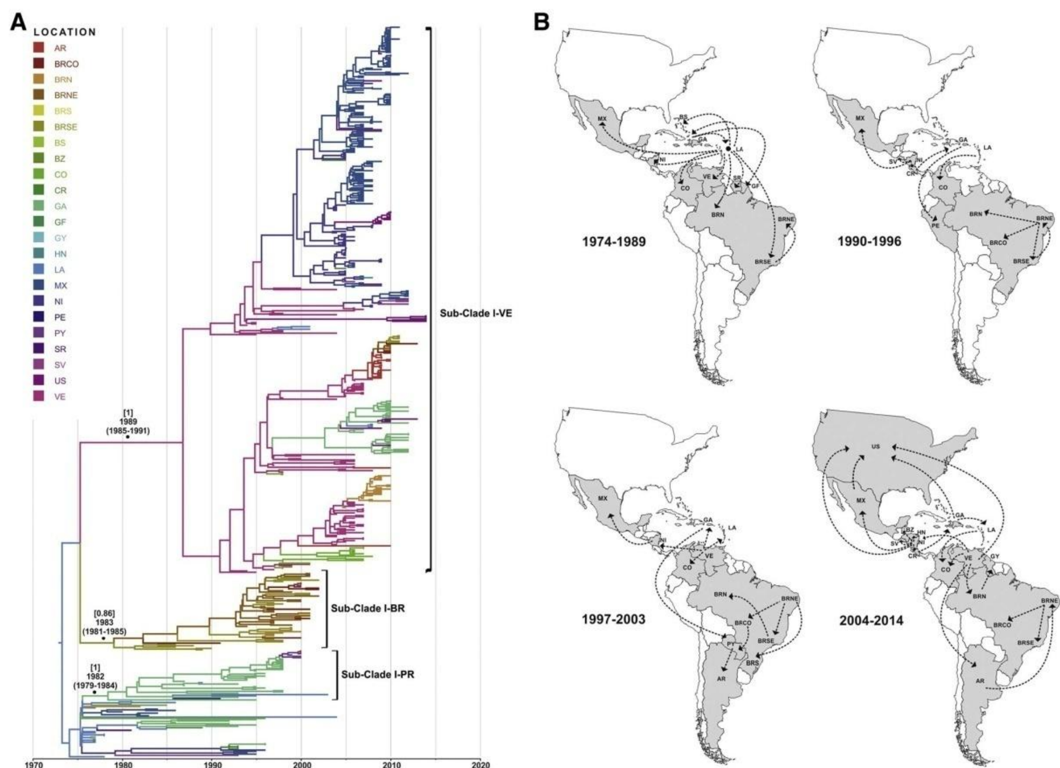

Fig. 4. Spatiotemvalue poral dynamics of dissemination of DENV-1 genotype V clade I in the Americas. (A) Time-scaled Bayesian MCC tree of 554 clade I E gene sequences. Three American sub-clades (I-PR, I-BR, I-VE) are identified. Branches are colored according to the most probable location (legend shown on the left side) of their parental node inferred by discrete phylogeographical analysis. The *PP* support (value in [ ]) and the T_{MRCA} (95% HPD) are only represented for those nodes corresponding to the ancestors of main clade I sub-clades. All horizontal branch lengths are drawn to a scale of years. The tree is automatically rooted under the assumption of a relaxed molecular clock. (AR: Argentina, BRCO: Brazil Central-West, BRN: Brasil North, BRNE: Brazil Northeast, BRS: Brazil South, BRSE: Brazil Southeast, BS: Bahamas, BZ: Belize, CO: Colombia, CR: Costa Rica, GA: Greater Antilles, GF: French Guiana, GY: Guyana, HN: Honduras, LA: Lesser Antilles, MX: Mexico, NI: Nicaragua, PE: Peru, PY: Paraguay, SR: Suriname, SV: El Salvador, US: United States, VE: Venezuela.) (B) Viral migration events occurred at four time periods between 1974 and 2014 are summarized in the map. Lines between locations represent branches in the Bayesian MCC tree along which location's transitions occur. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

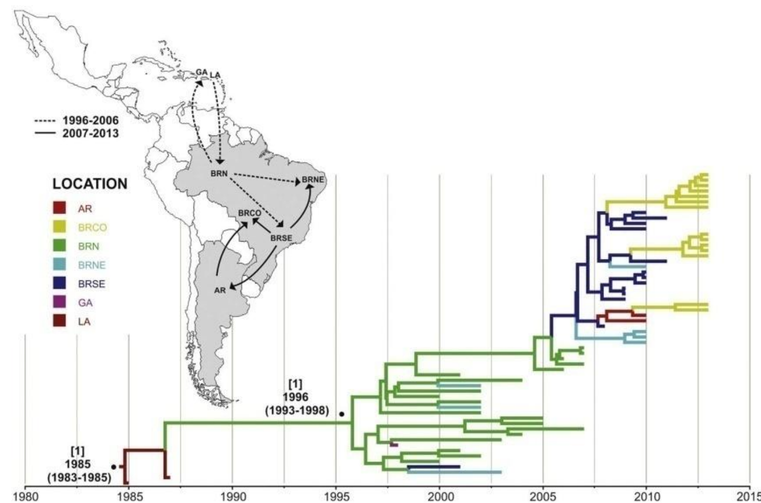

Fig. 5. Spatiotemvalue poral dynamics of dissemination of DENV-1 genotype V clade II in the Americas. Time-scaled Bayesian MCC tree of 58 clade II E gene sequences is shown. Branches are colored according to the most probable location (legend shown on the left side) of their parental node inferred by discrete phylogeographical analysis. The *PP* support (value in [ ]) and the T_{MRCA} (95% HPD) are only represented at key nodes. All horizontal branch lengths are drawn to a scale of years. The tree is automatically rooted under the assumption of a relaxed molecular clock. (AR: Argentina, BRCO: Brazil Central-West, BRN: Brasil North, BRNE: Brazil Northeast, BRSE: Brazil Southeast, GA: Greater Antilles, LA: Lesser Antilles.) Viral migration events occurred between 1996–2006 and 2007–2013 are summarized in the map with dashed and solid lines, respectively. Lines between locations represent branches in the Bayesian MCC tree along which location's transitions occur. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A previous study conducted by our group supports that the phylogeographic pattern of DENV-2 in the Americas could be explained by a unidirectional short-distance transmission model in which the virus moved outward from the Caribbean to near continental regions which behave as secondary hubs of dissemination to other neighboring mainland countries (Mir et al., 2014). This model seems to explain the early dissemination dynamics of DENV-1 in the Americas during the 1970s and 1980s. Since the middle 1990s, however, the central starting point of DENV-1 dissemination in the Americas moved out from the Caribbean to Venezuela, establishing a new directional flux of DENV-1 from Venezuela to the Caribbean and other mainland countries that was not previously observed for DENV-2. This demonstrates that the overall pattern of DENV dissemination in the Americas could greatly vary across distinct serotypes and that different geographic regions may act as a primary source for the maintenance and spread of this virus in the continent.

Our results further revealed that the phenomenon of lineage replacement across successive DENV-1 epidemic outbreaks was a common characteristic in several American countries. In Brazil, the clade I-BR was the most prevalent lineage until the early 2000s, when it became apparently extinct and was replaced by clade I-VE introduced from Venezuela and Argentina in the second half of the 2000s and by the clade II previously introduced from the Caribbean around the middle 1990s. The clade I-PR was the most prevalent lineage in Puerto Rico until the late 1990s, when it seemed to become extinct and was replaced by the clade I-VE introduced from Venezuela at the early 2000s. Several early clades disseminated from the LA circulate in Central America and Mexico until the middle 1990s, but were also replaced by the I-VE lineages introduced from Venezuela around 2000. The only exception to this trend seems to be Venezuela where the lineage I-VE circulates continuously from the late 1980s to the late 2000s, without evidence of lineage replacement.

Brazil has contributed to the largest proportion (usually >50%) of DENV positive cases detected in the Americas across different DF/DHF epidemics (WHO, 2012). Despite this great contribution to DF/DHF epidemics and the high number of DENV sequences available from this country, phylogeographic analyses conducted here and in a previous study (Mir et al., 2014) revealed that Brazil was not a major epicenter of new DENV lineages nor a major reservoir for DENV persistence at the regional level. According to these reconstructions, the main reservoirs of DENV-1 and DENV-2 in the Americas over the last 20–30 years were Venezuela and Puerto Rico, respectively; while most DENV-1 and DENV-2 strains introduced in Brazil were only dispersed within the country or to neighboring southern countries (Argentina, Bolivia and Paraguay) and seem to become extinct after a period of 10–20 years.

The DENV-1 dataset here used only comprises a minor fraction of all DENV-1 cases which occurred in the Americas since the late 1970s and sampling efforts were not continuous in both time and space, certainly leading to important unbalances between countries. Some lineages mainly associated to a single country like I-PR and I-BR, for example, may have been also circulating in other countries that were not properly sampled at that time period and the ancestral root locations in our Bayesian phylogeographic reconstructions may have been biased toward those countries that were overrepresented (Gubler, 2011). The countries that contribute with most DENV-1 sequences in our dataset (Mexico and Brazil), however, were not those pointed as the most important source locations of viral dissemination (LA, GA, Venezuela and Nicaragua). Furthermore, the phenomena of spatial heterogeneity and replacement of DENV-1 lineages were detected across heavily sampled countries (Puerto Rico, Brazil, and Venezuela). Thus, although some observations described here could be highly susceptible to sampling heterogeneity and should be thus interpreted with caution, major findings are quite robust to this limitation.

In conclusion, this study shows that DENV-1 genotype V diversity in the Americas resulted from the regional evolution of two independent founder strains introduced from India around the early 1970s and the early 1980s. The first strain introduced was responsible for most DENV-1 genotype V infections detected in the Americas, while the second one seems to be mostly restricted to Brazil. The Lesser Antilles

islands were the primary source of DENV-1 lineages disseminated in the Americas until the middle 1980s; after which Venezuela and Nicaragua became the most important hubs of viral maintenance and dissemination in the continent. Although several DENV-1 lineages established successful outbreaks in different American countries during the 1980s, they were later disseminated and became extinct with very different dynamics. Understanding the factors that determine the long-term persistence and replacement of DENV-1 lineages in the Americas will be of paramount importance for the implementation of control strategies aimed to limit the viral dispersion in the continent.

Authors contributions

Conceived and designed the experiments: FBN FBS GB. Performed the experiments: FBN DM GB. Analyzed the data: FBN DM GB. Wrote the paper: FBN DM FBS GB.

Competing interest

The authors have declared that no competing interests exist.

The following are the supplementary data related to this article.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.09.025.

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4.2.1 Dados suplementares relacionados ao artigo 2

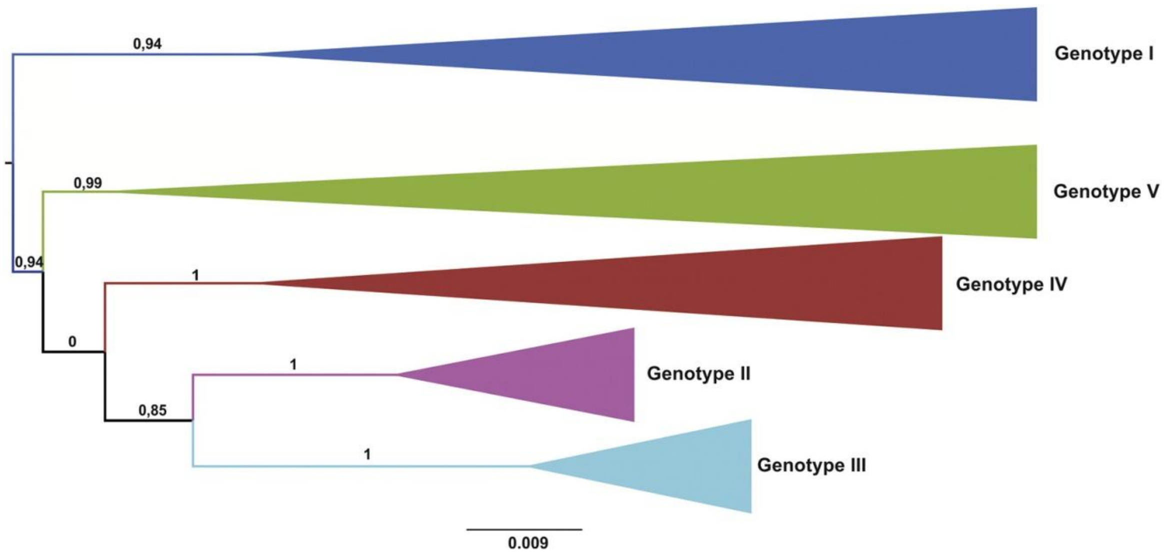

Fig. S1. Neighbor Joining tree of 3575 DENV-1 E gene sequences circulating globally. DENV-1 genotypes I, II, III, IV and V are identified. For visual clarity, strains from each genotype are shown collapsed. Only aLTR support values of major branching are shown. All horizontal branch lengths are drawn to a scale of nucleotide substitutions per site.

Table S1. Sequences of the DENV-1 global dataset.

Devido à grande quantidade de páginas deste arquivo, segue o endereço eletrônico no qual encontra-se disponível:

https://ars.els-cdn.com/content/image/1-s2.0-S1567134816304087-mmc1.pdf

Table S2. Complete dataset and subset of Genotype V DENV-1.

Location	Country / Sampling dates	Complete dataset Genotype V	Subset Genotype V
AO	Angola / 1988 - 2013	06	06
AS	BN (Brunei) / 2005	01	01
	MY (Malaysia) / 2010 - 2013	16	16
	SG (Singapore) / 1990 - 2014	36	36
CAM	CR (Costa Rica) / 1993-2005	04	03
	MX (Mexico) / 1980 - 2012	221	09
	SV (El Salvador) / 1993 - 2012	05	01
CAR	BS (Bahamas) / 1977	01	01
	GA (Greater Antilles) / 1977 - 2012	65	22
	JM (Jamaica) / 1977	*(01)*	*(01)*
	PR (Puerto Rico) /1986 - 2012	*(64)*	*(21)*
	LA (Lesser Antilles) / 1977 - 2008	25	17
	AW (Aruba) / 1985 - 2004	*(02)*	*(01)*
	BB (Barbados) / 1995 - 2003	*(05)*	*(01)*
	GD (Granada) / 1977 - 1981	*(09)*	*(09)*
	MQ (Martinique) / 1989 - 2008	*(04)*	*(01)*
	TT (Trinidad and Tobago)/1978 - 1986	*(03)*	*(03)*
	VG (British Virgin Islands) /1985 - 1987	*(02)*	*(02)*
CN	China / 2009 - 2014	17	17
IC	BD (Bangladesh) / 2009	01	01
	IN (India) / 1962 - 2011	36	36
	LK (Sri Lanka) / 2004	01	01
	NP (Nepal) / 2010	11	11
IND	KM (Comoro Island) / 1993	01	01
	RE (Reunion Island) / 2004	02	02
KR	South Korea / 2004 - 2006	02	02
MM	Myanmar / 1971 - 1998	07	07
PF	French Polynesia / 1989	01	01
SA	Saudi Arabia / 1994	03	03
SAM	AR (Argentina) / 2000 - 2010	27	02
	BR (Brazil) / 1982 - 2013	144	63
	CO (Colombia) / 1985 - 2008	20	01
	GF (French Guiana) / 1989	01	01
	PE (Peru) / 1991	01	01
	PY (Paraguay) / 1999 - 2000	05	-
	SR (Suriname) / 1981	01	01
	VE (Venezuela) / 1994 - 2008	81	06
TH	Thailand / 1980 - 1983	06	06
WA	CI (Ivory Coast) / 1985 - 1998	02	02
	NG (Nigeria) / 1968	01	01
	Others / 1996 - 2014	85	-
Total		**836**	**278**

Table S3. Complete dataset and subset Clade I and Clade II of Genotype V DENV-1.

Location	Country / Sampling dates	Complete dataset Genotype V	Subset Clade I	Subset Clade II
AO	Angola / 1988 - 2013	06	-	-
AR	Argentina / 2000- 2010	27	25	02
BD	Bangladesh / 2009	01	-	-
BN	Brunei / 2005	01	-	-
BR	Brazil / 1982 - 2013	144	90	53
BRCO	*Brazil: Central-Western*	*(24)*	*(10)*	*(14)*
BRSE	*Brazil: Southeastern*	*(42)*	*(29)*	*(13)*
BRS	*Brazil: South*	*(01)*	*(01)*	-
BRNE	*Brazil: Northeastern*	*(34)*	*(27)*	*(07)*
BRN	*Brazil: North*	*(43)*	*(23)*	*(19)*
BS	Bahamas / 1977	01	01	-
BZ	Belize / 2005	01	01	-
CI	Ivory Coast / 1985-1998	02	-	-
CN	China / 2009 - 2014	17	-	-
CO	Colombia / 1985-2008	20	20	-
CR	Costa Rica / 1993-2005	04	04	-
GF	French Guiana / 1989	01	01	-
GY	Guyana / 2008	01	01	-
HN	Honduras / 2008	01	01	-
IN	India / 1962-2011	36	-	-
JP	Japan / 1999	01	-	-
KM	Comoro Island / 1993	01	-	-
KR	South Korea / 2004 - 2006	02	-	-
LK	Sri Lanka / 2004	01	-	-
MM	Myanmar / 1971 - 1998	07	-	-
MX	Mexico / 1980 - 2012	221	149	-
MY	Malaysia / 2010 - 2013	16	-	-
NG	Nigeria / 1968	01	-	-
NI	Nicaragua / 1996 - 2009	51	51	-
NP	Nepal / 2010	11	-	-
PE	Peru / 1991	01	01	-
PF	French Polynesia / 1989	01	-	-
GA	Greater Antilles / 1977-2010	71	70	01
	DO (Dominican Republic) / 2007	*(01)*	*(01)*	-
	HT (Haiti) / 2010	*(05)*	*(05)*	-
	JM (Jamaica) / 1977	*(01)*	*(01)*	-
	PR (Puerto Rico) / 1986-2012	*(64)*	*(63)*	*(01)*
PY	Paraguay / 1999-2000	05	05	-
RE	Reunion Island / 2004	02	-	-
SA	Saudi Arabia / 1994	03	-	-
SG	Singapore / 1990 - 2014	36	-	-
SR	Suriname / 1981	01	01	-
SV	El Salvador / 1993 - 2012	05	05	-
TH	Thailand / 1980 - 1983	06	-	-
US	United States / 2009 - 2014	24	24	-
VE	Venezuela / 1994-2008	81	81	-
LA	Lesser Antilles / 1977-2008	25	23	02
	AW (Aruba) / 1985-2004	*(02)*	*(02)*	-
	BB (Barbados) / 1995-2003	*(05)*	*(05)*	-
	GD (Granada) / 1977-1981	*(09)*	*(09)*	-
	MQ (Martinique) / 1989-2008	*(04)*	*(04)*	-
	TT (Trinidad and Tobago) / 1978-1986	*(03)*	*(03)*	-
	VG (British Virgin Islands) / 1985-1987	*(02)*	-	02
Total		**836**	**554**	**58**

4.3 Artigo 3. DENV-1 Genotype V in Brazil: Spatiotemporal dispersion pattern reveals continuous co-circulation of distinct lineages until year of 2016.

Relação do Manuscrito com os Objetivos:

Objetivo específico 4: Realizar a vigilância molecular das diferentes linhagens de DENV-1 cocirculantes entre 2012-2016, período após a reemergência deste sorotipo no Brasil.

Submetido para publicação em: Scientific Reports

Fator de impacto: 4.259

Resumo:

O DENV-1 permaneceu como o sorotipo prevalente entre os anos de 2012 a 2016 no Brasil. Em período anterior, durante a reemergência no país, foi identificado a cocirculação de distintas linhagens. Neste estudo realizamos a vigilância molecular continuada após o período de reemergência (entre 2012 a 2016), cobrindo os 30 anos de circulação do DENV-1 no país. A Análise filogenética permitiu a confirmação da presença continuada do genótipo V, bem como três linhagens distintas cocirculantes. A caracterização molecular do gene E apresentou duas novas substituições de aminoácidos anteriormente não identificadas. Análises filogeográficas mostraram que um grande fluxo de migrações tem ocorrido entre o Brasil e a Argentina nos últimos 10 anos.

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1 # DENV-1 Genotype V in Brazil: Spatiotemporal dispersion pattern reveals
2 continuous co-circulation of distinct lineages until 2016

3

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17

18 ## Summary

19 In Brazil, DENV-1 introduced in the 80's, remained the prevalent serotype from 2012 to
20 2016. After its re-emergence in the country in 2009, the co-circulation of different viral
21 lineages was identified, however, its transmission dynamics afterwards, was not fully
22 characterized. In this study, we performed the continuous molecular surveillance after
23 the reemergence period (2012 to 2016), covering the 30 years of circulation of DENV-1
24 in Brazil. Phylogenetic analysis allowed confirmation of the continued presence of
25 genotype V, as well as three distinct co-circulating strains. The molecular

26 characterization of the E gene presented two new amino acid substitutions previously
27 unidentified in the country. Phylogeographic analysis has shown that a large flow of
28 migrations has occurred between Brazil and Argentina in the last 10 years.
29

30 Introduction

31 The dengue virus (DENV) belongs to the family *Flaviviridae*, genus *Flavivirus*, and
32 presents four serotypes antigenically distinct, DENV-1 to DENV-4 [1]. They are
33 classified as arboviruses, since its maintenance in nature occurs through a transmission
34 cycle of vertebrate hosts and hematophagous arthropods [2], most prevalently in tropical
35 and subtropical areas worldwide [3].
36

37 Dengue is one of the main public health problems in the world, with relevant social and
38 economical impact due to the increased geographic extension, number of cases and
39 disease severity [4] and, in Brazil, DENV activity has grown significantly since the
40 introduction of DENV-1 in Rio de Janeiro (RJ) in 80's. This serotype re-emerged in
41 2009 after approximately eight years without being related to epidemics, causing in
42 2010, more than one million probable cases, with the highest hospitalization rate
43 reported in the country, especially in those over 60 years old. In the last 32 years, more
44 than twelve million dengue cases have been reported in Brazil [5,6].
45

46 After reemergence period (2009-2010), the DENV-1 and DENV-4 were responsible for
47 a high number of cases in the following years, reviewed in dos Santos et al. [7], being the
48 DENV-1 prevalent in years of 2014 to 2016, representing proximally 90% of isolated
49 cases in country [8,9]. This serotype continue to be identified until today [10].
50

51 Phylogenetic analysis of DENV-1 since its introduction in the country, characterized
52 those viruses as belonging to genotype V, with its origin in the Caribbean and
53 Venezuela and showed that several independent introductions occurred over time with
54 high genotype variability [11-17].
55
56 Molecular characterization and evolutionary studies can be important tools to monitor
57 the introduction and understanding of the viruses spread in a region, as well as to
58 predict possible epidemiological consequences of such events. In this scenario, the
59 DENV serotypes surveillance is of great relevance, as it may provide information on the
60 spread of potentially virulent strains, as well as, to assess their impact on the population
61 during an outbreak. Here, we performed the molecular characterization and investigated
62 the spatiotemporal patterns of DENV-1 lineages emergence and dissemination in Brazil
63 during 30 years, including strains from 2012 to 2016, representative of its post-
64 reemergence and intense circulation in the country.
65

66 Results

67 Phylogenetic analysis of DENV-1 strains based on Envelope gene

68 For phylogenetic analysis of current DENV-1 lineages isolated from patients from 2012
69 to 2016 in Brazil, the envelope (E) gene sequencing of viral strains (n = 30), of four
70 distinct geographic region: Southeast (n= 20), Central-Western (n= 4), North (n= 1) and
71 Northeast (n= 5) (Table 1), was performed. The Maximum Likelihood (ML)
72 phylogenetic analysis of 717 E gene sequences of DENV-1 genotype V available in
73 Genbank, additionally to thirty sequences generated in this study, and sequences
74 representing each DENV-1 genotype (I to IV), totalizing 751 sequences, showed that all
75 strains currently belong to genotype V, but grouped in two distinct clades, previously

76 identified by the our group, as Clade I and Clade II into the cosmopolitan clade of
77 genotype V (Figure 1A).
78
79 In order to analyze the branches that actually had recent Brazilian sequences, we
80 separated from Clade I, the subset corresponding to Sub-Clade I, containing 240
81 sequences and submitted to a new ML analysis to identify the specific branches with
82 Brazilian representatives (Figure 1B). From this analysis, three subsets were separated
83 for phylogeographic reconstruction: Sub-Clade Ia (n= 106), Sub-Clade Ib (n= 56) and
84 Clade II (n= 116) (Table S2).
85

86 Dispersion of Sub-Clade Ia and Ib of DENV-1 genotype V in Brazil

87 The Sub-Clade I was constituted with 240 sequences representative of 11 countries with
88 1 to 66 sequences each, include current Brazilian strains (n=48) from four locations.
89 The Brazilian sequences were distributed according the geographic regions as follows:
90 Midwest (*n*= 4), Southeast (*n*= 18), Northeast (*n*= 7) and North (*n*= 19) (Table S2, Sub-
91 Clade I). Moreover, the Sub-Clades Ia and Ib identified within this Sub-Clade were
92 submitted separately to a Bayesian phylogeographic analysis.
93
94 The Sub-Clade Ia analysis showed that DENV-1 strains reached Argentina around 2008
95 from Venezuela (posterior state probability [PSP] = 0.99). From Argentina this clade
96 spread to Brazil, to the Northeast, Midwest and Southeast regions. This viral variant
97 remained circulating until after reaching the Southeast region approximately in 2010,
98 coming from Argentina [PSP = 0.94]. Since then, introductions in the Midwest,
99 Northeast and Argentina have occurred. An interesting spread was observed from the
100 Midwest region to China [PSP = 0.7] around the year 2015 (Figure 2A).

101

102 Sub-Clade Ib presented an introduction route in North Brazil region directly from
103 Venezuela around 2007 [PSP = 0.99] and it seems to have remained circulating only in
104 the North of the country, with a point-scattered event for the French Guiana in 2008
105 [PSP = 0.99] and in 2012, for Argentina and the Northeast of the country [PSP = 0.57]
106 (Figure 2B).

107

108 ## Dispersion of Clade II of DENV-1 genotype V in Brazil

109 The Clade II comprised the Subset-Clade II, containing 116 sequences from four
110 countries, including Brazil, with 67 sequences divided into four locations, and
111 represented by 1 to 46 sequences each. The Brazilian sequences were distributed
112 according to geographic regions as follows: Midwest (n= 17), Southeast (n= 21),
113 Northeast (n= 10) and North (n= 19) (Table S2, Subset-Clade II).

114

115 The subset was also subjected to a Bayesian phylogeographic analysis and the results
116 showed that this subset appears to have arisen in the Caribbean Lesser Antilles (LA),
117 represented here, by sequences from the Virgin Islands (VG), in the mid-1980s
118 [PSP=0.97] and remained without identified circulation until 2000. This clade arrived in
119 Brazil in the mid-1990s [PSP= 0.95] by the North region and spread for that region until
120 the mid-2000s, with occasional migration events to the Greater Antilles (GA)
121 (represented here by the Puerto Rico [PR] strain) and to Northeast and Southeast
122 regions of country. However, around 2005 and 2006-7, this clade arrived in the
123 Southeast [PSP= 0.99] and Northeast regions [PSP= 0.78], respectively. From the
124 Southeast region the clade spread to two directions: 1) For Argentina [PSP= 0.99] and
125 then for Midwest [PSP= 0.63], for Argentina in two other moments [PSP= 0.78 and

126 0.98] and for Northeast [PSP= 0.98] and, 2) For Northeast [PSP= 0.97], Argentina
127 [PSP= 1] and Midwest [PSP≥ 0.94]. From the Midwest, this clade spread for the
128 Southeast region of Brazil and Argentina, then back to the Midwest region again [PSP=
129 1]. A large flow of migrations between the Southeast, Midwest and Argentina seems to
130 be occurring since 2010. Of the 30 sequences generated in this study, fourteen
131 representing the Southeast, Midwest and Northeast regions of Brazil form 2013 to 2016,
132 belong to this clade and were involved in this migration dynamics (Figure 3).
133

134 Molecular characterization of DENV-1 strains based on E gene

135 The sequences alignment demonstrated different patterns of nucleotides responsible for
136 conserving some amino acid in domains I, II, III and stem region of the E gene among
137 the strains of the different clusters. In order to identified those differences, we used a
138 strain from the period of DENV-1 introduction in Brazil, for comparison purposes
139 (Table 2).
140

141 In domain I, at position E_{297}, the amino acid threonine (T) was observed in all 30
142 sequences studied, different from the 80's sequence which has a methionine (M) at that
143 same position. In domain II, we identified the Alanine (A) at position E_{230} in only two
144 of our sequences studied, belonging to the Sub-Clade Ib. All other sequences have a T
145 at this position. Analyzing all 240 sequences of the Sub-Clade I, only those two
146 sequences and one from Argentina from 2016 (KX768377), presented this amino acid.
147

148 Further conserved amino acids were observed in the domain III (E_{338}, E_{361} and E_{394}) and
149 stem region (E_{428} and E_{436}). The sequences from Sub-Clade Ia presented in E_{394} an
150 Arginine (R) in the place of Lysine (K) like the other sequences of Sub-Clade Ib and

151 Clade II. However, Clade II sequences showed the greatest differences between shared
152 amino acids, with a Leucine (L) at E_{338} and E_{428} and an Isoleucine (I) at E_{436}, amino
153 acids conserved in all clades. Additionally, six sequences from this study from 2014 and
154 2015, representative from the Southeast and Northeast regions, share an R in E_{361} with
155 eleven sequences from Argentina from 2016, differentiating those from the other
156 sequences from Clade II and Sub-Clade I dataset, which have a K at the same position.
157

158 ## Discussion and Conclusion

159 The Sub-Clade Ia had Argentina as the gateway to Brazil, spreading to the Northeast,
160 Midwest and Southeast regions of the country. The observation of Argentina as an
161 ancestor for the Brazilian strains, corroborate our previous study[15]. With the
162 introduction in the Southeast region, we observed most of the dispersions of the viral
163 strain within Brazil, as well as returning to Argentina, which circulated at least until
164 2016.
165

166 The viral spread from the Midwest region to China, probably refers to an imported case.
167 At Genbank, that Chinese strain does refer to an imported case study, but it has not been
168 published. However, the results obtained here suggest this probable importation from
169 Brazil, more specifically, from the Midwest region.
170

171 Sub-Clade Ib presented an introduction route in Northeast Brazil starting directly from
172 Venezuela, as already demonstrated previously[15]. This clade seems to have remained
173 circulating only in the North of the country, with a punctual dispersal event for the
174 French Guianas. However, we observed a more recent movement of this clade to the
175 Northeast region of Brazil and to Argentina. Tittarelli and collaborators studying

