INSTITUTO OSWALDO CRUZ

CURSO DE PÓS-GRADUAÇÃO EM BIOLOGIA PARASITÁRIA

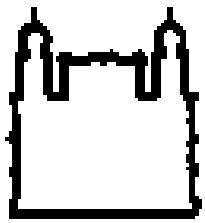
Vigilância virológica dos vírus dengue: genotipagem e caracterização molecular de vírus isolados em mosquitos naturalmente infectados e humanos,

1986-2011

MÁRCIA GONÇALVES DE CASTRO

Rio de Janeiro

2012



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Márcia Gonçalves de Castro

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Orientadora: Drª. Flávia Barreto dos Santos

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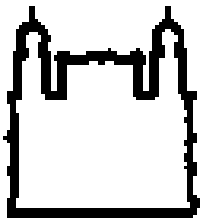

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Trabalho realizado nos Laboratórios de Transmissores de Hematozoários e de Flavivírus do Instituto Oswaldo Cruz (IOC) na Fundação Oswaldo Cruz sob a orientação da Dra. Flávia Barreto dos Santos e colaboração da Dra. Rita Maria Ribeiro Nogueira e Dr. Ricardo Lourenço de Oliveira com o apoio da Fundação Oswaldo Cruz (FIOCRUZ), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Aos meus sobrinhos Michelle, Jonathan e Max Junior.

"Se você quer transformar o mundo, experimente primeiro promover o seu aperfeiçoamento pessoal e realizar inovações no seu próprio interior."

(Dalai-Lama)

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SUMÁRIO

Publicações durante a vigência do doutorado relacionadas à infecção experimental com o vírus dengue e a biologia do *Ae.aegypti*.

- ✓ First record of *Aedes albopictus* (Díptera: Culicidae) in the state of Roraima, Brazil. Acta Amazonica. 2008; 38: 357-60.
- ✓ Seasonal dynamics of *Aedes aegypti* (Diptera: Culicidae) in the northernmost state of Brazil: a likely port-of-entry for dengue vírus 4.
- ✓ *Aedes aegypti* and *Aedes albopictus* spatial distribuition pattern in a habitat transition zone in Rio de Janeiro, Brazil.
- ✓ Dengue infection increases the locomotor activity of *Aedes aegypti* females.
- ✓ Potential impact of a presumed increase in the biting activity of dengue-virus-infected *Aedes aegypti* (Diptera: Culicidae) females on virus transmission dynamics.

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Siglas e Abreviaturas

3´NC	3´não codificante (3´non-coding region)
5´NC	5´não codificante (5´non-coding region)
3'SL	3' stem-loop
C	Proteína estrutural do capsídeo do vírus
cDNA	Ácido desoxirribonucleico complementar
CS1	Sequência conservada 1 (conserved sequence – CS1)
CS2	Sequência conservada 2 (conserved sequence – CS2)
d.C.	Depois de Cristo
DC	Dengue clássico (classic dengue)
DC-SIGN	Ligante de molécula de adesão intercelular não integrina específica de DC (*DC-specific ICAM-grabbing nonintegrin*)
DENCO	do inglês *Dengue Control*
DENV	Vírus dengue (dengue virus)
DENV-1	Sorotipo 1 do vírus dengue (dengue virus serotype 1)
DENV-2	Sorotipo 2 do vírus dengue (dengue vírus serotype 2)
DENV-3	Sorotipo 3 do vírus dengue (dengue vírus serotype 3)
DENV-4	Sorotipo 4 do vírus dengue (dengue vírus serotype 4)
DNA	Ácido desoxirribonucleico (deoxyribonucleic acid)
E	Proteína do envelope (envelope protein)
ECP	Efeito citopático (cytopathic effect)
E/NS1	Proteína do envelope/Proteína não estrutural 1
ELISA	Ensaio imunoenzimático (*enzime linked imunosorbent assay*)
FHD	Febre hemorrágica da dengue
ICAM-3	Molécula de adesão intercelular 3 (*Intercellular adhesion molecule 3*)
IFN	Interferon
IgG	Imunoglobulina G (Immunoglobulin G)
IgM	Imunoglobulina M (Immunoglobulin M)
IgG-Elisa	Teste imunoenzimático Elisa-IgG (immunological tests IgG-Elisa
Kb	Kilobase
kDa	Kilodálton

MAC-ELISA	Ensaio imunoenzimático para captura de anticorpos IgM
M	Proteína de membrana (membrane protein)
MV	Máxima verossimilhança *(* ML - *Maximum Likelehood)*
N-terminal	Amino-terminal
NS	Proteína não estrutural (non-structural protein NS)
NS1	Proteína não estrutural 1 (non-structural protein NS1)
NS2A	Proteína não estrutural 2A (non-structural protein NS2A)
NS2B	Proteína não estrutural 2B (non-structural protein NS2B)
NS3	Proteína não estrutural 3 (non-structural protein NS3)
NS4A	Proteína não estrutural 4A (non-structural protein NS4A)
NS4B	Proteína não estrutural 4B (non-structural protein NS4B)
NS5	Proteína não estrutural 5 (non-structural protein NS5)
NTPase	Nucleosídeo 5'-trifosfatase
OMS	Organização Mundial de Saúde (World Health Organization – WHO)
ORF	Fase aberta para leitura (*Open reading frame*)
Pb	Pares de bases
PCR	Reação em cadeia pela polimerase (*polymerase chain reaction*)
PrM/M	Proteínas estruturais Pré-membrana/Membrana
RdRP	RNA polimerase RNA dependente
RE	Retículo endoplasmático
RCS2	Sequência repetida conservada
RNA	Ácido ribonucleico
RTPase	RNA 5'-trifosfatase
RT-PCR	Transcrição reversa seguida da reação em cadeia pela polimerase
SCD	Síndrome do Choque por Dengue
SVS	Secretária de Vigilância em Saúde
VR	Região variável (variable region – VR)

RESUMO

O dengue tem se apresentado como um grave problema de saúde pública no Brasil, razão pela qual, vários estudos têm sido realizados com o intuito de esclarecer aspectos da epidemiologia dessa doença em diferentes localidades, com histórias distintas de circulação dos diferentes sorotipos dos vírus dengue (DENV). A implantação de um Programa de vigilância entomológica e virológica e, que desde 1986 visa detectar e monitorar a circulação dos sorotipos e genótipos DENV, resultou em distintas oportunidades, no isolamento de amostras de DENV de vetores e de casos humanos permitindo a caracterização molecular e a análise filogenética, fornecendo informações relevantes para a compreensão da interação vetor- vírus- humanos. O entendimento da variação genética no vírus quando este replica em mosquitos, e como essas variações atuam durante a transmissão entre humanos e mosquitos permanecem desconhecidos. Portanto, visando contribuir para um melhor conhecimento dos DENV e sua interação com o mosquito vetor, realizamos neste trabalho, a caracterização molecular e análise filogenética de DENV isolados de mosquitos naturalmente infectados e de casos humanos, provenientes de epidemias ocorridas entre 1986 e 2011 no Brasil. Foi demonstrado que os métodos moleculares foram fundamentais por facilitarem a rápida identificação dos vírus e consequentemente o monitoramento dos genótipos circulantes. A RT-PCR para a triagem de DENV em vetores se mostrou uma ferramenta útil para a vigilância virológica, com taxas de detecção que variaram de 0,78% a 25% no período estudado. A análise filogenética dos DENV-1 isolados de mosquitos e humanos mostrou que o genótipo V (Américas/África) continua o mesmo circulante desde a sua introdução, porém foi demonstrada a co-circulação de duas novas linhagens (II e III) no período de 2009 a 2011. O sequenciamento do genoma completo de DENV-3 isolado a partir de *Ae. aegypti* naturalmente infectados no Rio de Janeiro (RJ), assim como a análise da região 3´NC de vírus isolados em mosquitos e humanos, caracterizou estes vírus como pertencentes ao GIII e revelou a presença de inserções e deleções na região 3´NC do genoma. As deleções observadas na região 3´NC resultaram em estruturas secundárias porém nem todas as cepas com inserções nesta região apresentaram estrutura similar substituições exclusivas à cepa de DENV-3 isolada em mosquito foram observadas no gene NS5, incluindo a substituição que resultou na formação de um códon de terminação. O teste comercial Simplexa™ Dengue Real Time RT-PCR, disponível recentemente, foi utilizado pela primeira vez para detecção dos DENV e se mostrou um método molecular alternativo para as vigilâncias entomológica e virológica. O RT-PCR em Tempo Real possibilitou, pela primeira vez, a quantificação de DENV-1 e DENV-4 em fêmeas individuais naturalmente infectadas ($1,6 \times 10^4$ cópias/mL e $1,08 \times 10^3$ cópias/mL, respectivamente). Considerando-se o elevado índice de infestação por *Ae. aegypti* em todo o país, o estudo da caracterização dos DENV circulantes torna-se de grande importância no conhecimento da relação vírus-vetor pela análise da variabilidade genética, dispersão e persistência de genótipos durante a transmissão destes vírus.

ABSTRACT

Dengue has been a major public health problem in Brazil with several studies performed aiming to elucidate the disease epidemiology in geographically distinct areas with different dengue viruses (DENV) circulation. The establishment of an entomological and virological program since 1986 with the objective of detecting and monitoring DENV serotypes and genotypes resulted in distinct opportunities in DENV isolation from vectors and human cases, allowing the molecular characterization and phylogenetic analysis, providing relevant information for the understanding of the vector-virus-humans interactions. The understanding of the virus genetic variability when it replicates on mosquitoes and how those variations act during the transmission between humans and mosquitoes is not fully understood. Therefore, aiming to contribute for a better understanding of DENV and its interactions with the mosquito vector, we performed in this study the molecular characterization and phylogenetic analysis of viruses isolated from naturally infected mosquitoes and human cases, from epidemics occurred between 1986 and 2011 in Brazil. It has been shown that the molecular techniques were essential for allowing the rapid identification of the viruses and consequently the monitoring of the circulating genotypes. The RT-PCR for DENV screening in vectors has shown to be a useful tool for the virological surveillance, with detection rates varying from 0.78% to 25% in the studied period. The phylogenetic analysis from DENV-1 isolated from mosquitoes and human cases showed that genptype V (America/Africa) is still the same genotype circulating since this serotype introduction, however it was demonstrated the co-circulation of two distinct new viral lineages (II and III) from 2009 to 2011. The complete genome sequencing of a DENV-3 isolated from naturally infected *Ae. aegypti* in Rio de Janeiro (RJ) and the analysis of the 3´UTR region from viruses isolated from mosquitoes and humans, has characterized those viruses as belonging to genotype III (GIII) and revealed the presence of insertions and deletions in the 3´UTR region of the genome. The deletions observed in the 3´UTR region resulted in similar secondary structures, however not all strains with insertions were similar in structure. Exclusive substitutions to the DENV-3 isolated from the mosquitoes were observed in NS5, including a substitution leading to a stop codon formation. The Simplexa™ Dengue Real Time RT-PCR commercial kit, recently available, was used for the first time for DENV detection and it has been shown to be an alternative molecular method for the entomological and virological surveillances, The Real Time RT-PCR has allowed, for the first time the DENV-1 and DENV-4 quantification in single *Ae. aegypti* naturally infected (1.6×10^4 copies/mL e 1.08×10^3 copies/mL, respectively). Considering the high *Ae. aegypti* infestation index in the country, the characterization of DENV circulating is very relevant for the understanding of the virus-vector relations by the analysis of the genetic variability, spread and persistence of genotypes during the transmission of those viruses.

1. Introdução

1.1. Dengue: Aspectos Históricos

Durante a Dinastia Chin, nos anos de 265 a 420 d.C., foram descritos os sintomas de uma enfermidade que os chineses chamaram de "veneno da água", associando insetos voadores e água. Estes achados foram formalmente editados em uma enciclopédia médica chinesa durante a Dinastia Tang, no ano de 610 d.C., e a Dinastia Norte Sung, no ano de 992 d.C. (Gubler 1998, 2006).

Os primeiros relatos de grandes epidemias de uma doença que possivelmente tratava-se de dengue datam de 1779 e 1780 e foram relatadas em três continentes, Ásia, África e América do Norte, porém, há registros da ocorrência desta doença no século III. Outros possíveis surtos de dengue ocorreram em 1635, nas Índias Ocidentais, e em 1699, no Panamá (McSherry 1982).

A origem da palavra dengue, segundo Vambery (apud Siler et al 1926), seria do árabe arcaico, significando fraqueza (astenia). Nas línguas portuguesa e espanhola, que possuem influência moura, existe a palavra dengue significando afetação (Figueiredo e Fonseca 1996), referindo-se aos sintomas da doença ou ao comportamento praticado em virtude dela. O nome poderia ter surgido em Zanzibar, durante uma epidemia que ocorreu em 1870, e estaria relacionado à frase nativa *Ki-denga Pepo*, que significa pancada ou golpe dado por um mau espírito, provocando ataque doloroso semelhante à câimbra (Halstead 1971, Figueiredo e Fonseca 1996, Gubler e Kuno 1997).

Por outro lado, a origem do vírus dengue (DENV) é objeto de muita discussão. Uma das hipóteses seria que o vírus teria evoluído nos mosquitos antes de se adaptar aos primatas não humanos e humanos. A transmissão do DENV pelo *Aedes (Stegomyia) aegypti* (Linnaeus 1762) foi demonstrada em 1906 por Bancroft, sendo confirmado por estudos subsequentes (Siler et al 1926, Rosen et al 1954). A partir do século XVII, devido ao transporte de escravos oriundos da África, o *Ae. aegypti* foi levado para os demais continentes e as epidemias de dengue tornaram-se mais freqüentes e disseminadas (Holmes et al 1998).

As primeiras amostras dos DENV foram isoladas em 1944 durante a Segunda Guerra Mundial, a partir de soros de soldados que contraíram a infecção em Calcutá (Índia), Nova Guiné e Havaí (Sabin 1952). Os vírus provenientes da Índia, do Havaí e de uma das cepas de Nova Guiné foram antigenicamente semelhantes e denominados DENV-1. Atualmente, a cepa Havaí é considerada amostra protótipo. Outras cepas de Nova Guiné apresentaram características antigênicas diferentes, permitindo a identificação de outro sorotipo, que foi classificado como DENV-2, hoje considerado protótipo. Posteriormente, dois novos vírus foram isolados durante uma epidemia ocorrida em Manila (1953). Estes vírus foram classificados como DENV-3 e DENV-4 (Hammon et al 1960) e as cepas H87 (DENV-3) e H241 (DENV-4) consideradas protótipos.

Foi, contudo, após a II Guerra Mundial que ficou evidente a mudança no comportamento da doença. O crescimento populacional, a urbanização descontrolada, a falta de programas efetivos para o controle do vetor e o aumento das viagens comerciais contribuíram para a expansão geográfica do mosquito transmissor e do vírus, permitindo o estabelecimento de uma pandemia que persiste até hoje (Gubler 1997).

Do ponto de vista geográfico, o DENV é o arbovírus mais difundido no mundo, sendo encontrado em áreas tropicais e subtropicais, em mais de 100 países e onde 2.5 bilhões de pessoas vivem em áreas endêmicas com a co-circulação de vários sorotipos, (Guzman e Kouri 2002, Gubler 2006, Rico-Hesse 2007, Burdino et al 2011).

O aumento na incidência e distribuição geográfica do dengue tem levado a transmissão do vírus com uma periodicidade marcante em surtos graves em quase todas as áreas endêmicas e hiperendêmicas. Essa dinâmica pode ser também constatada a partir da análise comparativa de sequências dos genes virais de amostras obtidas, em uma escala espaço-temporal. Tais comparações consistem em uma poderosa ferramenta para se entender os mecanismos evolutivos, ecológicos e epidemiológicos de emergência de epidemias e da dinâmica da transmissão do vírus (Bennett et al 2010).

1.2. Ciclos de Transmissão do Dengue

O homem, os primatas não-humanos e os mosquitos do gênero *Aedes* são os hospedeiros naturais do DENV, sendo o homem o único a desenvolver a forma clínica da doença (Gubler 1998, 2002).

Os quatro sorotipos de DENV são mantidos em dois ciclos de transmissão distintos: o silvestre e o humano (Figura 1). Os ciclos silvestres têm sido documentados no Sudeste da Ásia e na África, e envolvem várias espécies de primatas inferiores e mosquitos de três subgêneros do gênero *Aedes* (*Stegomyia*, *Finlaya* e *Diceromyia*). Até a presente data, não existem evidências do ciclo de transmissão silvestre nas Américas (Gubler e Kuno 1997, Vasilakis et al 2010, Chen e Vasilakis, 2011).

Atualmente, quase todas as infecções humanas são devidas a cepas de DENV que circulam exclusivamente nos ambientes doméstico e peri-domésticos nos trópicos, onde os humanos servem como os hospedeiros de amplificação e de reservatório. No ciclo humano, mosquitos *Ae. aegypti* transmitem o DENV (Mattingly 1957), enquanto que outras espécies, tais como *Ae. albopictus* e *Ae. polynesiensis*, servem como vetores secundários (Gubler e Rosen 1976, Gubler e Kuno 1997, Lambretchs et al 2010, Chen e Vasilakis 2011).

O isolamento dos quatro sorotipos de DENV em diversos mamíferos de florestas tais como morcegos, roedores e marsupiais provavelmente representam conseqüências do ciclo de transmissão humano (de Thoisy et al 2004, 2009).

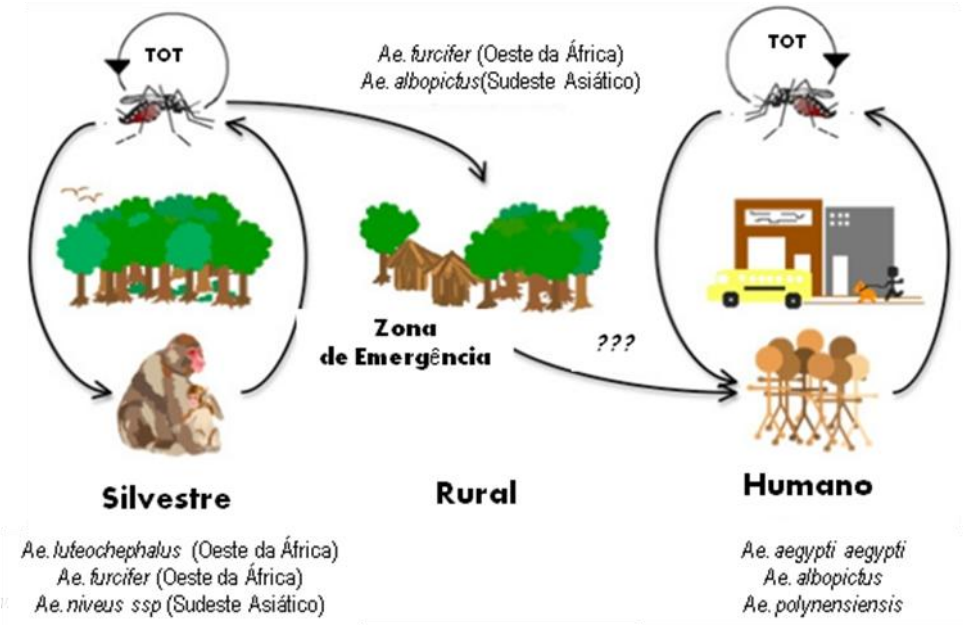

Figura 1: Ciclo de transmissão dos vírus dengue (DENV). Ciclo silvestre e zona de emergência onde o ciclo silvestre entra em contato com populações humanas em áreas rurais no Oeste da África e Sudeste Asiático (Adaptado de Chen e Vasilakis, 2011). TOT: transmissão transovariana.