176 isolates from Argentina during the epidemic that occurred in early 2016 highlights that a
177 sample of their study was related to samples from the North region of Brazil [18].
178
179 The Clade II migrations occurred until the mid-2000s after reaching the Southeast
180 region of the country. From the Southeast region, this clade dispersed directly to
181 Argentina, to the Northeast and also remained in the Southeast region. It reached the
182 Midwest directly through the Southeast and through Argentina, migrating again from
183 east to the Midwest. A large flow of migrations between the Southeast, the Midwest and
184 Argentina seems to be occurring during the decade of 2010.
185
186 The molecular characterization of the 30 strains allowed the identification of different
187 nucleotides patterns responsible for the conservation of some amino acids in the
188 domains I, II, III and stem-region of the E gene among the different strains and the
189 changes in domain II and one in domain III, were not previous identified. In domain I,
190 at position E_{297}, the amino acid T was observed in all 30 sequences studied, different
191 from the sequence of the 80s. In domain II, we identified an A at position E_{230} in only
192 two of our studied sequences, belonging to Sub-Clade Ib. All other sequences have a T
193 at this position. Analyzing all 240 sequences of Clade I, only those two sequences and
194 one from Argentina 2016 had this amino acid. During a study conducted in Argentina
195 from December 2015 to April 2016, this amino acid was not reported[18]. In fact, in all
196 the dataset analyzed here, the only sequences that present this shared amino acid are two
197 of our study detected in 2014 and 2015 and the one from Argentina, detected afterwards
198 in that country. Preserved amino acids were observed in domain III and in the stem
199 region. The sequences of Sub-Clade Ia presented in E_{394} an R instead of K, like the

200 other sequences of Sub-Clade I and Clade II. However, Clade II sequences showed the
201 largest differences among shared amino acids.
202
203 Recently, Dutra and collaborators investigated circulating DENV-1 strains in an
204 epidemic in the state of Minas Gerais, and reported the co-circulation of two distinct
205 lineages in 2013 and five independent introductions of genotype V in the country since
206 1982 [19]. The nomenclature of the different DENV-1 lineages circulating in Brazil is yet
207 to be defined. In our study, we designated the clades with roman numerals I and II, and
208 letters for the lineages within a clade. However, Dutra et al. [19] suggest the
209 standardization of BR1 to BR5 to the lineages, according to the chronological order of
210 introduction in country.
211
212 In conclusion, our data show that DENV-1 remains circulating in the country through
213 three distinct lineages, introduced by independent pathways in the last two decades. One
214 of the lineages seems to be restricted to the North region of the country, while the other
215 two are more dispersed by Northeast, Southeast and Midwest. Continuous surveillance
216 is necessary, as new viral variants may arise by local evolution or by introductions from
217 other countries. New viral strains may present nucleotide and/or amino acid changes in
218 viral genome important for infection and replication, impacting the dynamics of disease
219 maintenance and transmission.
220

221 ## Materials and methods

222 ### Ethical statement

223 The strains analyzed in this study belong to a previously gathered collection from the
224 Flavivirus Laboratory, IOC/FIOCRUZ, Rio de Janeiro, Brazil, obtained from human

225 serum from an ongoing Project approved by resolution number CSN196/96 from the
226 Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05) and collections
227 during a cross-sectional and observational study performed by the Viral Imunnology
228 Laboratory, IOC/FIOCRUZ, Rio de Janeiro, Brazil approved by the Oswaldo Cruz
229 Foundation Ethic Committee (CAAE 57221416.0.1001.5248). Samples were chosen
230 anonymously, based on the laboratorial results and clinical manifestations available on
231 the Laboratory database.
232

233 Dengue viral strains

234 The DENV-1 strains (*n*= 30) analyzed were detected in serum samples from patients
235 positive for dengue by virus isolation and/or RT-PCR, received at the Flavivirus
236 Laboratory (LABFLA), IOC/FIOCRUZ, Regional Reference Center for Dengue and
237 Yellow Fever Diagnosis and Viral Immunology Laboratory (LIV), IOC/FIOCRUZ.
238 Viral strains were selected according to the year of isolation and state of origin. Fifteen
239 strains were derived of the original isolate on cell culture, two after one passage of the
240 original isolate on cell culture and thirteen were analyzed directly from the serum
241 sample (Table 1). Virus isolation was performed by inoculation into C6/36 *Aedes*
242 *albopictus* cell line [20] and isolates were identified by indirect fluorescent antibody test
243 (IFAT) using serotype-specific monoclonal antibodies [21].
244
245

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

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

250 Lanciotti et al.[22] that detects all four serotypes simultaneously in a semi-nested
251 procedure, generating amplification products with specific size in base pairs (bp) of
252 each DENV serotype, was used to confirm the DENV-1 strains positivity.
253

254 ## Dengue virus genome amplification and sequencing

255 For E gene sequencing of DENV-1, three primers pairs (sets 2–4) were used to amplify
256 overlapping fragments of approximately 900 bp according as previously described by
257 our group [16]. Sequencing was performed on an ABI 3730 DNA Analyzer, Applied
258 Biosystems®, California, USA [23] and the sequences generated were deposited on
259 GenBank (Table 1). The sequences' analysis was performed using the Bioedit
260 (http://www.mbio.ncsu.edu/bioedit/bioedit.html), the sequences' identity by BLAST
261 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and alignments by CLUSTAL OMEGA
262 (https://www.ebi.ac.uk/Tools/msa/clustalo/).
263

264 ## Sequences dataset

265 Complete E gene (1485 bp) sequences of DENV-1 genotype V (Cosmopolitan clade)
266 with known sampling date and location available at GenBank by January 2018 were
267 downloaded and, a final dataset of 747 DENV-1 sequences covering a total of 31
268 countries and spanning a period of 70 years (1977 to 2016) was analyzed, including the
269 30 sequences derived from this study. From this dataset, 171 sequences are from Brazil
270 and are distributed according the geographic regions as follows: Midwest (n= 27),
271 Southeast (n= 62), South (n= 1), Northeast (n= 38) and North (n= 42) and one with no
272 information (Table S2). GenBank accession numbers, countries of origin and year of
273 isolation of all included sequences are shown in Table S1. Nucleotide sequences were

274 aligned using MAFFT v6.902b program and the alignments may be available from the
275 authors upon request.

276

277 DENV-1 Genotype V phylogenetic analysis

278 Phylogenetic relationships among genotype V sequences and sequences representing
279 genotypes I, II, III and IV were resolved using a Maximum Likelihood (ML) tree
280 inferred with PhyML [24], under the GTR + I + Γ4 model of nucleotide substitution as
281 determined by automatic model selection by SMS: Smart Model Selecion in PhyML [25]
282 and the SPR branch-swapping heuristic tree search algorithm. A second phylogenetics
283 analysis was performed in Sub-Clade I of genotype V, using a ML tree inferred with
284 PhyML, under the TN93 + I + Γ4 model of nucleotide substitution as determined by
285 automatic model selection by SMS: Smart Model Selecion in PhyML. The reliability of
286 the phylogenies was estimated with the approximate likelihood-ratio (aLRT) SH-like
287 test [26] and trees were visualized with FigTree v1.4.2 program [27].

288

289 Spatiotemporal dispersion of current lineages of DENV-1 genotype V in 290 Brazil

291 The rate of nucleotide substitution per site per year (subs./site/year), the time to the
292 most recent common ancestor (T_{MRCA}) and the spatial diffusion were jointly estimated
293 for the DENV-1 genotype V lineages using the Bayesian Markov Chain Monte Carlo
294 (MCMC) statistical framework implemented in the BEAST v1.8 package [28] with
295 BEAGLE [29] to improve run performance. The DENV-1 genotype V sequences were
296 subdivided into three subsets that contained Brazilian sequences until 2016: Sub-Clade
297 Ia (n= 106), Sub-Clade Ib (n= 56) and Clade II (n= 116) (Table S2). The spatiotemporal
298 scale of evolutionary process for analysis was directly estimated from the sampling

299 dates of the sequences using the GTR + Γ4 (Sub-Clade Ia), TN93 + Γ4 (Sub-Clade Ib)
300 and TN93 + I + Γ4 (Clade II) nucleotide substitution model as determined by
301 jModelTest program [30], a relaxed uncorrelated lognormal molecular clock model [31], a
302 Bayesian Skyline coalescent tree prior [32] and reversible discrete phylogeography model
303 [33]. MCMC was run sufficiently long to ensure stationary and convergence of parameters
304 was assessed by calculating the Effective Sample Size (ESS) using TRACER v1.6
305 (http://tree.bio.ed.ac.uk/software/tracer/). Maximum clade credibility (MCC) trees were
306 summarized using TreeAnnotator v1.8 and visualized with FigTree v1.4.2.
307

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313 fellowships.
314

315 ## Author contributions

316 FBN and FBS designed the study. TMAS participated in the samples collection in
317 Amapá and Mato Grosso do Sul in 2014 to 2016. NRCF and JBS participated in the
318 samples collection in Mato Grosso do Sul in 2013. FBN, TMAS, TCC, NRCF, JBS
319 performed the laboratory diagnosis. MCT assisted in the bioinformatics analysis. ILCR,
320 SFA, RMRN, AMBF, FBS provided biological samples for the study. FBS, RMRN and
321 AMBF sponsored the experiments. FBN implement the study. FBN and FBS analyzed
322 the data and wrote the paper. All authors read and approved the final version of the
323 paper.

324

Competing interest

326 The authors have declared that no competing interests exist

327

Data Availability

329
330 All data generated during this study are included in this published article. The
331 alignments may be available from the authors upon request.

332

Additional Information

334 Table S1

335 DENV-1 Genotype V (Cosmopolitan clade) dataset.

336 Table S2

337 Complete dataset, subsets Clade I and Clade II of Genotype V (clade cosmopolitan) DENV-1.
338

Table 1. DENV-1 strains (n=30) used in this study for envelope (E) gene (1,485 nucleotides) sequencing, 2012 to 2016, Brazil.

ID samples	Year	Country region: state	Origin of strain	Genbank Accession number
2071/2012/BR/RJ/2012	2012	Southeast: RJ	Isolated (C6/36)	MH401971
2612/2012/BR/RJ/2012	2012	Southeast: RJ	Isolated (C6/36)	MH401972
3239/2012/BR/RJ/2012	2012	Southeast: RJ	Isolated (C6/36)	MH401973
3246/2012/BR/RJ/2012	2012	Southeast: RJ	Isolated (C6/36)	MH401974
3599/2012/BR/RJ/2012	2012	Southeast: RJ	Isolated (C6/36)	MH311981
Lac1/BR/RJ/2012	2012	Southeast: RJ	Serum	MH401990
Lac5/BR/RJ/2012	2012	Southeast: RJ	Serum	MH401991
Lac16/BR/RJ/2012	2012	Southeast: RJ	Serum	MH401992
92/2013/BR/RJ/2013	2013	Southeast: RJ	Isolated (C6/36)	MH401977
7276/2013/BR/MS/2013	2013	Midwest: MS	Serum	MH401975
7436/2013/BR/MS/2013	2013	Midwest: MS	Serum	MH401976
05/2014/BR/RJ/2014	2014	Southeast: RJ	Isolated (C6/36)	MH401978
478/2014/BR/RJ/2014	2014	Southeast: RJ	Isolated (C6/36)	MH401979
494/2014/BR/SE/2014	2014	Northeast: SE	Isolated (C6/36)	MH401980
283/2017/BR/CE/2014	2014	Northeast: CE	Serum	MH401987
288/2017/BR/CE/2014	2014	Northeast: CE	Serum	MH401988
297/2017/BR/CE/2014	2014	Northeast: CE	Isolated (C6/36)	MH401989
30/2015/BR/RJ/2015	2015	Southeast: RJ	Isolated (C6/36) (passage 1)	MH401981
134/2015/BR/RJ/2015	2015	Southeast: RJ	Isolated (C6/36) (passage 1)	MH401982
148/2015/BR/SE/2015	2015	Northeast: SE	Isolated (C6/36)	MH401986
738/2015/BR/RJ/2015	2015	Southeast: RJ	Isolated (C6/36)	MH401983
2072/2015/BR/RJ/2015	2015	Southeast: RJ	Isolated (C6/36)	MH401984
3171/2015/BR/RJ/2015	2015	Southeast: RJ	Isolated (C6/36)	MH401985
Lac7/BR/RJ/2015	2015	Southeast: RJ	Serum	MH401993
67/2015/BR/AP/2015	2015	North: AP	Serum	MH401997
Lac4/BR/RJ/2016	2016	Southeast: RJ	Serum	MH401994
Lac8/BR/RJ/2016	2016	Southeast: RJ	Isolated (C6/36)	MH401995
Lac9/BR/RJ/2016	2016	Southeast: RJ	Serum	MH401996
KCMM25/BR/MS/2016	2016	Midwest: MS	Serum	MH401998
VAOR28/BR/MS/2016	2016	Midwest: MS	Serum	MH401999

ID: Identification; AP: Amapá; BR: Brazil; CE: Ceará; MS: Mato Grosso do Sul; RJ: Rio de Janeiro; SE: Sergipe.

Table 2. Amino acid conserved of the distinct Brazilian DENV- 1 genotype V lineages based on analysis of the envelope (E) gene.

ID/country/state/year	E gene (position)/substitution of amino acid							Clade cosmopolitan (Genotype-V)* Clade
	Domain II	Domain I	Domain III			Stem		
	E_{230}	E_{297}	E_{338}	E_{361}	E_{394}	E_{428}	E_{436}	
HQ026760/BR/RJ/1986	**T**	**T**	**S**	**K**	**K**	**V**	**V**	Sub-Clade I BR*
2071/2012/BR/RJ/2012	.	**M**	.	.	**R**	.	.	Sub-Clade I a
2612/2012/BR/RJ/2012	.	**M**	.	.	**R**	.	.	Sub-Clade I a
3239/2012/BR/RJ/2012	.	**M**	.	.	**R**	.	.	Sub-Clade I a
3246/2012/BR/RJ/2012	.	**M**	.	.	**R**	.	.	Sub-Clade I a
3599/2012/BR/RJ/2012	.	**M**	.	.	**R**	.	.	Sub-Clade I a
05/2014/BR/RJ/2014	.	**M**	.	.	**R**	.	.	Sub-Clade I a
148/2015/BR/SE/2015	.	**M**	.	.	**R**	.	.	Sub-Clade I a
Lac1/BR/RJ/2012	.	**M**	.	.	**R**	.	.	Sub-Clade I a
Lac5/BR/RJ/2012	.	**M**	.	.	**R**	.	.	Sub-Clade I a
Lac16/BR/RJ/2012	.	**M**	.	.	**R**	.	.	Sub-Clade I a
Lac7/BR/RJ/2015	.	**M**	.	.	**R**	.	.	Sub-Clade I a
Lac4/BR/RJ/2016	.	**M**	.	.	**R**	.	.	Sub-Clade I a
Lac8/BR/RJ/2016	.	**M**	.	.	**R**	.	.	Sub-Clade I a
VAOR28/BR/MS/2016	.	**M**	.	.	**R**	.	.	Sub-Clade I a
297/2017/BR/CE/2014	**A**#	**M**	.	.	.	.	.	Sub-Clade I b
67/2015/BR/AP/2015	**A**#	**M**	.	.	.	.	.	Sub-Clade I b
92/2013/BR/RJ/2013	.	**M**	**L**	.	.	**L**	**I**	Clade 2
7276/2013/BR/MS/2013	.	**M**	**L**	.	.	**L**	**I**	Clade 2
7436/2013/BR/MS/2013	.	**M**	**L**	.	.	**L**	**I**	Clade 2
134/2015/BR/RJ/2015	.	**M**	**L**	.	.	**L**	**I**	Clade 2
2072/2015/BR/RJ/2015	.	**M**	**L**	.	.	**L**	**I**	Clade 2
3171/2015/BR/RJ/2015	.	**M**	**L**	.	.	**L**	**I**	Clade 2
KCMM25/BR/MS/2016	.	**M**	**L**	.	.	**L**	**I**	Clade 2
Lac9/BR/RJ/2016	.	**M**	**L**	.	.	**L**	**I**	Clade 2
283/2017/BR/CE/2014	.	**M**	**L**	**R**	.	**L**	**I**	Clade 2
288/2017/BR/CE/2014	.	**M**	**L**	**R**	.	**L**	**I**	Clade 2
478/2014/BR/RJ/2014	.	**M**	**L**	**R**	.	**L**	**I**	Clade 2
494/2014/BR/SE/2014	.	**M**	**L**	**R**	.	**L**	**I**	Clade 2
30/2015/BR/RJ/2015	.	**M**	**L**	**R**	.	**L**	**I**	Clade 2
738/2015/BR/RJ/2015	.	**M**	**L**	**R**	.	**L**	**I**	Clade 2

T: threonine; S: serine; K: lysine; V: valine; A: alanine; M: methionine; L: leucine; R: arginine; I: isoleucine. *Classified according de Bruycker-Nogueira et al., 2016. #In these samples and one reference of the Argentina (KX768377). In others sequences of this clade the amino acid found is Threonine (T).

346 Figures legends

347

348 Figure 1. ML tree of E gene sequences of DENV-1 Genotype V. The aLRT support
349 values of major branching are shown. (A) ML tree of 751 E gene sequences. (B) ML
350 tree of 240 E gene sequences of Clade I.

351

352 Figure 2. Time-scaled Bayesian Maximum Clade Credibility tree for the Sub-Clade Ia
353 and Ib of E gene DENV-1 Genotype V. (A) Sub-Clade Ia (n= 106 sequences). (B) Sub-
354 Clade Ib (n= 56 sequences). Branches are colored according to the most probable
355 location (legend shown on the left side) of their parental node inferred by discrete
356 phylogeographical analysis. Posterior probability - *PP* values in [ ] and T_{MRCA} (95%
357 HPD) are represented in node the of branches. All horizontal branch lengths are drawn
358 to a scale of years. The tree is automatically rooted under the assumption of a relaxed
359 molecular clock. AR: Argentina; CO: Colombia; GY; French Guyana; VE: Venezuela;
360 CN: China; BRN: North of Brazil; BRNE: Northeast of Brazil; BRSE: Southeast of
361 Brazil; BRCO: Central-Western of Brazil. Viral dispersal pattern are showed in map.
362 Lines between locations represent branches in the Bayesian MCC tree along which
363 location transitions occurs. Star symbol: samples this study.

364

365 Figure 3. Time-scaled Bayesian Maximum Clade Credibility tree for the 116 sequences
366 of Clade II of E gene DENV-1 Genotype V. Branches are colored according to the most
367 probable location (legend shown on the left side) of their parental node inferred by
368 discrete phylogeographical analysis. Posterior probability - *PP* values in [ ] and T_{MRCA}
369 (95% HPD) are represented in node the of branches. All horizontal branch lengths are

370 drawn to a scale of years. The tree is automatically rooted under the assumption of a
371 relaxed molecular clock. AR: Argentina; GA (PR): Greater Antilles - Puerto Rico; LA
372 (VG): Lesser Antilles - Virgins Island; BRN: North of Brazil; BRNE: Northeastern of
373 Brazil; BRSE: Southeast of Brazil; BRCO: Central-Western of Brazil. Viral dispersal
374 pattern are showed in map. Lines between locations represent branches in the Bayesian
375 MCC tree along which location transitions occur. Star symbol: samples this study.
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418 *chikungunya e febre pelo vírus Zika até a Semana Epidemiológica 16,*
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435 history and spatiotemporal dynamics of DENV-1 genotype V in the Americas.
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442 circulation of two distinct lineages in 2013 and multiple introductions of dengue
443 virus in Goiás, Brazil. *Infect Genet Evol* **43**, 130-134,
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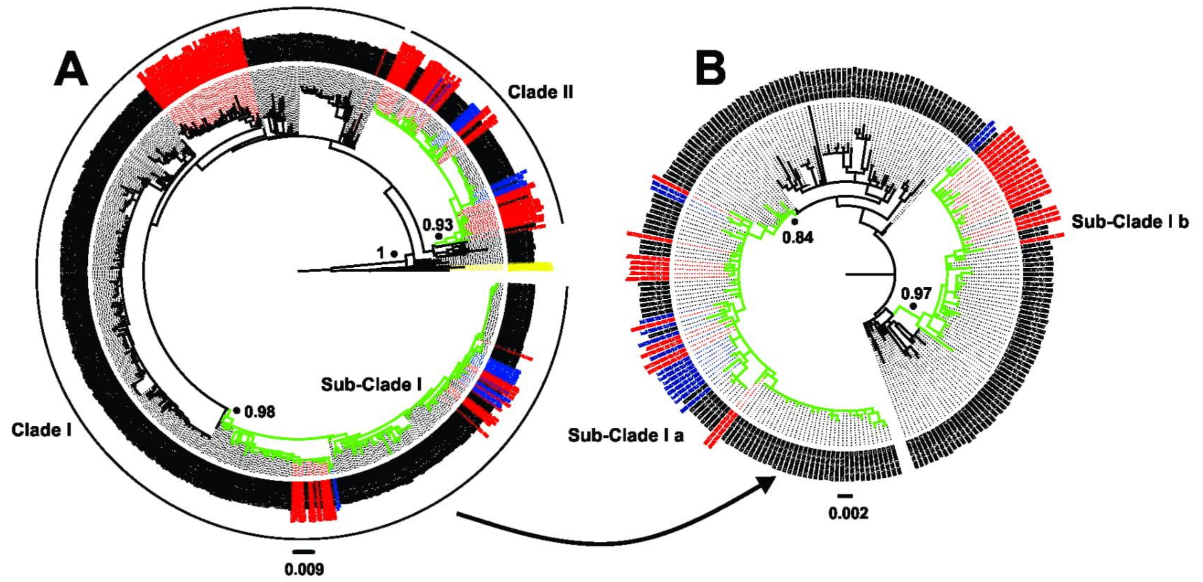
485 doi:10.1371/journal.pcbi.1000520 (2009).

486

487

488


Identification
Branches with BR current sequences
Sequences BR
Sequences BR of this study (2012-2016)
Outgroup (Genotype I, II, III and IV)
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Clade II
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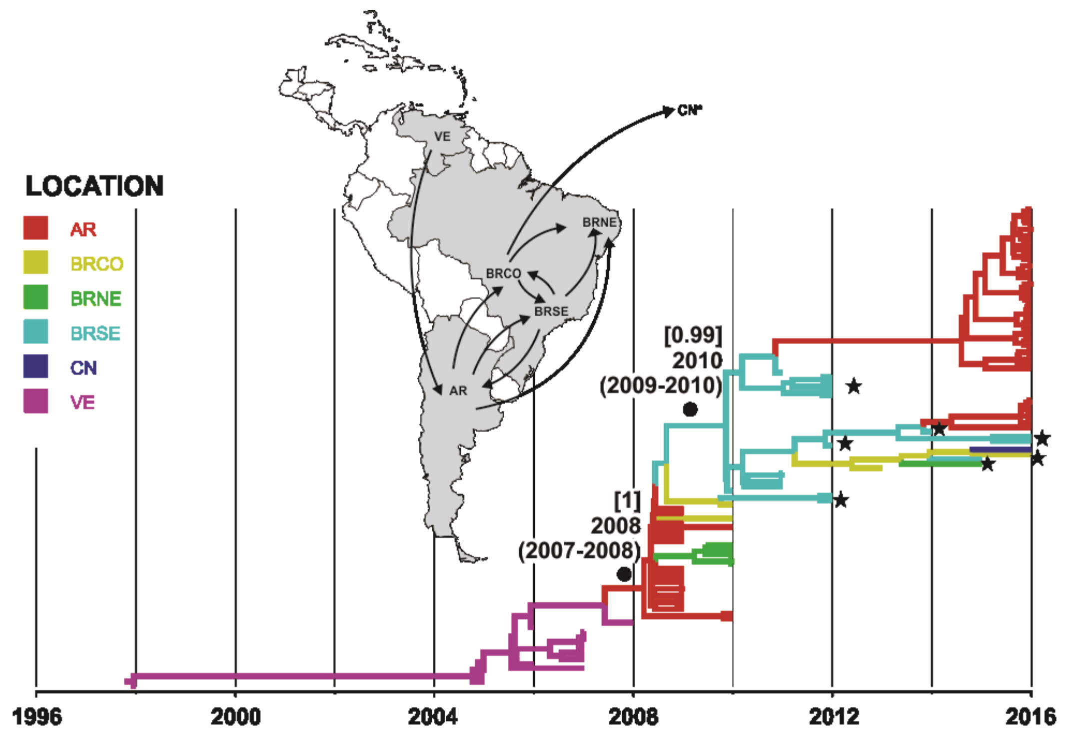
1
Sub-Clade I
0.98
Clade I
0.009
B
0.84
Sub-Clade I b
0.97
Sub-Clade I a
0.002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BRSE
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(2009-2010)
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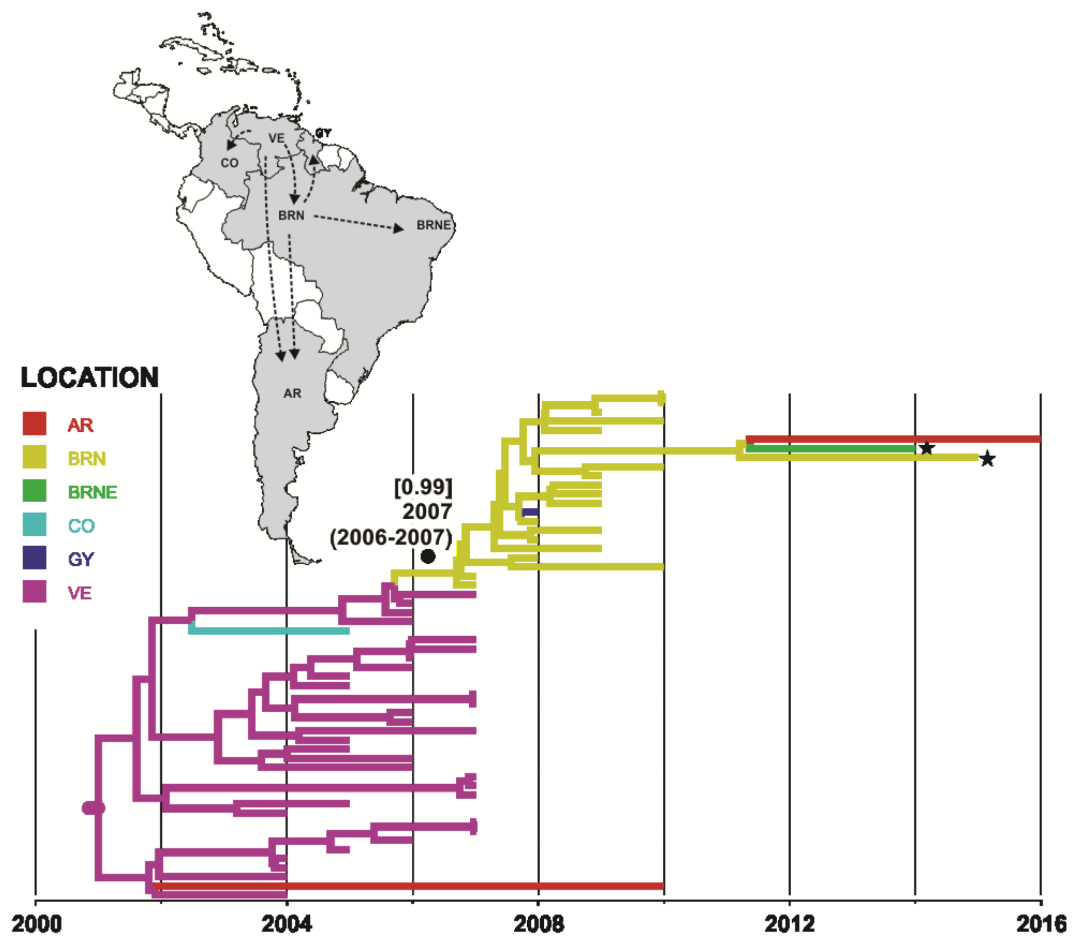
2008
(2007-2008)
1996
2000
2004
2008
2012
2016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(2006-2007)
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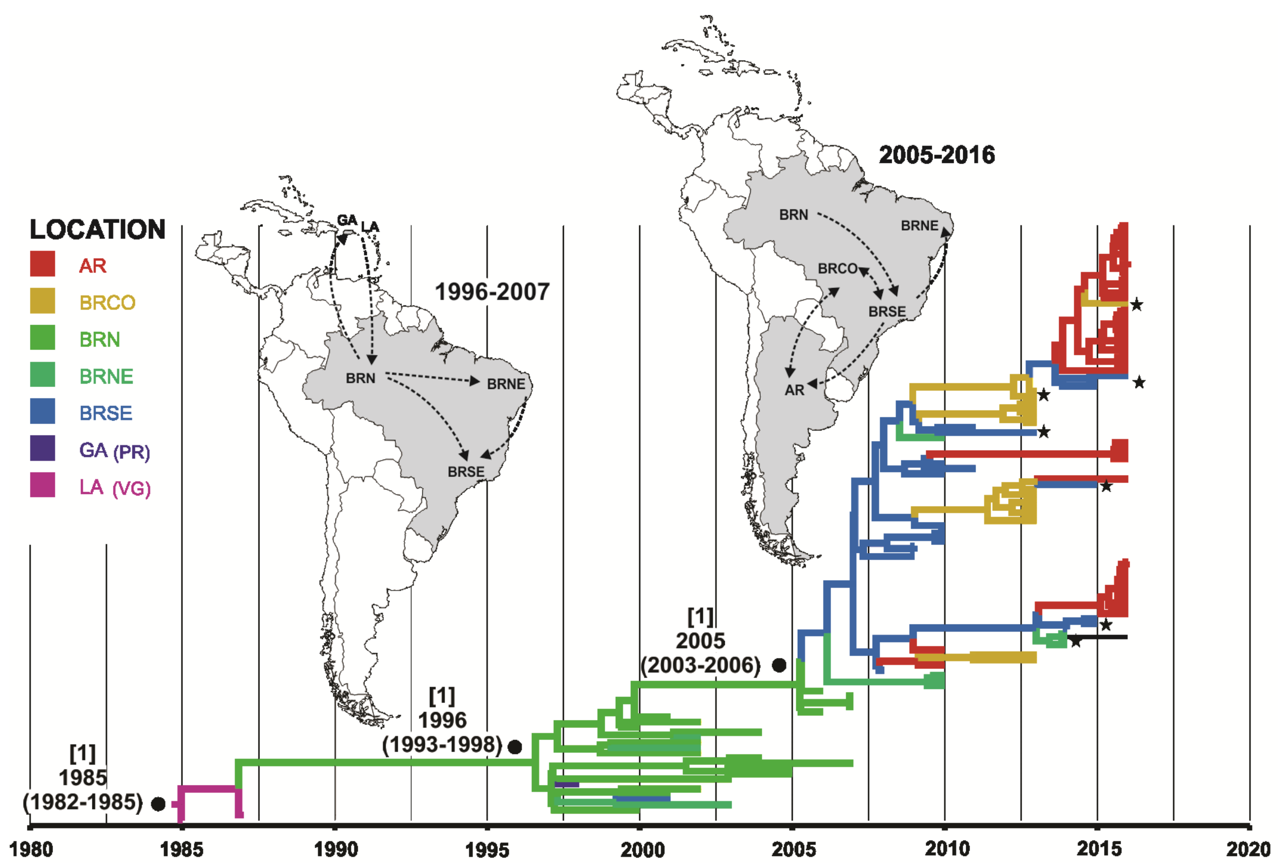
2012
2016



LOCATION
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BRN
BRNE
BRSE
GA (PR)
LA (VG)
1996-2007
2005-2016
GA
LA
BRN
BRNE
BRSE
BRCO
AR
[1]
1985
(1982-1985)
[1]
1996
(1993-1998)
[1]
2005
(2003-2006)
1980
1985
1990
1995
2000
2005
2010
2015
2020

4.3.1 Dados suplementares relacionados ao artigo 3

Table S1. Sequences of the DENV-1 Genotype V (clade cosmopolitan) dataset.

	ID	Genbank	Country	Year
1.	AF514889/AR/2000	AF514889	Argentina	2000
2.	AY206457/AR/2000	AY206457	Argentina	2000
3.	AF514885/AR/2000	AF514885	Argentina	2000
4.	AF514876/AR/2000	AF514876	Argentina	2000
5.	KC692510/AR/2009	KC692510	Argentina	2009
6.	KC692497/AR/2009	KC692497	Argentina	2009
7.	KC692502/AR/2009	KC692502	Argentina	2009
8.	KC692509/AR/2009	KC692509	Argentina	2009
9.	KC692506/AR/2009	KC692506	Argentina	2009
10.	KC692501/AR/2009	KC692501	Argentina	2009
11.	KC692508/AR/2009	KC692508	Argentina	2009
12.	KC692499/AR/2009	KC692499	Argentina	2009
13.	KC692500/AR/2009	KC692500	Argentina	2009
14.	KC692503/AR/2009	KC692503	Argentina	2009
15.	KC692495/AR/2009	KC692495	Argentina	2009
16.	KC692496/AR/2009	KC692496	Argentina	2009
17.	KC692505/AR/2009	KC692505	Argentina	2009
18.	KC692504/AR/2009	KC692504	Argentina	2009
19.	KC692507/AR/2009	KC692507	Argentina	2009
20.	KC692498/AR/2009	KC692498	Argentina	2009
21.	KC692511/AR/2010	KC692511	Argentina	2010
22.	KC692513/AR/2010	KC692513	Argentina	2010
23.	KC692517/AR/2010	KC692517	Argentina	2010
24.	KC692516/AR/2010	KC692516	Argentina	2010
25.	KC692514/AR/2010	KC692514	Argentina	2010
26.	KC692512/AR/2010	KC692512	Argentina	2010
27.	KC692515/AR/2010	KC692515	Argentina	2010
28.	KX768341/AR/2016	KX768341	Argentina	2016
29.	KX768342/AR/2016	KX768342	Argentina	2016
30.	KX768347/AR/2016	KX768347	Argentina	2016
31.	KX768348/AR/2016	KX768348	Argentina	2016
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33.	KX768343/AR/2016	KX768343	Argentina	2016
34.	KX768349/AR/2016	KX768349	Argentina	2016
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38.	KX768345/AR/2016	KX768345	Argentina	2016
39.	KX768354/AR/2016	KX768354	Argentina	2016
40.	KX768353/AR/2016	KX768353	Argentina	2016
41.	KX768352/AR/2016	KX768352	Argentina	2016
42.	KX768359/AR/2016	KX768359	Argentina	2016
43.	Y283848/AR/2016	Y283848	Argentina	2016
44.	KX768344/AR/2016	KX768344	Argentina	2016
45.	KX768350/AR/2016	KX768350	Argentina	2016
46.	KX768356/AR/2016	KX768356	Argentina	2016
47.	KX768357/AR/2016	KX768357	Argentina	2016
48.	Y283851/AR/2016	Y283851	Argentina	2016
49.	KX768355/AR/2016	KX768355	Argentina	2016
50.	KX768362/AR/2016	KX768362	Argentina	2016
51.	KX768360/AR/2016	KX768360	Argentina	2016
52.	KX768361/AR/2016	KX768361	Argentina	2016
53.	KX768339/AR/2016	KX768339	Argentina	2016
54.	Y283852/AR/2016	Y283852	Argentina	2016
55.	KX768340/AR/2016	KX768340	Argentina	2016
56.	KX768338/AR/2016	KX768338	Argentina	2016
57.	KX768364/AR/2016	KX768364	Argentina	2016
58.	KX768366/AR/2016	KX768366	Argentina	2016

59.	KX768363/AR/2016	KX768363	Argentina	2016
60.	KX768365/AR/2016	KX768365	Argentina	2016
61.	KX768375/AR/2016	KX768375	Argentina	2016
62.	KX768376/AR/2016	KX768376	Argentina	2016
63.	KX768370/AR/2016	KX768370	Argentina	2016
64.	KX768367/AR/2016	KX768367	Argentina	2016
65.	KY283849/AR/2016	KY283849	Argentina	2016
66.	KX768372/AR/2016	KX768372	Argentina	2016
67.	KX768369/AR/2016	KX768369	Argentina	2016
68.	KX768368/AR/2016	KX768368	Argentina	2016
69.	KX768373/AR/2016	KX768373	Argentina	2016
70.	KX768374/AR/2016	KX768374	Argentina	2016
71.	KX768371/AR/2016	KX768371	Argentina	2016
72.	KX768377/AR/2016	KX768377	Argentina	2016
73.	KX768380/AR/2016	KX768380	Argentina	2016
74.	KX768385/AR/2016	KX768385	Argentina	2016
75.	KX768384/AR/2016	KX768384	Argentina	2016
76.	KX768382/AR/2016	KX768382	Argentina	2016
77.	KX768381/AR/2016	KX768381	Argentina	2016
78.	KY283853/AR/2016	KY283853	Argentina	2016
79.	KX768383/AR/2016	KX768383	Argentina	2016
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81.	KX768410/AR/2016	KX768410	Argentina	2016
82.	KX768409/AR/2016	KX768409	Argentina	2016
83.	KX768378/AR/2016	KX768378	Argentina	2016
84.	KX768416/AR/2016	KX768416	Argentina	2016
85.	KX768407/AR/2016	KX768407	Argentina	2016
86.	KX768417/AR/2016	KX768417	Argentina	2016
87.	KX768399/AR/2016	KX768399	Argentina	2016
88.	KX768402/AR/2016	KX768402	Argentina	2016
89.	KX768412/AR/2016	KX768412	Argentina	2016
90.	KX768415/AR/2016	KX768415	Argentina	2016
91.	KX768419/AR/2016	KX768419	Argentina	2016
92.	KX768406/AR/2016	KX768406	Argentina	2016
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98.	KX768418/AR/2016	KX768418	Argentina	2016
99.	KX768389/AR/2016	KX768389	Argentina	2016
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107.	KX768398/AR/2016	KX768398	Argentina	2016
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119.	JN379486/BB/1995	JN379486	Barbados	1995
120.	JN379487/BB/1999	JN379487	Barbados	1999
121.	JN379470/BB/2001	JN379470	Barbados	2001
122.	JN379471/BB/2001	JN379471	Barbados	2001
123.	JN379472/BB/2003	JN379472	Barbados	2003
124.	JN379474/BZ/2005	JN379474	Belize	2005
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177.	JX669474/BRNE/PE/2001	JX669474	Brazil	2001
178.	AF513110/BRS/PR/2001	AF513110	Brazil	2001
179.	JX669473/BRNE/PE/2001	JX669473	Brazil	2001
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183.	FJ850075/BRN/BR/2002	FJ850075	Brazil	2002
184.	HM450093/BRNE/BR/PI/2002	HM450093	Brazil	2002
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190.	HM450091/BRCO/BR/MT/2002	HM450091	Brazil	2002
191.	HM450090/BRCO/BR/MT/2002	HM450090	Brazil	2002
192.	FJ850077/BRN/BR/2003	FJ850077	Brazil	2003
193.	HM450096/BRNE/BR/MA/2003	HM450096	Brazil	2003
194.	HM450097/BRN/BR/PA/2004	HM450097	Brazil	2004
195.	FJ850081/BRN/BR/2004	FJ850081	Brazil	2004
196.	HM450099/BRN/BR/AP/2005	HM450099	Brazil	2005
197.	FJ850084/BRN/BR/2005	FJ850084	Brazil	2005
198.	FJ850087/BRN/BR/2006	FJ850087	Brazil	2006
199.	HM450100/BRN/BR/PA/2006	HM450100	Brazil	2006
200.	FJ850090/BRN/BR/2007	FJ850090	Brazil	2007
201.	HM450101/BRN/BR/PA/2007	HM450101	Brazil	2007
202.	HM450077/BRN/BR/PA/2007	HM450077	Brazil	2007
203.	HM450078/BRN/BR/PA/2007	HM450078	Brazil	2007
204.	HM450102/BRN/BR/RR/2007	HM450102	Brazil	2007
205.	HM450103/BRN/BR/AM/2007	HM450103	Brazil	2007
206.	GU131863/BRSE/BR/SP/2008	GU131863	Brazil	2008
207.	KF444789/BRN/BR/RR/2008	KF444789	Brazil	2008
208.	FJ850093/BRN/BR/2008	FJ850093	Brazil	2008
209.	HM450104/BRN/BR/RR/2008	HM450104	Brazil	2008
210.	HM043709/BRSE/BR/ES/2009	HM043709	Brazil	2009
211.	HQ026761/BRSE/BR/RJ/2009	HQ026761	Brazil	2009
212.	HM043710/BRSE/BR/RJ/2009	HM043710	Brazil	2009
213.	KF444783/BRN/BR/RR/2009	KF444783	Brazil	2009
214.	KF444782/BRN/BR/RR/2009	KF444782	Brazil	2009
215.	KF444791/BRN/BR/RR/2009	KF444791	Brazil	2009
216.	KF444781/BRN/BR/RR/2009	KF444781	Brazil	2009
217.	KF444784/BRN/BR/RR/2009	KF444784	Brazil	2009
218.	KF444785/BRN/BR/RR/2009	KF444785	Brazil	2009
219.	KF444780/BRN/BR/RR/2009	KF444780	Brazil	2009
220.	KF444790/BRN/BR/RR/2009	KF444790	Brazil	2009
221.	JX669464/BRNE/BR/2010	JX669464	Brazil	2010
222.	JX669461/BRNE/BR/2010	JX669461	Brazil	2010
223.	JX669465/BRNE/BR/2010	JX669465	Brazil	2010
224.	JN982362/BRNE/BR/CE/2010	JN982362	Brazil	2010
225.	KF672759/BRSE/BR/RJ/2010	KF672759	Brazil	2010
226.	HQ026762/BRSE/BR/RJ/2010	HQ026762	Brazil	2010
227.	HQ696614/BRSE/BR/RJ/2010	HQ696614	Brazil	2010
228.	HQ696613/BRSE/BR/RJ/2010	HQ696613	Brazil	2010
229.	KF719187/BRSE/BR/ES/2010	KF719187	Brazil	2010
230.	KF672785/BRSE/BR/ES/2010	KF672785	Brazil	2010
231.	KF444787/BRN/BR/RR/2010	KF444787	Brazil	2010
232.	KF444788/BRN/BR/RR/2010	KF444788	Brazil	2010

233.	KF444786/BRN/BR/RR/2010	KF444786	Brazil	2010
234.	JN713897/BRN/BR/RR/2010	JN713897	Brazil	2010
235.	KF444792/BRN/BR/RR/2010	KF444792	Brazil	2010
236.	JQ015185/BRNE/BR/AL/2010	JQ015185	Brazil	2010
237.	JQ015184/BRNE/BR/AL/2010	JQ015184	Brazil	2010
238.	JX669463/BRNE/PE/2010	JX669463	Brazil	2010
239.	JX669466/BRNE/PE/2010	JX669466	Brazil	2010
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241.	HQ696612/BRCO/MS/2010	HQ696612	Brazil	2010
242.	KF672768/BRCO/BR/MS/2010	KF672768	Brazil	2010
243.	KF672786/BRSE/BR/RJ/2010	KF672786	Brazil	2010
244.	KF672784/BRSE/BR/RJ/2011	KF672784	Brazil	2011
245.	KF672767/BRSE/BR/RJ/2011	KF672767	Brazil	2011
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247.	KF672760/BRSE/BR/RJ/2011	KF672760	Brazil	2011
248.	JN122281/BRSE/RJ/2011	JN122281	Brazil	2011
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250.	KF672766/BRSE/BR/RJ/2011	KF672766	Brazil	2011
251.	2071/BRSE/BR/RJ/2012	MH401971	Brazil	2012
252.	3599/BRSE/BR/RJ/2012	MH311981	Brazil	2012
253.	2612/BRSE/BR/RJ/2012	2612/BRS	Brazil	2012
254.	Lac1/BRSE/BR/RJ/2012	MH401990	Brazil	2012
255.	3239/BRSE/BR/RJ/2012	MH401973	Brazil	2012
256.	3246/BRSE/BR/RJ/2012	MH401974	Brazil	2012
257.	Lac16/BRSE/BR/RJ/2012	MH401992	Brazil	2012
258.	Lac5/BRSE/BR/RJ/2012	MH401991	Brazil	2012
259.	7436/BRCO/BR/MS/2013	MH401976	Brazil	2013
260.	7276/BRCO/BR/MS/2013	MH401975	Brazil	2013
261.	92/BRSE/BR/RJ/2013	MH401977	Brazil	2013
262.	KP858112/BRCO/BR/GO/2013	KP858112	Brazil	2013
263.	KP858113/BRCO/BR/GO/2013	KP858113	Brazil	2013
264.	KP858117/BRCO/BR/GO/2013	KP858117	Brazil	2013
265.	KP858109/BRCO/BR/GO/2013	KP858109	Brazil	2013
266.	KP858115/BRCO/BR/GO/2013	KP858115	Brazil	2013
267.	KP858111/BRCO/BR/GO/2013	KP858111	Brazil	2013
268.	KP858118/BRCO/BR/GO/2013	KP858118	Brazil	2013
269.	KP858110/BRCO/BR/GO/2013	KP858110	Brazil	2013
270.	KP858105/BRCO/BR/GO/2013	KP858105	Brazil	2013
271.	KP858106/BRCO/BR/GO/2013	KP858106	Brazil	2013
272.	KP858108/BRCO/BR/GO/2013	KP858108	Brazil	2013
273.	KP858116/BRCO/BR/GO/2013	KP858116	Brazil	2013
274.	KP858119/BRCO/BR/GO/2013	KP858119	Brazil	2013
275.	KP858114/BRCO/BR/GO/2013	KP858114	Brazil	2013
276.	KP858107/BRCO/BR/GO/2013	KP858107	Brazil	2013
277.	283/BRNE/BR/CE/2014	MH401987	Brazil	2014
278.	288/BRNE/BR/CE/2014	MH401988	Brazil	2014
279.	478/BRSE/BR/RJ/2014	MH401979	Brazil	2014
280.	494/BRNE/BR/SE/2014	MH401980	Brazil	2014
281.	297/BRNE/BR/CE/2014	297/BRNE	Brazil	2014
282.	05/BRSE/BR/RJ/2014	MH401978	Brazil	2014
283.	2072/BRSE/BR/RJ/2015	MH401984	Brazil	2015
284.	3171/BRSE/BR/RJ/2015	MH401985	Brazil	2015
285.	134/BRSE/BR/RJ/2015	MH401982	Brazil	2015
286.	30/BRSE/BR/RJ/2015	MH401981	Brazil	2015
287.	738/BRSE/BR/RJ/2015	MH401983	Brazil	2015
288.	67/BRN/BR/AP/2015	67/BRN/B	Brazil	2015
289.	148/BRNE/BR/SE/2015	MH401986	Brazil	2015

290.	Lac7/BRSE/BR/RJ/2015	MH401993	Brazil	2015
291.	Lac9/BRSE/BR/RJ/2016	MH401996	Brazil	2016
292.	KCMM25/BRCO/BR/MS/2016	MH401998	Brazil	2016
293.	Lac4/BRSE/BR/RJ/2016	MH401994	Brazil	2016
294.	Lac8/BRSE/BR/RJ/2016	MH401995	Brazil	2016
295.	VAOR28/BRCO/BR/MS/2016	MH401999	Brazil	2016
296.	GQ868601/VG/1985	GQ868601	British Virgin Islands	1985
297.	JF804023/VG/1987	JF804023	British Virgin Islands	1987
298.	KX372687/CN/2016	KX372687	China	2016
299.	AF425616/CO/1985	AF425616	Colombia	1985
300.	AF425617/CO/1996	AF425617	Colombia	1996
301.	KJ189302/CO/1998	KJ189302	Colombia	1998
302.	GQ868560/CO/1998	GQ868560	Colombia	1998
303.	GQ868559/CO/1998	GQ868559	Colombia	1998
304.	KJ189303/CO/1998	KJ189303	Colombia	1998
305.	GQ868561/CO/1999	GQ868561	Colombia	1999
306.	GU131948/CO/2001	GU131948	Colombia	2001
307.	KJ189304/CO/2005	KJ189304	Colombia	2005
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309.	GQ868564/CO/2006	GQ868564	Colombia	2006
310.	GQ868563/CO/2006	GQ868563	Colombia	2006
311.	GQ868565/CO/2006	GQ868565	Colombia	2006
312.	GU131949/CO/2006	GU131949	Colombia	2006
313.	GQ868568/CO/2007	GQ868568	Colombia	2007
314.	GQ868567/CO/2007	GQ868567	Colombia	2007
315.	GQ868569/CO/2007	GQ868569	Colombia	2007
316.	GQ868566/CO/2007	GQ868566	Colombia	2007
317.	JF804015/CO/2007	JF804015	Colombia	2007
318.	GQ868570/CO/2008	GQ868570	Colombia	2008
319.	AY153755/CR/1993	AY153755	Costa Rica	1993
320.	AY153756/CR/1993	AY153756	Costa Rica	1993
321.	AY153757/CR/1993	AY153757	Costa Rica	1993
322.	JF804016/CR/2005	JF804016	Costa Rica	2005
323.	JF804017/DO/2007	JF804017	Dominican Republic	2007
324.	JN819417/SV/1993	JN819417	El Salvador	1993
325.	EU448414/SV/2006	EU448414	El Salvador	2006
326.	JX891659/SV/2012	JX891659	El Salvador	2012
327.	JX891661/SV/2012	JX891661	El Salvador	2012
328.	JX891660/SV/2012	JX891660	El Salvador	2012
329.	AF226687/GF/1989	AF226687	French Guiana	1989
330.	JN379480/GD/1977	JN379480	Granada	1977
331.	AF425618/GD/1977	AF425618	Granada	1977
332.	JN379476/GD/1977	JN379476	Granada	1977
333.	JN379479/GD/1977	JN379479	Granada	1977
334.	JN379478/GD/1977	JN379478	Granada	1977
335.	JN379477/GD/1977	JN379477	Granada	1977
336.	JN379482/GD/1978	JN379482	Granada	1978
337.	JN379481/GD/1978	JN379481	Granada	1978
338.	JN379484/GD/1981	JN379484	Granada	1981
339.	JN415506/GY/2008	JN415506	Guyana	2008
340.	JF969282/HT/2010	JF969282	Haiti	2010
341.	JF969280/HT/2010	JF969280	Haiti	2010
342.	JF969281/HT/2010	JF969281	Haiti	2010
343.	JF969283/HT/2010	JF969283	Haiti	2010
344.	JF969284/HT/2010	JF969284	Haiti	2010
345.	JF967804/HN/2008	JF967804	Honduras	2008
346.	JF297572/IN/1962	JF297572	India	1962
347.	JF297573/IN/1962	JF297573	India	1962
348.	JF297576/IN/1963	JF297576	India	1963
349.	JQ922544/IN/1963	JQ922544	India	1963

350.	JF297577/IN/1963	JF297577	India	1963
351.	JF297574/IN/1963	JF297574	India	1963
352.	JF297575/IN/1963	JF297575	India	1963
353.	DQ016653/IN/2003	DQ016653	India	2003
354.	DQ016654/IN/2003	DQ016654	India	2003
355.	DQ016655/IN/2003	DQ016655	India	2003
356.	JF297583/IN/2005	JF297583	India	2005
357.	EU448413/IN/2006	EU448413	India	2006
358.	JN903578/IN/2007	JN903578	India	2007
359.	AF425621/JM/1977	AF425621	Jamaica	1977
360.	JF804018/MQ/1989	JF804018	Martinique	1989
361.	JN022597/MQ/2008	JN022597	Martinique	2008
362.	JN022598/MQ/2008	JN022598	Martinique	2008
363.	JN022599/MQ/2008	JN022599	Martinique	2008
364.	AF425623/MX/1980	AF425623	Mexico	1980
365.	DQ341188/MX/1982	DQ341188	Mexico	1982
366.	AF425624/MX/1983	AF425624	Mexico	1983
367.	DQ341189/MX/1984	DQ341189	Mexico	1984
368.	DQ341190/MX/1984	DQ341190	Mexico	1984
369.	DQ341191/MX/1986	DQ341191	Mexico	1986
370.	DQ341192/MX/1994	DQ341192	Mexico	1994
371.	DQ341193/MX/1995	DQ341193	Mexico	1995
372.	DQ341194/MX/1995	DQ341194	Mexico	1995
373.	GU131958/MX/2006	GU131958	Mexico	2006
374.	GQ868499/MX/2006	GQ868499	Mexico	2006
375.	KF955415/MX/2006	KF955415	Mexico	2006
376.	HM171568/MX/2006	HM171568	Mexico	2006
377.	HM171570/MX/2006	HM171570	Mexico	2006
378.	KF955417/MX/2006	KF955417	Mexico	2006
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380.	HM171567/MX/2006	HM171567	Mexico	2006
381.	HM171566/MX/2006	HM171566	Mexico	2006
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385.	HM171558/MX/2006	HM171558	Mexico	2006
386.	GQ868498/MX/2006	GQ868498	Mexico	2006
387.	GU131960/MX/2007	GU131960	Mexico	2007
388.	KJ189321/MX/2007	KJ189321	Mexico	2007
389.	GU131966/MX/2007	GU131966	Mexico	2007
390.	HM171565/MX/2007	HM171565	Mexico	2007
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392.	HM171561/MX/2007	HM171561	Mexico	2007
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395.	GQ868503/MX/2007	GQ868503	Mexico	2007
396.	KF955443/MX/2007	KF955443	Mexico	2007
397.	GQ868509/MX/2007	GQ868509	Mexico	2007
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408.	GQ868505/MX/2007	GQ868505	Mexico	2007
409.	GQ868502/MX/2007	GQ868502	Mexico	2007

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459.	KJ189342/MX/2009	KJ189342	Mexico	2009
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474.	JQ920429/MX/2010	JQ920429	Mexico	2010
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476.	JQ065922/MX/2010	JQ065922	Mexico	2010
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518.	GQ199873/NI/2004	GQ199873	Nicaragua	2004
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523.	KF955403/NI/2005	KF955403	Nicaragua	2005
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525.	EU482617/NI/2005	EU482617	Nicaragua	2005
526.	EU596502/NI/2005	EU596502	Nicaragua	2005
527.	FJ024478/NI/2005	FJ024478	Nicaragua	2005
528.	EU482619/NI/2005	EU482619	Nicaragua	2005
529.	FJ547089/NI/2005	FJ547089	Nicaragua	2005

530.	FJ850114/NI/2005	FJ850114	Nicaragua	2005
531.	KF955409/NI/2005	KF955409	Nicaragua	2005
532.	FJ432721/NI/2005	FJ432721	Nicaragua	2005
533.	FJ432720/NI/2005	FJ432720	Nicaragua	2005
534.	FJ850113/NI/2005	FJ850113	Nicaragua	2005
535.	JN819402/NI/2005	JN819402	Nicaragua	2005
536.	EU482615/NI/2005	EU482615	Nicaragua	2005
537.	FJ024485/NI/2005	FJ024485	Nicaragua	2005
538.	FJ024480/NI/2005	FJ024480	Nicaragua	2005
539.	EU482618/NI/2005	EU482618	Nicaragua	2005
540.	FJ182002/NI/2005	FJ182002	Nicaragua	2005
541.	KF955404/NI/2005	KF955404	Nicaragua	2005
542.	EU596503/NI/2005	EU596503	Nicaragua	2005
543.	EU596504/NI/2005	EU596504	Nicaragua	2005
544.	FJ024481/NI/2005	FJ024481	Nicaragua	2005
545.	EU482616/NI/2005	EU482616	Nicaragua	2005
546.	FJ024484/NI/2005	FJ024484	Nicaragua	2005
547.	FJ873814/NI/2005	FJ873814	Nicaragua	2005
548.	FJ024423/NI/2005	FJ024423	Nicaragua	2005
549.	FJ562104/NI/2006	FJ562104	Nicaragua	2006
550.	JN819403/NI/2006	JN819403	Nicaragua	2006
551.	FJ024479/NI/2006	FJ024479	Nicaragua	2006
552.	FJ547068/NI/2006	FJ547068	Nicaragua	2006
553.	FJ810419/NI/2006	FJ810419	Nicaragua	2006
554.	FJ898433/NI/2007	FJ898433	Nicaragua	2007
555.	KF955410/NI/2008	KF955410	Nicaragua	2008
556.	GQ199859/NI/2008	GQ199859	Nicaragua	2008
557.	GQ199858/NI/2008	GQ199858	Nicaragua	2008
558.	GQ199857/NI/2008	GQ199857	Nicaragua	2008
559.	FJ547088/NI/2008	FJ547088	Nicaragua	2008
560.	JF937635/NI/2009	JF937635	Nicaragua	2009
561.	JQ287666/NI/2009	JQ287666	Nicaragua	2009
562.	JF937645/NI/2009	JF937645	Nicaragua	2009
563.	JF937644/NI/2009	JF937644	Nicaragua	2009
564.	AY277664/PY/1999	AY277664	Paraguay	1999
565.	AF514878/PY/2000	AF514878	Paraguay	2000
566.	AY277659/PY/2000	AY277659	Paraguay	2000
567.	AY277666/PY/2000	AY277666	Paraguay	2000
568.	AF514883/PY/2000	AF514883	Paraguay	2000
569.	AF425626/PE/1991	AF425626	Peru	1991
570.	FJ562106/PR/1986	FJ562106	Puerto Rico	1986
571.	FJ410190/PR/1987	FJ410190	Puerto Rico	1987
572.	FJ478458/PR/1987	FJ478458	Puerto Rico	1987
573.	FJ410187/PR/1992	FJ410187	Puerto Rico	1992
574.	FJ410186/PR/1992	FJ410186	Puerto Rico	1992
575.	FJ547087/PR/1992	FJ547087	Puerto Rico	1992
576.	FJ410184/PR/1993	FJ410184	Puerto Rico	1993
577.	FJ410183/PR/1993	FJ410183	Puerto Rico	1993
578.	FJ410185/PR/1993	FJ410185	Puerto Rico	1993
579.	FJ562105/PR/1993	FJ562105	Puerto Rico	1993
580.	FJ410175/PR/1994	FJ410175	Puerto Rico	1994
581.	FJ410179/PR/1994	FJ410179	Puerto Rico	1994
582.	FJ205875/PR/1995	FJ205875	Puerto Rico	1995
583.	FJ410181/PR/1995	FJ410181	Puerto Rico	1995
584.	KF955439/PR/1995	KF955439	Puerto Rico	1995
585.	FJ410180/PR/1995	FJ410180	Puerto Rico	1995
586.	FJ410174/PR/1995	FJ410174	Puerto Rico	1995
587.	FJ390374/PR/1995	FJ390374	Puerto Rico	1995
588.	FJ205874/PR/1995	FJ205874	Puerto Rico	1995
589.	FJ547086/PR/1995	FJ547086	Puerto Rico	1995

590.	KF921911/PR/1996	KF921911	Puerto Rico	1996
591.	FJ410182/PR/1996	FJ410182	Puerto Rico	1996
592.	FJ410189/PR/1996	FJ410189	Puerto Rico	1996
593.	FJ478457/PR/1996	FJ478457	Puerto Rico	1996
594.	FJ410188/PR/1996	FJ410188	Puerto Rico	1996
595.	JF804021/PR/1998	JF804021	Puerto Rico	1998
596.	KF955438/PR/1998	KF955438	Puerto Rico	1998
597.	EU482567/PR/1998	EU482567	Puerto Rico	1998
598.	FJ205873/PR/1998	FJ205873	Puerto Rico	1998
599.	FJ390380/PR/1998	FJ390380	Puerto Rico	1998
600.	KC812277/PR/1998	KC812277	Puerto Rico	1998
601.	EU482592/PR/1998	EU482592	Puerto Rico	1998
602.	KF955437/PR/1998	KF955437	Puerto Rico	1998
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604.	FJ205872/PR/1998	FJ205872	Puerto Rico	1998
605.	FJ410173/PR/1998	FJ410173	Puerto Rico	1998
606.	FJ390379/PR/1998	FJ390379	Puerto Rico	1998
607.	EU482591/PR/2006	EU482591	Puerto Rico	2006
608.	JF804022/PR/2007	JF804022	Puerto Rico	2007
609.;	KJ189363/PR/2010	KJ189363	Puerto Rico	2010
610.	KJ189360/PR/2010	KJ189360	Puerto Rico	2010
611.	KJ189361/PR/2010	KJ189361	Puerto Rico	2010
612.	KJ189367/PR/2010	KJ189367	Puerto Rico	2010
613.	KJ189365/PR/2010	KJ189365	Puerto Rico	2010
614.	KJ189366/PR/2010	KJ189366	Puerto Rico	2010
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616.	KJ189362/PR/2010	KJ189362	Puerto Rico	2010
617.	JX402207/PR/2010	JX402207	Puerto Rico	2010
618.	JX402211/PR/2010	JX402211	Puerto Rico	2010
619.	JX402209/PR/2010	JX402209	Puerto Rico	2010
620.	JX402208/PR/2010	JX402208	Puerto Rico	2010
621.	JX402210/PR/2010	JX402210	Puerto Rico	2010
622.	JX402212/PR/2010	JX402212	Puerto Rico	2010
623.	JX402213/PR/2010	JX402213	Puerto Rico	2010
624.	KJ189358/PR/2012	KJ189358	Puerto Rico	2012
625.	KJ189355/PR/2012	KJ189355	Puerto Rico	2012
626.	KJ189352/PR/2012	KJ189352	Puerto Rico	2012
627.	KJ189357/PR/2012	KJ189357	Puerto Rico	2012
628.	KJ189356/PR/2012	KJ189356	Puerto Rico	2012
629.	KJ189353/PR/2012	KJ189353	Puerto Rico	2012
630.	KJ189350/PR/2012	KJ189350	Puerto Rico	2012
631.	KJ189354/PR/2012	KJ189354	Puerto Rico	2012
632.	KJ189359/PR/2012	KJ189359	Puerto Rico	2012
633.	KJ189351/PR/2012	KJ189351	Puerto Rico	2012
634.	DQ285554/RE/2004	DQ285554	Reunion Island	2004
635.	DQ285559/RE/2004	DQ285559	Reunion Island	2004
636.	KY829115/SB/2016	KY829115	Saint Barthelemy	2016
637.	M87512/SG/1990	M87512	Singapore	1990
638.	EU081258/SG/2005	EU081258	Singapore	2005
639.	JN379485/SR/1981	JN379485	Suriname	1981
640.	AF425631/TT/1978	AF425631	Trinidad and Tobago	1978
641.	JN379483/TT/1981	JN379483	Trinidad and Tobago	1981
642.	AF425639/TT/1986	AF425639	Trinidad and Tobago	1986
643.	JQ425067/US/2009	JQ425067	United States	2009
644.	JQ425061/US/2009	JQ425061	United States	2009
645.	JQ425063/US/2009	JQ425063	United States	2009
646.	JQ425068/US/2009	JQ425068	United States	2009
647.	JQ425062/US/2009	JQ425062	United States	2009
648.	JQ425064/US/2009	JQ425064	United States	2009
649.	JQ425066/US/2009	JQ425066	United States	2009