1.3. Vetores

Os vetores *Ae. aegypti* e *Ae. albopictus* (*Skuse,1894*), pertencentes ao subgênero *Stegomyia*, naturais do Velho Mundo, invadiram vários países fora de sua distribuição zoogeográfica original, incluindo o Brasil (Consoli e Lourenço-de-Oliveira 1994).

No Brasil, a distribuição do *Ae. aegypti* está associada aos ambientes alterados pelo homem, sendo, essencialmente, um mosquito do peridomicílio e domicílio humano (Christophers 1960). É uma espécie muito abundante em áreas urbanas e suburbanas, onde se observa concentração populacional humana elevada e cobertura vegetal moderada (Braks et al 2003). O *Ae. aegypti* possui hábitos diurnos, alimentando-se de sangue e colocando seus ovos, preferencialmente, ao amanhecer e próximo ao crepúsculo vespertino. As fêmeas colocam seus ovos preferencialmente em criadouros artificiais, como pneus usados, garrafas, latas, potes, vasos de planta e reservatórios de água destampados (caixas d'água, cisternas, tonéis), contendo água parada (Christophers 1960).

As populações de *Ae. aegypti* que existem no Brasil são muito suscetíveis aos DENV e ao vírus da Febre Amarela (Lourenço-de-Oliveira et al 2004). Já foram isolados em adultos e larvas deste mosquito coletados em várias partes do Brasil: DENV-1 (Nogueira et al 1988, Serufo et al 1993, Degallier et al 2000), DENV-2 e DENV-3 (Lourenço-de-Oliveira et al 2002, Pinheiro et al 2005, Figueiredo et al 2010, Castro dados não publicados) e DENV-4 (Osanai et al 1983, Castro dados não publicados)

A espécie *Ae. albopictus* foi primeiramente encontrado no Brasil na década de 1980 (Consoli e Lourenço-de-Oliveira 1994). Sua distribuição também está associada à presença humana, contudo, é um mosquito que se espalha nos ambientes urbano, suburbano e rural com considerável cobertura vegetal, não dependendo dos locais de grande concentração humana, como o *Ae. aegypti* (Hawley 1988, Braks et al 2003). *Aedes albopictus* apresenta ampla valência ecológica, evidenciada pela capacidade de colonizar os mais variados tipos

de recipientes, naturais e artificiais (Hawley 1988). A espécie ainda não foi incriminada como vetor natural do dengue no Brasil (Degallier et al 2003), apesar de já ter sido comprovado que, em condições de laboratório, as populações brasileiras desta espécie têm a capacidade de se infectar e transmitir o DENV e Febre Amarela (Miller e Ballinger 1988, Lourenço-de-Oliveira et al 2003, Castro et al 2004).

1.4. Interação Vetor-Vírus

Assim como em outras arboviroses, a dinâmica da replicação do DENV em mosquitos infectados oralmente é dependente da temperatura ambiente, de características genéticas tanto das populações dos mosquitos, quanto das amostras circulantes do vírus, e do título viral no sangue ingerido pelo vetor (Rodhain e Rose 1997). No caso de *Ae.albopictus*, onde há transmissão natural pela bactéria endossinbionte *Wolbachia*, verificou-se redução da taxa de infecção e disseminação do vírus dengue (Kittayapong et al 2002).

A infecção do mosquito pelos DENV se dá principalmente por via oral, também podendo ocorrer por vias secundárias, através da transmissão vertical do vírus de uma fêmea infectada para seus descendentes (Khin e Than 1983, Hull et al 1984, Angel e Joshi 2008).

Até poucos dias após a ingestão do vírus, a replicação deste fica normalmente limitada às células epiteliais do intestino médio do mosquito, invadindo, em seguida, outros tecidos, como o proventrículo, corpos gordurosos, hemócitos e tecidos nervosos essencialmente através do sistema traqueolar (Salazar et al 2007). Posteriormente, o vírus pode ser encontrado em grandes quantidades no cérebro, gânglios torácicos e abdominais e glândula salivar, além dos órgãos já supracitados (Rodhain e Rose 1997, Salazar et al 2007, Vazeille et al 2010).

O mosquito se infecta ao ingerir sangue de um indivíduo durante o período de viremia (cerca de cinco dias após a picada infectante) e pode transmitir a doença para um indivíduo susceptível depois de um período de incubação extrínseco de oito a 14 dias dependendo da temperatura ambiente. Uma vez infectado, o mosquito transmite o vírus durante toda a sua vida (Halstead 2008), e é capaz de transmitir o vírus a diversas pessoas, devido a sua capacidade de se alimentar em múltiplos indivíduos para completar o ciclo

gonotrófico. A quantidade de vírus no sangue do hospedeiro é crucial para o sucesso da infecção do mosquito (Gubler et al 2007).

1.5. Vírus Dengue e sua Organização Genômica

Os DENV pertencem ao gênero *Flavivírus*, família *Flaviviridae*, e podem ser encontrados na natureza como quatro sorotipos antigenicamente distintos, designados de DENV-1, DENV-2, DENV-3 e DENV-4 (Sabin 1952, Hammon et al 1960, Westaway et al 1985, Lindebach et al 2007). A infecção com um sorotipo confere imunidade permanente apenas contra este sorotipo, não conferindo imunidade cruzada para os outros sorotipos. Desse modo, indivíduos que vivem em áreas endêmicas de dengue podem se infectar com mais de um sorotipo ao longo da vida (Gubler 1998). São vírus esféricos, envelopados e com cerca de 40 a 50 nanômetros de diâmetro. O RNA é envolto por um nucleocapsídeo de simetria icosaédrica, composto por uma única proteína, a proteína de capsídeo (C) e circundada por uma bicamada lipídica associada às proteínas de membrana (M) e envelope (E) (Figura 2).

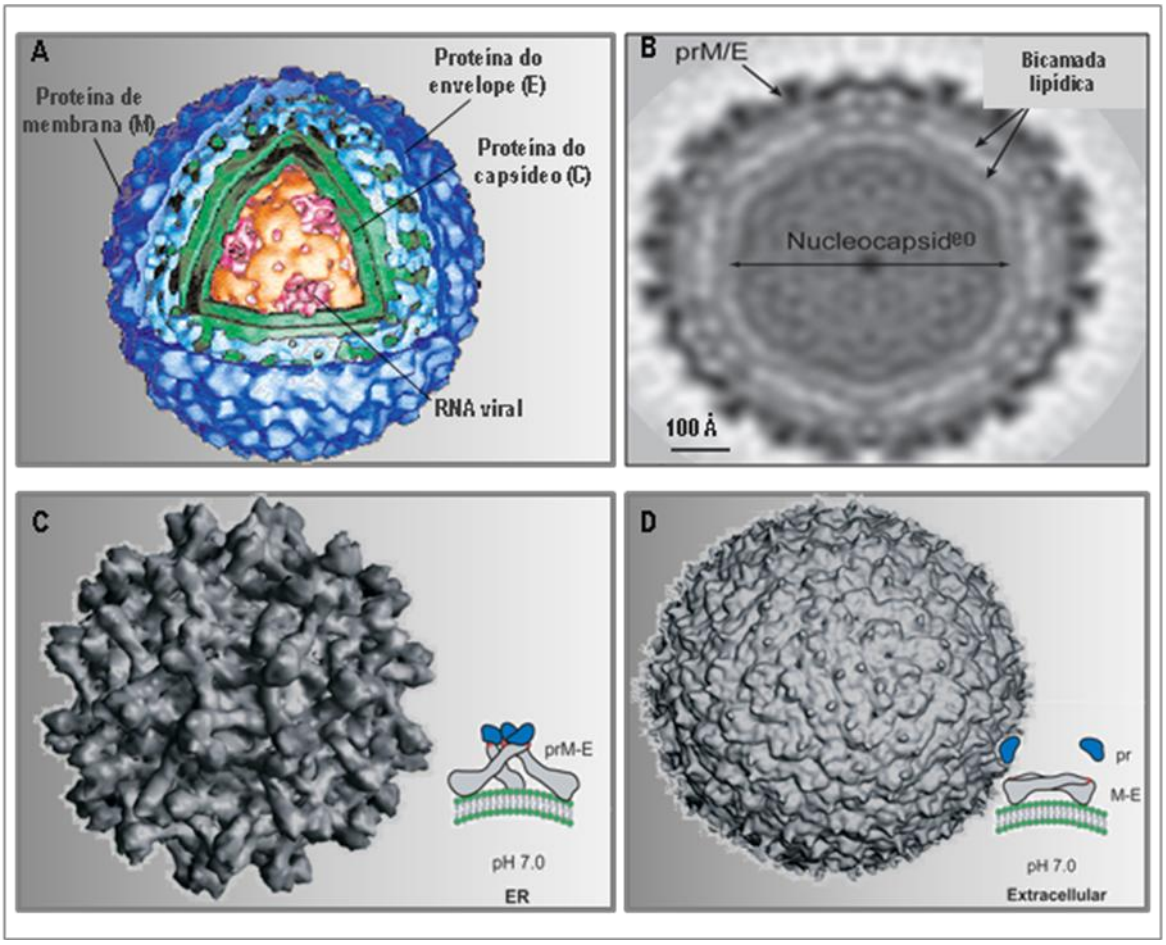

Figura 2: Vírus dengue. **(A)** Representação esquemática e **(B)** crioelectromicroscopia da partícula do vírus dengue (DENV). Organização das proteínas estrururais do envelope (E), de membrana (M) e do capsídeo (C). **(C)** Crioelectromiscropia da partícula viral imatura em pH neutro onde a proteína E existe como heterodímero com a prM. **(D)** Crioelectromicroscopia da partícula madura, após clivagem mediada por furina, o vírus maduro é liberado no meio extracelular e o peptídeo pr é liberado da partícula madura. Adaptado de http://www.sciencecentric.com/news/08032735-findings-reveal-how-dengue-virus-matures-becomes-infectious.html, acesso em 16/01/2012 e Perera e Kuhn, 2008.

O genoma é composto por uma molécula única de RNA de cadeia simples, de polaridade positiva, com aproximadamente 11.000 nucleotídeos, e uma única fase aberta de leitura (ORF - o*pen reading frame*) codificando uma poliproteina precursora. Proteases virais e do hospedeiro clivam a poliproteina cotraducionalmente para gerar o conjunto de proteínas estruturais [capsideo (C), precursora da proteína de membrana (prM), envelope (E)] e não-estruturais [NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5] (Figura 3).

Dentre as proteínas que compõem a partícula viral, a proteína C, de 11 kDa é de caráter básico, é a responsável por conferir a forma esférica à partícula viral. Além disso, na exposição do domínio de ligação da proteína E ao receptor celular (Heinz e Allison 2003), evitando que a proteína E sofra reorganização estrutural nos compartimentos acidificados da célula, e que ocorra a fusão prematura com membranas celulares durante a liberação da partícula viral (Zhang *et al* 2003; Heinz e Allison 2003).

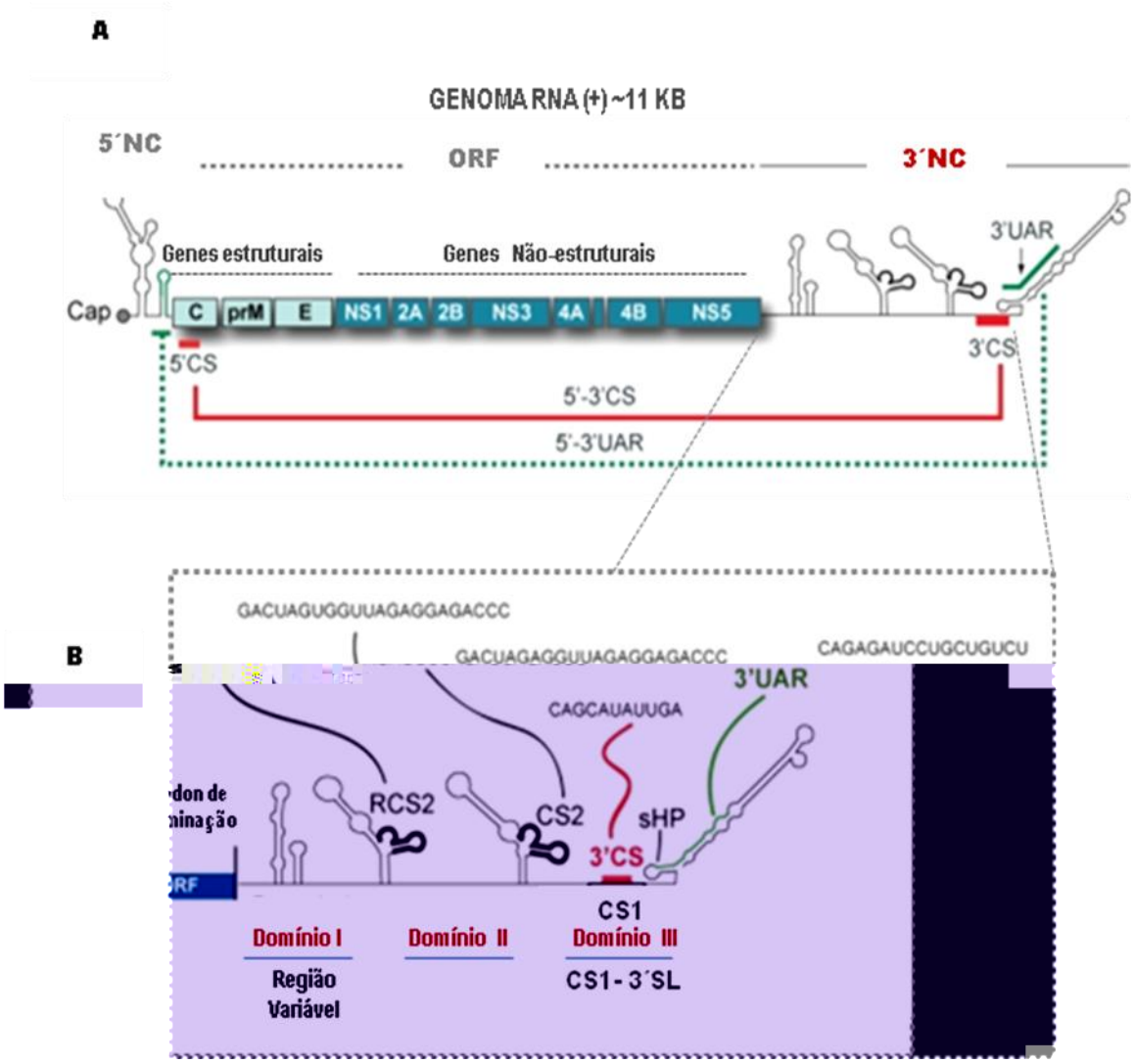

Figura 3: Organização do genoma do vírus dengue (DENV). **(A)** Regiões 5´ e 3´ não codificantes (NC) e a fase aberta de leitura (ORF) indicando os genes estruturais (C, prM, E) e não-estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5). **(B)** Esquema representativo dos elementos constituintes da região 3´NC dos DENV . As estruturas previstas para os três domínios estão indicados: domínio I (região variável [VR]), domínio

II e domínio III (sequência conservada CS1 e 3´stem-loop [3´SL]). Adaptado de Gebhard et al 2011.

A proteína precursora de membrana (prM) é glicosilada, tem 26 kDa, é clivada durante a replicação viral por uma protease do tipo furina, gerando a proteína estrutural M de 8kDa, que, juntamente com a proteína E, forma o revestimento externo da partícula viral (Chambers et al 1990; Kuhn et al 2002).

A proteína E é a principal proteína de superfície do virion, pois contém determinantes antigênicos que induzem à produção de anticorpos neutralizantes, interage com receptores celulares e participa do processo de fusão de membranas da célula e do envelope viral (Modis et al 2004). Estudos têm mostrado que mutações no gene viral que codifica a proteína E podem interferir na virulência (Uzcategui et al 2001, Leitmeyer et al 1999). Por meio de estudos de cristalografia, foi possível identificar que a proteína E possui três domínios: o primeiro (I) central, o segundo (II) contém a região de dimerização e o peptídeo de fusão, e o terceiro (III) apresenta o sítio de ligação ao receptor celular (Rey et al 1995, Chen et al 1997, Huang et al 2004, Modis et al 2005). Na partícula viral madura esta proteína se organiza em forma de homodímero (Modis et al 2004).

As proteínas não estruturais (NS) são encontradas dentro de células infectadas como parte de um complexo envolvido na replicação do RNA (Lai e Putnak 2006). A proteína NS1 (46 kDa) pode ser encontrada associada à célula, como possível cofator na replicação viral na superfície celular (Mackenzie et al 1996), onde pode estar envolvida na transdução de sinal (Jacobs et al 2000), ou na forma extracelular no hospedeiro vertebrado (Libraty et al 2002). A liberação desta proteína no meio extracelular induz forte produção de anticorpos específicos (não-neutralizantes), que são importantes para o diagnóstico. Os níveis de NS1 no soro de pacientes têm sido correlacionados com gravidade de doença (Avirutnan et al 2006). Além disso, essa proteína pode ter relevância na patogênese da dengue (Alcon-Leponder et al 2005, Libraty et al 2002, Clyde et al 2006), uma vez que anticorpos anti-NS1 podem reagir de forma cruzada com fibrinogênio, trombócitos e células endoteliais humanas (Falconar 1997).

A proteína NS3 (68 kDa) é a maior proteína citoplasmática associada com a membrana do retículo endoplasmático rugoso (RER) via interação com a NS2B (Arias

1993, Chambers et al 1990). É uma proteína multifuncional, apresenta as atividades de protease, helicase, nucleotídeo 5'-trifosfatase (NTPase) e RNA 5'-trifosfatase (RTPase) (Falgout et al 1991), fundamentais na replicação viral e na reação de *capping* do RNA viral (Chen et al 1997, Benarroch et al 2004).

A função das proteínas NS2A, NS4A e NS4B não está completamente elucidada. No entanto, elas parecem auxiliar na localização do RNA viral durante a sua síntese (Burke e Monath 2001). Além disso, a NS2A é necessária para a clivagem proteolítica da região C-terminal da NS1 (Falgout et al 1989). Estudos prévios demostraram que a NS4B e, com menor importância, a NS2A e NS4A, são capazes de bloquear a via de interferon (IFN) (Munoz-Jordan et al 2003, 2005).

A NS5 (103 kDa), a maior e mais conservada proteína NS dos flavivírus, apresenta caráter básico e atividade de RNA polimerase dependente de RNA (RdRP, replicase viral) (Kamer et al 1984, Chambers et al 1990) e de metiltransferase (Koonin 1993, Lindenbach et al 2007), essenciais para a replicação do vírus (Khromykh et al 2000). Foi demonstrado que durante a replicação viral, a proteína NS5 migra para o núcleo das células e induz a transcrição e a tradução da interleucina 8, um importante mediador na patogenia da infecção (Medin et al 2005).