650.	JQ425065/US/2009	JQ425065	United States	2009
651.	JQ045563/US/2010	JQ045563	United States	2010
652.	JQ045562/US/2010	JQ045562	United States	2010
653.	JQ045561/US/2010	JQ045561	United States	2010
654.	JQ675358/US/2010	JQ675358	United States	2010
655.	JQ045564/US/2010	JQ045564	United States	2010
656.	JF519855/US/2010	JF519855	United States	2010
657.	JQ425072/US/2010	JQ425072	United States	2010
658.	JQ425071/US/2010	JQ425071	United States	2010
659.	JQ425070/US/2010	JQ425070	United States	2010
660.	JQ425069/US/2010	JQ425069	United States	2010
661.	KJ415284/US/2013	KJ415284	United States	2013
662.	KM458188/US/2014	KM458188	United States	2014
663.	KM458186/US/2014	KM458186	United States	2014
664.	KM458189/US/2014	KM458189	United States	2014
665.	KM458187/US/2014	KM458187	United States	2014
666.	KM458190/US/2014	KM458190	United States	2014
667.	AF425636/VE/1994	AF425636	Venezuela	1994
668.	AF425637/VE/1994	AF425637	Venezuela	1994
669.	AF425632/VE/1995	AF425632	Venezuela	1995
670.	AF425638/VE/1995	AF425638	Venezuela	1995
671.	AF425633/VE/1995	AF425633	Venezuela	1995
672.	AF425635/VE/1995	AF425635	Venezuela	1995
673.	GU056030/VE/1997	GU056030	Venezuela	1997
674.	GU056029/VE/1997	GU056029	Venezuela	1997
675.	AF425634/VE/1997	AF425634	Venezuela	1997
676.	FJ639735/VE/1997	FJ639735	Venezuela	1997
677.	GU056033/VE/1998	GU056033	Venezuela	1998
678.	FJ639741/VE/1998	FJ639741	Venezuela	1998
679.	FJ639740/VE/1998	FJ639740	Venezuela	1998
680.	GU056031/VE/1998	GU056031	Venezuela	1998
681.	GU056032/VE/1998	GU056032	Venezuela	1998
682.	FJ639743/VE/1999	FJ639743	Venezuela	1999
683.	GU131832/VE/2000	GU131832	Venezuela	2000
684.	KF955411/VE/2000	KF955411	Venezuela	2000
685.	GU131833/VE/2000	GU131833	Venezuela	2000
686.	GU131834/VE/2001	GU131834	Venezuela	2001
687.	FJ639802/VE/2004	FJ639802	Venezuela	2004
688.	FJ639794/VE/2004	FJ639794	Venezuela	2004
689.	FJ744701/VE/2004	FJ744701	Venezuela	2004
690.	FJ639797/VE/2004	FJ639797	Venezuela	2004
691.	FJ639796/VE/2004	FJ639796	Venezuela	2004
692.	JN819425/VE/2004	JN819425	Venezuela	2004
693.	GU131835/VE/2004	GU131835	Venezuela	2004
694.	GU131836/VE/2004	GU131836	Venezuela	2004
695.	KF955412/VE/2004	KF955412	Venezuela	2004
696.	GU131837/VE/2005	GU131837	Venezuela	2005
697.	FJ639808/VE/2005	FJ639808	Venezuela	2005
698.	JN819411/VE/2005	JN819411	Venezuela	2005
699.	FJ639813/VE/2005	FJ639813	Venezuela	2005
700.	KF955407/VE/2005	KF955407	Venezuela	2005
701.	JN819412/VE/2005	JN819412	Venezuela	2005
702.	FJ639812/VE/2005	FJ639812	Venezuela	2005
703.	FJ639811/VE/2005	FJ639811	Venezuela	2005
704.	JN819410/VE/2005	JN819410	Venezuela	2005
705.	FJ639814/VE/2005	FJ639814	Venezuela	2005
706.	FJ810415/VE/2005	FJ810415	Venezuela	2005
707.	KF955413/VE/2006	KF955413	Venezuela	2006
708.	HQ332178/VE/2006	HQ332178	Venezuela	2006
709.	HQ332177/VE/2006	HQ332177	Venezuela	2006

710.	JN819413/VE/2006	JN819413	Venezuela	2006
711.	FJ639823/VE/2006	FJ639823	Venezuela	2006
712.	FJ639820/VE/2006	FJ639820	Venezuela	2006
713.	FJ639824/VE/2006	FJ639824	Venezuela	2006
714.	JN819405/VE/2006	JN819405	Venezuela	2006
715.	HQ332180/VE/2006	HQ332180	Venezuela	2006
716.	FJ639818/VE/2006	FJ639818	Venezuela	2006
717.	FJ639821/VE/2006	FJ639821	Venezuela	2006
718.	KF955414/VE/2006	KF955414	Venezuela	2006
719.	FJ639819/VE/2006	FJ639819	Venezuela	2006
720.	FJ639815/VE/2006	FJ639815	Venezuela	2006
721.	GU131838/VE/2006	GU131838	Venezuela	2006
722.	HQ332181/VE/2006	HQ332181	Venezuela	2006
723.	GU131839/VE/2006	GU131839	Venezuela	2006
724.	HQ332182/VE/2006	HQ332182	Venezuela	2006
725.	JN819415/VE/2006	JN819415	Venezuela	2006
726.	FJ639806/VE/2007	FJ639806	Venezuela	2007
727.	JF804026/VE/2007	JF804026	Venezuela	2007
728.	FJ873809/VE/2007	FJ873809	Venezuela	2007
729.	FJ850101/VE/2007	FJ850101	Venezuela	2007
730.	EU482609/VE/2007	EU482609	Venezuela	2007
731.	FJ850099/VE/2007	FJ850099	Venezuela	2007
732.	JN819414/VE/2007	JN819414	Venezuela	2007
733.	HQ332179/VE/2007	HQ332179	Venezuela	2007
734.	FJ882579/VE/2007	FJ882579	Venezuela	2007
735.	GU131842/VE/2007	GU131842	Venezuela	2007
736.	FJ850100/VE/2007	FJ850100	Venezuela	2007
737.	EU482610/VE/2007	EU482610	Venezuela	2007
738.	GU131840/VE/2007	GU131840	Venezuela	2007
739.	FJ850102/VE/2007	FJ850102	Venezuela	2007
740.	GQ199877/VE/2007	GQ199877	Venezuela	2007
741.	EU482611/VE/2007	EU482611	Venezuela	2007
742.	KF955441/VE/2007	KF955441	Venezuela	2007
743.	GU131841/VE/2007	GU131841	Venezuela	2007
744.	FJ873810/VE/2007	FJ873810	Venezuela	2007
745.	HQ332183/VE/2007	HQ332183	Venezuela	2007
746.	FJ850104/VE/2008	FJ850104	Venezuela	2008
747.	FJ850103/VE/2008	FJ850103	Venezuela	2008

Table S2. Complete dataset, subsets Clade I and Clade II of Genotype V (clade cosmopolitan) DENV-1.

Location	Country / Sampling dates	Complete dataset Genotype V (clade cosmopolitan)	Subset Clade I	Subset Sub-Clade Ia	Subset Sub-Clade Ib	Subset Clade II
AR	Argentina / 2000- 2016	115	65	62	2	46
BR	Brazil / 1982 - 2016	171	48	28	20	67
BRCO	*Brazil: Central-Western*	*27*	*4*	*4*	-	*17*
BRSE	*Brazil: Southeastern*	*62*	*18*	*18*	-	*21*
BRS	*Brazil: South*	*1*	-	-	-	-
BRNE	*Brazil: Northeastern*	*38*	*7*	*6*	*1*	*10*
BRN	*Brazil: Northern*	*42*	*19*	-	*19*	*19*
	No information	*1*	-	-	-	-
AW	Aruba / 1985-2004	2	-	-	-	-
BB	Barbados / 1995-2003	5	-	-	-	-
BS	Bahamas / 1977	1	-	-	-	-
BZ	Belize / 2005	1	-	-	-	-
CN	China / 2016	1	1	1	-	-
CO	Colombia / 1985-2008	20	18	-	-	-
CR	Costa Rica / 1993-2005	4	-	-	1	-
DO	Dominican Republic / 2007	1	1	-	-	-
GD	Granada / 1977-1981	9	-	-	-	-
GF	French Guiana / 1989	1	-	-	-	-
GY	Guyana / 2008	1	1	-	1	-
HN	Honduras / 2008	1	-	-	-	-
HT	Haiti / 2010	5	5	-	-	-
IN	India / 1962-2007	13	-	-	-	-
JM	Jamaica / 1977	1	-	-	-	-
MQ	Martinique / 1989-2008	4	3	-	-	-
MX	Mexico / 1980 - 2012	149	-	-	-	-
NI	Nicaragua / 1996 - 2009	51	-	-	-	-
PE	Peru / 1991	1	-	-	-	-
PR	Puerto Rico / 1986-2012	64	27	-	-	1
PY	Paraguay / 1999-2000	5	-	-	-	-
RE	Reunion Island / 2004	2	-	-	-	-
SB	Saint Barthelemy /2016	1	-	-	-	-
SG	Singapore / 1990 - 2005	2	-	-	-	-
SR	Suriname / 1981	1	-	-	-	-
SV	El Salvador / 1993 - 2012	5	-	-	-	-
TT	Trinidad and Tobago / 1978-1986	3	-	-	-	-
US	United States / 2009 - 2014	24	5	-	-	-
VE	Venezuela / 1994-2008	81	66	15	32	-
VG	British Virgin Islands / 1985-1987	2	-	-	-	2
Total		**747**	**240**	**106**	**56**	**116**

4.4 Artigo 4: First detection and molecular characterization of a DENV-1/DENV-4 co-infection during an epidemic in Rio de Janeiro, Brazil.

Relação do Manuscrito com os Objetivos:

Objetivo específico 4: Realizar a vigilância molecular do DENV-1 circulantes entre 2012-2016, período após a reemergência deste sorotipo no Brasil.

Publicado em: Clinical Case Report

Fator de impacto: não aplicado

Resumo: Na década de 1980, os vírus dengue tipo 1 (DENV-1) e tipo 4 (DENV-4) foram isolados pela primeira vez em Roraima (RR), região norte do Brasil. Entretanto, foi somente após a introdução do DENV-1 no estado do Rio de Janeiro (RJ), em meado da década de 80, que a dengue se tornou um problema de saúde pública em todo o país. Em 2009, este sorotipo reemergiu causando uma epidemia explosiva no país. O DENV-4, por outro lado, só foi detectado no RJ em 2011, após ter sido detectado em RR em 2010. Em 2012, o DENV-1 e o DENV-4 foram cocirculantes no país e responsáveis por um elevado número de casos notificados de dengue. Aqui, descrevemos a detecção e caracterização molecular de uma coinfecção por DENV-1/DENV-4 em amostra confirmada e isolada durante a epidemia de 2012 ocorrida no RJ. Além disso, foram identificados os Genótipo V e Genótipo II para DENV-1 e DENV-4, respectivamente.

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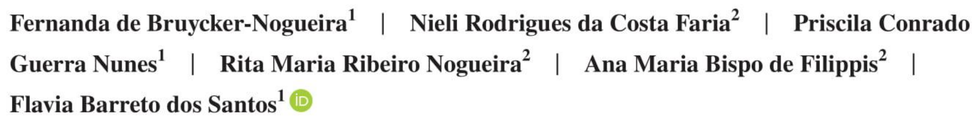

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Key Clinical Message

In the 80s, dengue viruses type 1 and 4 (DENV-1 and 4) were isolated in North region of Brazil. However, it was only after the DENV-1 introduction in the state of Rio de Janeiro (RJ) in mid-1980s, that dengue became a nationwide public health problem. In 2009, this serotype re-emerged causing an explosive epidemic in the country. DENV-4 was first detected in RJ in 2011 and in 2012, DENV-1 and 4 were co-circulating and responsible for a high number of cases notifications. Here, we describe the detection and molecular characterization of a DENV-1/4 co-infection in sample of 2012 in RJ.

KEYWORDS

co-infection, dengue virus 1, dengue virus 4, genotype II, genotype V

1 | INTRODUCTION

Dengue viruses (DENV 1-4) belong to the *Flaviviridae* family and *Flavivirus* genus and exist in either sylvatic or human transmission cycles, most prevalently in tropical and subtropical areas worldwide. The disease has become a major public health problem with relevant social and economical impact due to the increased geographic extension, number of cases, and disease severity.[1]

In Brazil, more than twelve million dengue cases have been reported in the last 32 years[2,3] and, the state of Rio de Janeiro (RJ) particularly, has been of major importance for the disease epidemiology as DENV-1 isolation and spread of DENV-1 in 1986. Outbreaks also occurred after the detection of DENV-2 in 1990, DENV-3 in 2000, and the re-emergence of DENV-2 in 2008. However, in 2009, DENV-1 re-emerged displacing DENV-2 and DENV-3 causing more than one million probable cases in 2010. DENV-4 was detected again in 2010 in

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Roraima (RR), 28 years after its first isolation, arriving in the state of RJ in 2011, causing with DENV-1, a high number of cases in the following years, reviewed in.[4] Here, we describe the detection and molecular characterization of a DENV-1/DENV-4 co-infection in a sample isolated in the epidemic occurred in RJ in 2012 and originally identified only as DENV-1.

2 | MATERIALS AND METHODS

2.1 | Dengue case investigation and ethical aspects

The dengue case analyzed here was received at the Flavivirus Laboratory (LABFLA), IOC/FIOCRUZ, Regional Reference Laboratory for Dengue and Yellow Fever Diagnosis. Cases are received as convenience sampling for diagnosis and investigation under approval by the resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05). The case was received accompanied by an investigation record and questionnaire containing the patient's demographic (age, gender, date of birth, address) and clinical (onset of disease and sign and symptoms) information.

2.2 | Viral isolation

Viruses isolation was performed by inoculating the original serum into C6/36 *Aedes albopictus* cell line[5] and identification by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies.[6] The volume of 25 μL of the supernatant from the original isolate was passaged in a new C6/36 cell monolayer in a 2-mL tube containing Leibovitz-15 medium with 2% fetal bovine serum and at 28°C.

2.3 | Reverse transcription followed by the polymerase chain reaction (RT-PCR)

The viral RNA was extracted from 140 μL of supernatant from the cultures isolated using the QIAmp Viral Mini Kit (Qiagen, Inc., Hilden, Germany) according to the protocol described by the manufacturer and stored at −70°C. The detection of the viral serotypes was performed as described previously.[7] This protocol detects all four DENV serotypes simultaneously, in a semi-nested procedure, generating amplification products with specific size in base pairs (bp) of each serotype.

2.4 | Viral quantification

The viral quantification of the isolated sample and the first passage in cell culture were measured by real-time RT-PCR according to the protocol described previously.[8] The Taqman assay was performed in duplicate, using primers and probes for DENV-1 and DENV-4, separately.

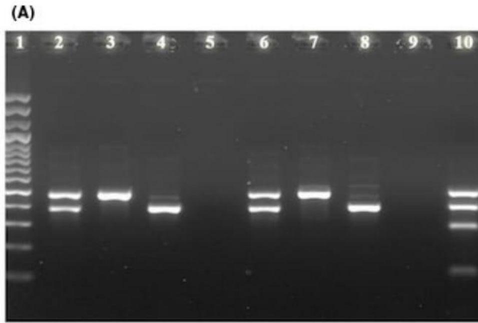
2.5 | Dengue virus genome amplification, sequencing, and phylogenetic analysis

For the envelope (E) gene (1485 bp) sequencing of DENV-1 and DENV-4, two primers pairs for each virus were used to amplify overlapping fragments of approximately 1300 bp. The oligonucleotides primers forward (f) and reverse (r) used for the amplification of the E gene of Brazilian DENV-1 (D1) and DENV-4 (D4) were: fD1-2A 5′-tgacctatgggacgtgttctca-3′ and rD1-3B 5′-ggcgcatctgttccttcgta-3′, fD1-3A 5′-gacgcgaactttgtgtgtcg-3′ and rD1-4B 5′-ccaatggctgctgacagtctt-3′, fD4-3 5′-gtcatgtatgggacatgcaccca-3′ and rD4-6 5′-cctgaaccaatggagtgttagt-3′, fD4-5 5′-ccaacagtacatttgccggagaga-3′ and rD4-8 5′-cccccatgtcttccatgaatat-3′. Five microliters of the extracted RNA was reverse transcribed into cDNA and amplified for sequencing using QIAGEN OneStep RT-PCR Kit (Qiagen, Inc., Germany) according to the protocol described by the manufacturer, in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). This amplicons were observed on 1% agarose gel and cut from the gel for purification a commercial gel extraction kit (Qiagen, Inc., Valencia, California, USA) according to the protocol described by the manufacturer.

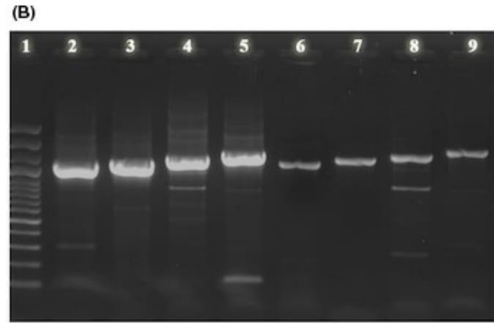
FIGURE 1 Electrophoresis analysis and amplification curve of DENV-1 and DENV-4 genome by RT-PCR for detection and genomic sequencing. A, Products amplified by RT-PCR (Lanciotti, 1992) for the confirmation of DENV-1 and DENV-4 isolated. Lanes: 1—molecular weight (100 bp); 2—original isolate with the mixture of all DENV-specific primers; 3—original isolate with the reaction mixture containing only the DENV-1 type -specific primer (TS1); 4—original isolate with the reaction mixture containing only the DENV-4 type-specific primer (TS4); 6—first viral passage in cell culture with the mixture of all DENV-specific primers; 7—first viral passage with DENV-1 type-specific primer (TS1); 8—first viral passage in cell culture with DENV-4 type-specific primer (TS4 9);—negative control (DNAse/RNAse free water); 10—mixture of positive controls (DENV-1 to 4). B, RT-PCR amplicons for sequencing from the original isolates and first passage. Lanes: 1—molecular weight (100 bp); 2 and 3—original isolate with primers D1-2a/D1-3b and D1-3a/D1-4b, respectively; 4 and 5—original isolate with primers D4-3/D4-6 and D4-5/D4-8, respectively; 6 and 7—first passage with primers D1-2a/D1-3b and D1-3a/D1-4b, respectively; 8 and 9—first passage with primers D4-3/D4-6 and D4-5/D4-8, respectively. C and D, DENV-1 and DENV-4 amplification curves in the original isolated, respectively. E and F, DENV-1 and DENV-4 amplification curves in the first passage, respectively

PCR products were sequenced in both directions using the BigDye Terminator Cycle Sequencing Ready Reaction version 3.1 kit (Applied Biosystems) and six primers, separately, generating fragments of approximately 900 bp. The primers used for sequencing reaction of D1 and D4 were: fD1-2A 5′-tgacctatgggacgtgttctca-3′, rD1-2B

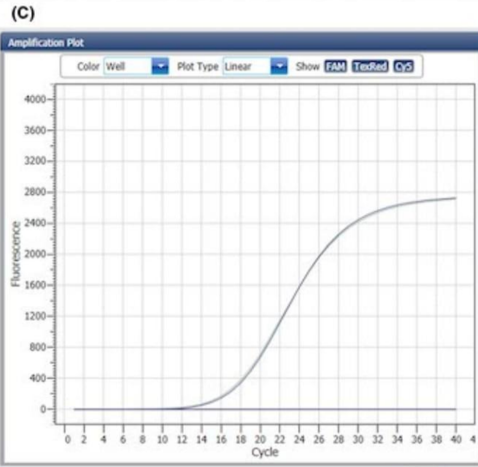
(A)



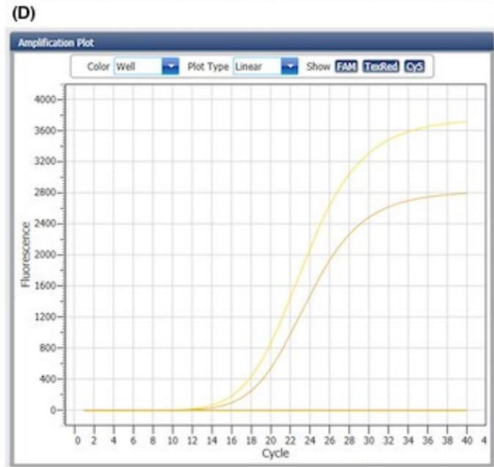
(B)



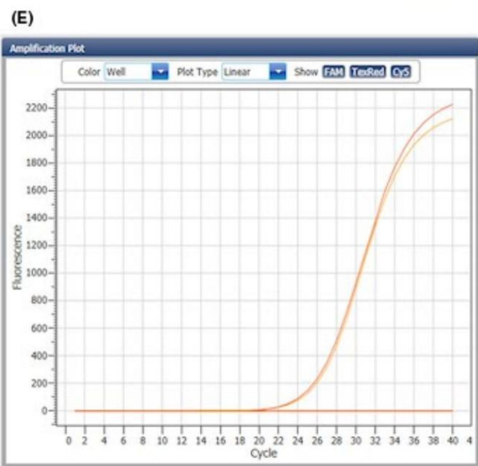
(C)



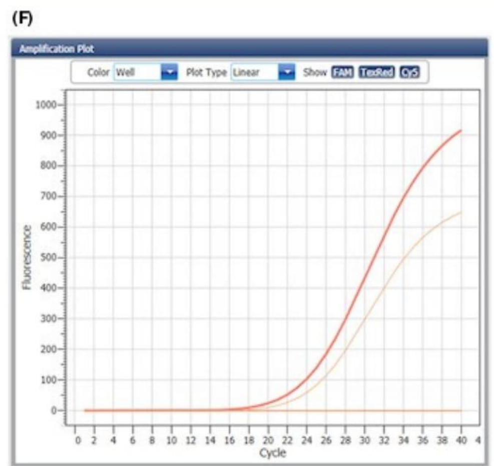
(D)



(E)



(F)



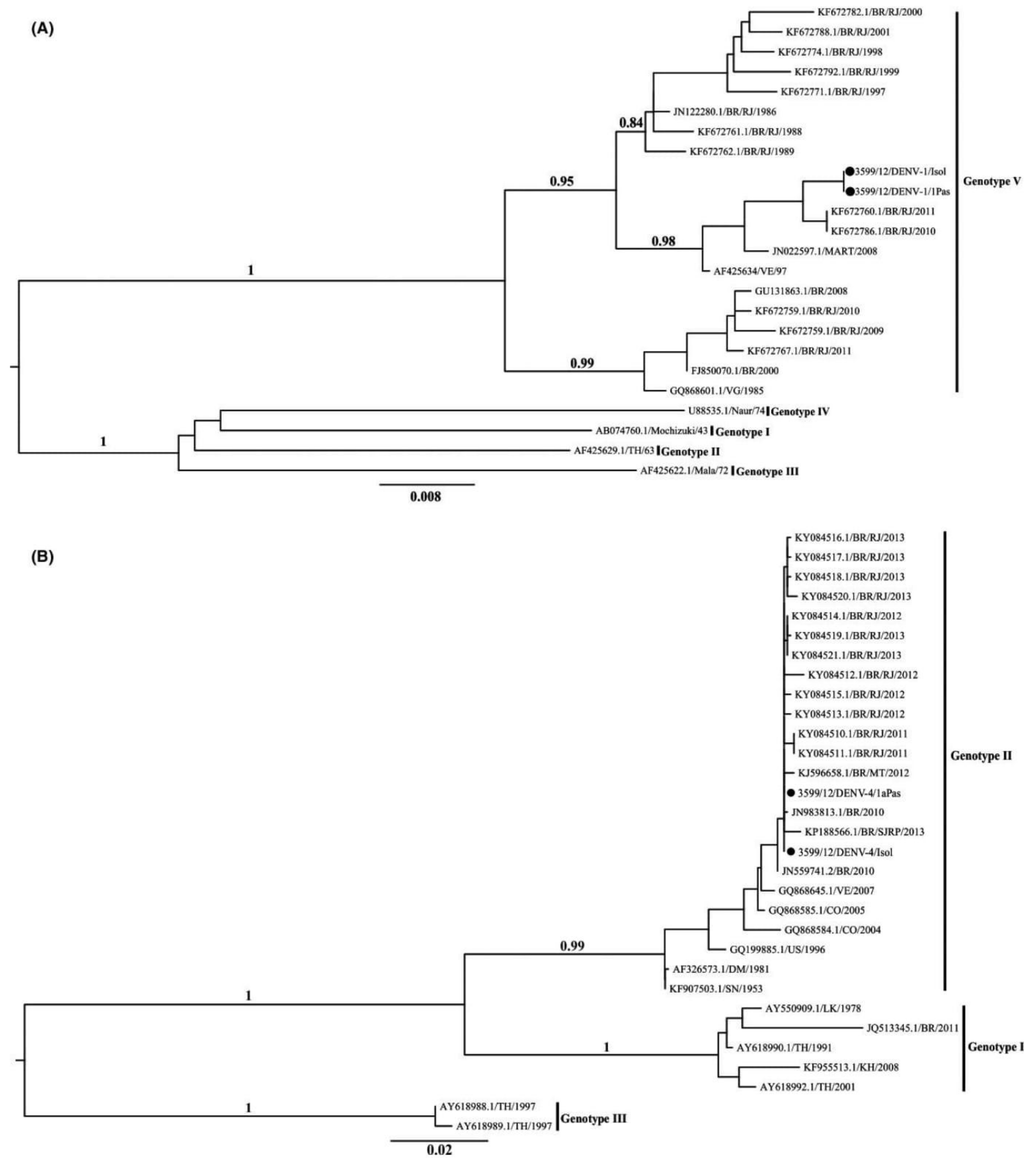


FIGURE 2 Maximum-likelihood tree of the envelope (E) gene of DENV-1 and DENV-4 sequences from a co-infection dengue case occurred in 2012, Rio de Janeiro, Brazil. The sequences analyzed are represented as black circles. DENV strains were named as follows: GenBank accession number/country/year. Only the aLRT support values of major clades are shown. The tree was rooted on the midpoint. All horizontal branch lengths are drawn to a scale of nucleotide substitutions per site as shown in the bar at the bottom

5′-cagtccaatgtgagggctcc-3′, fD1-3A 5′-gacgcgaactttgtgtgtcg-3′, rD1-3B 5′-ggcgcatctgttccttcgta-3′, fD1-4A 5′-ctgggatcacaagaaggagca-3′, rD1-4B 5′-ccaatggctgctgacagtctt-3′, fD4-3 5′-gtcatgtatgggacatgcaccca-3′, rD4-4 5′-gacctgggttcacaatcgagtgtt-3′, fD4-5 5′-ccaacagtacatttgccggagaga-3′, rD4-6 5′-cctgaaccaatggagtgttagt-3′, fD4-7 5′-ggacaacagtggtgaaagtcaagt-3′,

rD4-8 5′-cccccatgtcttccatgaatat-3′. The thermocycling conditions consisted of 40 cycles of denaturation (94°C/1 minute), annealing (60°C/2 minutes), and extension (72°C/3 minutes). Sequencing was performed on an ABI 3730 DNA Analyzer, Applied Biosystems,[9] and the sequences generated were deposited on GenBank (www.ncbi.nlm.nih.gov). The sequences analysis was performed using the Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), the sequences identity by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and alignments by CLUSTAL OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Phylogenetic relationships for both serotypes were analyzed using a maximum-likelihood (ML) tree inferred with PhyML,[10] under the TN93+G+Γ4 model of nucleotide substitution as determined by automatic model selection by SMS: Smart Model Selecion in PhyML[11] and the SPR branch-swapping heuristic tree search algorithm. The reliability of the phylogenies was estimated with the approximate likelihood-ratio (aLRT) SH-like test[12] and trees were visualized with FigTree v1.4.2 program.[13] Strains representative from the other DENV-1 and DENV-4 genotypes available in GenBank were also used for the analysis as outgroups.

3 | RESULTS AND DISCUSSION

The 46-year-old female patient (3 days of illness), presented fever, prostration, headache, myalgia, anorexia, nausea/vomiting, diarrhea, and platelet count of 106 000/mm^3 and a positive dengue NS1 analyzed by the Platelia© Dengue NS1 Ag-ELISA (Bio-Rad Laboratories, Marnes-La-Coquette, France).

The case analyzed here was originally misdiagnosed only as DENV-1, either by RT-PCR or viral isolation. However, when performing the RT-PCR from the cell culture inoculated with the patient's serum during a retrospective study, it was possible to identify the amplification of both fragments corresponding to the DENV-1 and DENV-4 genomes. In order to verify whether the result would be repeated, the isolated sample was submitted to a single passage on cell culture for further analysis by conventional and real-time RT-PCR techniques. The viral passage from the original isolate presented alterations in cell morphology, characterized by an initial cytopathic effect (CPE) after 6 days of incubation period at 28°C (data not shown). DENV-1 and DENV-4 genomes were recovered and detected by RT-PCR using the mixture of the four DENV primers and by reactions containing only DENV-1 and DENV-4 type-specific primers, separately (TS1and TS4, respectively), Figure 1A.

Both DENV-1 and DENV-4 genomes were also amplified by real-time RT-PCR, and the viral titers detected were 1,16E+06 copies/mL and 1,46E+06 copies/mL in the original isolate for DENV-1 and DENV-4, respectively, and 8,93E+03 copies/mL and 2,58E+04 copies/mL in the first passage (Figure 1C-F, respectively). It may be possible that DENV-4 may not have been identified from the patient's serum in the first analysis performed in 2012, due to low a viral load, which may have been increased in cell culture.

The amplicons generated for sequencing of the E gene of the two viral strains (Figure 1B) were used for genetic characterization and phylogenetic analysis. DENV-1 genotype V and DENV-4 genotype II were identified and, both are circulating in country (Figure 2). DENV-1 and DENV-4 genome sequences obtained here were deposited in GenBank under accession numbers MH311981 to MH311984.

In 2012 year, DENV-1 and 4 were prevalent throughout the country, including the Southeast region, in particular the state of RJ.[14]

Considering the current co-circulation of different arboviruses in the country, cases of co-infections may occur. In fact, co-infections by DENV-1 and 4 and other DENV serotypes have been previously reported in Brazil,[15,16] but until now, we were unaware of DENV serotypes co-infections reported during epidemics occurred in RJ.

Due to the emergence of major arboviruses, such as zika virus (ZIKV) and chikungunya virus (CHIKV) worldwide, co-infections have been recently reported. Despite rare events, DENV/ZIKV and ZIKV/CHIKV co-infections have been reported in humans.[17,18]

In Brazil, a recent study conducted by[19] during a dengue and zika epidemic in the state of Mato Grosso do Sul in 2016, identified patients co-infected with DENV/ZIKV. Another study by the same group, investigating the triple epidemic (dengue, zika, and chikungunya) occurred in RJ in 2016, also reported distinct arboviral co-infections: CHIKV/ZIKV, CHIKV/DENV, and DENV/ZIKV (manuscript in preparation).

It is unknown the consequences of a co-infection in the course of the disease for the patient, but the characterization of co-infections in a same patient with more than one virus circulating in a single viremia period, reinforces the role of virological and entomological surveillance. Whether the patient was bitten by a single mosquito carrying multiple viruses or by distinct mosquitoes, is unclear; however, it has been shown that co-infections, such as ZIKV/DENV, may strongly influence the vector competence.[20]

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CONFLICT OF INTEREST

The authors declare no conflict of interest exists.

AUTHORSHIP

FBN and FBS: designed the study. FBN, NRCF, and PCGN: implemented the study. FBN, NRCF, PCGN, and FBS: analyzed the data. FBN and FBS: wrote the manuscript. FBS, RMRN and AMBF: sponsored the experiments. All authors read and approved the final version of the manuscript.

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5 DISCUSSÃO

No Brasil, o DENV-1 foi detectado pela primeira vez em 1982, em Roraima, durante um surto que não se expandiu para outra regiões (Osanai *et al.*, 1983). Entretanto, em 1986, quando este sorotipo foi identificado no Rio de Janeiro, mais de 60.000 casos foram registrados, havendo disseminação do vírus pelo país (Schatzmayr *et al.*, 1986) tornando-se uma preocupação de saúde pública nacional (Nogueira *et al.*, 2007; Rodriguez-Barraquer *et al.*, 2011). Em 2009, quando este sorotipo voltou a circular expressivamente no país, causou uma importante epidemia em 2010 e, pela primeira vez, linhagens virais distintas foram relatadas (Dos Santos *et al.*, 2011).

Os DENV são altamente diversificados (Wang *et al.*, 2002; Aaskov *et al.*, 2006; Thai *et al.*, 2012) e esta diversidade genética foi descrita em diferentes genótipos dentro de cada sorotipo (Rico-Hesse, 1990; 2003; Weaver e Vasilakis, 2009). Estudos com o genótipo V do DENV-1 tem demonstrado que uma dinâmica de circulação e substituição de linhagens tem ocorrido em diversos países da América (Mendez *et al.*, 2010; Dos Santos *et al.*, 2011; Yamanaka *et al.*, 2011; Carneiro *et al.*, 2012; Drumond *et al.*, 2012; Añez e Rios, 2013; Muñoz-Jordán *et al.*, 2013; Tittarelli *et al.*, 2014; Dash *et al.*, 2015; Cunha *et al.*, 2016), e que múltiplos eventos de introdução têm ocorrido no Brasil.

No primeiro estudo (publicado no ano de 2015), com 48 sequências de DENV-1 representando cepas isoladas em 25 anos no Brasil (1986-2011), foi possível caracterizar linhagens distintas e sugerir que duas linhagens (aqui chamadas de 1a e 1b) estão mais relacionadas ao DENV-1 de origem latino-americana e à linhagem 2 com vírus de origem asiática. Porém, como o estudo foi conduzido utilizando análises filogenéticas, tais achados em relação à origem geográfica das linhagens não passam de especulações, necessitando de estudos filogeográficos para melhor estimar a origem das mesmas.

Substituições de aminoácidos foram observadas em todas as sequências analisadas, principalmente no domínio III do gene E, corroborando com estudos prévios (Carneiro *et al.*, 2012; Drumond *et al.*, 2012). Alterações no domínio III podem alterar na imunogenicidade da partícula viral, já que esta região possui o papel de ligação do vírus aos receptores celulares e é altamente indutora de resposta imunológica de anticorpos neutralizantes (Chen *et al.*, 1996; Drumond *et al.*, 2012), assim como na patogenicidade dos flavivírus (Goncalvez *et al.*, 2002; Carneiro *et al.*, 2012).

Como algumas regiões no domínio III são alvo de anticorpos neutralizantes em humanos, mutações nessa região podem ter importantes consequências funcionais (Thai *et al.*, 2012). Carneiro e colaboradores descreveram substituições de aminoácidos (aa) na posição E_{338} em quatro cepas de DENV-1 de pacientes com dengue grave (Carneiro *et al.*, 2012), no entanto, neste estudo, substituições nessa posição foram observadas em cepas isoladas de pacientes com todas as apresentações clínicas. As substituições M196V e T296P no gene E consideradas como marcadores de virulência (Bordignon *et al.*, 2008) não foram identificada nas cepas analisadas neste estudo.

Das três substituições observadas na região prM, uma única cepa representativa da linhagem 2 apresentou uma valina na posição prM_{29} em vez de uma alanina. Tal substituição foi descrita anteriormente quando cepas indianas foram comparadas com cepas globais (Kukreti *et al.*, 2009). Talvez o compartilhamento deste aa conservado em cepas indianas possa nos indicar um possível parentesco entre elas. Poderíamos ter maior embasamento se um número maior de amostras da linhagem 2 fossem submetidos ao sequenciamento completo do genoma.

As substituições nas posições $NS1_{84}$ e $NS1_{92}$ identificadas em duas estirpes da linhagem 1a são localizadas imediatamente antes ao epítopo linear ^{111}HKYSWK. Como a glicoproteína NS1 está envolvida nos estágios iniciais da replicação viral, seria importante determinar se essas diferenças entre as cepas estudadas afetariam a antigenicidade e a atividade biológica da NS1 (Dos Santos *et al.*, 2002). Um estudo conduzido por Suzuki e colaboradores demonstraram que um resíduo de tirosina (Y) na posição $NS1_{247}$, altamente conservado dos DENV-1, seria importante para a função da proteína (Suzuki *et al.*, 2007). Neste estudo, uma substituição foi observada em uma cepa da linhagem 1b, onde a tirosina foi substituída por uma fenilalanina (F). As substituições na $NS3_{358}$, $NS3_{437}$, $NS3_{465}$ e $NS3_{474}$ foram identificadas nas amostras analisadas, sendo três delas detectadas em uma cepa representativa da linhagem 2. Na $NS3_{465}$, um resíduo de asparagina foi detectado nos seis genomas completos analisados, diferenciando-os de uma cepa brasileira utilizada como referência, a BR/90 (Dos Santos *et al.*, 2002), até então a amostra brasileira mais antiga isolada de humanos no período da primeira epidemia com sequência nucleotídica disponível no Genbank. A partir deste estudo, quatro cepas da década de 1980 foram disponibilizadas, sendo duas C/prM/M/E e duas com genoma codificante completo.

Na NS4B, as únicas substituições conservadas observadas foram em $NS4B_{34}$ (R>H) e $NS4B_{90}$ (I>L) em duas cepas isoladas em 2000-2001, representativas da linhagem 1b. Esta proteína tem fortes interações com NS1 e está associada à replicação viral (Lindenbach *et al.*,

2013). Substituições na proteína NS5 podem afetar diretamente a replicação viral, uma vez que esta proteína atua como RNA polimerase - RNA dependente e, juntamente com a NS3, pode atuar tanto com atividade NTPase, quanto RTPase (Lindenbach *et al.*, 2013). As substituições em $NS5_{114}$ (V>I) e $NS5_{784}$ (I>V) observadas neste estudo foram prevalentes entre as linhagens, diferenciando-as novamente da BR/90. Duas cepas isoladas em 2000-2001 pertencentes à linhagem 1a, compartilham substituições de aa em $NS5_{629}$ (L>F) e $NS5_{635}$ (T>S), não detectadas nas outras linhagens. Embora várias substituições de aa tenham sido observadas entre as cepas estudadas, nenhuma alteração no caráter bioquímico foi identificada.

A análise de divergência nucleotídica e de aa mostrou que as linhagens são bastante semelhantes entre os grupos, e que as linhagens 1a e 1b estão mais relacionadas quando comparadas à linhagem 2. Esta divergência entre as linhagens é apresentada na árvore filogenética, mostrando que as mesmas se encontram em ramos distintos, com alto suporte de *bootstrap*.

A filogenia baseada no gene C não permitiu a genotipagem das cepas estudadas. A separação das linhagens 1a, 1b e 2 só foi possível pela análise do gene E ou da região codificadora completa do genoma. Além disso, o mesmo agrupamento foi observado analisando-se apenas o domínio III e a região *stem* da proteína E, demonstrando o fato de que as substituições nessa região estão associadas à separação das linhagens.

Estudos prévios têm descrito eventos de recombinação entre as cepas de DENV-1 (Tolou *et al.*, 2001; Aaskov *et al.*, 2007; Carvalho *et al.*, 2010; Chu *et al.*, 2013; Shin *et al.*, 2013), porém nenhuma evidência de recombinação foi observada na análise das 48 cepas brasileiras neste estudo.

A cocirculação de duas linhagens distintas no Brasil nos últimos anos tem sido caracterizada e o envolvimento de ambas as linhagens em casos brandos, graves e fatais demonstra que possivelmente não estão correlacionadas com algum desfecho da doença em particular. Não se sabe se a epidemia explosiva no Brasil em 2009 e 2010, causados principalmente pelo DENV-1, esteja relacionada à cocirculação das distintas linhagens. A suscetibilidade da população a um sorotipo que não circulou intensamente por quase uma década, e a ocorrência de infecções secundárias em um país hiperendêmico, também poderiam ter tido um papel na epidemiologia da doença. Devido à geografia e à epidemiologia da dengue no Brasil, a vigilância de populações virais, linhagens e genótipos é necessária, pois o impacto dessas substituições de linhagens e cocirculação ainda não são totalmente compreendidos.

A fim de entender um pouco mais da dinâmica do genótipo V do DENV-1 no Brasil, ampliamos nosso estudo para a reconstrução da história evolutiva deste genótiponas Américas, na tentativa de identificar a sua origem geográfica mais provável, há quanto tempo se encontra no continente americano e como se dispersou originando as mais distintas linhagens identificadas em diversos países americanos. Este genótipo é prevalente no continente americano, porém também abrange algumas sequências da África e uma parte da Ásia (Chen e Vasilakis, 2011). Para isto, o uso de um número elevado de sequências do genótipo V de distintas origens geográficas ao longo de aproximadamente 40 anos foram imprescindíveis para o estudo.

Nossa análise filogeográfica sustenta que a atual diversidade dos DENV-1 nas Américas resultou de duas introduções independentes do genótipo V da Índia por volta do início dos anos 70, e início dos anos 80, corroborando assim o estudo de Walimbe e colaboradores (Walimbe *et al.*, 2014). Ambas as cepas do genótipo V foram provavelmente introduzidas no continente americano através da região do Caribe e posteriormente disseminadas no continente, confirmando o papel central das ilhas do Caribe na dispersão inicial do DENV da Ásia para as Américas (Villabona-Arenas e Zanotto, 2011; Allicock *et al.*, 2012; Villabona-Arenas e Zanotto, 2013; Mir *et al.*, 2014).

A primeira cepa do genótipo V do DENV-1, foi identificada pela primeira vez nas Bahamas em 1977 e se espalhou rapidamente para outros países da América do Sul, Central e do Norte, originando a linhagem aqui designada como clado I. Com os resultados aqui apresentados, as Pequenas Antilhas (*Lesser Antilles* - LA) atuaram como fonte primária das cepas do clado I disseminadas nas Américas até meados da década de 1980, condizente com estudo anterior (Allicock *et al.*, 2012). A maioria das cepas do clado I disseminadas durante os anos 1970 e 1980 a partir da América Latina, resultou em surtos secundários de tamanhos pequenos e se extinguiu em meados da década de 1990. Duas cepas do clado I foram capazes de desencadear surtos secundários de grande porte em Porto Rico (linhagem I-PR) e no Brasil (linhagem I-BR) e persistiram como as linhagens predominantes de DENV-1 nesses países durante os anos 80 e 90. Essas linhagens, no entanto, permaneceram restritas aos seus países de origem e acabaram se tornando extintas no início dos anos 2000. A linhagem I-BR aqui denominada, refere-se à linhagem circulante do período da primeira epidemia de 1986 do Brasil, designada no primeiro estudo como linhagem 1a, baseado em análise filogenética.

Outro clado partiu das LA alcançando a Venezuela no final dos anos 80 e foi disseminado com sucesso dentro deste país, estabelecendo a linhagem I-VE. A linhagem I-VE

não permaneceu restrita à Venezuela, mas se dispersou com sucesso para outros países americanos e foi responsável por quase todas as infecções por DENV-1 detectadas na região após o ano de 2003. Entre meados da década de 1990 e meados da década de 2000 é possível identificar diferentes introduções da linhagem I-VE da Venezuela em outros países da América do Sul (Argentina, Brasil e Colômbia), América Central (Nicarágua), Caribe e também EUA. Desde meados dos anos 2000, a Nicarágua tornou-se um importante centro secundário de disseminação da linhagem I-VE para outros países da América Central e do Norte, levando ao estabelecimento de uma sub-linhagem regional, enquanto a Argentina e as Grandes Antilhas (GA) atuaram como pequenos centros de disseminação da linhagem I-VE para países vizinhos.

A segunda cepa do genótipo V, introduzida nas Américas por volta do início dos anos 80, foi identificada pela primeira vez nas Ilhas Virgens em 1985 e após uma década de circulação silenciosa chegou ao Brasil, originando a linhagem aqui designada como clado II. Esta linhagem corresponde à linhagem 2 designada no primeiro estudo.

Provavelmente, a circulação desse clado menor do genótipo V nas Américas permaneceu não reconhecida por um período tão longo devido à escassez de sequências de DENV-1 disponíveis no Caribe, particularmente nas ilhas das Pequenas Antilhas, durante os anos 80 e 90. De acordo com nossas estimativas, o clado II foi introduzido do Caribe na região Norte do Brasil em meados da década de 1990 e depois disseminado para as regiões Sudeste, Nordeste e Centro-Oeste, e da região Sudeste para a Argentina.

Um estudo anterior conduzido por nosso grupo sustenta que o padrão filogeográfico do DENV-2 nas Américas poderia ser explicado por um modelo unidirecional de transmissão de curta distância no qual o vírus se deslocou do Caribe para regiões próximas ao continente que se comportam como centros secundários de disseminação para outros países vizinhos (Mir *et al.*, 2014). Este modelo parece explicar a dinâmica inicial de disseminação do DENV-1 nas Américas durante os anos 1970 e 1980. Desde meados da década de 1990, no entanto, o ponto central de partida da disseminação do DENV-1 nas Américas foi do Caribe para a Venezuela, estabelecendo um novo fluxo direcional do DENV-1 da Venezuela para o Caribe e outros países continentais, o que não foi observado para o DENV-2. Isso demonstra que o padrão geral de disseminação dos DENV nas Américas poderia variar muito entre diferentes sorotipos e que diferentes regiões geográficas podem atuar como uma fonte primária para a manutenção e propagação desse vírus no continente.

Nossos resultados revelaram ainda que o fenômeno de substituição de linhagem através de surtos epidêmicos de DENV-1 sucessivos era uma característica comum em vários

países americanos. No Brasil, o clado I-BR foi a linhagem mais prevalente até o início dos anos 2000, quando foi aparentemente extinto e substituído por duas linhagens I-VE introduzidas a partir da Venezuela e da Argentina na segunda metade da década de 2000, e pelo clado II, introduzido pelo Caribe em meados dos anos 90. O clado I-PR foi a linhagem mais prevalente em Porto Rico até o final da década de 1990, quando parece ter sido extinta e substituída pelo clado I-VE introduzido pela Venezuela no início dos anos 2000. Vários clados iniciais disseminados das LA circularam na América Central e no México até meados da década de 1990, mas também foram substituídos pelas linhagens I-VE introduzidas da Venezuela por volta de 2000. A única exceção a essa tendência parece ser a Venezuela, onde a linhagem I-VE circula continuamente do final dos anos 80 até o final dos anos 2000, sem evidência de substituição de linhagem. Podemos especular que um dos principais fatores para a persistência a longo prazo do DENV é a disponibilidade de uma grande população humana suscetível, somada a alta infestação vetorial, mas nossas observações contradizem essa hipótese.

O Brasil contribui anualmente com a maior proporção (normalmente > 50%) dos casos positivos de DENV notificados nas Américas (WHO, 2012; PAHO, 2018), e as análises filogeográficas realizadas neste estudo e em um estudo anterior (Mir *et al.*, 2014), no entanto, revelaram que o Brasil não foi um grande epicentro das novas linhagens de DENV-1 e DENV-2 disseminadas em nível regional. A maioria das cepas de DENV-1 e DENV-2 introduzidas no Brasil foram dispersadas apenas no país ou em países vizinhos da América do Sul (Argentina, Bolívia e Paraguai) e parecem extintas após um período de 10 a 20 anos. De acordo com essas reconstruções, os principais reservatórios do DENV-1 e DENV-2 nas Américas nos últimos 20-30 anos foram Venezuela e Porto Rico, respectivamente; países que abrigam uma população de indivíduos suscetíveis, muito inferiores ao Brasil.

Paralelo ao estudo sobre a história evolutiva do genótipo V nas Américas, amostras provenientes de diferentes estados brasileiros dos anos de 2012-2016 foram sequenciadas com o intuito de dar continuidade ao estudo de vigilância molecular das diferentes linhagens já reconhecidas como circulantes no Brasil, pois as sequências mais recentes do país no nosso estudo anterior datavam de 2013, do estado de Goiás.

O DENV-1 permaneceu sendo o sorotipo prevalentemente identificado até o ano de 2016, correspondendo a aproximadamente 90% dos casos isolados. Em 2016, o DENV-1 completou 30 de atividade no país, desde a primeira epidemia em 1986.

A partir da análise filogenética por máxima verossimilhança, foi possível confirmar que as amostras do período pós reemergência continuavam pertencentes ao genótipo V e se

distribuíam em três ramos distintos. Um desses ramos se refere ao clado II, e os dois outros, pertencentes ao clado I. De acordo com nosso estudo anterior, esses dois ramos dentro do clado I pertencem ao clado I - VE que foi introduzido no Brasil em meados dos anos 2000 de forma independente, aqui denominados Sub-Clado Ia e Sub-Clado Ib.

Realizamos a análise filogeográfica dos três distintos grupos com o objetivo de acompanhar o padrão de disseminação destas linhagens até o ano de 2016.

Os Sub-Clados Ia e Ib parecem ter alcançado o Brasil por volta dos anos de 2007-2008 quase simultaneamente, porém por vias distintas, como já previamente mostrado em nosso estudo das Américas. Conduzindo as análises até 2016 destacamos dois padrões regionais entres estas duas linhagens.

O Sub-Clado Ia teve a Argentina como ancestral desta linhagem no Brasil, se disseminando para as regiões Nordeste, Centro-Oeste e Sudeste do país. A Argentina como ancestral das cepas brasileiras, corroboram com nosso estudo anterior. Com a introdução na região Sudeste observamos a maior parte das dispersões da cepa viral dentro do Brasil, assim como retornando para Argentina, que foram circulantes até o ano de 2016, pelo menos. A disseminação pontual da região Centro-Oeste para a China provavelmente se refere a um caso importado. No Genbank, esta cepa chinesa se refere a um estudo de caso importado, porém não houve publicação do mesmo. Porém, podemos dizer que este caso importado foi proveniente do Brasil, provavelmente da região Centro-Oeste.

O Sub-Clado Ib apresentou uma rota de introdução no Norte do Brasil partindo direto da Venezuela, assim como já demonstramos em nosso estudo anterior. Este clado parece ter se mantido circulante somente no Norte do país, com um evento pontual de dispersão para as Guianas Francesas. Porém, observamos uma movimentação mais recente deste clado para a região Nordeste do Brasil e para a Argentina. Tittarelli e colaboradores (Tittarelli *et al.*, 2017) estudando isolados da Argentina durante a epidemia ocorrida no início de 2016 destaca que um amostra do seu estudo estava relacionada com amostras da região Norte do Brasil.

O clado II apresentou o mesmo padrão de dispersão já descrito no nosso estudo anterior, destacando as migrações ocorridas até meado dos anos 2000 após alcançar a região Sudeste do país. A partir da região Sudeste, este clado se dispersou diretamente para a Argentina, para o Nordeste e se manteve na região Sudeste. Alcançou o Centro-Oeste diretamente pelo Sudeste e através da Argentina, migrando novamente deste para o Centro-Oeste. Um grande fluxo de migrações entre o Sudeste, o Centro-Oeste e a Argentina parece ter ocorrido durante a década de 2010.

A caracterização molecular das 30 cepas permitiu identificar diferentes padrões de nucleotídeos responsáveis pela conservação de alguns aminoácidos nos domínios I, II, III e região-*stem* do gene E entre as diferentes linhagens. A alteração no domínio II e uma no domínio III não havia sido identificada previamente.

No domínio I, na posição E_{297}, o aminoácido treonina (T) foi observado em todas as 30 sequências estudadas, diferente da sequência dos anos 80 que possui uma metionina (M) nesta posição, como já descrito anteriormente. No domínio II, identificamos uma alanina (A) na posição E_{230} em apenas duas das nossas sequências estudadas, pertencentes ao Sub-Clado Ia. Todas as outras sequências têm um T nesta posição. Analisando todas as 240 sequências do Subset-Clade I, apenas estas duas sequências e uma da Argentina do ano de 2016 (número de acesso do Genbank: KX768377) possuem este aminoácido. Tittarelli e colaboradores durante um estudo realizado na Argentina entre dezembro de 2015 a abril de 2016 não destacou este aminoácido em sua amostra (Tittarelli *et al.*, 2017). De fato, dentre todo o banco de dados as únicas sequências que apresentam este aminoácido compartilhado são duas do nosso estudo e esta da Argentina, sendo as nossas datadas de 2014-2015, portanto precede à da Argentina no tempo.

Aminoácidos conservados foram observados no domínio III (E_{338}, E_{361} e E_{394}) e na região *stem* (E_{428} e E_{436}). As sequências do Sub-Clado Ib apresentaram em E_{394} uma Arginina (R) no lugar de Lisina (K), como as outras sequências de Sub-Clado I e Clado II. No entanto, as sequências de Clado II apresentaram as maiores diferenças entre os aminoácidos compartilhados, tendo o aminoácido Leucina (L) nas posições E_{338} e E_{428} e uma Isoleucina (I) em E_{436}, aminoácidos conservados em todo o clado, como descrito por nosso grupo anteriormente. Além desses aminoácidos, uma parte do clade II (seis sequências deste estudo dos anos de 2014 e 2015 das regiões sudeste e nordeste) compartilham um R na E_{361} com 11 sequências da Argentina no ano de 2016, diferenciando-as da outras sequências no conjunto de dados Clade II e Sub-Clade I, que têm um K nesta posição. Este aminoácido foi descrito por Tittarelli e colaboradores em 10 amostras agrupadas com sequências brasileiras (Tittarelli *et al.*, 2017).

Recentemente, Dutra e colaboradores investigaram cepas circulantes em uma epidemia ocorrida na região centro-oeste do estado de Minas Gerais, em Divinópolis, apresentando a cocirculação de duas linhagens distintas em 2013. E assim como observamos em nosso estudo, apontaram para cinco introduções independentes para o genótipo V no país, desde o ano de 1982 (Dutra *et al.*, 2017).

A nomenclatura das diferentes linhagens dos DENV-1 no Brasil ainda se encontra sem um padrão. No nosso estudo designamos como clados acrescido de numeração romana I e II e letras para as linhagens dentro do clado I. Porém, Dutra e colaboradores (Dutra *et al.*, 2017) sugerem a padronização das linhagens com BR1 a BR5 de acordo com a ordem cronológica de introdução no país.

Durante a vigilância molecular do DENV-1 no período de 2012-2016, foi identificado que, dentre as 30 amostras, uma se tratava de uma coinfecção por DENV-1/DENV-4. Este caso foi inicialmente diagnosticado apenas como DENV-1, por RT-PCR e isolamento viral. No entanto, ao realizar a RT-PCR a partir da cultura de células inoculadas com o soro do paciente durante estudo retrospectivo, foi possível identificar a amplificação de ambos os fragmentos correspondentes aos genomas de DENV-1 e DENV-4. Para verificar se o resultado seria repetido, a amostra isolada foi submetida a uma passagem em cultura de células para posterior análise por técnicas de RT-PCR convencionais e em tempo real. A passagem viral do isolado original apresentou alterações na morfologia celular, caracterizada por um efeito citopático inicial (CPE) após seis dias do período de incubação a 28ºC. Os genomas de DENV-1 e DENV-4 foram recuperados e detectados por RT-PCR usando tanto a mistura dos quatro primers para os DENV, quanto contendo apenas primers específicos do tipo DENV-1 e DENV-4, separadamente. Os genomas DENV-1 e DENV-4 também foram amplificados por RT-PCR em tempo real, e os títulos virais quantificados.

A análise filogenética permitiu a identificação do genótipo V do DENV-1 e o genótipo II do DENV-4, ambos circulantes no país (De Bruycker-Nogueira *et al.*, 2016). No ano de 2012, o DENV-1 e 4 foram prevalentes em todo o país, incluindo a região Sudeste, em especial o estado do RJ (Svs/Ms, 2012). Considerando a atual cocirculação de diferentes arboviroses no país, casos de coinfecção podem e têm ocorrido. De fato, as coinfecções por DENV-1 e 4 e outros sorotipos de DENV foram relatadas anteriormente no Brasil (Colombo *et al.*, 2013; Martins *et al.*, 2014), porém até o momento, desconhecíamos algum caso de coinfecção por sorotipos de DENV relatadas durante as epidemias ocorridas no Rio de Janeiro.

Devido à emergência de importantes arbovírus com caráter epidêmico explosivo, como o vírus zika (ZIKV) e o vírus chikungunya (CHIKV) em todo o mundo, coinfecções têm sido relatadas recentemente. Apesar de parecer eventos raros, coinfecções por DENV/ZIKV e ZIKV/CHIKV têm sido relatadas em humanos (Dupont-Rouzeyrol et al., 2015; Furuya-Kanamori et al., 2016; Waggoner et al., 2016; Zambrano et al., 2016; Iovine et al., 2017; Kaur et al., 2017).

No Brasil, um estudo recente realizado por Azeredo e colaboradores durante uma epidemia de dengue e zika no estado de Mato Grosso do Sul em 2016, identificou pacientes coinfectados com DENV/ZIKV (Azeredo *et al.*, 2018). Outro estudo do mesmo grupo, investigando uma tripla epidemia (dengue, zika e chikungunya) ocorrida no RJ em 2016, também relatou coinfecções por arbovírus distintos: CHIKV/ZIKV, CHIKV/DENV e DENV/ZIKV (manuscrito em preparação).

Desconhece-se as consequências de uma coinfecção no curso da doença para o paciente, mas a caracterização de coinfecções em um mesmo paciente com mais de um vírus circulante em um único período de viremia, reforça o papel da vigilância virológica e entomológica. Se o paciente foi picado por um único mosquito portador de múltiplos vírus ou por mosquitos distintos, não está claro, no entanto, tem sido demonstrado que coinfecções, como ZIKV/DENV, podem influenciar fortemente a competência do vetor (Chaves et al., 2018).

Assim como foi identificado a coinfecção de sorotipos virais em nossas amostras estudadas, entendemos que durante uma infecção o hospedeiro pode apresentar variantes de um mesmo sorotipo viral, sendo analisado, por sequenciamento nucleotídico método de Sanger, somente aquele que se encontra em quantidade prevalente. Com o advento das novas plataformas de sequenciamento como, por exemplo, o sequenciamento de nova geração (NGS do inglês, *next generation sequencing*), é possível estudar a diversidade de variantes virais desconhecidas presentes no hospedeiro. Outra ferramenta que pode ser utiliza é a RT-PCR em tempo real método Taqman, utilizando sondas específicas para cada linhagem já caracterizada molecularmente.

Estudos *in vitro* analisando duas linhagens cocirculantes em São José do Rio Preto, SP, em cultura de células VERO, C6/36 e Huh7 demonstrou que uma das linhagens cocirculantes tem maior *fitness* em células de origem humana, de mosquito e de primata não-humano, tendo maior taxa de replicação e disseminação no hospedeiro, podendo ser mais facilmente transmitida ao mosquito vetor (Pinheiro *et al.*, 2018). Porém, visto que hoje reconhecemos três linhagens cocirculantes no país, sendo todas detectadas na região Nordeste em um curto espaço de tempo (2014-2015), e duas delas distruibuídas pela região Sudeste e Centro-Oeste, entender o papel das linhagens em infecção mistas *in vitro* poderia elucidar alguns questionamentos quanto a permanência da cocirculação de múltiplas linhagens em uma mesma região geográfica, e no caso de infecção mista em paciente qual seria o comportamento de uma linhagem frente à outra.

Diante destes resultados, destacamos a importância do Brasil como um grande receptor e mantenedor das cepas virais que foram responsáveis por diferentes epidemias ao longo destes 30 anos. Diferentes eventos de introdução de linhagens de DENV-1 ocorreram no território brasileiro, principalmente a partir das regiões Norte e Sudeste. Tal fato trás um alerta para que estas regiões sejam monitoradas com maior intensidade, visto que são fortes portas de entrada no país. A cocirculação continuada de diferentes linhagens do DENV-1 até o ano de 2016 reforça a importância da vigilância epidemiológica, virológica e molecular, dos DENV, pois assim como novas linhagens vêm alcançando o país, outros genótipos poderão ser introduzidos e disseminados dentre a população.

6 CONCLUSÕES

- A análise filogenética do DENV-1 brasileiro confirmou a circulação do genótipo V no país, e a análise do domínio III do gene E e da região-*stem* foi suficiente para diferenciar as distintas linhagens identificadas;

- Alterações nucleotídicas gerando padrões de aminoácidos conservados entre as linhagens foram responsáveis pela diferenciação das linhagens;

- A diversidade do genótipo V do DENV-1 nas Américas resultou da evolução local a partir de duas cepas introduzidas da Índia por volta do início dos anos 70 e início dos anos 80, independentemente.

- A primeira cepa introduzida nas Américas foi responsável pela maioria das infecções pelo genótipo V do DENV-1, enquanto a segunda parece estar restrita principalmente ao Brasil.

- As Pequenas Antilhas foram a principal fonte de linhagens de DENV-1 disseminadas nas Américas até meados da década de 1980; e Venezuela e Nicarágua se tornaram posteriormente os centros mais importantes de manutenção e disseminação viral no continente.

- Embora várias linhagens de DENV-1 tenham estabelecido surtos de sucesso em diferentes países da América durante os anos 80, elas foram posteriormente disseminadas e extintas com dinâmicas muito diferentes.

- Após o período de reemergência do Brasil, diferentes linhagens do genótipo V continuaram cocirculando no país até o ano de 2016, pelo menos.

- Duas novas substituições de aminoácidos foram identificados, uma localizada no domínio II do gene E pertencente à amostras recentes do Brasil e Argentina. E outra no domínio III, sendo encontrada em amostras brasileiras e argentinas.

- O evento de dispersão do Sub-Clado 1a para China provavelmente está relacionado a um caso pontual de viagem.

  A detecção de uma amostra coinfectada com DENV-1 e DENV-4 no Rio de Janeiro, ressalta a importância da vigilância molecular constante não só dos sorotipos, mas como dos genótipos e linhagens.

- O Brasil foi e continua sendo um importante receptor e mantenedor dos DENV-1 ao longo do tempo.

7 PERSPECTIVAS

Realizar um estudo sobre o *fitness* viral das diferentes linhagens cocirculantes no Brasil utilizando células C6/36, Vero e Huh7. Para isto, as massas virais de representantes de cada linhagem já foram estabelecidas, apresentando o título de 10E6 cópias/mL.

Desenvolver um protocolo de RT-PCR para genotipagem e linhagem para o DENV-1. Primers específicos para os genótipos e linhagens já foram construídos e adquiridos, aguardando o início dos testes.

Estabelecer um protocolo de pirosequenciamento como alternativa ao sequenciamento de Sanger para triagem das amostras a serem completamente ou parcialmente sequenciadas.