A região codificadora é flanqueada por regiões não traduzidas conservadas (5'NC e 3'NC), que parecem desempenhar importante papel na modulação da replicação e tradução do genoma viral (Alvarez et al 2006, Chiu et al 2005, Alvarez et al 2005, Gamarnik 2010, Iglesias e Gamarnik 2011).

A região 5'NC é relativamente curta (95-132 bases), enquanto a região 3'NC é usualmente mais longa e demonstra extensiva heterogeneidade no tamanho e na sequência entre espécies virais diferentes e mesmo entre cepas diferentes da mesma espécie (Mandl et al 1993, Wallner et al 1995). De acordo com as estruturas secundárias previstas, a região 3´NC dos DENV pode ser dividida em três domínios (Figura 3B). O dominío I, que esta localizado imediatamente após o códon de terminação do gene NS5, é considerado a região mais variável (VR). Sua heterogeneidade é devida a mutações em sua sequência nucleotídica, tornando esta região um bom marcador para estudos evolutivos dos DENV (Shurtleff et al 2001, Klungthong et al 2008, Pankhong et al 2009). Mutações e deleções

dentro desta região podem alterar a infectividade, reduzir a eficiência na replicação viral (Men et al 1996, Mandl et al 1998, Shi et al 2002) e influenciar na virulência e patogenicidade dos DENV (Leitmeyer et al 1999, Cologna e Rico-Hesse 2003, Clyde et al 2006, Silva et al 2008). Estudos prévios já demonstraram deleções e variações nucleotídicas dentro da VR de cepas de um mesmo sorotipo (Aquino et al 2006, Roche et al 2007, Vasilakis et al 2008). O domínio II é moderadamente conservado, contendo vários *hairpins* ou grampos onde estão localizadas as sequências altamente conservadas (CS1 e CS2) e a sequência repetida conservada (RCS2). O domínio III é a região mais conservada da região 3´NC, compreendendo uma CS1 seguido de um *stem-loop* (3´SL) terminal (Hahn et al 1987, Proutski et al 1997, Shurtleff et al 2001, Zhou et al 2006, Silva et al 2008, Gamarnik 2010, Iglesias e Gamarnik 2011, Gebhard et al 2011).

1.6. Filogenia e Evolução dos Vírus Dengue

Assim como ocorre com outros vírus RNA, os DENV exibem um alto grau de variabilidade genética devido à falta do mecanismo de correção da RNA polimerase viral durante a replicação, as rápidas taxas de replicação, ao grande tamanho populacional e pressão frente à reposta imunológica do hospedeiro (Twiddy et al 2003).

Historicamente, as variantes dentro de cada sorotipo de DENV foram classificadas de formas distintas, acompanhando o progresso tecnológico.

Estudos da década de 70 apontaram a existência de variantes antigênicas dentro dos DENV-3, e ainda que, cepas de DENV-3 de Porto Rico e do Taiti eram antigênica e biologicamente distintas das cepas da Ásia (Russel e McCown 1972).

Na década de 80, baseado na técnica de "fingerprinting" do RNA, o termo "topotipo" foi utilizado para definir cinco variantes genéticas dentro do DENV-2 (Repik et al 1983, Trent et al 1990). Técnicas de hibridização de cDNA-RNA, hibridização utilizando oligonucleotídeos sintéticos e análise de produtos de RT-PCR, digeridos por endonucleases de restrição que também foram utilizadas para demonstrar a existência de variabilidade genética dentro de cada sorotipo dos DENV (Blok et al 1984, Blok 1985, Kerschner et al 1986, Vorndam et al 1994a 1994b).

Nos anos 90, a utilização de métodos de sequenciamento do genoma viral e análise filogenética permitiram a classificação dos DENV em grupos geneticamente distintos ou

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

Em uma revisão sobre a evolução dos DENV, analisando seqüências parciais do gene E, Rico-Hesse (2003) descreveu cinco genótipos para DENV-1, quatro para DENV-2 e DENV-3 e três genótipos para DENV-4.

Recentemente, revisões acerca de análises filogenéticas baseadas no sequenciamento completo do gene E determinaram cinco genótipos para os DENV-1 (a) Genótipo I, representado por cepas do Sudeste Asiático, China, e Leste da África; (b) Genótipo II, representado por cepas da Tailândia isoladas nas décadas de 1950 e 1960; (c) Genótipo III, representado pelas cepas selvagens coletadas na Malásia; (d) Genótipo IV, representado pelas cepas das Ilhas do Oeste do Pacífico e Austrália; e (e) Genótipo V, representado por todas as cepas coletadas nas Américas, no Oeste Africano, e algumas cepas coletadas na Ásia (Weaver e Vasilakis 2009, Chen e Vasilakis 2011).

Para os DENV-2 foram descritos seis genótipos: (a) Genótipo Asiático I, representado pelas cepas da Malásia, Tailândia, Camboja, Myanmar, Vietnã e Austrália; (b) Genótipo Asiático II, representado pelas cepas da China, Taiwan, Sri Lanka, Filipinas; (c) Genótipo Cosmopolita, representado por cepas da Austrália, Leste e Oeste Africano, Ilhas dos oceanos Pacífico e Índico, Subcontinente Indiano e Oriente Médio; (d) Genótipo Americano, representado pelas cepas da América Latina e por cepas isoladas nas décadas de 1950 e 1960 no Caribe, Subcontinente Indiano e Ilhas do Pacífico; (e) Genótipo Sudeste Asiático/Americano, representado por cepas da Tailândia e Vietnã e por cepas isoladas nas Américas nos últimos 20 anos e (f) Genótipo Selvagem, representado por cepas isoladas em humanos, mosquitos silvestres, ou macacos sentinelas do Oeste Africano e Sudeste Asiático (Lewis et al 1993, Rico-Hesse et al 1997, Twiddy et al 2002, Wang et al 2002, Rico-Hesse 2003, Ong et al 2008, Zaki et al 2008, Weaver e Vasilakis 2009, Chen e Vasilakis 2011).

Para os DENV-3 que inicialmente foram agrupados em cinco genótipos baseados na técnica de "fingerprinting" (Trent et al 1990), análises subsequentes baseadas nas seqüências dos genes prM/M/E (Lanciotti et al 1994) e pelo sequenciamento completo do

gene E (Rico-Hesse 2003) e do genoma viral completo (Chao et al 2005) caracterizaram quatro genótipos distintos para este sorotipo. Atualmente, o DENV-3 está classificado em cinco genótipos: (a) Genótipo I, representado por cepas da Indonésia, Malásia, Filipinas e isolados de Ilhas do Pacífico Sul e Brasil (Figueiredo et al 2008); (b) Genótipo II, representado por cepas do Sudeste asiático; (c) Genótipo III, representado por cepas da Ásia, Leste da África e Américas; (d) Genótipo IV, representado por cepas de Porto Rico e uma cepa de 1965 isolada no Taiti e (e) Genótipo V, representado inicialmente por três amostras, de Filipinas (1956), Japão (1973), China (1980) (Weaver e Vasilakis 2009, Chen e Vasilakis 2011) e cepas do Brasil do período de 2002 a 2004 (Nogueira et al 2008, Araújo et al 2009). No entanto, ficou sugerido que as cepas brasileiras incluídas neste genótipo constituíam contaminações laboratoriais devido à alta similaridade com cepas protótipos representantes deste grupo (Chen e Vasilakis 2011).

Para os DENV-4, foram caracterizados quatro genótipos distintos baseados na análise da seqüência do gene E (Lanciotti et al 1997, AbuBakar et al 2002, Foster et al 2003) ou no sequenciamento completo do genoma viral (Klungthong et al 2004): (a) Genótipo I representados por cepas da Tailândia, Filipinas, Sri Lanka, casos importados do Japão, China e mais recentemente as cepas isoladas no Brasil; (b) Genótipo II representado pelas cepas do Sudeste da Ásia, China, Ilhas do Pacífico, Austrália, Caribe e Américas; (c) Genótipo III, representados por cepas recentes da Tailândia que eram diferentes das demais (Klungthong et al 2004) e (d) Genótipo IV, representado pelas cepas selvagens da Malásia (Weaver e Vasilakis 2009, Chen e Vasilakis 2011).

É importante ressaltar que a classificação dos DENV em genótipos dentro de cada sorotipo está sendo constantemente revisada, uma vez que o sequenciamento e análises evolutivas são aperfeiçoados e os bancos de dados de genomas expandem. Atualmente, não há um consenso na utilização das diferentes classificações descritas para os genótipos de DENV. Portanto, durante a apresentação dos resultados deste estudo, a classificação adotada para a genotipagem dos DENV brasileiros na tabela 1 será de acordo com a descrita por Chen e Vasilakis (2011) (Tabela 1).

Tabela 1: Classificação genotípica dos vírus dengue (DENV) baseada na análise filogenética do sequenciamento do gene que codifica para a proteína E, de acordo com Chen e Vasilakis (2011).

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

Métodos utilizados para inferir as taxas de evolução dos DENV foram baseados em medidas de distâncias genéticas simples e análises de regressão (Zanotto et al 1996, Lanciotti et al 1997, Wang et al 2000) e as estimativas de evolução para estes vírus foram de 6-8x10^{-4} substituições/sítio/ano. Métodos de Máxima Verossimilhança (MV) aplicados a seqüências do gene E resultaram em estimativas similares para as taxas evolutivas dos DENV (Twiddy et al 2003). Taxas estimadas para os quatro sorotipos de DENV variam de 4,55x 10^{-4} para DENV-1 a 11,58 x 10^{-4} para o DENV-3. Além disso, comparações estatísticas sugerem que o DENV-3 está evoluindo mais rapidamente do que os DENV-1 e DENV-2 (Weaver e Vasilakis 2009).

1.7. Replicação dos Vírus Dengue

Os DENV replicam no citoplasma celular, após período de latência de 12 a 16 horas, nas células dos vertebrados (Figura 4). A replicação viral ocorre no local da inoculação provalmente nas células retículos-endoteliais, células de Langerhans ou nos

fibroblastos que se encarregam de levar o vírus até os linfonodos regionais, favorecendo sua disseminação no sangue, preferencialmente nos monócitos (Rothman 1997).

Os DENV entram na célula por ligação específica da proteína E com receptores celulares, incluindo DC-SIGN (ICAM-3) (Lozach et al 2005) e receptores de manose, incluindo os monócitos, macrófagos e células dendríticas (Miller et al 2008), seguido do processo de endocitose (Chambers 1990). O pH ácido induz a fusão do envelope do vírion com a membrana celular. Após o desnudamento, o RNA viral é liberado no citoplasma. As proteínas virais são produzidas como parte de uma única e longa poliproteína viral de mais de três mil aminoácidos que é clivada pela combinação de proteases virais e do hospedeiro. As proteínas estruturais e não estruturais são codificadas na porção N-terminal da poliproteína. A replicação inicia com síntese da fita negativa de RNA, que serve como molde para a síntese de fitas positivas de RNA da progênie. A montagem das partículas virais ocorre em íntima associação com o retículo endoplasmático (RE) das células hospedeiras. A formação das partículas virais ocorre pelo revestimento do nucleocapsídeo viral com as proteínas do envelope, ancoradas na membrana do RE celular. Os vírus brotam para dentro do lúmen do RE adquirindo uma membrana lipídica (envelope), assim, os vírus se mantêm solúveis nas cisternas do RE e as partículas virais são liberadas pela via exocítica através do sistema de Golgi (Lindenbach et al 2007).

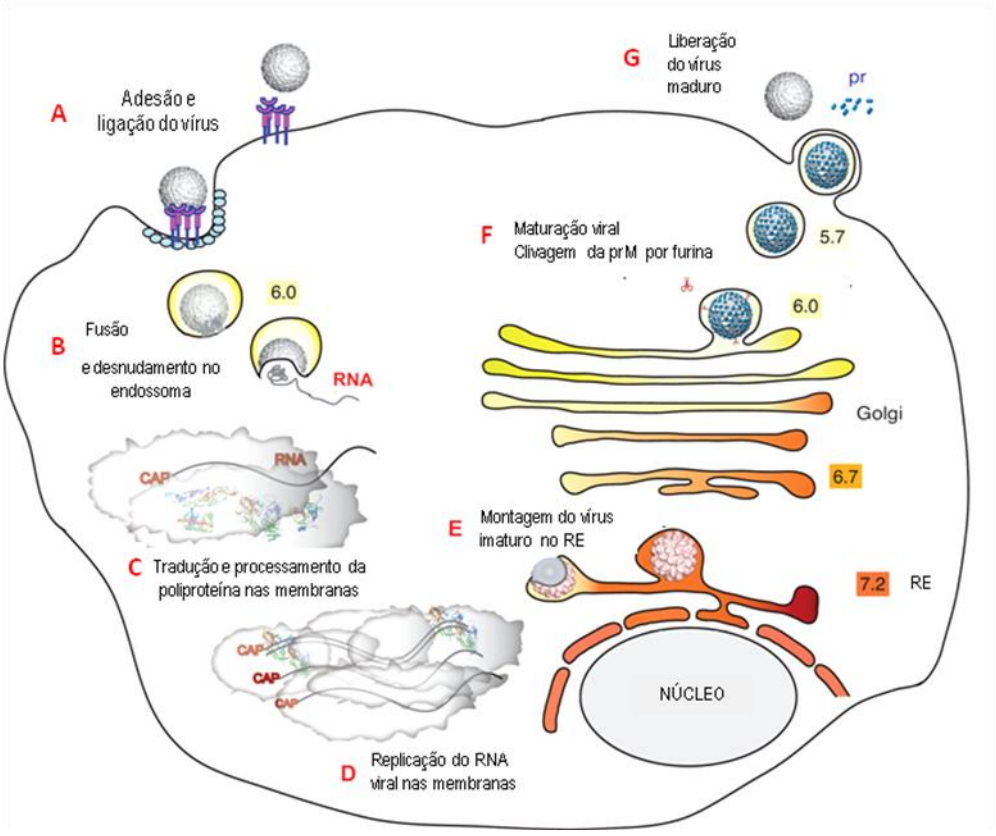

Figure 4: Representação esquemática da replicação dos vírus dengue (DENV). **(A)** Partículas virais se ligam a moléculas de superfície e receptores celulares e são internalizados por endocitose. **(B)** No pH ácido do endossomo, glicoproteínas virais mediam a fusão de membranas viral e celular, permitindo o desnudamento do virio e liberação do RNA no citoplasma. **(C)** O RNA viral é traduzido em uma poliproteína que é processada por proteases virais e celulares. **(D)** Proteínas não estruturais replicam o RNA genômico. **(E)** A montagem viral ocorre na membrana do retículo endoplasmático (RE), onde a proteína do capsídeo e o RNA viral são envelopados pela membrana do RE e glicoproteínas para formar partículas imaturas. **(F)** Partículas virais imaturas são transportadas através da via secretória. Em pH ácido do trans-Golgi, a clivagem da prM mediada pela furina leva a maturação viral. **(G)** O vírus maduro é liberado no citoplasma. Números nas caixas coloridas se referem ao pH dos respectivos compartimentos (Adaptado de Perera et al 2008).

1.8. Manifestações Clínicas e Classificação dos Casos de Dengue

Segundo a Organização Mundial de Saúde (OMS), as infecções pelos DENV apresentam um grande espectro de doença, variando desde infecções clinicamente inaparentes e brandas (dengue clássico [DC]) a formas graves e fatais (febre hemorrágica do dengue [FHD]) e síndrome do choque por dengue [SCD] (WHO 1997).

No DC a febre é geralmente de início súbito, com duração de, em média, 2 a 7 dias, com sintomas que podem incluir cefaleia, dor retro-orbital, sintomas gastrointestinais, mialgias, artralgia, rash e anorexia, náuseas, vômito e diarréia que podem ser observados por 2 a 6 dias (Souza et al 2008). As manifestações hemorrágicas, apesar de incomuns no DC podem ocorrer em alguns indivíduos, sendo mais comuns as petéquias, equimoses, epistaxe, hemorragia gengival e metrorragias (Cunha e Nogueira 2005). A forma clássica é autolimitada e resulta numa completa recuperação (Nishiura e Halstead 2007).

A forma mais grave do dengue é a FHD/SCD, que possui 4 graus de gravidade, sendo observada mais frequentemente em infecções secundárias e definida por febre, tendências hemorrágicas, trombocitopenia e extravasamento de plasma. Os Graus I e II são considerados os Graus mais brandos da FHD, enquanto os graus III e IV representam a evolução da doença para o estágio de choque (SCD).

Contudo, visando orientar médicos na identificação precoce de casos graves, a WHO propôs uma nova classificação dos casos de dengue, a partir da iniciativa intitulada *Dengue Control* (DENCO). Observações preliminares do DENCO confirmaram que utilizando um conjunto de parâmetros clínicos e/ou laboratoriais, é possível a distinção entre pacientes apresentando dengue grave ou não (WHO 2009). Os critérios para a classificação dos casos de dengue (com ou sem sinais de alerta) e dengue grave estão apresentados na Figura 5.

Figura 5: Classificação dos casos de dengue e níveis de gravidade, de acordo com observações do DENCO. Adaptado de WHO 2009.

1.9. Diagnóstico laboratorial

O diagnóstico laboratorial das infecções por DENV é realizado pelo isolamento e identificação do vírus, pela demonstração de antígenos e/ ou do ácido nucléico viral e pela demonstração de anticorpos específicos, imunoglobulinas G e M (IgG e IgM). Estas três metodologias englobam o diagnóstico virológico, molecular e sorológico das infecções pelos DENV.

1.9.1. Isolamento Viral

A inoculação de soros de fase aguda da infecção em cultura ou células de mosquito *Ae. albopictus* (C6/36) tem sido o método mais utilizado para o isolamento viral e ainda permanece como o "padrão ouro" para a comparação com outros métodos (Igarashi 1978). O isolamento pode ser observado pela presença do efeito citopático (ECP) e/ou confirmado pela técnica de imunofluorescência indireta com a utilização de soros hiper imunes aos quatro sorotipos dos DENV para a detecção dos vírus. Para a identificação dos DENV, utilizam-se anticorpos monoclonais específicos para os quatro sorotipos (Gubler et al 1984).

1.9.2. ELISA

O ensaio imunoenzimático de captura de anticorpos de classe IgM (MAC-ELISA) tem sido o método de eleição para o diagnóstico das infecções pelos DENV nas últimas décadas (Kuno et al 1987, Nogueira et al 1993, De Simone et al 2004). É um método rápido, fácil de ser executado e tem se mostrado extremamente útil, tanto para o diagnóstico individual de dengue como para estudos epidemiológicos. A presença de anticorpos da classe IgM em única amostra de soro indica infecção ativa ou recente, contornando as dificuldades de obtenção de uma segunda coleta de sangue.

O teste imunoenzimático tem sido igualmente utilizado para a detecção de IgG (IgG-ELISA). De fácil e rápida execução, tem sido utilizado em larga escala para a caracterização do tipo de infecção para a dengue (primária ou secundária) de acordo com os títulos observados e os dias após o início dos sintomas (Miagostovich et al 1999).

Alguns kits comerciais para a captura da proteína NS1, tanto no formato de ELISA quanto no formato de teste imunocromatográfico rápido estão disponíveis e têm sido úteis para o diagnóstico precoce das infecções e casos fatais por DENV (Lima et al 2010, 2011).