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9 ANEXO

9.1 Nome e abreviatura dos aminoácidos

Aminoácido	Abreviatura de três letras	Abreviatura de uma letra	Cadeia lateral
Ácido aspártico	Asp	D	Ácida
Ácido glutâmico	Glu	E	Ácida
Alanina	Ala	A	Apolar
Cisteína	Cys	C	Apolar
Fenilalanina	Phe	F	Apolar
Glicina	Gly	G	Apolar
Isoleucina	Ile	I	Apolar
Leucina	Leu	L	Apolar
Metionina	Met	M	Apolar
Asparagina	Asn	N	Apolar
Valina	Val	V	Apolar
Triptofano	Trp	W	Apolar
Histidina	His	H	Básica
Lisina	Lys	K	Básica
Glutamina	Gln	Q	Básica
Prolina	Pro	P	Polar não-carregada
Arginina	Arg	R	Polar não-carregada
Serina	Ser	S	Polar não-carregada
Treonina	Thr	T	Polar não-carregada
Tirosina	Tyr	Y	Polar não-carregada

Fonte: (Alberts *et al.*, 2010).

10 PRODUÇÃO CIENTÍFICA

10.1 Primeira autoria

10.1.1 Molecular Biology Approaches for Dengue Diagnosis and Research in Brazil: An Overview (pp. 1-30) (Capítulo de livro).

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Chapter 1

MOLECULAR BIOLOGY APPROACHES FOR DENGUE DIAGNOSIS AND RESEARCH IN BRAZIL: AN OVERVIEW

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ABSTRACT

Dengue is a major public health in tropical and subtropical regions of the world and currently there are no specific therapies and vaccines available. In Brazil, explosive epidemics have been occurring since the 80's and over the years, the dramatic increase of dengue cases in the country has led to the establishment of a National Dengue Diagnosis Network in 1989 to monitor dengue viruses (DENV) transmission and spread as surveillance has been accepted as one of the most reliable tools for the prediction of dengue epidemics. The implementation of molecular techniques in the 90's was imperative for DENV diagnosis. The use of conventional reverse transcriptase-polymerase chain reaction (RT-PCR) technique, such as the one described by Lanciotti and colleagues and suggested by Pan American Health Organization is the most widely

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used protocol. The two steps semi-nested RT-PCR was used to confirm cases, deaths and for the surveillance of DENV in Aedes aegypti mosquitoes and it has contributed to the diagnosis during 28 years of dengue activity in Brazil. A generic RT-PCR that detects dengue and other flaviviruses was also used in some opportunities. Several protocols for real time RT-PCR (rtRT-PCR) using specific primers and probes for each serotype are available. The use of fluorescent probes allows the detection of the amplified products in real time, faster, with lower risk for contamination and higher sensitivity, without the need for electrophoresis. The technique is one of the most reliable for the quantification of viral load and, studies on patient's viremia and its association to a more severe disease and death was performed. Furthermore, rtRT-PCR protocols were also useful for viral detection and quantification on Aedes aegypti saliva. The molecular surveillance of DENV is very important to observe the introduction, spread and shifts of potentially virulent strains, genotypes and lineages as well as to assess their impact on the population during an outbreak. The partial and/or complete DENV genome sequencing has allowed studies on DENV genotypes surveillance and recombination by phylogenentic studies and molecular characterization. During an infection the host can present variants of a same infecting serotype, which may not be detected by the conventional Sanger's nucleotide sequencing method. By the recent advent of next generation sequencing (NGS), viral populations within a host are now more easily studied. Despite the several molecular tools available for DENV diagnosis and investigation, the establishment of new ones may improve even more the sensitivity of the viral detection and quality of data generated. Our experience has shown that the implementation of new techniques over the years has constituted an important strategy for the disease surveillance and research in Brazil.

Keywords: dengue, molecular biology, surveillance, diagnosis, research, Brazil

INTRODUCTION

Dengue viruses (DENV) exists in either sylvatic or human transmission cycles, which are most prevalent in tropical and subtropical areas, and it has become a major public health problem with relevant social an economical effect due to the increased geographic extension, number of cases and disease severity (Guzman and Harris, 2015).

DENV 1 to 4 belong to the family Flaviviridae and the genus Flavivirus (Lindenbach and Rice, 2007) and cause one of the most important infectious diseases in the world in terms of morbidity and mortality. In the last 50 years, the disease has gradually reached the status of a pandemic, hospitalizing more than 5 million children and resulting in more than 70,000 deaths (WHO, 2013). In Brazil, more than eight million cases have been reported since the 80's, with the years of 2002, 2008, 2010 and 2013 the most critical for the country (Silva Jr., 2013). The viral genome from 10.6 to 11 kb in size, encodes three structural proteins (capsid [C], membrane [M] and envelope [E] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) and is flanked by approximately 100 nucleotides (nts) at 5' untranslated region (UTR) and 388–462 nts at the 3' UTR (Shurtleff et al., 2001; Miller et al., 2010).

The dramatic increase of dengue cases in Brazil has led to the establishment and consolidation of a National Dengue Diagnosis Network in 1989 to monitor DENV transmission and spread as DENV surveillance has been accepted as one of the most important tools for the prediction of dengue epidemics. The laboratorial diagnosis can be

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carried out using several approaches for the detection of the virus, viral RNA, antigens or specific antibodies, or even a combination of these methods (revised in Lima et al., 2014). The implementation of molecular techniques in the 90's constituted an important advance in diagnosis for detecting the infecting DENVserotypes, identifying the viruses in cases negative by virus isolation, molecular characterizing and genotyping circulating strains and accessing its impact over the population. In this chapter, we aim to present an overview on the molecular biology approaches most commonly used for dengue surveillance in Brazil, presenting some experiences on its use for laboratorial diagnosis, molecular characterization, phylogeny and viral research.

Viral RNA Extraction for Molecular Detection

The extraction and purification of nucleic acids (RNA/DNA) in biological samples are important steps for efficacy in molecular detection and many protocols have been described. The first step is cell lysis, which is the disruption of the cell membrane that can be achieved by using chemical or physical methods that disrupt hydrophobic associations and destroy the lipid bilayer. Among the substances used are detergents, chaotropic agents, enzymes and acids and basic agents. In the physical methods, the cells may be subjected to mechanical shock, freeze-thaw, sonication and homogenization. The nucleic acid purification is performed to exclude cell debris and proteins and, organic solvents, such as phenol and chloroform, are the most commonly used. The precipitation step usually uses alcohol to decrease the water molecules around the nucleic acid.

The protocol described by Boom in 1990 is widely used for DNA/RNA extraction from samples of blood or urine, on a small scale. It uses conventional silica and can be performed manually or automated. The method is based on the lysing and nuclease inactivating properties of the chaotropic agent guanidine thiocyanate together with the binding properties of nucleic acids to the silica particles or diatoms in the presence of this agent. After the implementation of the molecular biology methods in the 90's in Brazil, this protocol was widely used to extract DENV RNA from clinical samples of dengue suspected cases. However, currently, there are several commercial kits for nucleic acid extraction based on organic extraction, silica-based technology, magnetic separation and ion change technology, used in routine and research laboratories.

Molecular Biology Applied to Laboratorial Diagnosis and Research: Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is based on the in vitro enzymatic amplification, of specific sequences of DNA (target) directed by oligonucleotide primers that results in the generation of an exponential number of copies. The technique consists in repeated cycles of nucleic acid denaturation, primers hybridization on the target and chain synthesis by a DNA polymerase. The DNA denaturation occurs at an elevated temperature, in order to convert a double-stranded nucleic acid to single-stranded. The hybridization of the primers occurs when the temperature is decreased below the dissociation temperature of the primers and the

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synthesis of the new DNA strand is performed by a thermostable DNA polymerase that incorporates the deoxynucleotide triphosphates in the free 3-OH end of the DNA duplex formed between the DNA-target and the initiator. The nucleotide sequence of the new chain is complementary to the target sequence and the number of copies of the generated product increases exponentially. Other variations of the polymerase chain reaction have also been developed, for instance, the RT-PCR, Semi-Nested-PCR and Nested PCR, Multiplex-PCR and Real-Time PCR (rtPCR).

The reverse transcriptase-polymerase chain reaction (RT-PCR) is used for the amplification of RNA, and to do so, the synthesis of a complementary DNA (cDNA) to RNA is needed by using a reverse transcriptase (RT) enzyme. The semi-nested- PCR and nested PCR result in amplicons obtained from a PCR reamplification. Semi-nested PCR uses a primer already employed previously in the PCR reaction while, the Nested- PCR uses for the amplification, a new pair of primers, different from those used previously in the PCR reaction. Those methods result in increased sensitivity and specificity, however it can also increase the risk of contamination by the manipulation of amplicons. In a multiplex-PCR, two or more primers are used, allowing the detection of different viruses and genotypes, however it may result in a lower sensitivity.

The RT-PCR protocol described by Lanciotti et al. (1992) and suggested by Pan American Health Organization is the most widely used protocol for dengue molecular diagnosis. The viral RNA may be detected in serum samples, plasma, infected cells, mosquitoes, fresh tissues and tissues formalin-fixed. In a first step, consensus primers (D1 and D2) are used to anneal to C and prM genes of any of the four DENV serotypes and amplify a 511-bp product in a RT reaction. After a second round of amplification (nested PCR) with type-specific primers (TS1 to TS4, for DENV-1 to 4, respectively), DNA products specific for DENV type are generated.

The protocol by Lanciotti et al. (1992) was established in 1995 in Brazil as a rapid and specific molecular approach for detecting and typing DENV in the Regional Reference Laboratory at the Oswaldo Cruz Institute, Rio de Janeiro and allowed the identification of DENV-1 and DENV-2 in 41% of previously confirmed dengue cases (Miagostovich et al., 1997). In Brazil, DENV-3 was first isolated in 2000 from a patient and from naturally infected Aedes aegypti collected during an entomological surveillance and by using RT-PCR and/or virus isolation, DENV-3 was identified in 13% of the cases. The RT-PCR played a definitive role in surveillance as a rapid diagnostic tool that guided the implementation of control measures as local authorities were notified of confirmed DENV-3 cases. Furthermore, in combination with virus isolation, RT-PCR increased case confirmation due to DENV infections. The implementation of RT-PCR in 1995 constituted an important advance in diagnosis by detecting the infecting DENV type and identifying DENV in cases negative by virus isolation. In some years, RT-PCR identified the infecting DENV type in 40% of cases where the virus was not isolated. From 1997 to 2011, virus isolation and RT-PCR identified the infecting DENV type in a total of 4,990 dengue cases. In the same period, RT-PCR confirmed 24.7% (1,840/7,441) of the dengue cases analyzed (reviewed in dos Santos et al., 2013). The first DENV-4 cases reported in Rio de Janeiro in 2011 were recovered by using RT-PCR from infected patients and from an individual Aedes aegypti female collected in the field (Castro et al., 2012). In a recent study analyzing the epidemiological and laboratorial aspects of the emergence an re-emergence of the disctinct DENV seroytpes in Rio de Janeiro, from 2010 to 2012, the RT-PCR confirmed 56.3% (1,022/1,814) of the cases tested and when

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the co-circulation of the four serotypes was reported for the first time in the State (Heringer et al., 2015).

However, other RT-PCR protocols for molecular detection of DENV were previously described and are available. The protocol described by Morita et al. (1994), for instance, used serum from dengue infected patients directly subjected to the reverse transcription and amplification, without RNA extraction, and the viral was detected in 3 hours, suggesting that the direct RT-PCR procedure greatly facilitates the rapid diagnosis of dengue infection. Harris et al. (1998) adapted the nested RT-PCR protocol described by Lanciotti et al. (1992), for a multiplex reaction in a single tube in order to maximize the simplicity and minimize the risk of sample cross-contamination. The same showed an equivalent sensitivity to the two-step protocol. Wang et al. (2000) developed a competitive RT-PCR method to quantify the RNA of the four DENV serotypes. A pair of primers was designed in a highly conserved region of the C gene and the assay sensitivity was estimated to be 10-50 copies of RNA per reaction.

REAL-TIME PCR

The Real-Time PCR (rtPCR) is an amplification system based on the conventional PCR approach, but with an analysis in real time, where primers and specific probes for each DENV serotype or hybridizing to a conserved region of all serotypes may be used. The use of fluorescence in probe, allows the detection of the products, as they are produced, in real time, without the need of a post-reaction electrophoresis. The emission of fluorescent compounds generates a signal that increases in direct proportion to the amount of the PCR product. The point that detects the cycle in which the reaction reaches the threshold of the exponential phase, is called Ct (Cycle Threshold), and allows the exact nucleic acid quantification. A low Ct value indicates a higher concentration of the initial target. The amplification efficiency in rtPCR should be 90 to 100%, and can be calculated using data collected from a standard curve using the following formula: Efficiency = [10 (-1/slope)] – 1. The slope is the real indicator of amplification and represents the angular coefficient of the straight line formed by the points of the standard curve. A high efficiency is associated with approximately 3.32 slope for each 10-fold dilution of the target.

Two distinct systems are commonly used. The SYBR Green binds nonspecifically within the double-stranded DNA and the TaqMan system, uses probes doubly marked by distinct fluorescent dyes, a reporter (e.g., FAM) and a quencher (e.g., TAMRA). When the two dyes are close, and the probe still intact, the quencher absorbs the energy released by reporter, thus avoiding the emission of fluorescence. The Taq polymerase 5'exonuclease activity degrades the probe during the PCR amplifiction, separating the fluorophores, resulting in the release of fluorescence.

In 2003, the rtRT-PCR was established in the Regional Reference Laboratory at the Oswaldo Cruz Institute, Rio de Janeiro as a novel research tool for DENV detection and quantification in dengue suspected cases. The rtRT-PCR also yielded the highest positivity rate in detecting DENV-3 RNA in tissues from those fatal cases (de Araújo et al., 2009a). The amount of DENV-3 RNA in 42 patients with fatal and non-fatal outcomes during the 2002 epidemic in Rio de Janeiro and its correlation with primary or secondary infection was

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evaluated and a significantly higher virus titer was found in the samples from fatal cases. Moreover, as more than half of fatal cases were primary infections, antibody enhancement alone would not explain the deaths (de Araújo et al., 2009b).

A SYBR Green onestep rtRT-PCR protocol described by Dos Santos et al. (2008) was developed for the early diagnosis of dengue, and presented a higher sensitivity compared to serology, standard virus isolation and the qualitative RT-PCR. However, despite a lower cost, the assay is less specific than the TaqMan system. Several commercial rtRT-PCR kits for the detection of DENV in clinical samples are available and have been evaluated previously (Najioullah et al., 2014).

Protocols detecting one DENV serotype (de Oliveira Poersch et al., 2005) or all four serotypes in a single reaction have been described (Johnson et al. 2005; Kong et al., 2006; Santiago et al., 2013). A study performed in dengue suspected cases assisted in hospitals and clinics from Goiânia, Central Brazil, evaluated a commercial rtRT-PCR kit that proved to be feasible for confirming dengue cases in a timely manner, however stressed that the cost would pose a burden (Levi et al., 2007). In Rio de Janeiro, Brazil, a study by de Araújo et al. (2009a) reported an increased sensitivity of rtRT-PCR in confirming dengue infection in tissues from fatal cases during a DENV-3 epidemic occurred in 2002. Viral genome quantification is also possible by using rtRT-PCR protocols and dengue viral quantitation by using rtRT-PCR was used to determine the correlation between the amount of viral particles and the disease severity. In that same epidemic, de Araújo et al., (2009b) reported a higher viral load in fatal cases due to DENV-3 when compared to classic dengue cases. In an investigation performed in Sao Paulo, Brazil, DENV-2 and DENV-3 were the serotypes recovered in the saliva and urine from patients by rtRT-PCR (Poloni et al., 2010). An rtRT-PCR procedure for detection and quantification of DENV- 1 to -3 successfully identified distinct DENV strains in clinical samples and mosquitoes in Brazil (Conceição et al., 2010). By using rtRT-PCR, Nunes (2012) correlated DENV viral load with disease severity in patients infected by two distinct DENV-2 lineages from the Southeast Asian genotype circulating in Brazil in 90's and 2000's. Patients infected by lineage II had a viral load significantly higher than patients infected by the lineage I, moreover, severe cases from lineage II had a viral load higher than the classic cases form that same lineage. The diagnostic performance of a rtRT-PCR protocol and of serological methods for dengue diagnosis was evaluated in a real epidemic occurred in Belo Horizonte, Minas Gerais, Brazil (Ferraz et al., 2013). The rtRT-PCR protocol by Johnson et al. (2005) was used along with other diagnostic tests during dengue cases surveillance performed in Rio Grande do Sul, South Brazil, from 2007 to 2013, to characterize the epidemiological patterns of the disease in a region with recent autochtonous cases (Tumioto et al., 2014).

GENETIC VARIABILITY AND PHYLOGENY OF DENGUE VIRUSES: TARGET GENES AND RESOURCES

RNA viruses have genetic variability due to the high degree of mutation associated with the error-prone RNA-dependent RNA polymerase, which does not have proof reading capacity and is known to produce approximately one mutation per round of genome replication (Steinhauer et al.1992; Drake, 1993). In DENV, this results in the existence of genetic variability of the four antigenically distinct serotypes (Holmes and Twiddy, 2003).

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The first genetic evidence for differences between DENV serotypes were determined by RNA fingerprinting technique, using enzymes to digest the RNA strain (Rico-Hesse, 2003; Chen and Vasilakis, 2011). However, with the improvement of molecular techniques, whole genome sequences could be generated by the reverse transcription of the viral RNA into a complementary DNA (cDNA) and multiple round of amplification by RT-PCR. The sequencing of the viral genome allowed, with greater efficiency and accuracy, the characterization of DENV strains and improved evolutionary relationships between serotypes. The term "genotype" was defined as the grouping of DENV with nucleotide divergence $\geq$6% for a given region of the genome (Rico-Hesse, 1990; Chen and Vasilakis, 2011). The four DENV seroytpes share amino acids similarities ranging from 60-70% over the E gene and within the same serotype homologous amino acids present in a ratio of 90% or more. However, within each serotype differences of 6% and 3% at the nucleotide and amino acid, respectively are observed (Pierson and Diamond, 2013). Given the reported genetic diversity, genomic sequencing techniques aim to analyze the nucleotide sequences and characterize genotypes and possible viral variants within the same genotype.

The advances on molecular techniques, reagents and bioinformatic softwares allowed the analysis of longer fragments and larger sequences subsets, more and more frequently availble on public gene banks online such as the GenBank® (http://www.ncbi.nlm.nih.gov, European Nucleotide Archive (http://www.ebi.ac.uk/ena) and DNA Data Bank of Japan (http://www. ddbj.nig.ac.jp/). The bioinformatics tools have also contributed in phylogeographic studies allowing analysis on the DENV evolutionary patterns and dynamics. Some softwares for editing sequences include, for instance, Chromas® 2.4.3 (http://www. technelysium.com.au) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), for the analysis of nucleotide and amino acid identities, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), nucleotide and amino acid alignments, CLUSTAL OMEGA (http://www. ebi.ac.uk/Tools/msa/clustalo/) and MAFFT (http://www.ebi. ac.uk/Tools/msa/mafft/), phylogeny by MEGA 6 (http://www. megasoftware. net; Tamura et al., 2013), PhyML3.0 (http://www.atgc-montpellier.fr/phyml/; Guindon et al., 2010), MrBayes (http://mrbayes.sourceforge.net/; Ronquist and Huelsenbeck, 2003), evolutionary and phylogeographic analysis, BEAST (http://beast. bio.ed.ac.uk/; Drummond et al., 2012).

Dengue Viruses Genotypes

Currently, DENV genotyping and phylogenetic analysis are based on the genomic sequencing of the E gene, the most widely used, since this structural gene is the more likely to go under nucleotide and amino acid changes, as is associated with the immunogenicity of the virus particle and the host antibody response. The complete genome analysis is usually performed when a more detailed molecular characterization is intended, allowing the analysis of other structural and non-structural genes besides the untranslated regions (UTR's), involved in viral replication (Rico-Hesse, 2003; Weaver and Vasilakis, 2009).

Phylogenetic studies based either on the partial and/or complete viral genomic sequences have contributed for the understanding of DENV evolution and diversity, including recombination events. The phylogeny beased on the analysis of the E/NS1 junction or E gene characterized five genotypes for DENV-1: Genotype I, representing strains from Southeast

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Asia, China and East Africa; Genotype II, representing strains from Thailand collected in the 1950s and 1960s; Genotype III, representing a wild strain isolated in Malaysia; Genotype IV, representing strains from Western Pacific Islands and Australia; and Genotype V, representing all strains from the Americas, West Africa, and some strains from Asia (Rico-Hesse, 1990; Weaver and Vasilakis, 2009; Chen and Vasilakis, 2011).

The nucleotide sequence analysis of the E gene characterized six genotypes for DENV-2: Asian 1, represented by strains form Malaysia and Thailand, Asian 2 represented by strains from Vietnam, China, Taiwan, Sri Lanka and the Philippines; Cosmopolitan Genotype, including strains of wide geographical distribution and strains from Australia, East and West Africa, the Indian and Pacific Islands, Indian Subcontinent and the Middle East; American Genotype, represented by strains from Latin America and the oldest strains of the Caribbean, Indian Subcontinent and the Pacific Islands from the 1950s and 1960s; Southeast Asian/American Genotype, including strains from Thailand, Vietnam and from the Americas in the last two decades, approximately; and Wild Genotype, representing strains of human, wild mosquitoes, or sentinel monkeys in West Africa and South East Asia (Rico-Hesse et al., 1997; Twiddy et al., 2002; Weaver and Vasilakis, 2009; Chen and Vasilakis, 2011).

Originally, four distinct genotypes were characterized for DENV-3 (Lanciotti et al., 1994) and were corroborated by other analysis based on the partial or complete genome sequencing (Chao et al. 2005; King et al., 2008; Araújo et al., 2009a; Amarilla et al. 2009; Ramirez et al., 2010; Sharma et al., 2011). However, a detailed analysis of DENV-3 sequences available on GenBank identified five genoytpes as follows: Genotype I, represented by Southeast Asia, mainly Indonesia, Singapore, Malaysia, the Philippines, Taiwan and South Pacific Islands; Genotype II, representing strains from Thailand (1962 until recently), a single strain from Singapore (1995), one from Indonesia (1998), Taiwan, Vietnam, Bangladesh, Cambodia, China, Japan and Myanmar; Genotype III, represented by strains from Sri Lanka, India, Japan, Taiwan, Singapore, Samoa, East Africa, Central and South America, the Caribbean and a couple of imported strains to Europe; Genotype IV, represented by strains from Puerto Rico (1960s -1970s) and Tahiti and Genotype V, represented by the DENV-3 prototype strain from the Philippines (H87), Japan, China (1987-2009) and strains from Brazil sampled in the early 2000s (Chen and Vasilakis, 2011).

For DENV-4, only two distinct genotypes were initially characterized by the analysis of limited E gene sequences (Lanciotti et al., 1997). However, broader analysis reported four genotypes: Genotype I, represented by strains from Philippines, Thailand, Vietnam, Myanmar, Malaysia, Sri Lanka, India and imported cases in Japan, China and Brazil, including the This genotype includes the prototype isolated in the Philippines in 1956 (H241); Genotype II, represented by strains from Southeast Asia, China, Western Pacific Ocean Islands, Australia, the Caribbean, Americas and Easter Island; Genotype III, represented by Thai strains (1997- 2001) and Genotype IV, represented by sylvatic strains isolated from sentinel monkeys in Malaysia in 1970's (Klungthong et al., 2004; Villabona-Arenas et al., 2011; Chen and Vasilakis, 2011).

The existence of lineages within the distinct DENV genotypes, presenting distinct geographic and temporal relationships has been reported (Myat Thu et al., 2005; Kukreti et al., 2009) in the Americas and Asia (Zhang et al., 2005; Carrillo-Valenzo et al., 2010; Duong et al., 2013; Lambrechts et al., 2012; Shin et al., 2013; Zhang et al., 2014) and, the term "lineage" has been used to characterize those viruses clustered in clades in a taxonomic level beneath genotype (Mendez et al., 2010). Those genetically distinct lineages may temporally

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emerge or disappear on a regular basis (Drumond et al., 2012; Choudhury et al., 2014) however, the mechanisms involving lineages replacement, emergences or disappearances are not fully known.

The DENV evolution has had a major impact on its virulence to humans and epidemiology of the disease worldwide. Phylogenetic studies allow the correlation between specific genotypes and disease severity. Despite the complex set of factors that contribute to the epidemiology, studies have suggested that specific viral structures may contribute to the increased replication in human target cells and enhance the transmission by the vector mosquito. Moreover, the patients immune status and possibly genetic characteristics of the host may also be implicated in the disease severity (Rico-Hesse, 2003).

DENGUE MOLECULAR EPIDEMIOLOGY IN BRAZIL

In Brazil, more than eight million dengue cases were reported over the past 29 years (Silva Jr, 2013) since DENV-1 introduction and spread in 1986. After epidemics caused by the introduction of DENV-2 in 1990, DENV-3 in 2000 and the re-emergence of DENV-2 in 2008, DENV-1 re-emerged in 2009, displacing DENV-2 and DENV-3 and caused more than 1 million probable cases in 2010 (reviewed in dos Santos et al., 2013). Even after the DENV-4 introduction in 2010 in a fully susceptible country, DENV-1 was responsible for outbreaks over two million suspected cases between 2010 and 2011. Only in 2015, a total of 1,006,414 cases and 377 deaths were reported in the country until May (SVS, 2015). The hyperendemic scenario in Brazil, with the co-circulation of the for DENV serotypes, resulting in distinct epidemiological patters with and increased number of cases, severe disease in children, high hospitalization rates in elderly enphazises the need for serotypes/genotypes surveillance as some are associated with greater disease severity, as those of Asian origin. DENV molecular characterization and phylogenetic studies has been performed and described by many research groups in Brazil, by the partial (E gene) or complete genome sequencing (dos Santos et al., 2002a; Miagostovich et al., 2006; Araújo et al., 2009a; Carvalho et al., 2010; dos Santos et al., 2011; Faria et al., 2013; Drumond et al., 2012; 2013).

Molecular Epidemiology of Brazilian DENV-1

DENV-1 was first introduced in Brazil, by the state of Rio de Janeiro in 1986, resulting in the spread of the virus and consequently epidemics in several states of the country. All published studies so far describe the detection of the genotype V (Americas/Africa), the only circulating since its introduction, Figure 1. A study by dos Santos et al. (2002b) analyzing the complete genome sequences of DENV-1 isolates from 1990 and 2001 in Brazil, structural genes remarkably conserved among the strains isolated in northeastern Brazil in 1997 (BR/97), in the southern region in 2001 (BR/01) and in Rio de Janeiro in 1990 (BR/90), while the non-structural genes presented 27 amino acid changes, previously identified in strains from Nauru Island, Asia and Africa. Among the amino acid changes, nine were unique to strains BR/97 and BR/01, suggesting a local evolution of the Brazilian strains. A comparative analysis of DENV-1 complete genome sequences circulating in the Americas, including two

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Brazilian strains isolated in Brasilia, Federal District, in 2001 and demonstrated the existence of a Latin American monophyletic cluster, subdivided into two sub-clusters. Furthermore, three recombinant events were reported and the study suggested that possible recombination between DENV-1 could be underestimated (Carvalho et al., 2010). In the early 2000s, DENV-1 circulated silently in Brazil, re-emerging in 2009 and accounting for a serious epidemic in 2010. During this period, dos Santos et al. (2011) performing molecular epidemiological surveillance of DENV-1 isolated in the state of Rio de Janeiro, reported for the first time, the circulation of three distinct viral lineages. The analysis of 10 isolates showed that DENV-1 circulating during the period of this serotype re-emergence (2009-2011) still belonged to genotype V (Americas/Africa), but clustering in two distinct clades, different from that observed by the strains circulating in the 80's. Those observations were confirmed by Drumond et al. (2012) when analyzing the population dynamics from DENV-1 genotype V in Brazil. The co-circulation and replacement of distinct lineages was confirmed and it was shown that DENV-1 was introduced during four different events. The first DENV-1 introduction occurred in 1984-85, the second in 1997-99, and the third and fourth from 2004 to 2007. Morever, a potentially recombinant strain was reported and the study reinforced the importance of molecular surveillance of circulating genotypes in the country. The study by Carneiro et al. (2012) reported the DENV-1 variability by analyzing the genes encoding the structural proteins C/prM/M/E of 34 strains isolated during epidemics occurred in Brazil from 1994 to 2011, confirmed the circulation of genotype V and also reported the different introductions and lineages replacements. The temporal divergence analysis indicated that the circulating lineages in Brazil emerged from an ancestral lineage approximately 44.35 years ago. Although studies on circulating DENV-1 in Brazil are complementary, indicating the same genotype and reaffirming circulation, co-circulation and lineages replacements, Nogueira (2013) conducted a retrospective study on DENV-1 circulating in 25 years in Brazil in the states of Rio de Janeiro, Espirito Santo, Minas Gerais, Mato Grosso do Sul, Alagoas, Ceará, Piauí and Rio Grande do Norte. Phylogeny and molecular characterization based on analysis of E gene of 48 DENV-1 strains and the complete coding region of six, confirmed genotype V as previously reported. The three clades characterized compromised strains isolated from 1986 to 2002 (lineage 1a), a second clade represented by viruses isolated from 2009 to 2011 and a representative strain isolated in 2002 (lineage 2) and a clade represented by strains isolated from 2010 and 2011 (lineage 1b). Furthermore, the lineages 1a and 1b were more related with the American strains and lineage 2 with Asian strains. Amino acid substitutions were observed in ectodomains I and III of the E protein and were associated to the lineages separation. The phylogenetic analysis based on the nucleotide sequence of the ectodomain III and steam region was sufficient for differentiation of the three lineages, greatly contributing to the genotypes surveillance by using a target of approximately 500 base pair fragment. The role of air transportation in DENV-1 and 3 dissemination in Brazil, was recently stressed by analyzing complete viral genomes (n = 287), including Brazilian DENV-1 isolates (n = 42). The three lineages from genotype V characterized were estimated to have been introduced in the country in 1985, 1995 and 2006 and it suggested that lineages I and II originated from Caribbean and lineage III from Venezuela (Nunes et al., 2014).

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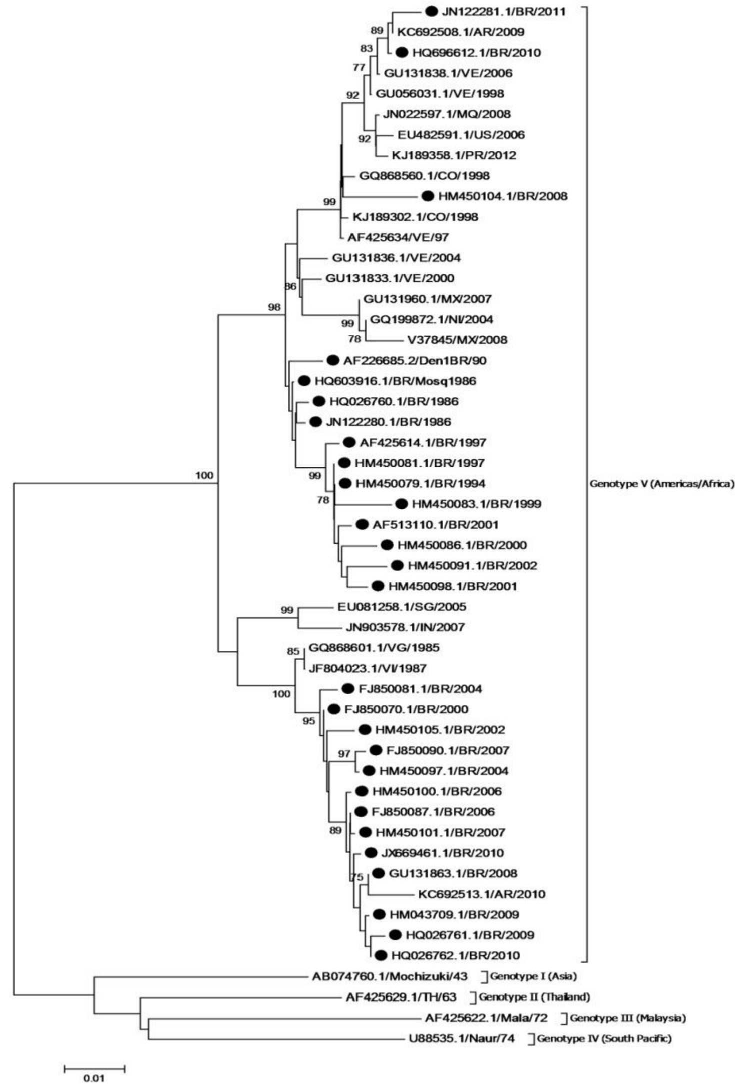

Figure 1. Phylogenetic analysis based on the E gene of representative DENV-1 strains. Neighbor joining method, TN93+G model with correction value of 0.26. Bootstrap test (1000 replicates) is shown next to the branches. Black circles represent the Brazilian DENV-1. DENV strains were named as follows: GenBank accession number/country/year.

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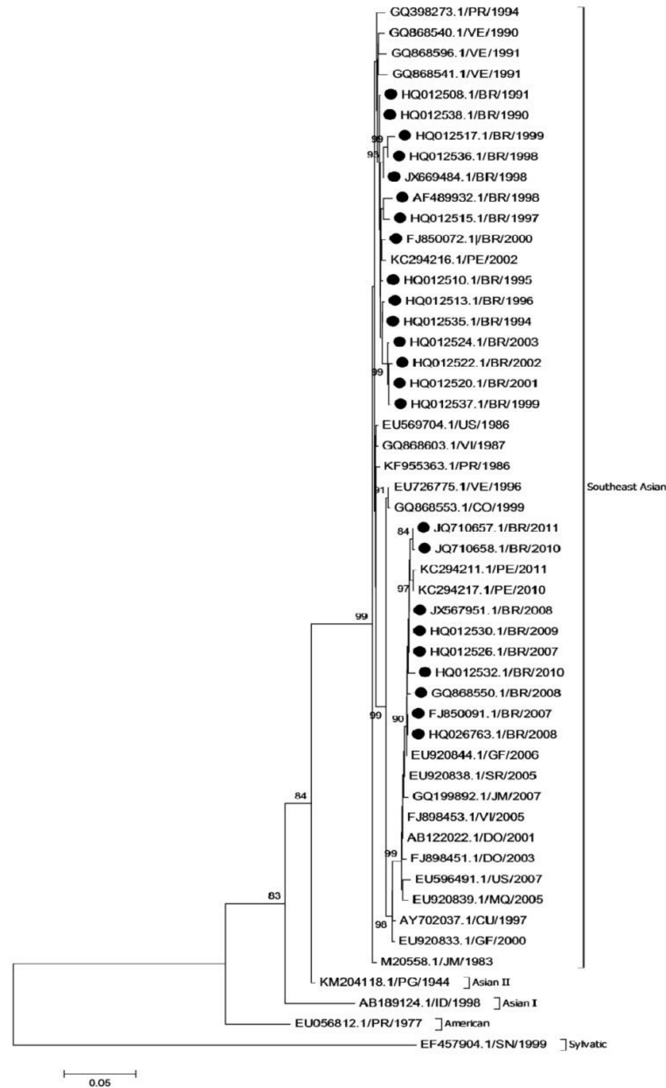

Figure 2. Phylogenetic analysis based on the E gene of representative DENV-2 strains. Neighbor joining method, TN93+G model with correction value of 0.24. Bootstrap test (1000 replicates) is shown next to the branches. Black circles represent the Brazilian DENV-2. DENV strains were named as follows: GenBank accession number/country/year.

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Molecular Epidemiology of Brazilian DENV-2

In Brazil, DENV-2 was first isolated in the city of Niterói, Rio de Janeiro in 1990, and first cases of dengue hemorrhagic fever/dengue shock syndrome (FHD/DSS) (Nogueira et al., 1990) were reported. By sequencing the E gene from DENV-2 isolates from distinct geogaphical regions in Brazil from 1990 to 1995, Miagostovich et al. (1998) reported the circulation of the genotype III (Southeast Asia-American genotype), a genotype associated with increase disease severity. Those data were corroborated by dos Santos et al. (2002a) by analysing the full length genome of a brazilian DENV-2 isolated in 1998 in the the state of Rio de Janeiro. After a low circulation with other DENV serotypes, DENV-2 reemerged in 2007 causing a large epidemic in 2008 in Brazil, with 632,680 dengue cases and 561 deaths (SVS, 2014a; 2014b). The epidemic was characterized by an increase number of severe cases in children under 15 years old (Teixeira et al., 2009). Phylogeny of DENV-2 strains isolated during the re-emergence period (2007-2008) showed the same genotype (Southeast Asia-American) circulating since this serotype introduction in 1990, howerver the emerging viruses grouped in a distinct monophyletic group. The circulating viruses in the 1990s were classified as lineage I and those from the re-emergence period (2007-2008), as lineage II (Oliveira et al., 2010). Analyzing the E gene of DENV-2 isolated from eighteen patients, Romano et al. (2010) reported the reintroduction of this serotype on the coast of São Paulo and confirmed the circulating genotype. Furthermore, it was shown that the strains were related to those from Rio de Janeiro isolated in 2007 and 2008 and, the most common ancestor dated from about 40 years ago. The Southeast region of the country was strongly affected during the re-emergence of DENV-2, and as studies performed in Rio de Janeiro and São Paulo, Dettogni et al. (2012) characterized the strains isolated in the state of Espirito Santo in 2009, confirming the previous observations. The molecular characterization and phylogeny of 34 strains isolated during the twenty years of DENV-2 activity (1990 to 2010), representing six states in Brazil, identified two epidemiologically distinct groups: one represented by strains isolated from 1990 to 2003 and of isolates from 2007 to 2010. The analysis of the E gene and the complete coding region of strains representative of distinct clinical manifestations, showed no mutations on the viral genome associated to the disease severity, however all strains presented an asparagine (N) in position E_{390}, a genetic marker for virulence. The Southeast Asia-American genotype was confirmed as the only detected in 20 years and the viruses isolated between 1990 to 2003 were grouped in the lineage I and isolates as from the re-emergence in 2007 grouped in the lineage II, corroborating Oliveira et al. (2010) and Romano et al. (2010). Moreover, strains from lineage II had a high similarity with the strain isolated from the Dominican Republic in 2001 and, the high divergence with the strains isolated in the 1990's, suggested that the new strain did not evolved locally, but was probably introduced from the Caribbean (Faria et al., 2013). The study by Drumond et al. (2013) described the dynamics, molecular characterization and phylogeny of the DENV-2 lineages belonging to the Southeast Asia-American genotype based on whole genomes from viruses isolated in in Sao Jose do Rio Preto, Sao Paulo in 2008. Brazilian strains were subdivided into three lineages grouped into two clades. It was estimated that lineage 1, represented by strains with broader geographic region and higher genetic diversity, was introduced in 1988-1989. Lineage 2 was estimated to have been introduced in Northern Brazil, probably from Puerto Rico between 1998 and 2000 and lineage 3, the latest to emerge in the country, during parallel events. A report by Nunes et al. (2014), described the results on the analysis of the complete genomes of 294 DENV-2

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clearly evidencing three different lineages from this serotype in Brazil. The estimated time between the introductions of different lineages is eight years (between the lines I and II) and approximately seven years (between strains II and III), and the lineage II replaced by lineage III. The results also highlighted the northern region of Brazil as a entrance and dispersal door of this serotype in the country, especially those originating from the Caribbean. Barcelos Figueiredo et al. (2014) reconstructed the phylogeographic history of DENV-2 in Brazil by analyzing the gene E of viruses isolated during an outbreak in the state of Piauí, in 2006-2007, and pointed out that the introduction and spread of those viruses in Brazil followed one Caribbean route. As Drummond et al. (2013), the authors demonstrated that lineage 2 (represented by strains of period 2007-2011) may have been introduced in Brazil from the Caribbean islands through two parallel events: one in the Southeast (lineage 2a) and another in the North (lineage 2b). This study confirmed the occurrence of the first co-circulation spatiotemporal of two DENV-2 different lineages probably introduced independently to the state of Piaui. The Caribbean islands are the main source of DENV-2 in Brazil and the Northeast seems to be an important route of introduction and spread of this virus in the country. Figure 2 presents the phylogenetic analysis on representative strains of DENV-2, circulating in Brazil.

Molecular Epidemiology of Brazilian DENV-3

The DENV-3 was first isolated in the municipality of Nova Iguaçu, Rio de Janeiro in the end of 2000 and the introduction of this new serotype resulted in the following year, in co-circulation of the three serotypes (DENV-1, DENV-2 and DENV-3), and in the largest and most severe epidemic reported in the country so far in the year 2002 (De Simone et al., 2004; Nogueira et al., 2002).

The genetic characterization of a DENV-3 isolated in Rio de Janeiro in 2001 was performed and published by Miagostovich et al. (2002). In 2006, the first complete genome genetic characterization of a DENV-3 isolated from the liver of a fatal case occurred in 2002 in Rio de Janeiro was reported and the phylogeny confirmed the genotype III (Indian Subcontinent). The genome molecular characterization identified an insertion of 11 nucleotides in the 3 'UTR of the viral genome, charcteristic of DENV-3 of Asian origin and also found in strains isolated in Martinique (Miagostovich et al., 2006). In the same year, Aquino et al. (2006) published a study on the molecular epidemiology of DENV-3 from Brazil and Paraguay isolated between 2002 and 2004, based on the analysis of the E gene and 5' and 3' UTRs. Genotype III was confirmed and reported as a common genotype in the Americas, probably originating in Southeast Asia. The 5' and 3'UTRs were highly conserved between samples studied, but two Brazilian isolates of the North region presented an eight nucleotide deletion compared to the other 26 virus analyzed. Phylogenetic analysis indicated that the two strains isolated in the North in 2002 and 2004 were more related to isolates from Martinique than to the Brazilian strains isolated in Rio de Janeiro and it was suggested that those strains did not evolved locally, but were probably introduced from the Caribbean, indicating at least two DENV-3 introductions in Brazil. In 2008, Barcelos Figueiredo et al. (2008) by the phylogenetic analysis based on a conserved fragments of the genome and on a more variable region reported the circulation of a new DENV-3 genotype (genotype I) in strains isolated in the state of Minas Gerais from DF, DHF and fatal cases. In the same year, a

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study by Nogueira et al. (2008) described the co-circulation of genotypes III and V in North Brazil (Acre and Rondônia) during epidemics occurred from 2002 to 2004. Araújo et al. (2009b) performed the evolutionary history reconstruction and phylogeography of the three major DENV-3 genotypes, estimating their dispersion, based on the analysis of the E gene of 200 strains isolated from 31 different countries around the world in a 50-year period (1956-2006). The phylogenetic analysis revealed a geographical subdivision of DENV-3 in several specific clades in each country. Migration patterns of the main DENV-3 genotypes showed that genotype I was mostly circulating in the maritime region of Southeast Asia and the South Pacific, genotype II remained within continental areas of Southeast Asia, while genotype III was propagated throughout Asia, East Africa and the Americas. Estimates of evolutionary rates revealed no significant differences between the main DENV-3 genotypes and the average rate of evolution in endemic areas with long-term transmission was similar to that observed in the Americas, which have suffered a more recent spread of dengue. Estimated origins of DENV-3 was around 1890, and the emergence of the current diversity of the major genotypes, between 1960 and 1970, coinciding with the population growth, urbanization and the description of the first cases of DHF by DENV-3 in Asia. Due to the controversy in the DENV-3 genoytpes classification described in Brazil, genotype I by Barcelos Figueiredo et al. (2008) and genotype V by Nogueira et al. (2008), Araújo et al. (2009a) performed a DENV-3 phylogenetic perspective study circulating in the country. The phylogenetic analysis and nucleotide distance based on the E gene of 103 strains from 30 different countries around the world, supported the DENV-3 subdivision into five distinct genotype and confirmed the classification of the new genotype from South America as genotype V. Alfonso et al. (2012) analyzed the phylogenetic relationships of DENV-3 isolated in Brazil and Paraguay with viruses isolated around the world, and the dynamics of evolutionary divergence of the serotype based on the complete coding region. The data obtained indicated that two groups of the genotype III circulated during 2002-2009 in Brazil, being separated as two sub-lines A and B, suggesting different introduction events different regions of the country. In the same year, de Araújo et al. (2012) published a study on the origin and evolution of DENV-3 genotype III in Brazil by analyzing the E gene of 107 sequences isolated from 2001 to 2009 in several regions of the country. The analysis revealed that at least four introductions of genotype III occurred in Brazil, represented by the presence of four distinct phylogenetic lineages and highlighted the importance of the Caribbean to the frequent introduction of strains in Brazil, and suggested that the North and Southeast regions are of great importance to the introduction and spread of DENV-3 strains. Aware that the DENV genetic diversity plays an important role in the disease immunopathogenesis, and that the polymorphisms associated with adaptive response is relevant to vaccine development, Villabona-Arenas et (2013) reported the adaptive changes of DENV-3 during an epidemic in Sao Jose do Rio Preto, in 2006 and 2007. In order to characterize the role of possible genetic changes that would interfere with the immune escape, the study sequenced the entire coding region from thirty-three DENV-3 isolates. Based on the evolutionary history, at least two different DENV-3 introductions were identified in the city, and ten polymorphic sites over significant positive pressure detected were detected. Furthermore, the major polymorphic sites were found in the E, NS1, NS2A and NS5 genes, suggesting that those could be experiencing recent adaptive changes. Figure 3 presents the phylogeny based on the E gene analysis from representative Brazilian DENV-3 strains.

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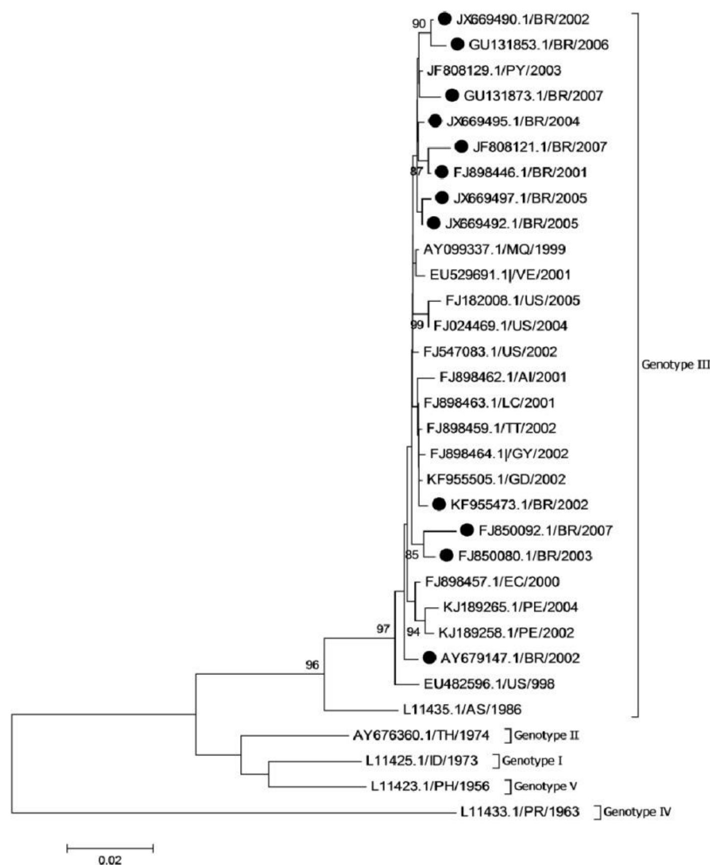

Figure 3. Phylogenetic analysis based on the E gene of representative DENV-3 strains. Neighbor joining method, TN93+G model with correction value of 0.22. Bootstrap test (1000 replicates) is shown next to the branches. Black circles represent the Brazilian DENV-3. DENV strains were named as follows: GenBank accession number country/year.

Molecular Epidemiology of Brazilian DENV-4

The DENV-4 reemerged in July of 2010 in the city of Roraima, northern Brazil, after about 30 years since its introduction in 1981-82 (Temporao et al., 2011). This serotype spread throughout the country and in 2011, co-circulated with DENV-1, causing an epidemic of 764,032 reported cases. In that same year, de Souza et al. (2011) analyzed the phylogenetic

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relationships based on the E gene of six DENV-4 strains isolated in the states of São Paulo, Paraná and Rio Grande do Sul and reported the genotype II circulating in the country. Nunes et al. (2012) studied the phylogeography of DENV-4 in Brazil based on the complete genome of sixteen isolates circulating in the North (Roraima, Amazonas, Pará) and Northeast (Bahia) regions, from 2010 and 2011. It was shown that, despite the confimation of genotype II, one DENV-4 strain isolated in Bahia belonged to genotype I. Furthermore, it was also revealed that genotype II was introduced more than three times in the last decade, two from Venezuela to Roraima and from Colombia to the Amazon. Analyzing ten DENV-4 isolates from Rio de Janeiro in 2011 and 2012, Campos et al. (2013) confirmed the circulation of genotype I in one strain and genotype IIb on the remainig strains, which were closely related to those isolated in Roraima in 2010 and Sao Paulo in 2011. The co-circulation of two distinct DENV-4 genotypes was demonstrated in country (Figure 4) and corroborated by the recent results described by Pinho et al. (2015), also analyzing strains from Bahia. It was shown that the Brazilian strains were similar to the circulating strains in Vietnam between 2004 and 2008, suggesting that those were derived from this variant of genotype I.

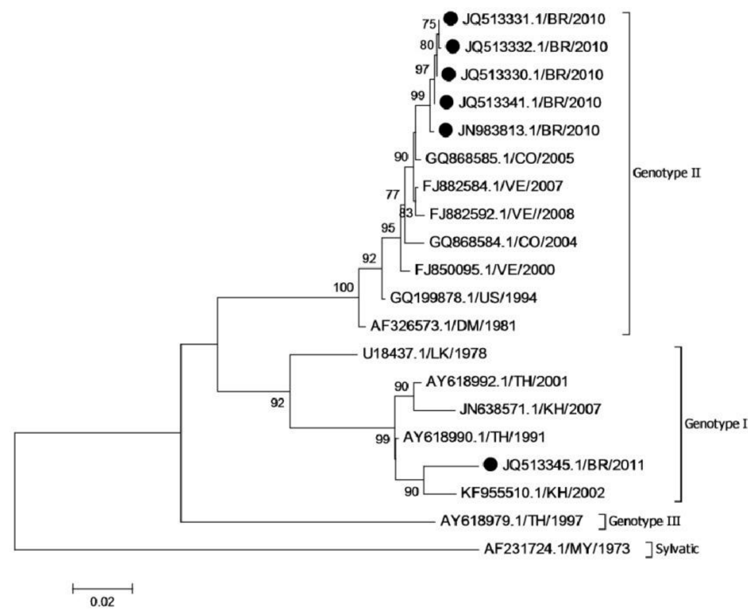

Figure 4. Phylogenetic analysis based on the E gene of representative DENV-4 strains. Neighbor joining method, TN93+G model with correction value of 0.29. Bootstrap test (1000 replicates) is shown next to the branches. Black circles represent the Brazilian DENV-4. DENV strains were named as follows: GenBank accession number/country/year.

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Molecular Biology Approaches Applied to Dengue Virus Surveillance in Mosquitoes Vector

The broad spread of the mosquito vector Aedes aegypti has made dengue the most important human arbovirus disease in the world. Besides the man-mosquito-man cycle of dengue transmission, virus maybe vertically transmitted from a female infected to her offspring and by veneral transmission among mosquitoes. It is possible that the transovarial transmission represents a way of maintenance of the DENV in nature, because the virus can persist until the seventh generation in the mosquitoes tissues (Kow et al. 2001; Joshi et al. 2002; Le Goff et al. 2011).

The viral detection in mosquitoes highlights the position of the vector as a required element in the transmission cycle of DENV during an epidemic (Liotta et al. 2005) and the entomological surveillance of those viruses by rapid methods of detection and characterization is an important tool not only to help to predict the occurrence of explosive epidemics but also to detect the introduction of new virus and/or genotypes. DENV detection rates from Aedes mosquitoes by RT-PCR may vary depending on the geographical location, epidemiological background or the vector popula- tion. For instance, during a surveillance performed in Taiwan, only 0.2% of Aedes aegypti females were positive for DENV (Chen et al. 2010). On the other hand, in a surveillance performed in Mexico, 16.1% of the Aedes aegypti females collected in schools were infected with DENV (García-Rejón et al. 2011).

In Brazil, during an entomological surveillance performed in RJ in 2001, DENV-1 was detected in Aedes aegypti mosquitoes by RT-PCR, when the presence of DENV-3 was being investigated. Previous studies showed that 17% of the Aedes aegypti mosquitoes were infected in a DENV-3 surveillance performed in Manaus, North region of Brazil (Pinheiro et al. 2005). However, in Rio de Janeiro only 0.1% of adult mosquitoes was infected by DENV-3 during an entomological surveillance performed during the inter-epidemic year of 2006. A semi Nested RT-PCR specific to genus Flavivirus was performed during an entomological surveillance in 1,700 mosquitoes collected in the Northeast, Southeast and South regions of Brazil, from 1999 to 2005. DENV-1 to 3 were detected in 3.8% of the pools analyzed and one in a pool of Haemagogus leucocelaenus suggesting that this virus could be involved in a sylvatic cycle. DENV-3 was found infecting pools of Aedes albopictus larvae and phylogeny identified the DENV-3 genotype III (de Figueiredo et al., 2010). In Recife, Northeastern Brazil, despite the predominance of DENV-3 in human cases, 10% of the tested pools were infected, and both DENV-2 and DENV-1 were detected in mosquitoes (Guedes et al. 2010). During the DENV-4 entomological surveillance performed by RT-PCR in Roraima, North Brazil in 2010, after this serotype emergence, DENV-1 was also detected (Castro MG, personal communication). By using rtRT-PCR, Maciel-de-Freitas (2011) analyzed the survival rate, longevity and fecundity of Aedes aegypti females orally challenged with DENV-2 and demonstrated that infected mosquitoes from a long-established laboratory colony presented more viral RNA copies at death than those from the F1-generation of a field population from Rio de Janeiro, Brazil. In 2008, during an entomological surveillance performed in the district of Tancredo Neves Manaus, a total of 260 Aedes mosquitoes was tested by Nested RT-PCR. One Aedes aegypti pool was positive for DENV-4 and the genome sequencing characterized the DENV-4 genotype (Figueiredo et al., 2013). A study by Castro et al. (2012) characterized the first DENV- 4 cases occurred in Rio de Janeiro in 2011, and

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demonstrated the role of rapid molecular techniques, such as conventional RT-PCR and rtRT-PCR, in the entomological surveillance of the newly introduced DENV-4 in vector populations after it was isolated from humans. The rtRT-PCR was used to quantify the DENV-4 viral titre (1.08 x 10^3 copies/mL) from a single Aedes aegypti female mosquito that was naturally infected and individually macerated. In the study, the effectiveness of the Simplexa™ Dengue rtRT-PCR kit comercially available, for the detection and typing of DENV in Aedes aegypti, was also evaluated. A single female was positive for DENV-4 with a low Ct value (23.5), suggesting a high viral load. Natural vertical transmission of DENVV-2 and DENV-3 in Aedes aegypti and Aedes albopictus was reported in a study performed in 40 neighborhoods in Fortaleza, Ceará, Brazil from March 2007 to July 2009. A total of 3,417 Aedes aegypti and 336 Aedes albopictus was analyzed by virus isolation, RT-PCR and genome sequencing. The study reported Aedes albopictus infected by DENV-3, Aedes aegypti infected by DENV-2 and Aedes albopictus co-infected by DENV-2 and DENV-3 (Martins et al., 2012). Aiming to contribute for DENV phylogenetic and vector-virus-human host studies, de Castro et al. (2013) sequenced the entire genome of one DENV-3 isolated from naturally infected Aedes aegypti from RJ in 2001 and characterized the 3' UTR from strains isolated from mosquitoes and humans. The phylogeny characterized the DENV-3 isolated from both vector and human host as belonging to Genotype III despite the differences observed on the 3' UTR. In the DENV-3 isolated from the mosquito vector, an eight nucleotides deletion was observed within the eleven nucleotides insertion on the variable region from the 3'UTR. A study evaluated whether those distinct DENV-3 variants presenting those characteristics showed differences on the virus-vector interaction by determining the vector competence of two populations of Aedes aegypti. The results suggested that the experimental population proved to be competent to transmit the two DENV-3 strains. However, the viral dissemination in the mosquitoes body presented heterogeneously, suggesting that there are advantages for the strain with eleven nucleotides insertion in the 3'UTR, once it disseminated more rapidly (Chouin-Carneiro, 2014). Previous studies have suggested that the sequence and secondary structures of the 5'and 3'UTR of flaviviruses play an important role in viral replication and differences in these regions may influence viral virulence. Moreover, mutations and deletions within these regions may alter infectivity and reduce efficiency of viral replication. A study aiming to compare the DENV susceptibility of field-captured Aedes aegypti from nine distinct geographic areas of the city of Belo Horizonte in 2009 and 2011, analyzed adult females experimentally infected with DENV-2 and the virus was detected by rtPCR in body and head samples. Infection rate ranged from from 40% to 82.5% in 2009 and 60% to 100% in 2011, vector competence ranged from 25% to 77.5% in 2009 and 25% to 80% in 2011and disseminated infection rates oscillated from 68.7% to 100% in 2009 and 38.4% to 86.8 in 2011, concluding that Aedes aegypti populations from Belo Horizonte exhibited wide variation in vector competence to transmit DENV (Gonçalves et al., 2014). The natural transovarial transmission of DENV in Aedes aegypti from Cuiabá, Mato Grosso, Brazil was evaluated by using a multiplex semi-nested RT-PCR to detect the four DENV, Yellow Fever virus, West Nile virus and Saint Louis encephalitis virus in mosquitoes collected in 2012. DENV-4 was the only flavivirus detected in 16.0% of the mosquitoes pools tested, including males. Furthermore, the nucleotide sequences presented 96-100% homology with the DENV-4 strains form genotype II detected in Manaus, Amazonas (da Cruz et al., 2015).

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As shown previously, the molecular techniques used in several entomological surveillances were found be effective even when a new serotype was introduced or when a serotype re-emerged during a dengue epidemic of a different serotype.

NEW APPROACHES FOR DENGUE DIAGNOSIS AND RESEARCH: APTAMERS

With the technical development and new tools availability, several approaches for dengue diagnosis and research are currently under investigation. Some promising tools rely on molecular biology-based techniques. Aptamers, for instance, are single-stranded oligonucleotides that bind to its target with high affinity and specificity. In 1990, both the Gold (Tuerk and Gold, 1990) and Szostak groups (Ellington and Szostak, 1990), described a new method using combinatorial chemistry library of nucleic acids (DNA and RNA) to select RNA oligonucleotides that firm and selectively linked to certain targets. The technique known as "Systematic Evolution of Ligands by Exponential Enrichment" - SELEX - allows obtaining high affinity and specificity primers for any class of organic molecules. The oligonucleotide ligands generated by this technique are called aptamers derived from the Latin "aptus", able to bind to and "mers" derived from the Greek, meaning particle. The most revolutionary aspect of SELEX is the selection of binders by the use of oligonucleotide libraries chemically synthesized without the constraint of having to be selected, or produced in a living organism (Stoltenburg et al., 2007). In general, the SELEX process comprises three steps that are repeated to seek more nucleotides capable of binding to the target. In the first step, library selection begins with library generation of single stranded random nucleotides. In a second step, oligos must be incubated with a target molecule in the presence of a buffer and the desired temperature. These factors control the stringency of the binding process of the nucleic acid molecules of interest. Finally, in the third step, the library amplified by PCR to create a new library. This new and enriched oligonucleotide pool will be used in the binding reaction with the target in the next cycle. The last cycle is stopped after amplification and PCR products are cloned to characterize the aptamers. Aptamers have been mainly developed for applications such as diagnostics or therapeutics (Bunka and Stockley, 2006; Song et al., 2012). For dengue a modular biosensor that is able to quickly identify sequences associated with the virus genome has been developed. The biosensor was able to detect sequences derived from each of the four DENV serotypes with a high degree of specificity. Along with sequences specific for each serotype, a sequence for pan-Dengue detection of all serotypes was produced (Fletcher et al., 2012). Furthermore, several DNA aptamers that bind to various important arboviruses with potential diagnostic biosensor applications haave been developed (Bruno et al., 2012). The aptamers enables direct application in the detection and quantification of molecules and its advantages over alternative methods include being a relatively simple technique, requiring simple equipment for its isolation, large number of alternative molecules that can be sorted and its chemical simplicity (James, 2000).

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NEXT GENERATION SEQUENCING

As previously reported, RNA viruses are known to rapidly adapt facing selective pressure from the host immune system and/or antivirals mainly due to the error-prone characteristic of the RNA-dependent RNA polymerase. More recently, using next-generation sequencing (NGS), the generated viral populations can be identified and quantified. NGS and also known as deep sequencing has recently emerged as one of the most promising tools for genome sequencing, evolution, ecology, discovery and transcriptomics (Tang and Chiu, 2010; Radford et al., 2012; Chiu, 2013).

This platform involves the analysis of millions of nucleic acid–derived sequences present in clinical samples to detect sequences related to particular pathogens. Due to its unbiased nature, NGS allows the identification of new viruses, emerging viruses, which can be highly diverse, with high rates of mutation and recombination. Moreover, one can now accurately quantify distinct viral populations and track the changes to these populations over the course of a single infection (Chiu, 2013). Currently, there are many NGS platforms to choose from, depending on the read length and depth, such as, the 454 Sequencing (Roche Diagnostics), SOLiD sequencing (Life Technologies), Illumina and Ion Torrent. A recent study compared three NGS platforms for metagenomic sequencing and identification of pathogens in blood and reported that the Roche-454 Titanium platform detected DENV at titers as low as $1X10^{2.5}$ pfu/mL, corresponding to maximum $5.4X10^{4}$ genome copies/ml. The Ion Torrent and Illumina platforms, enabled detection of viral genomes at concentrations as low as $1X10^{4}$ genome copies/mL and platform-specific biases were evident in sequences read distributions and viral genome coverage. Overall, the platforms performed well for the pathogen identification from blood. However, unlike the identification, the pathogen characterization is likely to require higher titers, multiple libraries and/or multiple sequencing runs (Frey et al., 2014). The NGS approach has many advantages over more traditional methods for virus detection, such as PCR, ELISA and virus isolation. Those may include speed, the ability to detect non-culturable pathogens or an unknown pathogen in a clinical sample. However, difficulties associated to the intensive computational analysis, data storage, lack of reference genomes for comparison in some cases, the need of high coverage to detect some pathogens at very low levels in a clinical specimen and high cost, are some challenges still to be faced.