1.9.3. Transcrição reversa seguida pela reação em cadeia da polimerase (RT-PCR)

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, Figueiredo et al 1997, De Paula et al 2002). Esses protocolos detectam e caracterizam a presença do vírus nos casos suspeitos, podendo identificar o sorotipo infectante (DENV-1, 2, 3, 4). Esta metodologia tem sido importante tanto para a clínica quanto para a vigilância virológica, além de confirmar o diagnóstico em situações onde o material disponível não é adequado para o isolamento viral. Atualmente, a WHO recomenda o protocolo descrito por Lanciotti et al (1992) o qual, permite detectar os quatro sorotipos de DENV simultaneamente em um procedimento "semi-nested".

Diversos protocolos para a detecção e quantificação dos DENV têm sido propostos desde o surgimento dessa nova tecnologia (Drosten et al 2002, Jhonson et al 2005), os quais têm possibilitado maior rapidez dos resultados, além de maior sensibilidade.

1.9.4. Imunohistoquímica

A técnica de imunohistoquímica pode ser de grande utilidade para a detecção dos DENV em tecidos infectados. Baseia-se na conjugação de distintos marcadores, com moléculas de imunoglobulina, que com auxílio de um substrato específico localiza o antígeno tecidual. A disponibilidade de anticorpos para uso em tecidos fixados em formol e conservados em blocos de parafina permitem o estudo de blocos arquivados por longos períodos, principalmente em casos fatais (Miagostovich et al 1997).

1.10. Epidemiologia do Dengue

O aumento das epidemias de dengue ocorridas nos últimos vinte e cinco anos deve-se à intensificação do processo de urbanização em países tropicais e subtropicais, à

incapacidade de controlar os mosquitos vetores por parte desses países e à facilidade com que se faz a introdução de vírus por meios de transporte rápido de um continente para outro (Gubler 2002). Como conseqüência, houve um aumento da distribuição geográfica dos DENV assim como da infestação do principal vetor o *Ae. aegypti*, principalmente em regiões tropicais e subtropicais, as quais se tornaram hiperepidêmicas (Gubler 2005). Todos esses fatores conduziram à emergência de epidemias de febre hemorrágica do dengue (FHD) em áreas que incluem os países da Ásia, Ilhas do Pacífico, África e as Américas (Gubler 1998, 2005). Estima-se que atualmente, 2,5 bilhões de pessoas vivam em mais de 100 países e áreas endêmicas onde os DENV podem ser transmitidos. Cerca de 50 milhões de infecções por dengue e 500.000 casos de FDH ocorrem todos os anos, destes 22.000 resultam em óbito principalmente, infantil. Atualmente, a doença está dispersa em regiões das Américas Central e do Sul, partes do Caribe, Sul e Sudeste da Ásia, África e Pacífico Sul (Figura 6; WHO 2010).

Figura 6: Áreas com risco de transmissão e expansão de dengue, 2010 (Adaptado de http://gamapserver.who.int/mapLibrary/Files/Maps/Global_DengueTransmission_ITHRiskMap.png, acesso em 15 de Janeiro de 2012).

1.10.1. Dengue nas Américas

Após as primeiras descrições de dengue ocorridas no século XVIII foram registradas, a partir de 1896, nos Estados Unidos e em países do Caribe, inúmeras epidemias em intervalos irregulares. Os sorotipos envolvidos nestas epidemias não eram conhecidos até 1953, quando foram isoladas as primeiras amostras de DENV-2 em Trinidad (Anderson et al 1956). Dez anos depois, foram isoladas as primeiras amostras de DENV-3 em Porto Rico, sendo estes dois sorotipos responsáveis pelas epidemias ocorridas no continente na década de 1960 (Gubler 1992). Ainda nesta década, houve uma tentativa de erradicação do mosquito *Ae. aegypti* das Américas. Porém, com a descontinuidade do programa de erradicação no início da década de 1970 houve uma reinfestação deste vetor pelo continente (Schliesman e Calheiros 1974, Pinheiro 1989, Gubler 1997).

Em 1977, o DENV-1 foi introduzido nas Américas causando uma extensa epidemia (Gubler 1992). No mesmo período, o DENV-3 apresentou pouca atividade na região com o registro de nenhum isolamento ocorrido em 1978, em Porto Rico (Anonymus 1995).

Em 1981, o DENV-4 foi introduzido em São Bartolomeu, causando surtos no Caribe, México, países da América Central e América do Sul, tornando-se endêmico na região (Pinheiro, 1989). Durante este mesmo ano, uma nova variante do DENV-2 foi introduzida no continente americano, causando em Cuba, a primeira epidemia de Febre Hemorrágica por Dengue (FHD)/Síndrome do Choque por Dengue (SCD) das Américas (Kouri et al 1986, Pinheiro e Corber 1997).

No período de 1989 a 1990, ocorreu na Venezuela a segunda maior epidemia de FHD/SCD das Américas, com cerca de 22 mil casos, onde co-circularam os DENV-1, DENV-2 e DENV-4 (WHO 1997, Pinheiro e Chuit 1998).

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

Em 2002, mais de 30 países latino-americanos registraram mais de 1 milhão de casos de Dengue Clássico (DC), sendo que cerca de 750 mil casos ocorreram no Brasil. Esse número apresentou uma queda significativa entre 2003 e 2005, voltando a crescer a partir de 2006.

Em 2009, um total de 917.311 casos de dengue, incluindo 25.268 casos graves de dengue e 371 óbitos, co-circulação dos 4 sorotipos virais foram registrados em Porto Rico, El Salvador, Nicaragua, Peru e Venezuela (WHO 2009).

No ano de 2011 até a 31ª semana epidemiológica, foram registrados 890.756 casos de dengue, 10.840 casos graves e 488 óbitos nas Américas, sendo o cone Sul responsável pelo maior número de casos: 750.946 casos de DC, 8199 casos de dengue grave e 372 óbitos. Somente o Brasil notificou 715.666 casos de dengue, 8.104 casos graves e 310 óbitos (PAHO 2011).

Durante as últimas quatro décadas, o continente Americano evoluiu de uma situação não-endêmica para hiperendêmica, onde se observou um aumento na densidade vetorial, a co-circulação de múltiplos sorotipos de DENV, a endemicidade de FHD e em um aumento na frequência da atividade dos DENV (Gubler 2002). Nos primeiros cinco anos da década atual, a média anual de casos foi praticamente o dobro daquela registrada na década anterior (Nathan e Dayal-Drager 2006).

1.10.2. Dengue no Brasil

As primeiras referências sobre o dengue no Brasil são do século XIX (Meira 1916). Reis (1846) relatou quadro clínico semelhante ao dengue em casos de um surto de doença febril ocorrido em Curitiba. Mariano (1917) relatou uma epidemia cuja clínica se assemelha ao dengue que teria acontecido em 1846, no Rio de Janeiro, e outra ocorrida em 1916, no Rio Grande do Sul. Embora epidemias de febre semelhante ao dengue tivessem sido relatadas no Brasil durante o século XIX, foi Pedro (1923) quem pela primeira vez descreveu claramente a doença, durante uma epidemia que ocorreu em Niterói e no Grande Rio.

No inicio do século XX, o Brasil passou por campanhas de luta contra a espécie *Ae. aegypti*, vetor da febre amarela urbana e também do dengue. Esta ação brasileira de

controle do *Ae. aegypti* que começou com Emilio Ribas, em 1903, e Oswaldo Cruz, em 1904, teve grande êxito mais tarde com apoio e coordenação da Organização Pan-Americana da Saúde (OPAS) e da Fundação Rockefeller (Benchimol 2001). Assim, esta espécie foi considerada erradicada no Brasil em 1956. Por isso, é provável que não tenham relatos de epidemias de dengue no país a partir da década de 1950.

Anticorpos contra dengue foram encontrados em moradores de pequenas cidades da Amazônia brasileira por Causey e Theiler (1958) o que levou esses autores a sugerir que o vírus havia circulado nessa região no início do século XX. No final dos anos 1960 e início da década seguinte, o *Ae. aegypti* passou a ser encontrado em alguns estados do Norte e Nordeste do Brasil (Consoli e Lourenço-de-Oliveira 1994). Contudo, nenhum registro de dengue no país foi realizado antes dos anos 80.

Após décadas sem casos de dengue no Brasil, o primeiro surto ocorreu em Boa Vista, Roraima (1981-1982), causado pelos sorotipos 1 e 4. A transmissão permaneceu restrita à cidade de Boa Vista (Osanai et al 1983). Quatro anos mais tarde, o DENV-1 foi isolado em Nova Iguaçu, no estado do Rio de Janeiro (Schatzmayr et al 1986), e após 1987, a doença tornou-se endêmica em vários estados, como no Rio de Janeiro, São Paulo, Minas Gerais, Ceará (Degallier et al 1996, Nogueira et al 1999, Vasconcelos et al 2000). Em 1990, um novo surto ocorreu no Rio de Janeiro e em Niterói, no qual o DENV-2 foi isolado pela primeira vez no Brasil (Nogueira et al 1990). A introdução do DENV-3 ocorreu no ano de 2000, também no município de Nova Iguaçu, Rio de Janeiro, sendo este sorotipo isolado de mosquitos *Ae. aegypti* e de humanos durante grave epidemia na localidade (Nogueira et al 2001, Lourenço-de-Oliveira et al 2002).

A epidemia ocorrida no ano de 2002 foi caracterizada como a maior e mais grave epidemia do país devido ao DENV-3 (De Simone et al 2004, Nogueira et al 2002), na qual a metade dos casos fatais relacionava-se às infecções primárias por este sorotipo, dado indicativo de sua maior virulência o que modificou a epidemiologia do dengue no país, com características de hiperendemicidade (Nogueira et al 2005, Araújo et al 2009b, 2009c).

No período de 2002 a 2005, observou-se que ocorria a circulação do DENV-3 no estado do Rio de Janeiro, mas o número de casos notificados diminuiu neste período. Os anos de 2004 e 2005 foram considerados como interepidêmicos (Araújo et al 2006). No

final de 2005, houve a prevalência de atividade para o sorotipo DENV-3, e no ano de 2006, observou-se que houve uma maior atividade deste sorotipo comparada ao ano anterior (Nogueira et al 2007). A partir do mês abril de 2007, observou-se a re-emergência do DENV-2 e este sorotipo foi responsável pela ocorrência de uma grave epidemia no ano de 2008, com um total de 259.392 casos no estado do Rio de Janeiro que correspondeu a 35% dos 734.384 casos notificados no Brasil (PAHO 2008, SVS 2009).

No ano de 2011 até a 26ª semana epidemiológica, foram notificados 715.666 casos de dengue no país e as atividades de monitoramento da circulação dos DENV demonstraram uma maior proporção de isolamentos do DENV-1 (SVS 2011).

O risco da introdução do DENV-4 no país era iminente, uma vez que este sorotipo circulava em países vizinhos, como a Venezuela e a Colômbia (Guzman e Kouri, 2002). Apesar de Figueiredo et al (2008) ter relatado a detecção de DENV-4 em pacientes residentes na cidade de Manaus, entre 2005 e 2007, estes dados não foram confirmados pelo Ministério da Saúde. Em julho de 2010, um caso de DENV-4 foi detectado em uma Unidade Sentinela de vigilância laboratorial de Roraima (RR), com o diagnóstico inicial realizado pelas técnicas de RT-PCR e isolamento viral. Em seguida, outros casos de DENV-4 foram detectados em RR. Em 2011, o DENV-4 foi isolado de casos provenientes dos estados de Amazonas, Rondônia, Pará, Piauí, Ceará, Pernambuco, Bahia, Rio de Janreiro e São Paulo (SVS 2010; 2011, Nogueira e Eppinghaus 2011).

Atualmente, os sorotipos DENV-1, 2, 3 e 4 circulam simultaneamente no Rio de Janeiro, o que reforça este Estado como o mais receptivo para introdução e disseminação de novos sorotipos e genótipos de dengue no Brasil (Miagostovich et al 1998, 2003; 2006, Duarte dos Santos et al 2000, dos Santos et al 2002, De-Simone et al 2004, Nogueira e Eppinghaus 2011).

1.10.2.1 Epidemiologia Molecular do Dengue no Brasil

Atualmente circulam no Brasil os genótipos: Américas/África (ou genótipo V) de DENV-1 (Duarte dos Santos 2002, dos Santos et al 2011), o Asiático/Americano do DENV-2 (dos Santos et al 2002) e do Subcontinente Indiano (ou genótipo III), para DENV-3 (Miagostovich et al 2006, Araújo et al 2009, Barcelos Figueiredo e colaboradores (2008) descreveram a detecção do genótipo I de DENV-3 no país. O sequenciamento do gene E e

análise filogenética das cepas de DENV-4 introduzidas em Roraima em 2010 caracterizou estes vírus como pertencentes ao Genótipo I de DENV-4 (de Melo et al 2009).

A análise filogenética de cepas de DENV-3 isoladas no Brasil do período de 2002 a 2004 caracterizou estas cepas como pertencentes ao Genótipo V (Nogueira et al 2008, Araújo et al 2009) representado inicialmente por três amostras, de Filipinas (1956), Japão (1973), China (1980) (Weaver e Vasilakis 2009, Chen e Vasilakis 2011). No entanto, ficou sugerido que as cepas brasileiras incluídas neste genótipo constituíam contaminações laboratoriais devido à alta similaridade com cepas protótipos representantes deste grupo (Chen e Vasilakis 2011).

A filogenia baseada na análise do gene E das cepas de DENV-2 isoladas na epidemia de 2008 demonstrou que, apesar destes vírus ainda pertencerem ao mesmo genótipo circulante anteriormente, porém de linhagens distintas, estas se agruparam formando um grupo monofilético distinto (Oliveira et al 2010). Esta re-emergência causou a mais grave epidemia de dengue registrada no país até então, com um maior número de hospitalizações e ocorrência de óbitos em crianças e adultos (SVS 2009).

1.10.2.2 Vigilância do Dengue

A vigilância epidemiológica, apesar de pouco sensível para detectar a circulação esporádica do vírus, é um componente fundamental do sistema de vigilância do dengue (Tauil 2002), para monitorar a ocorrência de casos de dengue na população. É também importante para estabelecer os critérios de confirmação e descarte da doença, permitindo comparação de dados provenientes de diversas fontes de notificação e entre mais de uma região ou país.

Para se tornar um componente de impacto no controle da doença, antecipando a transmissão epidêmica, a vigilância epidemiológica deve contar com apoio laboratorial, para detectar os sorotipos e variações genéticas circulantes (vigilância virológica) e classificar os casos da doença (vigilância clínica), principalmente em relação às formas graves (Gubler 1989, WHO 1996, Gubler 1998).

A prevenção e o controle das epidemias de dengue dependem fundamentalmente da redução da população do vetor no domicílio e peridomicílio, principais locais nos quais ocorre à transmissão. A eliminação dos reservatórios aquáticos que servem de habitat para

as larvas do *Ae. aegypti* constitui-se no mais efetivo modo de reduzir sua disseminação e reprodução nos centros urbanos (Gubler 1998). Neste contexto, a vigilância entomológica se torna um dos componentes fundamentais de um sistema de vigilância para prevenção e controle do dengue, sendo importante para orientar as intervenções (Gubler 1989). No entanto, a utilização de índices que detectam a densidade da infestação vetorial e o nível crítico para predição de epidemias ainda é controversa na literatura, visto que a multiplicidade de fatores envolvidos na transmissão da doença na população não são totalmente representados por estes indicadores (Kuno 1995, Gomes 1998, Gómez et al 2001).

Os índices rotineiramente utilizados na vigilância dos níveis de infestação pelo *Ae. aegypti* são conhecidos como índices de *Stegomyia* (revisados por Focks 2003). Estes índices desempenham papéis importantes na orientação dos principais programas de controle, no Brasil e demais países, onde a dengue é endêmica ou epidêmica, ou mesmo de campanhas de erradicação, como no caso da febre amarela urbana. No entanto, existem múltiplos fatores envolvidos na transmissão do dengue que não são totalmente cobertos por estes indicadores, como, por exemplo, a medida da abundância do adulto fêmea e a estimativa do risco de transmissão do dengue.

Alguns dos índices de *Stegomyia* mais empregados são o índice de infestação predial (IP) e o índice de Breteau (IB). O IP é definido como o percentual de imóveis positivos para vetores imaturos de *Ae. aegypti* em uma dada localidade, enquanto que o IB quantifica o número de recipientes contendo larvas de *Ae. aegypti* por 100 imóveis pesquisados (Honório 2009, Focks et al 2000, Focks 2003).

O levantamento de índice rápido de infestação por *Ae. aegypti*, ou LIRAa, por sua vez, é uma estratégia diferenciada de seleção de domicílios durante os inquéritos entomológicos de dengue que, resultará no cálculo do IP e do IB. Neste, emprega-se uma técnica de amostragem randômica na qual uma unidade de amostra corresponde a 9.000 ou 12.000 imóveis, onde 450 destes são sorteados e visitados pelos agentes de endemias para a busca de larvas ou pupas de *Ae. aegypti* (Coelho et al 2008).

No Brasil, foram poucos os estudos que tentaram correlacionar os índices de infestação vetorial com a ocorrência de transmissão de dengue. Os resultados observados tem se mostrado discordantes e inconsistentes para a construção efetiva de indicadores de

risco de transmissão desta arbovirose. A baixa associação entre os índices de infestação e o risco de transmissão pode ser atribuída: i) à baixa qualidade da coleta de dados entomológicos; ii) à subnotificação de casos; iii) e ao índice de infestação predial por ser inadequado para mensurar o nível de infestação, já que tenta inferir sobre a frequência de adultos, que é a fase responsável pela transmissão, a partir de coletas de larvas. Além disso, tais estudos foram baseados essencialmente em índices de infestação, em especial no IP, fornecidos pelas Secretarias de Saúde locais, cujos programas de controle do dengue podem sofrer periódicas interferências de ordem política. Possíveis falhas nas operações de campo também serão diretamente refletidas no cálculo do IP/IB (Honório 2009).

Por outro lado, alguns autores, na tentativa de associar os níveis de infestação e transmissão de dengue, sugerem que a capacidade vetorial do mosquito varie dependendo das condições ambientais, da longevidade, taxa de picada, susceptibilidade ao vírus, entre outros fatores. Sendo assim, não só a densidade de mosquitos como também o comportamento e a competência vetorial de suas populações, afetaria a transmissão e que esta, possa ser afetada por características da dinâmica populacional do vírus e não do vetor em si (Chevillon e Failloux 2003, Honório 2009). Além disso, a taxa de transmissão depende também de características da população humana, uma vez que os indivíduos podem ter diferentes graus de exposição e/ou susceptibilidade à infecção ou, ainda, se a soroprevalência for alta, a transmissão pode simplesmente não ocorrer, devido à chamada imunidade de rebanho (Halstead 2008).

Em condições naturais, a detecção de mosquitos *Ae. aegypti* infectados com DENV pode ser raro, mesmo em períodos epidêmicos (Lourenço-de-Oliveira et al 2002, Barbazan et al 2009). Além disso, a escassez dos trabalhos de campo para monitoramento da circulação viral em *Ae. aegypti* reside na complexidade logística dessa atividade, muito laboriosa e que requer alta disponibilidade de tempo e pessoal.