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10.1.2 Arboviruses in the context of large-events.

ARTICLE

Arboviruses in the context of large-events

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Human movement especially associated with major events can facilitate the transmission and spread of pathogens through importation by visitors in the host country or exportation of endemic pathogens to the countries of origin of visitors when returning home. The current scenario in Brazil is of a triple epidemic of Dengue, Chikungunya and Zika, including in the state of Rio de Janeiro, local headquarters of the Olympic Games which occurred in August, 2016. The complications arising from infections by Zika as microcephaly and Guillain-Barré syndrome, dengue and Chikugunya morbidity frames preoccupied participating countries as well as tourists who took part of the event. However, the possibility of introducing new non-current arboviruses in Brazil should be considered a risk factor, once the country has conditions to maintain and propagate new arboviruses, for both the vector and susceptible population, as occurred with the rapid dissemination of Chikungunya and Zika viruses.

Key words: Arbovirus, Tourism, Mega-event, Olympic Games, Brazil

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Arboviruses comprise a major group of viruses (RNA genome) maintained in nature through cycles involving arthropod vectors (mosquitoes and ticks, mainly) and susceptible vertebrate hosts. *Flaviviridae*, *Togaviridae*, *Bunyaviridae* are the main families of arboviruses that cause disease in humans. *Flavivirus*, *Alphavirus* and *Orthobunyavirus* are genera considered of great public health concern and of most important threats in tropical regions due to the rapid climate change, deforestations, population migration, disorderly occupation of urban areas and precarious health conditions that favor the amplification and viral transmission (Figueiredo 2007, Cleton et al. 2012, Rust 2012, Dash et al. 2013).

The Arbovirus Catalogue available at the Centers for Disease Control and Prevention (CDC) estimates that 537 species of arboviruses have been identified. These are widely distributed in the world, mainly concentrated in countries of sub-tropical and tropical climates. About 150 of these species are associated with human disease and zoonosis (Gubler 2001, Cleton et al. 2012).

In Brazil, several arboviruses have been implicated as causative agents of human diseases. Grouped by family, we highlight , *Flaviviridae* (*Flavivirus*): Yellow Fever virus (YFV), Dengue virus 1 to 4 (DENV-1 to DENV-4), Encephalitis Saint Louis virus (SLEV), Rocio virus (ROCV), Zika virus (ZIKV); *Togaviridae* (*Alphavirus*): Mayaro virus (MAYV), Chikungunya virus (CHIKV); *Bunyaviridae* (*Orthobunyavirus*): Oropouche virus (OROV). However, other arbovi-

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ruses have been identified in mosquito vectors and other animal reservoirs (birds, monkey, equine, alligator and sheep, for example) indicating the presence of these viruses in nature, such as Ilheus virus (ILHV), Bussuquara virus (BSQV), Cacipacoré virus (CPCV), Iguape virus (IGUV), West Nile virus (WNV), Naranjal-like virus (NJLV), Culex flavivirus virus (CXFV), Eastern Equine Encephalitis virus (EEEV), Nhumirim virus (NHUV) (Box 1) (Lopes et al. 2014, Pauvolid-Correa et al. 2014, Nunes et al. 2015, Zanluca et al. 2015, Pauvolid-Correa et al. 2015a, 2015b).

Brazil offers ideal conditions for the maintenance of several arboviruses, such as its large territory and cities with large population clusters associated with environmental conditions favorable to the development of the mosquito. In the past decades the country has been the main tourist destination of many travelers from various countries, which has brought concern to health authorities. Many of these travelers can not only serve as input sources of new arboviruses into the country, but also as sources of arboviruses circulating in Brazil to their countries of origin if the vectors are present. Airplane travels contribute to the rapid introduction of viruses into different countries around the world (Nunes et al. 2014).

Box 1. Main arboviruses described in vector and/or vertebrate hosts in Brazil.

Family	*Arbovirus*	*Regional distribution*	*References*
Flaviviridae	Cacipacore	North, Midwest	Figueiredo, 2000; Cruz et al., 2009; Batista et al., 2011; Batista et al., 2013; Casseb et al. 2014; Pauvolid-Corrêa et al., 2014.
	Dengue	North, Northeast, Midwest, Southeast, South	Osanai et al., 1983; Schatzmayr et al., 1986; Nogueira et al., 1990; 2001; 2007; De Castro et al., 2003; De Simone et al., 2004; Araújo et al., 2006; Feres et al., 2006; Araújo et al., 2009; Faria et al., 2010; 2016; Temporao et al., 2011; dos Santos et al., 2011; 2013; Castro et al., 2012; Macedo et al., 2013; Fares et al., 2015; Heinen et al., 2015a; Heringer et al., 2015; de Bruycker-Nogueira et al., 2015; 2016; SVS, 2016.
	Ilheus	North, Midwest	Iversson et al., 1993; Cruz et al., 2009; Pauvolid-Corrêa et al., 2013; 2014; Casseb et al., 2014.
	Rocio	North, Midwest, Southeast	Cruz et al., 2009; Casseb et al. 2014; Pauvolid-Corrêa et al., 2014; Silva et al., 2014.
	Saint-Louis Encephalitis	North, Midwest, Southeast, South	Rocco et al., 2005; Mondini et al., 2007; Cruz et al., 2009; Casseb et al., 2014; Pauvolid-Corrêa et al., 2010; 2014. Terzian et al., 2011; Rosa et al., 2013; Silva et al., 2014; Maia et al., 2014; Heinen et al., 2015b; Svoboda et al., 2014.
	West Nile	Northeast, Midwest	Pauvolid-Corrêa et al., 2011; 2014; Melandri et al., 2012; Ometto et al., 2013; Vieira et al., 2015b; 2015c.
	Yellow Fever	North, Midwest, Southeast, South	Araújo et al., 2002.; Filippis et al., 2001; 2004; de Filippis et al., 2002; Cruz et al., 2009; Chaves et al., 2009; Ribeiro et al., 2009; Lima et al., 2010; Souza et al., 2010; 2011; Almeida et al., 2012; 2014; Moreno et al., 2011; 2013; Câmara et al., 2013; Tranquilin et al., 2013; Mascheretti et al., 2013; Casseb et al., 2014; Romano et al., 2014; SVS, 2015.
	Zika	North, Northeast, Midwest, Southeast, South	Zanluca et al., 2015; Oliveira Melo et al., 2016; Cordeiro et al., 2016; Calvet et al., 2016; Brasil et al., 2016a; 2016b; Araújo et al., 2016; SVS, 2016.
Bunyaviridae	Oropouche	North, Northeast, Midwest, Southeast	Azevedo et al., 2007; Mourão et al., 2009; Bernardes-Terzian et al., 2009; Vasconcelos et al., 2009; 2011. Bastos et al., 2012; Cardoso et al., 2015.
Togaviridae	Chikungunya	North, Northeast, Midwest, Southeast, South	Albuquerque et al., 2012; Figueiredo & Figueiredo, 2014; Teixeira et al., 2015; Nunes et al., 2015. Conteville et al., 2016; SVS, 2016.
	Eastern Equine Encephalitis	North, Northeast, Midwest, Southeast; South	Iversson et al., 1993; Fernandéz et al., 2000; Pauvolid-Corrêa et al., 2010; Silva et al., 2011; de Novaes Oliveira et al., 2014;
	Mayaro	North, Midwest, Southeast* (*imported case of Mato Grosso do Sul)	Coimbra et al., 2007; Azevedo et al., 2009; Mourão et al., 2012; Figueiredo & Figueiredo, 2014. Vieira et al., 2015a; Pauvolid-Corrêa et al., 2015a; Serra et al., 2016.

In this context, epidemiological surveillance, use of diagnostic instruments able to identify and characterize infectious agents at an early stage, and implementation of control actions to ensure conditions that minimize the risk of infection in both the local population and visitors, are extremely important. In relation to control, it is important to consider that in cases of outbreaks, controlling of notified cases alone is not enough to stop pathogens from spreading. It is essential that surveillance units acting as sentinels for the early detection of newly introduced arboviruses are established.

During events, whether sporting, cultural, religious, ecological, and recreational or business, it is essential to consider the possibility of increased pathogen movement, since mobilization of human population tends to be stimulated (Nunes et al. 2014). This becomes even more worrying in mass events such as Rock in Rio, World Youth Day and sporting events such as the Soccer World Cup and the Summer Olympic Games, the latter being the largest and longest

sporting event, held in the Rio de Janeiro, Brazil, 2016.

With a high influx of people from many parts of world, a good portion of tourists do not limit their participation to Olympic events. Recreational activities such as ecotourism in the municipality of Rio de Janeiro and small trips to nearby municipalities can facilitate tourist contact with native viruses, as well as the spread of other non-occurring arboviruses in our region (Box 2). Tourists may serve as asymptomatic carriers of viruses or be still in the prodromal phase of the disease. Several publications involving mega events and arboviruses in Brazil have been published in recent years (Box 3). In the face of dengue epidemics over several decades, most of the publications until 2014 analyzed the spread of dengue viruses through exportation from country to country through tourism during big events. In 2015, the Chikungunya and Zika epidemics were established in Brazil, and some authors have hypothesized about possible viral pathways in Brazil related to the occurrence of major events in the country.

One of the studies suggests that entrance of ZIKV in the country has possibly occurred during FIFA Confederations Cup in 2013 (Faria et al. 2016). However, another plausible possibility was that introduction of ZIKV occurred during the Va'a World Sprint Championship canoe race, held in 2014, when four participating Pacific countries (French Polynesia, New Caledonia, Cook Islands, and Easter Island) were facing a ZIKV epidemics (Musso 2015). It is noteworthy that in 2014 publication raised concern about the spread of Mayaro virus, a flavivirus circulating in the Midwest Region of Brazil (Slegers et al. 2014). In this scenario, in addition to the simultaneous movement of different arboviruses with a plurality and similarity of clinical manifestations, the lack of strict preventive measures, and specific therapy, little engagement in disclosure of educational measures and application of health measures, point to a vulnerability and the possibility that arboviruses endemic in certain regions could be rapidly identified in other regions of the world. Viral transmission by *Aedes aegypti*, a vector widely associated to human activities, present in large urban centers such as Rio de Janeiro, the availability of artificial oviposition sites and maintenance of the vector, viruses

Box 2. Arboviruses of medical importance related to travel without occurrence in Brazil.

Family	*Arbovirus*	*Vector*	*Geographic Distribution*
Flaviviridae	Alkhurma hemorrhagic fever	Tick	Western Asian
	Japanese encephalitis	Mosquito	South and South-East Asia,Oceania
	Kyasanur Forest disease	Tick	South-East and Western Asian
	Murray Valley	Mosquito	Oceania
	Powassan	Tick	North American
	Tick-borne encephalitis	Tick	Central, Northern and Eastern Europe, and Asia
Bunyaviridae	Bunyamwera	*Mosquito*	Sub-Saharan Africa
	Bwamba	*Mosquito*	Sub-Saharan Africa
	Crimean-Congo hemorrhagic fever	Tick	South-East and Eastern Europe, Africa, Asian
	Guaroa	*Mosquito*	Central and South America
	Ilesha	*Mosquito*	Sub-Saharan Africa
	Jamestown Canyon	*Mosquito*	North American
	La Cross	*Mosquito*	North America
	Ngari	*Mosquito*	Sub-Saharan Africa
	Rift Valley fever	*Mosquito*	Africa, Western Asia
	Sandfly fever	*Sandfly*	Southern Europe, Northern Africa, Asia
	Tahyna	*Mosquito*	Europe, Asia, Africa
	Tataguine	*Mosquito*	Sub-Saharan Africa
	Toscana	*Sandfly*	Southern Europe
Togaviridae	Barmah Forest	*Mosquito*	Australia
	O'Nyong-nyong	*Mosquito*	Sub-Saharan Africa
	Ross River	*Mosquito*	Oceania
	Sindbi	*Mosquito*	Northern Europe, Asia, Africa, Oceania

Font: Cleton et al., 2012; Pastula et al., 2016.

Box 3. Publications on arboviruses and large sport events in Brazil.

Title	*Author*	*Year*
Zika is not a reason for missing the Olympic Games in Rio de Janeiro: response to the open letter of Dr Attaran and colleagues to Dr Margaret Chan, Director - General, WHO, on the Zika threat to the Olympic and Paralympic Games.	Codeço et al.	2016
Travelers to the FIFA world cup 2014 in Brazil: Health risks related to mass gatherings/sports events and implications for the Summer Olympic Games in Rio de Janeiro in 2016	Eberhardt et al.	
The risk of dengue for non-immune foreign visitors to the 2016 summer olympic games in Rio de Janeiro, Brazil	Ximenes et al.	
Rapid Spread of Zika Virus in The Americas - Implications for Public Health Preparedness for Mass Gatherings at the 2016 Brazil Olympic Games.	Pertersen et al.	
Potential exposure to Zika virus for foreign tourists during the 2016 Carnival and Olympic Games in Rio de Janeiro, Brazil	Burattini et al.	
A crucial time for public health preparedness: Zika virus and the 2016 Olympics, Umrah, and Hajj	Elachola et al.	
Countdown to the 2016 Olympic Games: A travel medicine checklist	Patel et al.	
Entry routes for Zika virus in Brazil after 2014 world cup: New possibilities	Salvador & Fujita.	2015
Dengue transmission during the 2014 FIFA World Cup in Brazil	Aguiar et al.	
Dengue, chikungunya and Zika and mass gatherings: What happened in Brazil, 2014	Gautred & Simon.	
Recommendations for Chilean travelers to the FIFA World Cup 2014 in Brazil	Perret & Weitzel.	2014
Travel to Brazil: analysis of data from the Boston Area Travel Medicine Network (BATMN) and relevance to travelers attending world cup and olympics.	Iliaki et al.	
The 2014 FIFA World Cup: communicable disease risks and advice for visitors to Brazil--a review from the Latin American Society for Travel Medicine (SLAMVI).	Gallego et al.	
Risk of symptomatic dengue for foreign visitors to the 2014 FIFA World Cup in Brazil	Massad et al.	
Risk of Dengue for Tourists and Teams during the World Cup 2014 in Brazil	van Panhuis et al.	
Persisting arthralgia due to Mayaro virus infection in a traveler from Brazil: Is there a risk for attendants to the 2014 FIFA World Cup?	Slegers et al.	
Illness in travelers returned from Brazil: the GeoSentinel experience and implications for the 2014 FIFA World Cup and the 2016 Summer Olympics.	Wilson et al.	
Health risks among travelers to Brazil: implications for the 2014 FIFA World Cup and 2016 Olympic Games	Wilson & Chen.	
Football fans and fevers: dengue and the World Cup in Brazil.	Harley & Viennet.	
Dengue outlook for the World Cup in Brazil: an early warning model framework driven by real-time seasonal climate forecasts.	Lowe et al.	
Dengue outlook for the World Cup in Brazil.	Massad et al.	

and human interaction is facilitated (Forattini 2002).

Tauil (2001) has highlighted population growth, migration, air travel, inadequate urbanization, poor functioning of health systems and population density as key factors to define standard viral transmission. Concern increases when a country is experiencing a triple epidemic (Dengue, Chikungunya and Zika), especially in Rio de Janeiro (RJ). The probable dengue cases in the country exceed 1 million (approximately 50% in the Southeast Region, being 47,000 of RJ), about 120,000 Zika occurrences (about 46,000 in the Southeast, 32,000 of RJ) and 64,000 of Chikungunya (about 4,000 in the Southeast, being 1,000 of RJ) (SVS 2016). As dengue epidemic, the intense expansion of reported cases of CHIKV and ZIKV infections, this latter associated with microcephaly and Guillain-Barré syndrome (Oliveira-Melo et al. 2016, Brazil et al. 2016), emerged as a major public health

problem, not only in Brazil but all over the world (Stahl et al. 2013, Vieira-Machado et al. 2014, Martelli et al. 2015, Cardona-Ospina et al. 2015).

Concern regarding infection of tourists with ZIKV has been demonstrated, especially in pregnant women, due to the possible neurological disorders occurring in the fetus. However, during the Olympic Games when we observed a large concentration of athletes, we believe that CHIKV infection is of greatest concern due to the morbidity associated to this disease. Symptoms of the infection were described by several authors as highly debilitating, since some individuals suffer intense pain, with persistent arthralgia and arthritis for months or even years (Borgherini et al. 2008, Lwande et al. 2015, Vijayan & Sukumaran 2016, Zeana et al. 2016). Given this fact, athletes who may be infected with CHIKV are at risk of having their athletic activities interrupted for undetermined period of time.

CONCLUSION

Several factors are related to the increasing number of cases of arboviral diseases in the world, including: (i) continued growth of the population, (ii) disorganized urbanization, (iii) failure in the control of arthropods such as mosquitoes and ticks, with expansion territorial vectors, (iv) increased travel, particularly by air, to the transport of people and goods; (v) deforestation and (vi) climate and environmental changes.

According to the WHO the incidence of dengue has increased 30 times in the last 50 years, with geographic expansion to new countries. An estimated of 50 to 100 million infections occur every year. The growing number of Dengue, Zika and Chikungunya cases in different Brazilian states, as well as Mayaro cases in states of the Midwest region of the country, confirms the potential simultaneous outbreak of these arboviruses; against which there are no effective antiviral drugs or vaccines available yet. As a result, there was an increasing risk that a large number of people can get infected by one of these arboviruses during the Olympic Games.

Finally, while the main concern is related to tourists at risk of acquiring the infection in Brazil, it is essential to consider the possibility of introducing new arboviruses associated with mosquitoes such as Japanese encephalitis (JBE) and Murray Valley (MVE) as well as those associated with ticks such as tick borne encephalitis (TBE) and the Kyasanur forest disease (KFD). contain a possible spread of an infectious disease.

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10.2 Co-autoria

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

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ORIGINAL ARTICLE

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

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

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

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

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

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

Introduction

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

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

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

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

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ORIGINAL ARTICLE

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

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

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

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

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

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

Introduction

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

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

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

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10.2.3 *First Report of the East-Central South African Genotype of Chikungunya Virus in Rio de Janeiro, Brazil.*

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Research Article
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First Report of the East-Central South African Genotype of Chikungunya Virus in Rio de Janeiro, Brazil

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Abstract

Background: Chikungunya virus (CHIKV) is an arbovirus that causes an acute febrile syndrome with a severe and debilitating arthralgia. In Brazil, the Asian and East-Central South African (ECSA) genotypes are circulating in the north and northeast of the country, respectively. In 2015, the first autochthonous cases in Rio de Janeiro, Brazil were reported but until now the circulating strains have not been characterized. Therefore, we aimed here to perform the molecular characterization and phylogenetic analysis of CHIKV strains circulating in the 2016 outbreak occurred in the municipality of Rio de Janeiro.

Methods: The cases analyzed in this study were collected at a private Hospital, from April 2016 to May 2016, during the chikungunya outbreak in Rio de Janeiro, Brazil. All cases were submitted to the Real Time RT-PCR for CHIKV genome detection and to anti-CHIKV IgM ELISA. Chikungunya infection was laboratorially confirmed by at least one diagnostic method and, randomly selected positive cases (n=10), were partially sequenced (CHIKV E1 gene) and analyzed.

Results: The results showed that all the samples grouped in ECSA genotype branch and the molecular characterization of the fragment did not reveal the A226V mutation in the Rio de Janeiro strains analyzed, but a K211T amino acid substitution was observed for the first time in all samples and a V156A substitution in two of ten samples.

Conclusions: Phylogenetic analysis and molecular characterization reveals the circulation of the ECSA genotype of CHIKV in the city of Rio de Janeiro, Brazil and two amino acids substitutions (K211T and V156A) exclusive to the CHIKV strains obtained during the 2016 epidemic, were reported.

Keywords: Brazil, Chikungunya, ECSA genotype, Rio de Janeiro, Surveillance

Introduction

Chikungunya virus (CHIKV) is an arbovirus belonging to the Togaviridae family, Alphavirus genus causing an acute febrile syndrome with severe and debilitating arthralgia [1,2,3,4]. The viral particle is approximately 60-70 nm in diameter and composed of an icosahedral capsid surrounded by a lipid envelope. The viral genome consists of a single stranded positive sense RNA of 12kb in length, which encodes four non-structural proteins (NSP1-4) and five structural proteins (C, E3, E2, 6K and E1) [5,6,7].

10.2.4 Dengue type 4 in Rio de Janeiro, Brazil: case characterization following its introduction in an endemic region.

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BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Dengue type 4 in Rio de Janeiro, Brazil: case characterization following its introduction in an endemic region

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Abstract

Background: Due to the populations' susceptibility, DENV-4 introduction in 2010 led to the occurrence of explosive epidemics in the following years in Brazil. In 2011, DENV-4 was identified in Rio de Janeiro (RJ) and it was prevalent in 2012 and 2013. Here, we aimed to characterize clinical, epidemiological and laboratorial aspects of DENV-4 cases after this serotype introduction in an endemic scenario.

Methods: Dengue suspected cases (n = 3727) were received and analyzed from January 2011 to December 2013, during outbreaks occurred in RJ, Brazil. Samples were submitted to virological, serological and molecular methods for case confirmation. DENV-4 cases (n = 705) were characterized according to the type of infection, disease severity and, viremia levels and NS1 antigenemia were accessed. Representative strains were partial sequenced for genotyping.

Results: DENV-4 was identified in 44.2% (705/1593) of dengue positive cases, virus isolated in 48.7% of the cases. Anti-DENV IgM was detected in 39.4% of the cases, however an increased detection was observed in cases with ≥4 days of symptoms (57.0%). NS1 antigen was identified in 41.5% of DENV-4 cases however, after immune complexes dissociation, the detection significantly increased (87.6%). Females were more affected than males, so did children aged 11–15 years old. Primary cases were more frequently observed than secondary ones and most of them were classified as dengue. No differences on NS1 antigenemia and viraemia within the groups were observed. Despite the higher frequency of severe disease on individuals >65 years old, no differences were observed among the groups and type of infection. However, DENV-4 fatal cases were more frequent on secondary infections (57.1%). DENV-4 Genotype II was identified with a probable origin from Venezuela and Colombia.

Conclusions: It has been shown that laboratorial diagnosis is still a reliable tool for the disease surveillance, detecting and confirming emerging epidemics. Despite the occurrence of secondary infections, most DENV-4 cases presented a mild disease. As RJ is endemic for dengue, high rates of secondary infections would be expected. Despite the existence of two genotypes, only Genotype II was identified in our study.

Keywords: Dengue virus type 4, Laboratorial diagnosis, Phylogeny, Endemic, Rio de Janeiro, Brazil

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10.2.5 *Analysis of Clinical and Laboratory Alterations Related to Dengue Case Severity: Comparison between Serotypes 2 and 4 in Brazil*

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Analysis of Clinical and Laboratory Alterations Related to Dengue Case Severity: Comparison between Serotypes 2 and 4 in Brazil

Nieli Rodrigues da Costa Faria,[1,2] Victor Edgar Fiestas Solorzano,[2] Luiz José de Souza,[3] Rita Maria Ribeiro Nogueira,[1] Fernanda de Bruycker-Nogueira,[1] Thaís Chouin-Carneiro,[1] Jaqueline Bastos Santos Simões,[1] Monique da Rocha Queiroz Lima,[1] Luzia Maria de Oliveira Pinto,[2] Claire Fernandes Kubelka,[2] Manoela Heringer,[1] Elzinandes Leal de Azeredo,[2]† and Flavia Barreto dos Santos[1]*†

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Abstract. The increase in severe dengue (SD) cases has caused great impact on public health and has concerned authorities of countries where the disease is endemic and epidemics reach high proportions. The recognition of progression signs of this severe disease during the initial febrile phase can be difficult, since the symptoms are often indistinguishable from other febrile diseases. The aim of this study was to evaluate the clinical manifestations and laboratory findings in patients from two dengue outbreaks and their association with the disease. The study was conducted in patients (n = 153) with signs and symptoms consistent with dengue occurred during two distinct epidemics, 2010 and 2013, in the city of Campos dos Goytacazes, Rio de Janeiro, Brazil. According to the 2009 World Health Organization criteria, patients were classified as dengue without warning signs ([DwoWS] 60.6%, 57/94), dengue with warning signs ([DwWS] 30.9%, 29/94), and SD (4.25%, 4/94). Patients with DwWS/SD presented lower platelet and leukocyte counts and higher transaminase levels when compared with the DwoWS ones. Interestingly, patients from the epidemic of 2010 caused by dengue virus 2 (DENV-2) had lower platelet counts than patients of the 2013 epidemic caused by DENV-4. Furthermore, plasma leakage, gastrointestinal bleeding, and pleural effusion, hallmarks for a more severe disease, were also more frequently observed in those cases. Although previous studies may have extensively reported the wide range of the clinical aspects of dengue, the characterization of DENV-4 is desirable considering the burden of the disease during epidemics, especially for the health units and hospitals performing patient's management.

INTRODUCTION

Dengue is currently considered globally as the most important mosquito-borne viral disease, and the incidence has grown dramatically around the world in recent decades. It is estimated that 3.9 billion people in 128 countries are at risk of dengue infection.[1] One recent estimate indicates that 390 million dengue infections occur per year, of which 96 million manifest clinically with any disease severity.[2]

Demographic changes, urbanization, and international travel contribute to the expansion of geographical areas where transmission occurs, and all four dengue virus (DENV) serotypes are now circulating in Asia, Africa, and the Americas.[3] Not only is the number of cases increasing as the disease spreads to new areas, but explosive outbreaks are also occurring. Currently, Brazil accounts for approximately 70.0% of reported cases of dengue in the Americas.[4]

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

In 2010 and 2013, a total of 1,011,548 and 1,452,489 dengue cases were reported in Brazil, with RJ representing 2.9% (29,824) and 14.7% (213,058) of those cases, respectively.[10]

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

The white blood cell (WBC) count can reveal a leukopenia and neutropenia, lymphocytosis with lymphocytic atypia, and monocytosis. Signs and symptoms such as severe abdominal pain and continuous, persistent vomiting, postural hypotension, hepatomegaly, mucosal bleeding, increased hematocrit, or abrupt drop of platelets, are considered as potential hallmarks of the disease severity.[13] Repeated monitoring of the platelet count and hematocrit is recommended, as an abrupt decrease in platelet counts is an alarm sign and a significant hematocrit increase is an indirect sign of plasma leakage.[14] In this study, we aimed to analyze the laboratory and clinical findings during two distinct epidemics that occurred in 2010 and 2013, caused by DENV-2 and DENV-4, respectively, in Campos dos Goytacazes, RJ, Brazil.

MATERIALS AND METHODS

Ethics statement. The samples were collected as part of an ongoing project approved by resolution number

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10.2.6 Dengue Virus Serotype 2 Established in Northern Mozambique (2015-2016).

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Dengue Virus Serotype 2 Established in Northern Mozambique (2015–2016)

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Abstract. After the report of an outbreak of dengue virus serotype 2 in 2014 in Nampula and Pemba cities, northern Mozambique, a surveillance system was established by the National Institute of Health. A study was performed during 2015–2016 to monitor the trend of the outbreak and confirm the circulating serotype of dengue virus (DENV). After the inclusion of consenting patients who met the case definition, samples from 192 patients were tested for the presence of nonstructural protein 1 antigen, and 60/192 (31%) samples were positive. Further analysis included DENV IgM antibodies, with 39 (20%) IgM positive cases. Reverse transcriptase (RT) PCR was performed for identification of the prevailing DENV serotype; 21/23 tested samples were DENV-2 positive, with DENV-2 present in both affected cities. When sequencing DENV, phenotype Cosmopolitan was identified. The surveillance indicates ongoing spread of DENV-2 in northern Mozambique 2 years after the first report of the outbreak.

Dengue is an expanding mosquito-borne viral disease, with an increasing impact on global health. Over 50% of the population in subtropical and tropical regions are at risk of contracting the disease.[1] Dengue virus (DENV) is considered to be the most important arbovirus based on its fast spread combined with its high burden of morbidity and mortality.[2]

DENV is a positive single-stranded RNA virus belonging to the genus flavivirus and the family *Flaviviridae*. It has four antigenically related but distinct serotypes DENV-1, DENV-2, DENV-3, and DENV-4.[2] DENV diversity is vast, and within each serotype, distinct genotypes can be identified and have been correlated with geographical distribution of the virus. An association between disease severity and genetical differences within the genotype has also been reported.[3]

The primary vector of the DENV is the *Aedes aegypti* mosquito, but it is also transmitted by *Aedes albopictus*. The disease is self-limiting but can sometimes be severe. Clinical symptoms include fever, headache, retro-orbital pain, nausea, vomiting, myalgia, arthralgia, thrombocytopenia, rash, and mucosal bleeding from the gums, nose, and the gastrointestinal system.[4] Dengue is endemic in parts of Africa, Asia, and the Americas with favorable conditions for vector multiplication, disease transmission, and spread. High temperature, heavy rainfall, inadequate waste disposal management, unplanned urbanization, high population density, traveling, and global mobility are some of the factors that have contributed to the increasing global spread of DENV.[5]

In Africa, simultaneous circulation of DENV-1, DENV-2, and DENV-3 has been described in Gabon.[6] DENV outbreaks have been reported from several countries in East Africa, the earliest reports dating back as far as 1823.[7] The first recorded outbreak of DENV in Mozambique occurred in Pemba between late 1984 and early 1985.[8] The circulating serotype in this outbreak was also the first documented case of DENV-3 in Africa. Not until about three decades later, in March 2014, a DENV outbreak was reported for the second time in Mozambique, again in the northern parts of the country.[9] The circulating serotype was identified as DENV-2. In 2014, the National Institute of Health in Mozambique established a surveillance system of DENV in the affected cities Nampula and Pemba, with the objective to monitor the trend in DENV prevalence and provide evidence for decision making on interventions for controlling DENV in northern Mozambique.

Nampula and Pemba are the capital cities of Nampula and Cabo Delgado provinces in northern Mozambique, with a population of approximately 470,000 in Nampula and 140,000 inhabitants in Pemba. Both cities are strategic urban centers and melting points of different cultures, with heavy traffic of people from and to different countries and booming commercial activities. The tropical climate is favorable for mosquitoes to breed especially during the rainy season from December to March.

A cross-sectional study on patients with suspected DENV infection was conducted at Nampula Central Hospital (NCH) and Pemba Provincial Hospital (PPH) from January 2015 until March 2016. Both hospitals are tertiary health facilities that offer health care services to a cross section of the society, ranging from high income earners to middle- and low-income earners.

To be eligible for inclusion in this study, patients had to have fever 37.5°C or above (axillary temperature) for a maximum of 7 days duration and of unknown etiology. Cases diagnosed with malaria, cellulitis, otitis media, and pneumonia, which are readily identifiable infections, were excluded. In addition, participants had to be above 5 years of age. A written informed consent was obtained from all patients, or parents of children, included at the emergency unit of NCH and PPH during the study period. For all consenting participants, a questionnaire on clinical and sociodemographic data was filled in by trained health personnel.

A total of 290 samples were collected from the 290 patients initially included in the study, (152 from Pemba and 138 from Nampula) between January 2015 and March 2016. Based on the availability of rapid tests at the emergency unit of both study sites (PPH and NCH), 192/290 patients were analyzed for nonstructural protein 1 (NS1) antigen, 108 from Pemba and 84 from Nampula. These 192 patients with available NS1 antigen results were then included in the further analysis according to the test algorithm in Figure 1.

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