A coleta de mosquitos para detecção dos DENV deve empregar ferramentas como o aspirador costal (Clark et al 1994), método apontado como o padrão-ouro para coleta de adultos, por capturar machos e fêmeas independentemente de sua idade fisiológica (Focks 2003). Alternativamente, pode-se utilizar armadilhas (Fávaro et al 2006, Gama et al 2007), que cobrem um maior número de residências em dada área geográfica, aumentando o sucesso de capturas (Focks 2003). No entanto, as inspeções

realizadas semanalmente nestas armadilhas, resultam em espécimes inviáveis para a detecção do material genético viral, devido à exposição dos mesmos a temperaturas inadequadas de conservação. Neste contexto, esta estratégia de monitoramento baseada em armadilhas pode dificultar os estudos de vigilância virológica em mosquitos. Portanto, a utilização de métodos de coleta de vetores mais eficientes combinados a técnicas de detecção viral sensível fornecem uma abordagem atrativa à vigilância entomológica.

2. Justificativa

O dengue tem se apresentado como um grave problema de saúde pública no Brasil, razão pela qual, vários estudos têm sido realizados com o intuito de esclarecer aspectos da epidemiologia dessa doença em diferentes localidades, com histórias distintas de circulação dos diferentes sorotipos de DENV (Teixeira et al 2002, Nogueira et al 1999, 2007).

Os padrões de transmissão do dengue são determinados pela combinação de fatores que envolvem o hospedeiro humano, o vírus, o vetor e o ambiente. Sendo assim, o monitoramento de fatores de risco de transmissão, pela vigilância epidemiológica é de grande importância na epidemiologia da doença. A vigilância epidemiológica do dengue, que envolve a vigilância entomológica e virológica, deve ser intensificada, principalmente, em períodos interepidêmicos, e pelo monitoramento contínuo de casos suspeitos e dos vetores das áreas de transmissão. Neste contexto, o constante monitoramento da circulação viral no vetor, nas suas formas imatura e alada, possibilita, não somente a detecção do vírus, mas também a detecção de transmissão vertical. Além disso, o monitoramento da infecção em mosquitos permite a detecção do aumento de circulação viral em período hábil para a efetivação de medidas de controle. A genotipagem dos vírus isolados destes vetores pode ainda fornecer informações relevantes na compreensão da interação genótipo/sorotipo – vetor.

A caracterização molecular de amostras de DENV isoladas no Brasil de áreas geograficamente e temporalmente distintas é uma ferramenta fundamental para a epidemiologia molecular, que por sua vez pode fornecer informações importantes sobre a introdução, dispersão e circulação de distintas linhagens destes vírus (Miagostovich et al 1998, 2003 e 2006, Duarte dos Santos et al 2000, Oliveira et al 2008, dos Santos et al 2002, 2011). Variações em sequências nucleotídicas em um genótipo de DENV podem ser responsáveis pelo aparecimento de cepas de vírus que se replicam com mais eficiência em humanos e em mosquitos, e produzem altas viremias em humanos facilitando a transmissão pelos vetores (Monath 1994). Porém, não se sabe até que ponto os mosquitos contribuem, evolutivamente, para a estabilidade genética ou para a seleção de variantes/mutações dominantes em determinada área.

O entendimento da variação genética no vírus quando replica em mosquitos, e como essas variações atuam durante a transmissão entre humanos e mosquitos permanecem desconhecidos. Porém, sabe-se que mutações que ocorram ao longo do genoma, assim como mutações nos genes virais estruturais e não-estruturais e na região 3´NC dos DENV já foram implicados como marcadores moleculares de virulência (Hurrellbrink e Mc Minn 2003). Portanto, visando contribuir para um melhor conhecimento dos DENV e sua interação com o mosquito vetor, realizamos neste trabalho, a caracterização molecular e estudos/ou análise filogenética de cepas de DENV isoladas de mosquitos naturalmente infectados e de casos humanos provenientes de epidemias ocorridas de 1986 a 2011 no Brasil.

3. Objetivos

3.1. Objetivo geral

Caracterização molecular e genotipagem de cepas de DENV isoladas de mosquitos *Ae. aegypti* e de casos humanos ao longo de epidemias ocorridas, entre 1986 e 2011, em áreas com diferentes índices de infestação do vetor e história pregressa de casos de dengue.

3.2. Objetivos específicos

- Realizar a análise filogenética baseada no gene E de cepa de DENV-1 isoladas durante o período estudado (1986 a 2011) – Trabalhos 1 e 2.
- Realizar o sequenciamento completo do genoma de DENV-3 isolado de mosquitos naturalmente infectados durante a epidemia ocorrida em 2001 no Rio de Janeiro – Trabalho 3
- Caracterizar a região 3'-não codificante (3'NC) de DENV-3 isolados de mosquitos e de casos humanos – Trabalho 3.
- Realizar a vigilância entomológica do DENV-4 pelo meio da utilização de metodologias moleculares – Trabalho 4.

4. Material e Métodos e Resultados

A metodologia e os resultados obtidos nesta tese estão apresentados sob a forma de manuscritos (aceito, submetidos para publicação).

4.1. First Report Of Multiple Lineages Of Dengue Viruses Type 1 In Rio De Janeiro, Brazil

Publicado no Virology Journal 2011, 8:387

4.2. Dengue Virus Type 1 From Field-Caught Vectors And Humans In Brazil: Phylogeny Reveals Different Lineages Of The American African Genotype In 25 Years

Submetido ao periódico "Plos One"

4.3. Molecular Differences Of The 3´Untranslated Region From Brazilian Dengue Virus Type 3 Isolated From Naturally Infected Mosquitoes And Humans

Submetido ao periódico "Virology Journal"

4.4. DENV-4 Entomological Surveillance In Rio De Janeiro: Role of Molecular Techniques Dengue Vírus Type 4 (DENV-4) in Niteroi, Rio de Janeiro: The Role of Molecular Techniques in the Laboratorial Diagnosis and Entomological Surveillance

Submetido ao periódico "Memórias do Instituto Oswaldo Cruz"

4.1. **Artigo 1**. First Report Of Multiple Lineages Of Dengue Viruses Type 1 In Rio De Janeiro, Brazil

Referência bibliográfica: Flavia B dos Santos, Fernanda B Nogueira, Márcia G Castro, Priscila CG Nunes, Ana Maria B de Filippis, Nieli RC Faria, Jaqueline BS Simões, Simone A Sampaio, Clarice R Santos and Rita Maria R Nogueira.

Situação do manuscrito. Publicado no periódico "Virology Journal".

Este artigo atende ao objetivo específico 1.

Resumo: Detectou-se múltiplas linhagens do vírus DENV-1 circulando no Rio de Janeiro e no país, após um período de baixa ou silenciosa co-circulação desde a sua entrada em 1986.

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VIROLOGY JOURNAL

SHORT REPORT Open Access

First report of multiple lineages of dengue viruses type 1 in Rio de Janeiro, Brazil

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Abstract

Background: In Brazil dengue has been a major public health problem since DENV-1 introduction and spread in 1986. After a low or silent co-circulation, DENV-1 re-emerged in 2009 causing a major epidemic in the country in 2010 and 2011. In this study, the phylogeny of DENV-1 strains isolated in RJ after its first introduction in 1986 and after its emergence in 2009 and 2010 was performed in order to document possible evolutionary patterns or introductions in a re-emergent virus.

Findings: The analysis of the E gene sequences demonstrated that DENV-1 isolated during 2009/2010 still belong to genotype V (Americas/Africa) but grouping in a distinct clade (lineage II) of that represented by earlier DENV-1 (lineage I). However, strains isolated in 2011 grouped together forming another distinct clade (lineage III).

Conclusions: The monitoring of DENV is important to observe the spread of potentially virulent strains as well to evaluate its impact over the population during an outbreak. Whether explosive epidemics reported in Brazil causec mainly by DENV-1 was due to lineage replacement, or due the population susceptibility to this serotype which has not circulated for almost a decade or even due to the occurrence of secondary infections in a hyperendemic country, is not clear. This is the first report of multiple lineages of DENV-1 detected in Brazil.

Keywords: Dengue virus type 1, multiple lineages, phylogeny, Rio de Janeiro

Findings

Dengue viruses (DENV) are the most important human arboviruses worldwide, transmitted by mosquitoes of the genus *Aedes* and currently it is estimated that 70 to 500 million dengue infections occur annually in 124 endemic countries. Nearly 3.6 billion people (55% of world popu-

rapid global spread of the four DENV serotypes (DENV-1 to 4) in the last 50 years resulted in the dispersal of genotypes associated with increased severity [2].

In Brazil, the State of Rio de Janeiro (RJ), in the Southeast region (Figure 1A) has been important to the epidemiology of dengue, with the introduction of DENV-1 in 1986, DENV-2 in 1990 and DENV-3 in 2000 [3]. The latter was prevalent in the majority of Brazilian States from 2002 to 2006 and, from 2007 to 2009 this serotype was displaced by DENV-2. In 2008, the Southeast and the Northeast regions were respon ble for approximately 80% of the cases reported in t most severe epidemic in the country, where DENV and DENV-3 were detected in 96.4% of the cases is lated. After a low or silent circulation, DENV-1 r emerged in the Southeast region in 2009 (Figure 1 isolations, displacing DENV-2 (30.5%) and DENV-3 (19.1%) [4].

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 (America/Africa) and the existence of lineages with distinct geographic and temporal relationships have been suggested [5,6]. Moreover, lineage turnover or replacements have become more frequently common in phylogenetic studies. The term "lineage" has been used non- officially to characterize those viruses clustered in clades in a taxonomic level beneath genotype [7].

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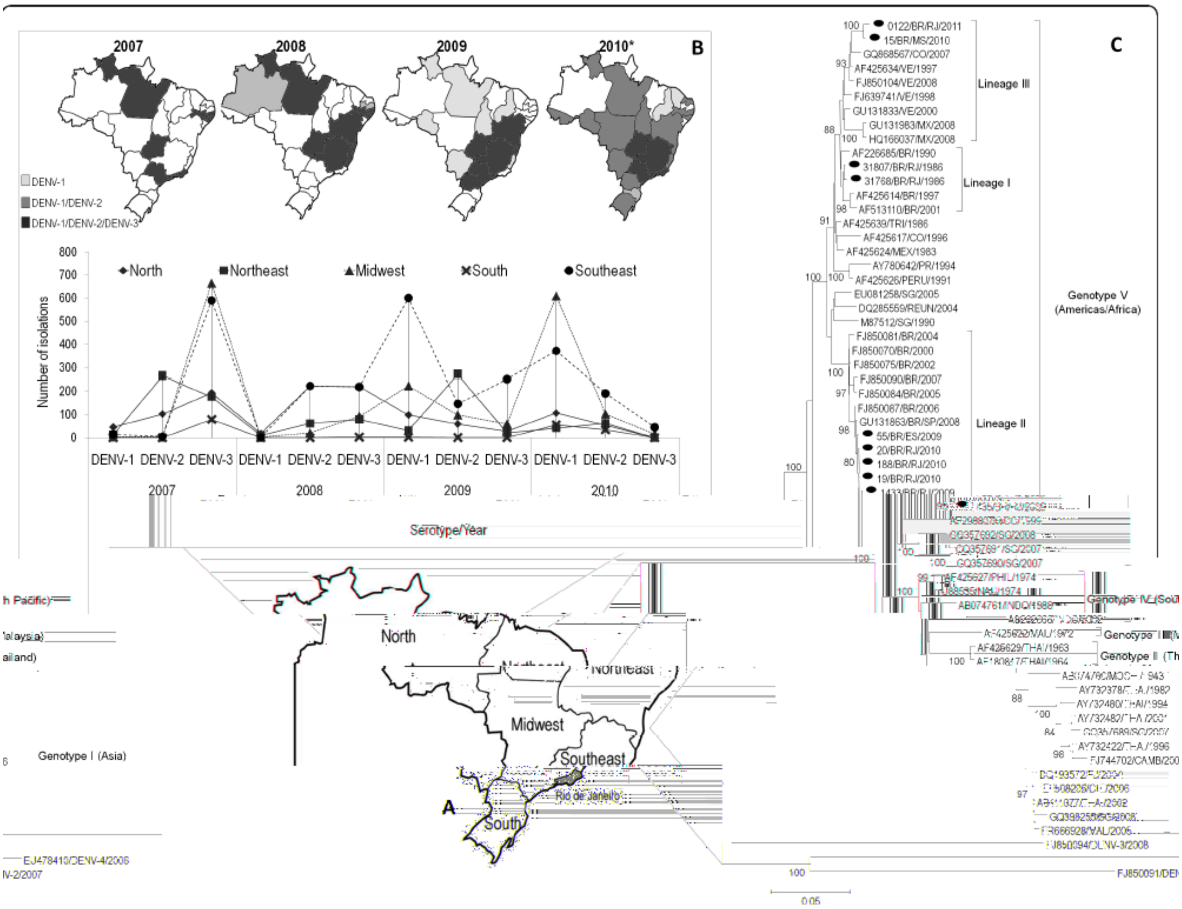

…uth. In gray, Rio de …' to 2010. (C): …niologically …replicate trees in …represent DENV-1

Figure 1 DENV-1 re-emergence in Brazil. (A): Brazil's five geographical regions: North, Northeast, Midwest, Southeast and So… Janeiro's localization. (B): Dengue viruses (DENV) serotypes replacements and DENV-1 emergence in Brazil, by region from 200… Neighbor-joining phylogenetic of ten complete envelope (E) gene sequences from DENV-1 isolated during two periods epider… distinct: 1986 (*n* = 2) when the serotype was first introduced and after its emergence in 2009-2011 (*n* = 8). The percentage of which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Black circle… sequences generated in this study. DENV strains used were named as follows: GenBank accession number/country/year.

…troduced by *in* …ltures we used … = 2], 2009 [n = …tracted directly … …m did not yield … …inoculation into …and isolates were …ibody test (IFAT) …tibodies [9]. RT- …from serum was …10]. For RT-PCR …extracted from directly from the

In this study, the phylogeny of DENV-1 strains isolated in RJ after its first introduction in 1986 and after its emergence in 2009 and 2010 was performed in order to document possible evolutionary patterns or introduc- … constitute an important tool to monitor the introduction and spread of viruses as well as to predict the potential epidemiological consequences of such events.

The strains analyzed in this study belong to a collection obtained from acute-phase human serum through the passive surveillance system performed by the Laboratory of Flavivirus, IOC/FIOCRUZ, Rio de Janeiro, Brazil, 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. To avoid mutations i… *vitro* passages of the virus in cell cu… DENV-1 strains (*n* = 10; from 1986 [n … 3], 2010 [n = 4] and 2011 [*n* = 1]) ex… … isolated from cell culture when ser… enough volume for RNA extraction.

DENV-1 isolation was performed b… C6/36 *Aedes albopictus* cell line [8]… identified by indirect fluorescent ant… using serotype-specific monoclonal a… PCR for detecting and typing DENV… performed as described previously … and sequencing the viral RNA wa… infected cell culture supernatant or

patients serum using QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's instructions and stored at -70°C for DENV typing and sequencing.

The sequencing reaction was performed by reverse transcription using 5 μL of extracted RNA in 25 μL of AccessQuick™ RT-PCR System (Promega Corporation) and specific oligonucleotides primers which sequences can be provided upon request, to amplify the C/prM/M/E region of 2,325 bp. Amplification was conducted using a Model 9700 thermal cycler (Applied Biosystems). PCR products were purified from using QIAquick Gel extraction Kit or QIAquick PCR purification Kit (Qiagen) and used as template for cycle sequencing. Sequencing reactions were performed as recommended in the BigDye Dideoxy Terminator sequencing kit (Applied Biosystems) and the products were analyzed using an automated 3130 DNA Sequencer (Applied Biosystems). Sequences for the complete E gene (1,485 nucleotides) were deposited in GenBank (http://www.ncbi.nlm.nih.gov).

The sequences multiple alignment was performed using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and the phylogenetic analysis by MEGA 4 software (http://www.megasoftware.net), using the "Neighbor-joining" method, according to the Tamura-Nei model, with a bootstrap of 1,000 replications. Strains representative from the five genotypes available in Genbank (http://www.ncbi.nlm.nih.gov) were used for the comparison, DENV-2, DENV-3 and DENV-4 strains were used as outgroup to root the trees.

The results based on the analysis of the E gene sequences have demonstrated that the DENV-1 strains isolated during 2009/2010 in RJ and one isolated in the State of Espirito Santo (ES) used for comparison purposes, still belong to genotype V (America/Africa) previously detected in the country, but grouping into a distinct clade (lineage II) of that represented by earlier Brazilian DENV-1 strains (lineage I) with a strong bootstrap support. In fact, the re-emergent DENV-1 was more closely related to strains isolated in Singapore in 1990 and in 2005, suggesting a probable Asian origin. However, one strain isolated in 2010 (15/BR/MS/2010) from a RJ resident who traveled to Mato Grosso do Sul (MS), Midwest region and one strain recently isolated in 2011 in RJ (0122/BR/RJ/2011) grouped together forming another distinct clade (lineage III), grouping with strains isolated in 2007 and 2008 in Colombia, Venezuela and Mexico, suggesting a Latin American origin for those strains (Figure 1C).

In spite of the continuous low circulation in the country, the low percentage of identity of the newly isolated viruses with those strains first introduced in the 80's suggest that the re-emergent DENV-1 did not evolved locally but occurred probably due to new lineages introductions in the country (Table 1). The analysis based on the E gene sequences from DENV-1 strains isolated in the Northern region of Brazil from 2000 to 2008 available on GenBank support the idea that those viruses could have been introduced earlier and their low or silent circulation could be due to the prevalent DENV-3/DENV-2 circulation during that time (unpublished data). The circulation of more than one DENV-1 "lineage" has been described in Asia [6] and in the Americas [7]. A previous study by Carrillo-Valenzo [11] recently reported multiple viral lineages introductions for each DENV serotype in Mexico with frequent lineage replacements. In fact, lineage replacements appear to be a more common observation than long term lineage persistence [12].

Lineage replacement occurs when an entire clade of viruses that has persisted in a particular locality for a period of time is not evident on a subsequent sampling, indicating that it has dropped dramatically in frequency, even experiencing extinction, and sometimes replaced by a new clade of viruses [13]. Despite this, the evolutionary processes controlling these events are not fully understood. Recently, it has been suggested that despite

Table 1 Sequences identity between Brazilian DENV-1 based on the E gene analysis (1,485 nucleotides)

DENV-1 Strains	55/2009[a]	1435/2009	1433/2009	15/2010	19/2010	20/2010	188/2010	0122/2011	31768/1986	31807/1986
55/2009	-	99,6[b]	99,6	95,8	99,8	99,8	99,8	95,6	96,8	96,9
1435/2009	**99,7**	-	100,0	95,6	99,7	99,7	99,7	95,4	96,6	96,7
1433/2009	**99,7**	**100**	-	95,6	99,7	99,7	99,7	95,4	96,6	96,7
15/2010	**99,1**	**98,9**	**98,9**	-	95,8	95,8	95,8	99,4	98,1	98,1
19/2010	**100**	**99,7**	**99,7**	**99,1**	-	100	100	95,6	96,8	96,9
20/2010	**100**	**99,7**	**99,7**	**99,1**	**100**	-	100	95,6	96,8	96,9
188/2010	**100**	**99,7**	**99,7**	**99,1**	**100**	**100**	-	95,6	96,8	96,9
0122/2011	**98,9**	**98,9**	**98,9**	**99,7**	**98,9**	**98,9**	**98,9%**	-	97,7	97,7
31768/1986	**99,1**	**98,9**	**98,9**	**99,5**	**99,1**	**99,1**	**99,1%**	**99,3**	-	99,6
31807/1986	**98,9**	**98,7**	**98,7**	**99,3**	**98,9**	**98,9**	**98,9%**	**99,1**	**99,7**	-

[a]: Brazilian strains analyzed in this study. Strain name followed by year of isolation; [b]: percentage of nucleotide identity as determined by BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html); [c]: percentage of amino acid identity (bold).

the endemicity of a particular serotype in a specific geographic region for a long period, different viral clades may be involved in that period [14]. It is known that the introduction of new DENV serotypes/genotypes/lineages is a major risk factor for dengue epidemics. In 2009/2010, 1,471,390 dengue fever (DF) suspected cases and 665 deaths were reported in Brazil, with DENV-1 causing epidemics in most states [4,15]

It is not clear whether the explosive epidemic reported in Brazil during 2009 and 2010 caused mainly by DENV-1 was due to this lineage replacement. The population susceptibility to this serotype which has not circulated for almost a decade and the occurrence of secondary infections in a hyperendemic country may also have played an important role in the disease epidemiology,. In this scenario, the monitoring of DENV is of great relevance to observe the spread of potentially virulent strains as well to evaluate its impact over the population during an outbreak.

Due to the Brazil's geography and dengue epidemiology, along with the fact the country has important tourist regions, a larger sampling analysis is suggested to better characterize those replacement events and lineage introductions in the country.

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Authors' contributions

FBS, RMRN and AMBF designed the study, FBN, NRCF, SAS, JBSS and CS performed the experiments, NCF and PCGN analyzed the data. FBS and FBN wrote the paper. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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4.2- **Artigo 2-** Dengue Virus Type 1 From Field-Caught Vectors And Humans In Brazil: Phylogeny Reveals Different Lineages Of The American African Genotype In 25 Years.

Referência Bibiográfica: Márcia Gonçalves de Castro, Fernanda de Bruycker Nogueira, Rita Maria Ribeiro Nogueira, Anielly Alves Ferreira, Nieli Rodrigues da Costa Faria, Priscila Conrado Guerra Nunes, Ricardo Lourenço-de-Oliveira, Flávia Barreto dos Santos.

Situação do manuscrito. Submetido ao periódico "PLoS **O**ne".

Este artigo atende ao objetivo específico 1.

Resumo: Desde a sua entrada no Rio de Janeiro em 1986 até 2001 o sorotipo DENV-1 (genótipo V) (America/África) circulava com a linhagem I. Após sua ressurgência em 2009, e com análise filogenética do gene E, foi possível constatar que o DENV-1 ainda pertencia ao genótipo V, porém amostras de 2009, 2010 e 2011, tanto de humanos, como do vetor, apresentou a co-circulação de mais duas linhagens (II e III). Também foi observado pela caracterização molecular que houve uma divergência maior entre as linhagens II e III, com substituições de aminoácidos principalmente na linhagem III.

Dengue Virus Type 1 from Field-Caught Vectors and Humans in Brazil: Phylogeny Reveals Different Lineages of the American African Genotype in 25 Years

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Running Title: Dengue 1 from Naturally Infected Vector and Humans

Key words: dengue virus type 1, *Aedes aegypti,* RT-PCR, Real Time qRT-PCR, multiple lineages, entomological surveillance, Brazil

Abstract

Background

Dengue viruses (DENV) replicate alternately on the mosquito vector (mainly *Aedes aegypti*) and human host. In Brazil, dengue became a major public health problem after DENV-1 introduction in 1986 in Rio de Janeiro and in 2009, this serotype re-emerged causing major epidemics in the country. Since then, a virological and entomological program was established for monitoring DENV in human sera and vectors and it has constituted an important tool for dengue epidemiology and vector-virus-host interactions studies.

Methods and Results

DENV-1 was identified by virus isolation and RT-PCR during the 1986, 2001 and 2010 entomological surveillances performed in Rio de Janeiro (RJ) and Roraima (RR) and the Real Time qRT-PCR detected $1.6x10^4$ copies/mL of DENV-1 in the macerate of a single *Ae. aegypti* female naturally infected. The phylogeny demonstrated that DENV-1 isolated from both field-caught vector and humans belong to genotype V (Americas/Africa), although the co-circulation of two distinct lineages (lineages II and III) was detected. A higher sequence divergence was observed between lineages II and III, and most amino acid substitutions were observed on domain III from E protein. Moreover, some residues were exclusive to some lineages, and may be predicted to be differentiating the three lineages.

Conclusions

The use of molecular techniques combined to virus isolation showed to be important approaches for the surveillance and molecular characterization studies of DENV from field-caught vectors. We detected DENV-1 in the vector after this serotype was first introduced during a virgin soil epidemic (1986), when it co-circulated with DENV-2 and DENV-3 (2001) and during the DENV-4 introduction (2010), demonstrating the co-circulation of two distinct lineages of the genotype V in Brazil. The molecular characterization showed sequence differences lineage-specific, independently in which host the virus was isolated. Moreover, we also determined the viral titer in a single *Ae. aegypti* female naturally infected.

Author Summary

Dengue became a major public health problem in Brazil and since 1986 a virological and entomological surveillance program has been established to monitor dengue activity. The entomological surveillance of DENV in *Ae. aegypti* mosquitoes and or/larvae may constitute an important tool for early prediction of dengue outbreaks. Furthermore, as lineage replacements have been considered as a major driver in dengue evolution in South America, phylogenetic studies from viruses isolated in human host and vectors may help to elucidate the genetic diversity and transmission dynamics in endemic and epidemic settings. Here, we detected and characterized DENV-1 isolated from both *Ae.aegypti* vector and human host isolated in 25 years in Brazil.

Introduction

Dengue viruses (DENV), *Flaviviridae* Family, comprehend four distinct serotypes (DENV-1 to 4) and are the most important human arboviruses worldwide. They are transmitted by the bite of mosquitoes belonging to genus *Aedes*, with *Aedes aegypti* (*Ae. aegypti*) being its main vector.

DENV evolution has been associated to its global expansion due to the widespread of its vector, increase of human population size, uncontrolled urbanization and expansion of international commerce and travel [1-3]. Currently, an estimated 2.5 billion people living in urban areas in tropical and sub-tropical countries in Southeast Asia, the Pacific and the Americas are at risk of infection [4]. However, if current global weather trends continue, it is predicted that 50-60% of world´s population may be at risk of dengue infection by 2085 [5].

In Brazil, the first outbreak after the reintroduction of *A. aegypti* in the country was caused by DENV-1 and DENV-4 in Boa Vista, State of Roraima (RR), in the Northwestern country's border, during 1981-1982 [6]. Four years later, DENV-1 caused the first epidemic in virgin soil in the municipality of Nova Iguaçu, State of Rio de Janeiro (RJ), Southeast region [7]. Since then, RJ has played an important role in dengue epidemiology in the country, with the first introduction of DENV-2 in 1990 [8] and DENV-3 in 2000 [9]. DENV-3 was prevalent in the majority of Brazilian States from 2002 to 2006 and, from 2007 to 2008 this serotype was displaced by DENV-2. In 2008 Brazil experienced a severe dengue epidemic mainly caused by DENV-2 with 806,036 cases reported and RJ alone was responsible for 255,818 cases. In 2009

DENV-1 re-emerged in the Southeast region and it was the serotype detected in 50.4% of the viral isolations, displacing DENV-2 and DENV-3 [10]. Despite the introduction of DENV-4 in RR in 2010 and its subsequent spread to other states of the country [11], DENV-1 was the most prevalent serotype, responsible for epidemics with more than 2 million cases reported in Brazil in 2010 and 2011 [12,13].

The DENV RNA encodes a single precursor polyprotein which is cleaved co- and post-translationally into three structural (C, prM/M and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) by viral and cellular proteases [14]. The DENV E protein (~495 amino acids) is composed by three domains, domain I which organizes the protein structure, domain II which bears the fusion loop and domain III, a highly immunogenic immunoglobulin (Ig)-like module thought to contain receptor binding sites and neutralizing epitopes [15-20].

Nucleic acid sequencing allowed the classification of DENV into genetically distinct groups or genotypes within each DENV serotype with sequence divergence not greater than 6% within the E/NS1 junction [21].This observation was latter confirmed by the complete E gene sequencing [22-35].

Currently, phylogenetic analysis based on the E gene sequences has demonstrated that 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 (America/Africa) [33]. Furthermore, the existence of lineages with distinct geographic and temporal relationships within each genotype have been observed [36-40]. Lineage turnover or replacements have become more frequently

common in phylogenetic studies and the term "lineage" has been used non- officially to characterize those viruses clustered in clades in a taxonomic level beneath genotype.

Here, we aimed to analyze DENV-1 strains circulating in field-caught vectors and humans in an epidemic-endemic scenario, during the last 25 years in Brazil. As the years passed by, the availability of new molecular techniques played an important role on DENV surveillance in humans and vectors.

Materials and methods

Ethical Statement

All human DENV-1 strains belong to a previously gathered collection from the Laboratory of Flavivirus, Instituto Oswaldo Cruz/Fundação Oswaldo Cruz (IOC/FIOCRUZ), Rio de Janeiro, Brazil, obtained from acute phase human serum through the passive surveillance system 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.

Mosquito larval and adult stages examined in this study were collected by the staff of the local State Dengue Control Program during the routine performed for the determination of house infestation index, entomological and virological surveillances. The Transmissores of Hematozoários Laboratory is a reference laboratory for dengue entomological surveillance, for the Brazilian Ministry of Health. During the routine surveillance, field workers do not get any personal information from house owners and

no report identifies the house address. Anonymity is respected and no special permission for house entrance for mosquitoes collection and larval site treatment is required.

Viruses

The DENV-1 samples used in this study were isolated from *Ae. aegypti* adult mosquitoes and/or larvae and human hosts naturally infected in Brazil during epidemics spanning 25 years (1986-2011). Six isolates (three from *Ae. aegypti* and three from humans) sequenced in the present study were also compared to other ten recently analyzed by our group [41]. Detailed information on the strains is provided on Table 1.

Entomological Surveillance Study Sites

The entomological surveillance is often performed by Transmissores de Hematozoários, IOC/FIOCRUZ during inter-epidemic and epidemic periods in areas of DENV cases introductions and emergences. Here, the municipality of Nova Iguaçu, RJ was selected in two opportunities for the entomological surveillance, after new DENV introductions the country in 1986 and 2001. Nova Iguaçu is 523km^2 in area the metropolitan region of RJ, composed by 69 neighborhoods and 767,505 i with a population density of 1,517 inhabitants per km^2. The 2010 ent surveillance was performed in Boa Vista, RR, aiming to detect DENV-introduction in the country. The city of Boa Vista is 5,687 km^2 in area loc North Region of RR with 290,741 inhabitants and a population density of 51 per km^2.

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Entomological Surveillance in 1986

During the 1986 epidemic occurred in the municipality of Nova Iguaçu, when DENV-1 was firstly introduced in RJ, a total of 120 *Ae. aegypti* females were field-caught in this epidemic area in May, one month after the first DENV-1 isolation from humans. Adult females were separated in pools of 30 (total of 4 pools) and submitted to virus isolation at that time [42].

Entomological Surveillance in 2001

Aiming to isolate DENV-3 from *Aedes* vectors, just after this serotype introduction in the country by the municipality of Nova Iguaçu, RJ, in December of 2000, an entomological surveillance was performed in 18 out of 69 neighborhoods. Collections were performed in artificial containers in the household from dengue suspected cases from January to September, 2001. *Ae. albopicuts* females (n=22), males (n=7) and larvae (n=15) and *Ae. aegypti* females (n=176), males (n=77) and larvae (n=2,181) were available. *Ae. albopictus* were separated in 8 pools composed by 1 to 10 specimen/pool. *Ae. aegypti* females and males were separated in 26 and 22 pools (1 to 10 specimen/pool), respectively and larval pools were separated in 198 pools (2 to 10 larvae/pool) . All pools were macerated and submitted to virus isolation and RT-PCR.

Entomological Surveillance in 2010

Aiming to isolate DENV-4 just after this serotype introduction in the country in July of 2010, an entomological surveillance was performed in Boa Vista, RR and *Ae. aegypti* larvae, adult females and males (n=3,705) were collected by Maciel-de-Freitas et al

(unpublished data) at dengue suspected cases neighborhoods. A total of 234 pools of larvae (n=2,340 larvae; 10 larvae/pool), 128 pools of females (n= 1,218 females), and 15 pools of males (n=147 males) collected in July and August were available. The 1,218 females were divided in pools as follows: 120 pools composed by10 females each, 1 pool with 4 females, 3 pools with 2 females each, 2 pools with 3 females and 2 females were individually macerated. Males were divided in 14 pools of 10 and 1 pool of 7. All pools or individual specimen were processed and initially submitted to RT-PCR. Virus isolation was attempted afterwards.

Human viral strains

Aiming to analyze geographically distinct DENV-1 strains and to better characterized DENV-1causing recent epidemics in Brazil, we sequenced three human DENV-1 strains isolated from dengue cases occurred in the States of Alagoas (AL) and Ceará (CE), Northeast region of the country in 2010, as the other ten human DENV-1 strains included for phylogenetic analysis were already representative by States from Southeast and Midwest regions of Brazil [41].

Preparation of vectors

The pools were macerated in 1 mL of Leibovitz L-15 medium (Sigma) plus antibiotics (penicillin-streptomycin, 10,000 units - Invitrogen) and centrifuged (6,000 rpm at 4° C for 30 min). Supernatant was transferred to an Eppendorf tube containing 100 mL of streptomycin / fungizone and penicillin, kept in an ice bath for 1 hour and centrifuged (3,000 rpm at 4° C for 15 min). Supernatant was transferred to an Eppendorf tube containing 0.3mL of fetal calf serum (Invitrogen) and frozen (-70 ° C) until use.

Virus isolation

Virus isolation was performed by inoculating C6/36 *Aedes albopictus* cell line culture [43] kindly provided by Laboratorio Central, Bahia (LACEN/BA) and isolates were identified by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies [44]. Briefly, patients` sera or mosquitoes' macerates were inoculated into C6/36 *Ae. albopictus* cell monolayers in Leibovitz L-15 medium (Sigma) supplemented with 2% fetal calf serum (Invitrogen) and 0.2 mM of nonessential amino acids (Invitrogen). Cells were incubated at 28° C for 5 to 7 days and daily observed for cytopathic effects. Infected supernatant was clarified by centrifugation and virus stocks stored in 1-mL aliquots at –70°C until use.

Viral RNA extraction

Viral RNA was extracted from original infected cell culture supernatant or directly from the adult mosquitoes or larvae macerates using QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's instructions and stored at -70^0 C for RT-PCR assay to serotype DENV and sequencing for genotyping viruses.
To avoid mutations introduced by *in vitro* passages of the virus in cell cultures, we used DENV-1 strains extracted directly from serum previously detected by RT-PCR or originally isolated from cell culture when serum did not yield enough volume for RNA extraction.

RT –PCR (Reverse transcriptase- polymerase chain reaction)

RT—PCR for detecting and typing DENV was performed as previously described [45]. Briefly, consensus primers were used to anneal to any of the four DENV types, and amplify a 511-bp product in a reverse transcriptase-polymerase reaction. A cDNA copy of a portion of the viral genome was produced in a reverse transcriptase reaction. After a second round of amplification (nested PCR) with type-specific primers, DNA products of unique size for DENV-1 (482 bp) were generated.

Real Time qRT –PCR (Real Time Quantitive Reverse Transcriptase-Polymerase Chain Reaction)

For viral quantification , the RNA from the original *Ae. aegypti* individually macerated was submitted to Real Time qRT-PCR according to the protocol described by [46]. Briefly, sense (DenS [GGATAGACCAGAGATCCTGCTGT]) and anti-sense primers (DenAs [CATTCCATTTTCTGGCGTTC plus CAATCCATCTTGCGGCGCTC], 1:1) and probe (DenP [CAGCATCATTCCAGGCACAG]) were used to amplify the 3´untranslated region of DENV genome. All 5'nuclease probes were labeled with 6-carboxyfluorescein at the 5' end and with 6-carboxy-*N,N,N',N'*-tetramethylrhodamine at the 3' end.

Sequencing

The sequencing reaction was performed by reverse transcription using 5 µL of extracted RNA in 25 µL of AccessQuick™ RT-PCR System (Promega Corporation) and specific oligonucleotides primers, which sequences can be provided upon request. Primers were

used to amplify approximately 900 base pair (bp) fragments with 200bp overlapping regions spanning the C/prM/M/E genes (2,325 bp). Fragment were sequenced in both directions and thermocycling conditions consisted of a single step of 42°C for 60 minutes and 40 cycles of desnaturation at 94°C (30 seconds), annealing at 56°or 63°C (60 seconds) depending on the set of primers, extension at 72° C (2 minutes) and a final extension at 72° C (10 minutes). Amplification was conducted using a Model 9700 thermal cycler (Applied Biosystems). PCR products were purified from 1.0% agarose gels using QIAquick Gel extraction Kit or QIAquick PCR purification Kit (Qiagen) and used as template for cycle sequencing. Sequencing reactions were performed as recommended by the BigDye Dideoxy Terminator sequencing kit (Applied Biosystems) and the products were analyzed using an automated 3130 DNA Sequencer (Applied Biosystems). Sequences for the complete E gene (1,485 nucleotides) were deposited in GenBank (www.ncbi.nlm.nih.gov).

Sequences and phylogenetic analysis

Sequence and similarity identity analysis was performed using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.htmL). The E genes (1,485bp) multiple alignment was performed using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and the phylogenetic analysis by MEGA 5 software (www.megasoftware.net), using the "Neighbor-joining" method, according to the Tamura-Nei model, with a bootstrap of 1,000 replications. Strains representative from the five genotypes available in Genbank

(www.ncbi.nlm.nih.gov) were used for the comparison, DENV-2, DENV-3 and DENV-4 strains were used as outgroup to root the tree.

Results

Entomological Surveillance in 1986, 2001 and 2010

During 1986, the Flavivirus Laboratory, IOC/FIOCRUZ, received a total of 1,629 dengue suspected cases from the State of RJ, in the first epidemic in virgin soil, with 594 DENV-1 isolations from human cases and 78 from Nova Iguaçu. During the entomological surveillance, one DENV-1 strain (BR/RJAeaegypti/86) was isolated out of four pools of 120 *A. aegypti* females analyzed. Isolations were performed by inoculation into C6/36 *Ae. albopictus* cells, with subsequent serotype identification by IFAT. The RT-PCR was recently performed in the original macerate, and DENV-1 was confirmed as the infecting serotype. As the nucleic acid extraction did not yield enough RNA for sequencing, the original macerate was re-inoculated for new virus isolation and extraction.

In 2001, a total of 4,834 dengue suspected cases from RJ were received in the Laboratory for diagnosis and, DENV were isolated in 10.2% (494/4,834) of the cases: 329 DENV-1, 116 DENV-2 and 49 DENV-3. In Nova Iguaçu, a total of 84 DENV-1, 33DENV-2 and 11 DENV-3 were isolated from human cases [47]. During the entomological surveillance in 2001, when DENV-1 co-circulated with DENV-2 and DENV-3, only one pool out of 198 (0.5%) composed by five *Ae. aegypti* larvae collected in Nova Iguaçu on August of 2001 was positive to DENV-1 by both cell

culture inoculation and RT-PCR. The infecting DENV-1 strain was designated as BR/RJ74342/01. DENV-3 was identified in three *Ae. aegypti* females (9 females/pool each) and were further investigated (Castro et al., in preparation). No DENV were detected on the *Ae aegypti* adult pools or on the *Ae. albopictus* pools.

During the DENV-4 entomological surveillance performed on RR, in 2010, only one female individually examined was positive to DENV-1 by RT-PCR. Quantitive RT-PCR (qRT-PCR) was performed for DENV-1quantification and the results obtained showed that a total of $1.6x10^4$ copies/mL of DENV-1 was detected in the macerate of the female. Subsequently, the single female macerate was submitted to virus isolation and the identification of the infecting DENV-1 (strain BR1107/11) was confirmed by IFAT. A total of 377 pools were tested and the DENV detection rate by RT-PCR considering all polls analyzed was 0.26% (1/377). However, considering only the adult female pools, the detection rate is increased to 0.78% (1/128).

Phylogeny of Dengue Virus Type 1 from field-caught vectors and humans

The E gene sequences (1,485 bp) analysis from viruses isolated in *Ae. aegypti* adult females and larvae and humans demonstrated that DENV-1 isolated in the last 25 years, representative of six different states in the Southeast, Midwest, Northeast and North regions of the country, still belong to genotype V (Americas/Africa), but the existence of three different lineages was observed. The DENV-1 strains BR/RJAeaegypti/86 and BR/RJ74342/01 respectively isolated from the female mosquitoes and larvae, in 1986 and 2001, grouped together on lineage I, formed by DENV-1human strains isolated in 1986, 1990, 1997 and 2001 (Figure1) . However, one DENV-1strain isolated from a human host in CE state in 2010 (strain BR594/11) grouped on Lineage II, with strains

isolated during DENV-1 re-emergence in 2009 and 2010. As previously observed (dos Santos et al., 2011), a third lineage (lineage III) was identified and represented by strains isolated in humans in 2010 and 2011. DENV-1 strains isolated from humans in AL in 2010 (strains BR1141/11 and BR1142/11) and from the female mosquito isolated in 2010 in RR (strain BR1107/11, grouped together on this lineage, with a strong bootstrap support. Interestingly, the sequence of one DENV-1 strain isolated in Brazil in 1982 available on Genbank, did not group with strains from lineage I, showing no relation to strains introduced in 1986 in RJ (Figure 1).

Molecular characterization of the Brazilian dengue virus type 1 envelope (E) gene sequences

The nucleotide identities observed for Brazilian DENV-1 lineages based on the analysis of the E gene ranged from 95.6% to 96.9% between lineages I and II and from 96.7% to 98.3% between lineages I and III. The lower similarities were observed between lineages II and III (95.4% to 95.8%), showing divergences ranging from 4.2% to 4.6%, depending on the strains compared (Table 2).

In order to better characterize the genetic differences among the Brazilian DENV-1, we compared the complete E gene sequences from the three distinct lineages and observed six substitutions: one on domain I and five on domain III. Some of the residues are exclusive to some lineages and may be predicted to be differentiating the three lineages. On E_{297} (domain I), lineages II and III share a methionine (M), while a threonine (T) is exclusive to lineage I. On E_{338}, a substitution (S→L) is observed only on lineage II, and it is exclusive to this lineage. On E_{346}, all three lineages mostly share a T, with

exception of two strains (BR1435/RJ/2009 and BR1433/RJ/2009) from lineage II isolated from humans in RJ in 2009 (T→N) and one strain (BR0122/11/RJ/2011) from lineage III isolated from a human host in RJ in 2011 (T→A). On E_{394}, one residue (R) is exclusive to lineage III, however the strain isolated from the single *Ae. Aegypti* female from RR in 2010, despite grouping on lineage III, it shares the same residue from lineages I and II (R→K). As shown in Table 3 and Figure 1, lineages I and III are more closely related sharing more common residues on the E gene. The most divergent lineage (lineage II), presents three exclusive residues to this lineage (E_{338}, E_{428} and E_{436}), all located on the domain III of the E gene.

As most of the substitutions on the three lineages were observed on this domain, we further analyzed this region aiming to predict whether this particular region would be able to differentiate the distinct lineages. The phylogenetic analysis based only on the 195 amino acids from the domain III showed reproducibly the existence of the three lineages with the same tree topology presented by the analysis of the complete E gene (Figure 2).

Discussion

In the past 25 years, a global increase in both *Ae. Aegypti* distribution and dengue epidemics have been reported [48,49]. In Brazil, the disease has become a serious public health problem in most large cities of the country, particularly RJ, considered the main entry point for DENV. The spread of the viruses within the city and to other States is favoured by the high population densities and the frequent movement of residents and

tourists entry [50,51]. Since 1986 a virological and entomological program was established for monitoring DENV in human sera and vectors [8,42,52,53].

Despite the presence of *Ae. albopictus*, only *Ae. aegypti* has been incriminated as natural vector of DENV in Brazil [50]. The entomological surveillance of DENV in adult as well as in immature mosquito stages constitutes an important tool for early prediction of dengue epidemics. Moreover, virological surveillance screening field-caught dengue vectors by using RT-PCR has been useful to early detect dengue outbreaks in endemic regions and/or for the detection of new DENV introductions [54-60]. In our study, RT-PCR was effective on detecting the infecting serotype in field-caught mosquitoes in 2001 and 2010 as a screening tool and also as a retrospective study tool, by detecting the RNA from a macerate from 1986, frozen for 24 years.

The DENV-1 detection rate by RT-PCR in mosquitoes isolated in 1986 during the entomological surveillance performed in the first epidemic in virgin soil in RJ was of 25%. Due to the epidemiological importance of RJ, a virological surveillance program was established in Nova Iguaçu, RJ, for monitoring DENV activity during inter-epidemic periods. This has led to the first isolation of DENV-3 in December of 2000 from a classic dengue fever case and from the *Ae. aegytpti* collected on the field [9,53]. However, before causing one of the most severe epidemics in 2002 [61], DENV-3 co-circulated in RJ with DENV-1 and DENV-2 [47] and the entomological surveillance also detected DENV-1 in one pool of larvae by RT-PCR and virus isolation.

In 2009, DENV-1 re-emerged in Brazil displacing DENV-2 and DENV-3 [10] and despite the introduction of DENV-4 in RR in 2010 and its subsequent spread to other

states of the country, DENV-1 was the most prevalent and responsible for epidemics with more than 2 million cases reported in Brazil in 2010 and 2011 [12,13].

Aiming to address the dispersal of DENV-4 in the city of Boa Vista, RR, where a few DENV-4 cases have been newly detected, an entomological surveillance performed in July and August 2010 (Maciel-de-Freitas at al., unpublished data) yielding 3,705 *Ae. aegypti* adult and larvae to be tested. Despite the recent introduction DENV-4, DENV-1 was the infecting serotype detected in one *Ae. aegypti* female, individually macerated and analyzed by RT-PCR (detection rate = 0.78% for adult mosquito females).

DENV detection rates on *Aedes* mosquitoes vectors by RT-PCR may vary depending on the geographic settings, epidemiological backgrounds and vector population. In Taiwan, only 0.2% of 43,133 *Ae. aegypti* females analyzed were positive for DENV [59]. Recently, it has been shown that 16.1% of the *Ae aegypti* females collected in Mexican schools were DENV infected [62]. In Brazil, previous studies have shown rates of 17% in a DENV-3 surveillance performed during an epidemic in the city of Manaus, North region of Brazil [56]. On the other hand, only 0.1% of adult mosquitoes were infected by the same serotype when an entomological surveillance was performed in RJ, during the inter-epidemic year of 2006 (Pereira et al., unpublished data). In Recife, Northeast region of the country 10% of pools tested were infected and, despite the prevalence of DENV-3 in human cases, DENV-2 and DENV-1 were also detected in mosquitoes, with the latter being the most frequently detected [60]. Interestingly, in two opportunities of our present study (2001 and 2010), DENV-1 was detected in mosquitoes when other serotypes were being investigated, both after new serotypes introductions. It has been

suggested that a prevalent serotype may persist for one or two years until replaced by a new one [54]. Even though PCR assays have proven to be an effective tool for virological surveillance of DENV on field-caught adult mosquitoes and immature forms, high detection rates and even co-infections should be addressed carefully and preferably confirmed by other techniques such as virus isolation and genome sequencing.

Real-Time RT-PCR techniques have been established as more rapid and sensitive for detecting and quantifying DENV in clinical samples [46,63-66]. Furthermore, it was employed to quantify DENV-2 in laboratory infected mosquitoes [67,68]. Moreover, this technique is already being used as the standard method for screening arboviruses in field-caught mosquitoes in Singapore [68]. Here, we used the Real-Time RT-PCR to quantify the DENV-1 titer (1.6×10^4 copies/mL) from a single *Ae. aegypti* female naturally infected and individually macerated. From our knowledge, this is the first study describing the DENV titer in a single *Ae. aegypti* female naturally infected in Brazil. This observation is quite relevant as the threshold of viremia in humans needed to infect mosquitoes has not been measured accurately [69].

Phylogenetic studies based on viruses isolated from both field-caught naturally infected mosquitoes and humans may contribute for a better understanding of vector-virus interactions. In this study, the phylogeny of DENV-1 strains isolated from DENV vectors and from human host was performed to characterize the genotype circulating for 25 years in Brazil. The analysis of the E gene sequences from viruses isolated in adult mosquitoes, larvae and human host demonstrated that DENV-1 representative of six different states in the Southeast, Midwest, Northeast and North regions of the country,

still belong to genotype V (Americas/Africa), but the existence of three different

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existence of multiple lineages of the Indian DENV-1 within the A

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DENV-1 and DENV-4 in Boa Vista, RR during 1981-1982 [6]. T

restricted to this remote city located in the sparsely populated Northwe

Brazilian Amazon. Four years later, DENV-1 caused the first

Southeastern Brazil, almost 5,000 km apart [7]. Interestingly, one D

available on Genbank, from a strain isolated in Brazil in 1982, di

strains from lineage I, showing no relation to strains introduced in 1

other hand, the two DENV-1 strains isolated from mosquitoes collect

apart (1986 and 2001), grouped together on lineage I, formed by DEN

isolated in 1986, 1990, 1997 and 2001. It may suggest distinct DENV-

RR (1981-1982) and RJ (1986), lineage replacement or even evoluti

with the acquisition of adaptive mutation in DENV-1 providin

transmission by mosquito. Indeed, while DENV-1 introduction in RR

constrained to one city, DENV-1 arrival in RJ four years latter was fo

state epidemic and wide spread in the country [71]. One DENV-1stra

human host in CE in 2010 grouped on lineage II, with strains isolate

emergence on 2009 and 2010. As previously shown [41] a third lin

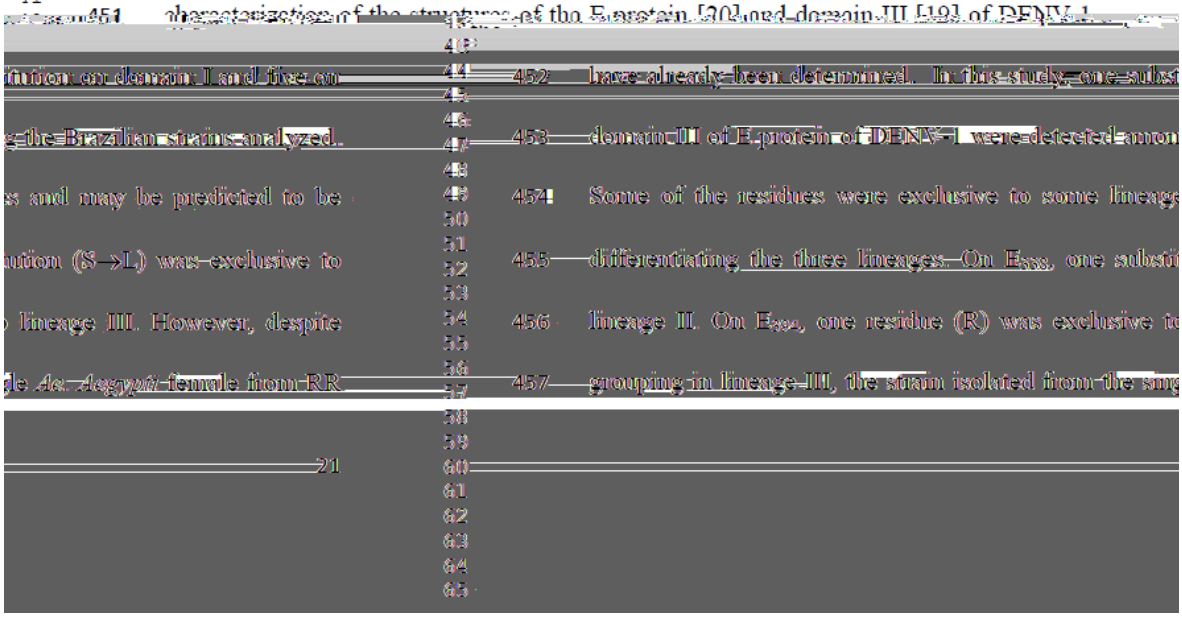
was identified and represented by strains isolated in humans in 2010 a

strains isolated from humans in AL and from the female mosquito is

RR grouped together on this lineage, with a strong bootstrap support. Furthermore, the lower nucleotide similarities were observed between lineages II and III (95.4% to 95.8%).

The mechanisms involved on lineage replacements are still not fully understood, despite some hypothesis raised [29] and the circulation of more than one DENV-1 "lineages" has been described in Asia [38] and in the Americas [72]. Multiple viral lineages introductions for each DENV serotype in Mexico with frequent lineage replacements was reported [73]. Recently, four distinct DENV-1 lineages with different dynamics were identified over a 10-year period in Cambodia [39] and constant viral introductions and *in situ* virus evolution are contributing to DENV diversity in Singapore [74].

The DENV E protein (~495 amino acids) is composed by three domains, domain I which organizes the protein structure, domain II which bears the fusion loop and domain III, a highly immunogenic immunoglobulin (Ig)-like module thought to contain receptor binding sites [15,16]. Neutralizing epitopes in domain III of DENV include residues 307, 333-351 and 383-389 [17,18]. Furthermore, a more detailed



in 2010 shares the same residue from lineages I and II (K). In fact, this may explain why this strain is clustered in an outside branch within lineage III (Figure 1). Sequencing of more DENV-1 strains is suggested to confirm this observation. On E_{428} and E_{436}, all strains from lineage II present exclusive residues are more closely related sharing more common residues on the E gene (Table 3 and Figure 1). The most divergent lineage (lineage II), presents three exclusive residues (E_{338}, E_{428} and E_{436}), all located on the domain III of the E gene. The phylogeny based only on the 195 amino acids from the domain III showed reproducibly the existence of the three lineages with the same tree topology presented by the analysis of the complete E gene.

Lineage replacements have been considered as a major driver in dengue evolution in South America [72,73,75,76] and Asia [38,39,74]. Phylogenetic studies from viruses isolated in human host and vectors may help to elucidate the genetic diversity and transmission dynamics in endemic and epidemic settings. Therefore, detecting viruses and providing sequences on both hosts, as obtained herein, would help on those studies. Moreover, as a key issue for dengue spread is the virus-vector interaction [77,78], the entomological surveillance of DENV in *Ae. aegypti* mosquitoes and or/larvae may constitute an important tool for early prediction of dengue outbreaks.

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Competing interest

The authors have declared that no competing interests exist.

Author Contributions

Conceived and designed the experiments: FBS, RMRN and RLO, performed the experiments: MGC, FBN, NRCF, PCGN, AAF, analyzed the data: MGC, FBS, contributed with reagents/materials/analysis tools: RMRN, RLO, wrote the paper: MGC, FBS.

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FIGURES LEGEND

Figure 1

plete envelope (E) gene sequences

quitoes and human cases from 1986

represent DENV-1 sequences generated in this study, open circles

equences published previously by our Group (dos Santos et al.,

mparison purposes. Strains representative from the five genotypes

(www.ncbi.nlm.nih.gov) were used for the comparison, DENV-2,

4 strains were used as outgroup to root the trees.The percentage of

ch the associated taxa clustered together in the bootstrap test (1000

next to the branches. DENV strains used were named as follows:

umber/country/year.

Figure 2

ylogenetic analysis of DENV-1 isolated from naturally infected

an cases from 1986 to 2011 based on the analysis of the envelope

5 residues, 588 base pairs). Black circles represent DENV-1

in this study, open circles represent DENV-1 sequences published

oup (dos Santos et al., 2011) and used for comparison purposes.

e from each of the five genotypes available in Genbank

ov) were used for the comparison. The percentage of replicate

associated taxa clustered together in the bootstrap test (1000

738 replicates) is shown next to the branches. DENV strains used were named as follows:

720 GenBank accession number/country/year

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Table 1: Brazilian dengue virus type 1 (DENV-1) isolated from naturally infected vectors and human cases, 1986 to 2011.

Strain	Origin State*	Year of isolation	Source	GenBank accession number	Reference
BR/RJAeaegypti/86	RJ	1986	Mosquitoes	HQ603916	This study
BR/RJ74342/01	RJ	2001	Mosquitoes	HQ603917	This study
BR1107/11	RR	2010	Mosquitoes	JN713897	This study
BR1141/11	AL	2010	human serum	JQ015184	This study
BR1142/11	AL	2010	human serum	JQ015185	This study
BR594/11	CE	2010	human serum	JN982362	This study
BR31768/86	RJ	1986	human serum	HQ026760	[41]
BR31807/86	RJ	1986	human serum	JN122280	[41]
BR0055/09	ES	2009	human serum	HM043709	[41]
BR1433/09	RJ	2009	human serum	HQ026761	[41]
BR1435/09	RJ	2009	human serum	HQ043710	[41]
BR0015/10	MS	2010	human serum	HQ696612	[41]
BR0019/10	RJ	2010	human serum	HQ026762	[41]
BR0020/10	RJ	2010	human serum	HQ696613	[41]
BR0188/10	RJ	2010	human serum	HQ696614	[41]
BR122/11	RJ	2011	human serum	JN122281	[41]

*RJ: Rio de Janeiro, RR: Roraima, AL: Alagoas, CE: Ceara, MS: Mato Grosso do Sul, ES: Espirito Santo).

Table 2: Sequences identity based on the E gene analysis between Brazilian DENV-1 lineages.

	Strains	Lineage I				Lineage II							Lineage III				
		Aeaegypti/86	BR31768	BR31807	74342mosq	BR0055	BR1433	BR1435	BR0019	BR0020	BR188	BR594	1107mosq	BR0015	BR1141	BR1142	BR0122
Lineage I	*Aeaegypti*/86*	-	99.7[a]	99.8	98.5	96.9	96.7	96.7	96.9	96.9	96.9	96.9	97.9	98.3	98.2	98.3	97.9
	BR31768	**100.0**[b]	-	99.7	98.4	96.8	96.6	96.6	96.8	96.8	96.8	96.8	97.7	98.1	97.9	98.0	97.7
	BR31807	**100.0**	**100.0**	-	98.5	96.9	96.7	96.7	96.9	96.9	96.9	96.9	97.8	98.2	98.1	98.1	97.8
	74342mosq	**100.0**	**100.0**	**100.0**	-	95.8	95.6	95.6	95.8	95.8	95.8	95.8	96.7	97.1	96.9	97.0	96.7
LineageII	BR0055	**99.1**	**99.1**	**99.1**	**99.1**	-	99.6	99.6	99.8	99.8	99.8	99.5	95.8	95.8	95.6	95.7	95.6
	BR1433	**98.9**	**98.9**	**98.9**	**98.9**	**99.7**	-	100.0	99.7	99.7	99.7	99.3	95.6	95.6	95.4	95.5	95.4
	BR1435	**98.9**	**98.9**	**98.9**	**98.9**	**99.7**	**100.0**	-	99.7	99.7	99.7	99.3	95.6	95.6	95.4	95.5	95.4
	BR0019	**99.1**	**99.1**	**99.1**	**99.1**	**100.0**	**99.7**	**99.7**	-	100.0	100.0	99.5	95.8	95.8	95.6	95.7	95.6
	BR0020	**99.1**	**99.1**	**99.1**	**99.1**	**100.0**	**99.7**	**99.7**	**100.0**	-	100.0	99.5	95.8	95.8	95.6	95.7	95.6
	BR188	**99.1**	**99.1**	**99.1**	**99.1**	**100.0**	**99.7**	**99.7**	**100.0**	**100.0**	-	99.5	95.8	95.8	95.6	95.7	95.6
	BR594	**99.1**	**99.1**	**99.1**	**99.1**	**100.0**	**99.7**	**99.7**	**100.0**	**100.0**	**100.0**	-	95.8	95.8	95.6	95.7	95.6
LineageIII	1107mosq	**99.7**	**99.7**	**99.7**	**99.7**	**99.3**	**99.1**	**99.1**	**99.3**	**99.3**	**99.3**	**99.3**	-	98.1	98.1	98.0	97.9
	BR0015	**99.5**	**99.5**	**99.5**	**99.5**	**99.1**	**98.9**	**98.9**	**99.1**	**99.1**	**99.1**	**99.1**	**99.7**	-	99.7	99.6	99.4
	BR1141	**99.5**	**99.5**	**99.5**	**99.5**	**99.1**	**98.9**	**98.9**	**99.1**	**99.1**	**99.1**	**99.1**	**99.7**	**100.0**	-	99.7	99.4
	BR1142	**99.5**	**99.5**	**99.5**	**99.5**	**99.1**	**98.9**	**98.9**	**99.1**	**99.1**	**99.1**	**99.1**	**99.7**	**100.0**	**100.0**	-	99.3
	BR0122	**99.3**	**99.3**	**99.3**	**99.3**	**98.9**	**98.9**	**98.9**	**98.9**	**98.9**	**98.9**	**98.9**	**99.5**	**99.7**	**99.7**	**99.7**	-

*Taxons name were shortened to fit in table. [a]percentage of nucleotide identity as determined by BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.htmL); [b]: percentage of amino acid identity (bold).

762 Table 3: Amino acid substitutions predicting to be differentiating the Brazilian dengue

763 virus type 1 (DENV-1) lineages based on the analysis of the envelope (E) gene.

E gene (position) /substitutions						Lineage	Strain*, Source	Origin State, Year of isolation
DI	DIII							
297	E_{338}	E_{346}	E_{394}	E_{428}	E_{436}			
T	S	T	K	V	V		BR/RJAeaegypti, vector	RJ, 1986
T	S	T	K	V	V	I	BR31768, human host	RJ, 1986
T	S	T	K	V	V		BR31807, human host	RJ, 1986
T	S	T	K	V	V		BR74342mosq, vector	RJ, 2001
M	**L**	T	K	**L**	**I**		BR0055, human host	ES, 2009
M	**L**	N	K	**L**	**I**		BR1433, human host	RJ, 2009
M	**L**	N	K	**L**	**I**		BR1435, human host	RJ, 2009
M	**L**	T	K	**L**	**I**	II	BR0019, human host	RJ, 2010
M	**L**	T	K	**L**	**I**		BR0020, human host	RJ, 2010
M	**L**	T	K	**L**	**I**		BR0188, human host	RJ, 2010
M	**L**	T	K	**L**	**I**		BR594/11, human host	CE, 2010
M	S	T	K	V	V		BR1107/11, vector	RR, 2010
M	S	T	**R**	V	V		BR0015, human host	MS, 2010
M	S	T	**R**	V	V	III	BR1141/11, human host	AL, 2010
M	S	T	**R**	V	V		BR1142/11, human host	AL, 2010
M	S	A	**R**	V	V		BR122/11, human host	RJ, 2011

aneiro, ES: Espirito Santo, CE: Ceará RR:

main I, DIII: domain III. T: Threonine, M:

e, K: Lysine, R: Arginine, V: Valine, I:

764 *Taxons name were shortened to fit table. RJ: Rio de J

765 Roraima, MS: Mato Grosso do Sul, AL: Alagoas. DI: do

766 Methionine, S:Serine, L: Leucine, N: Asparagine, A: Alanine

767 Isoleucine. On bold: residues exclusive to the lineage.

768

769

Figure
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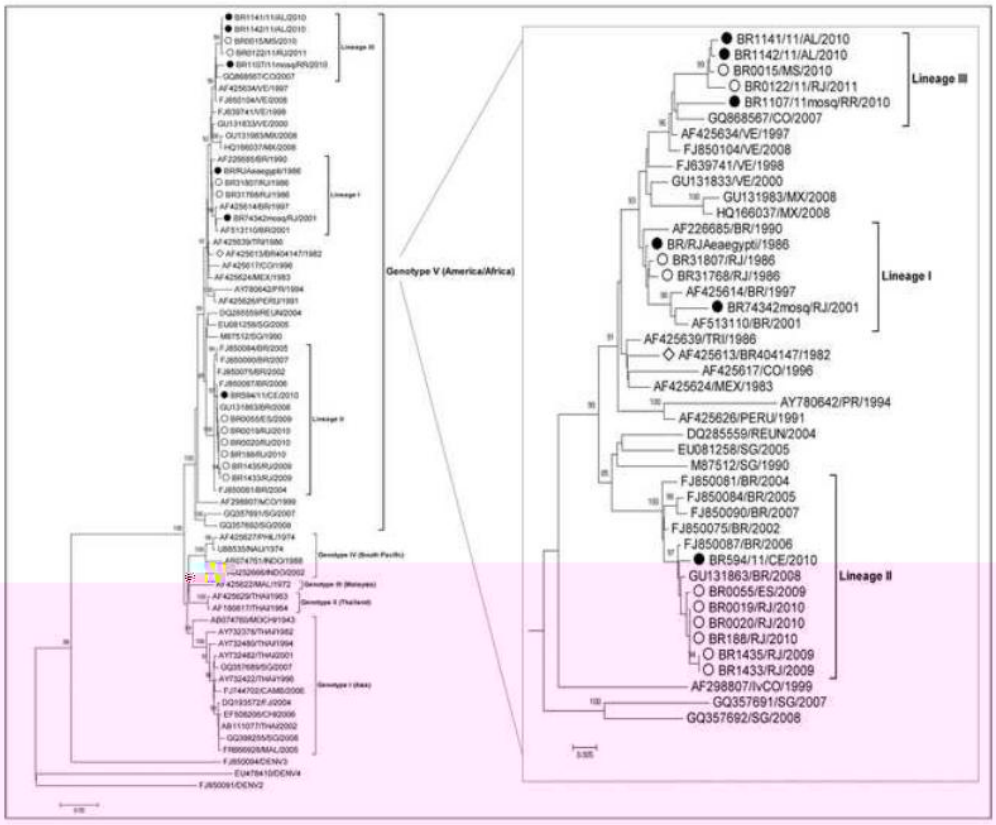

Figure
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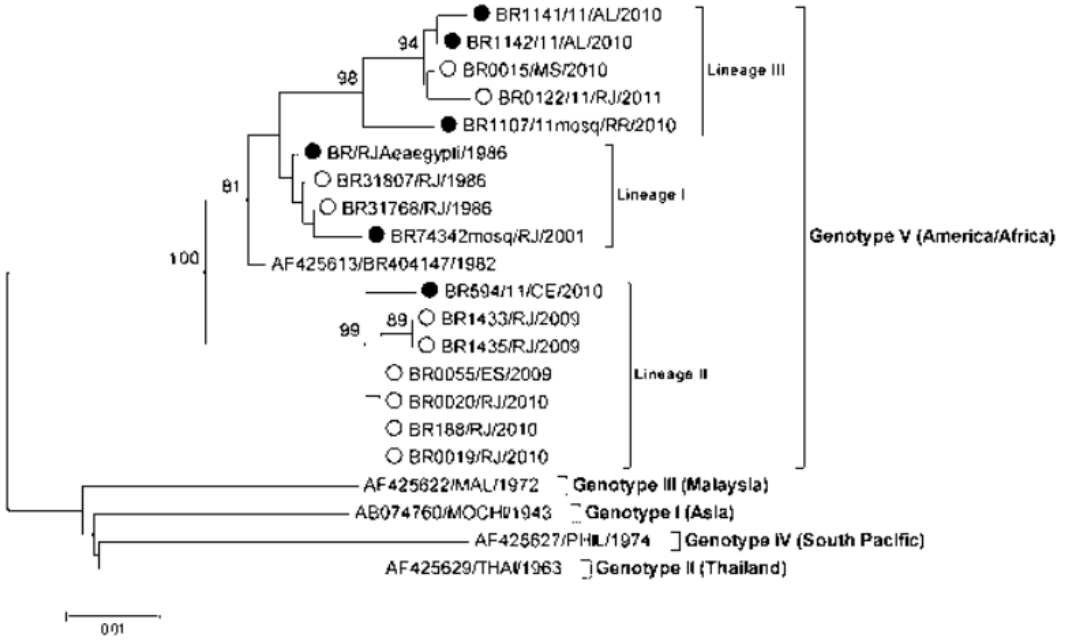

4.2. Molecular Differences Of The 3´Untranslated Region From Brazilian Dengue Virus Type 3 Isolated From Naturally Infected Mosquitoes And Humans

Referência Bibliográfica: Márcia Gonçalves de Castro, Fernanda de Bruycker Nogueira, Rita Maria Ribeiro Nogueira, Ricardo Lourenço-de-Oliveira, Flávia Barreto dos Santos.

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Molecular differences of the 3´untranslated region from Brazilian dengue virus type 3 isolated from naturally infected mosquitoes and humans

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Running Title: Molecular characterization of Dengue Virus Type 3 isolated from Naturally Infected Mosquitoes

Key words: Dengue virus type 3, *Aedes aegypti,* 3'UTR

SUMMARY

Background

Dengue, a mosquito-borne viral infection caused by one of the four dengue virus (DENV) serotypes (DENV-1 to 4), replicate alternately on the mosquito vector and human host and are responsible for infections throughout tropical and subtropical regions of the world. In Brazil, the disease has become a major public health problem and, the introduction of DENV-3 in 2000 in Rio de Janeiro (RJ) was associated with severe dengue epidemics. The potential emergence of strains associated with severe disease highlights the need for the surveillance of DENV in human host and vectors.

Methods

Aiming to contribute for DENV phylogenetic and vector-virus-human host studies, we 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.

Results

Substitutions exclusive to this strain and a substitution leading to a stop codon formation on the NS5 gene were observed. As an 8- nucleotides deletion was observed within the 11- nucleotides (nts) insertion on the variable region (VR) from the 3´UTR, we further sequenced other DENV-3 from both mosquitoes (n=3) and humans (n=10) isolated from 2001 to 2008 in RJ. The majority of DENV-3 from RJ analyzed was characterized by the 11-nts insertion in the VR of the 3´UTR, despite the observation of strains carrying the 8-nts deletion. The latter presented similar secondary structures

however, not all strains presenting the 11-nts insertion were similar in the secondary predicted structure.

Conclusions

The phylogeny based on the analysis of the complete genome and 3´UTR characterized the DENV-3 isolated from both vector and human host as belonging to Genotype III (GIII), despite the differences observed on the 3' UTR. Further studies are needed to address the role of those mutations in the transmission of the different viral populations and vector competence.

Background

Dengue is a mosquito-borne viral infection caused by one of the four dengue virus (DENV) serotypes (DENV-1 to 4), belonging to genus *Flavivirus*, family *Flaviviridae.* The viruses replicate alternately on the mosquito vector, mainly (*Ae. Aegypti*) and human host and are responsible for infections throughout tropical and subtropical regions of the world [1, 2].

The rapid global spread of the four DENV serotypes in the last 50 years resulted in the dispersal of genotypes associated with increased disease severity [3]. In Brazil, dengue has been a major public health problem since DENV-1 introduction and spread in 1986 [4], however the introduction of the genotype III of DENV-3, in December 2000, in Nova Iguaçu, State of Rio de Janeiro (RJ), caused one of the most severe epidemics reported in the country in 2002 [5-7]. Despite the co-circulation of DENV-1, DENV-2 and DENV-3 in that area, DENV-3 was the only serotype detected in pools of *Ae. aegypti* during an entomological surveillance performed [8].

Sequencing of distinct DENV genomic regions has identified five genotypes for DENV-3: Genotypes I to III (GI to GIII) which are responsible for most DENV-3 human infections and have been associated with both dengue fever (DF) and dengue haemorrhagic fever (DHF) epidemics in Southeast Asia, Indian Subcontinent, South Pacific and East Africa and Americas, and Genotypes IV and V (GIV and GV) which were not associated with DHF epidemics and are only represented by few early sequences from Americas, South Pacific and Asia [9-13].

The DENV genome is composed by a positive single-stranded RNA of approximately 11 kb in length with an open reading frame encoding for the viral polyprotein, which is cleaved into three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4 and NS5) flanked by 5´ and 3´ untranslated regions (UTRs) of about 100 and 400 nucleotidest, respectively [1]. The flaviviruses UTRs are predicted to form secondary stem-loop (SL) structures, which are highly conserved and play a role in viral replication [14-18].

According to predicted secondary structures, the DENV-2 3´UTR can be divided into

three domains [18]. The domain I, which is located immediately after the

NS5 stop codon, is considered the most variable region (VR) within the viral 3´UTR, a

s it shows large heterogeneity in both length and nucleotide sequences due to mutatio

ns of this sequence and can serve as a good marker for the evolution of DENV

[19-21]. Mutations and deletions within these regions may alter infectivity and reduce

efficiency of viral replication [22, 23] and differences between strains occurred in the

se regions may influence DENV virulence and pathogenicity [24-27]. Furthermore, del

etions and nucleotide variations were also described in the VR within the same serotyp

e [28-30]. Domain II is of moderate conservation, comprising several hairpins motifs a

and where conserved sequence (CS2) and repeated CS2 (RCS2) are present. Domain III i

s the most conserved region of the 3´UTR with CS1 followed by a terminal stem-loop (3´

´SL) [18].

It has been previously reported that DENV, particularly DENV-3, like o

ther RNA viruses, is present in human host as quasispecies, a population of close

ly related sequences [31]. However, the extent of DENV sequence variation in the

mosquito vector and the role of these quasispecies during transmission between h

uman and

[illegible] maintaining a more [illegible] 32].

[illegible] vector interactions, we [illegible] turally infected field- [illegible] comparison to other [illegible] based on best [illegible] complete genome sequence isolated from [illegible] g the first DENV-3 epidemic in Brazil.

mosquitoes may contribute to the DENV genetic stability, by homogenous viral population and/or selecting a dominant variant [

Here, aiming to contribute for the studies on human host-virus-v fully sequenced the genome of one DENV-3 isolated from na caught mosquitoes in RJ and characterized the viral 3´UTR in [illegible] DENV-3 isolated from [illegible] from our knowledge, this the first DENV-3 [illegible] mosquitoes durin

Methods

ent

NV-3 strains belong to a previously gathered collection from the avivirus, IOC/FIOCRUZ, RJ, Brazil obtained from acute phase human ıe passive surveillance system from an ongoing Project approved by ɛr CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee ? 274/05), Ministry of Health, Brazil.

amined in this study were collected by the staff of the Dengue Control for the determination of house infestation index, virological and ırveillance. No special permission or written consent is required for ɔr mosquito collection and larval site treatment.

Material and

Ethical Statem

All human DEN Laboratory of Fla serum through th resolution numbe in Research (CEP

Aedes aegypti ex State Program f entomological su house entrance fc

Viral strains

The DENV-3 strains isolated from *Ae. aegypti* adult mosquitoes (n=4) and human hosts (n=10) naturally infected in RJ, were collected from epidemics occurred from 2001 to 2008. The first Brazilian DENV-3 strain (BR74886/02) isolated from a human fatal case fully sequenced [33] was used for comparison purposes. Detailed information on the strains is provided on Table 1.

DENV-3 entomological surveillances

The threeDENV-3 strains isolated (BR73354/01, BR73356/01 and BR73636/01) in 2001 from naturally infected *Ae.aegypti* adult mosquitoes used in this study were collected during an entomological survey performed in 35 districts of Nova Iguaçu, RJ, from July 2000 to June 2001. The other DENV-3 strain (BR81200/06) was isolated from naturally infected *Ae.aegypti* adult mosquitoes collected during an entomological survey conducted on 7 districts with different infestation index, randomly chosen in the municipality of Rio de Janeiro, RJ, from March 2005 to February 2006. Briefly, adult mosquitoes were collected twice a week, alternately in the morning and in the afternoon with manual and battery backpack aspirators and with nets, both indoors and in the yards and gardens, close to the dwellings. Mosquitoes were identified, pooled according to gender, date, district of collection and stored in liquid nitrogen at the same day of collection. A total of 503 *Ae. aegypti* mosquitoes (352 females and 151 males) collected in 2000-2001 and 874 *Ae.aegypti* females collected in 2006 were polled (9-17mosquitoes/pool and 27 pools of 2–10 mosquitoes/pool, respectively) and processed for virus isolation and RT-PCR.

Preparation of vectors

Mosquitoes pools were macerated in 1 ml of Leibovitz L-15 medium (Sigma) plus antibiotics (penicillin-streptomycin, 10,000 units - Invitrogen) and centrifuged (6,000 rpm at 4 ° C for 30 min). Supernatant was transferred to an Eppendorf tube containing 100 mL of streptomycin / fungizone and penicillin, kept in an ice bath for 1 hour and centrifuged (3,000 rpm at 4 ° C for 15 min). Supernatant was transferred to an Eppendorf tube containing 0.3ml of fetal calf serum (Invitrogen) and frozen (-70 ° C).

Virus isolation

Virus isolation was performed by inoculation into monolayers of C6/36 *Aedes albopictus* cells [34] in Leibovitz L-15 medium (Sigma) supplemented with 2% fetal calf serum (Invitrogen) and 0.2 mM of nonessential amino acids (Invitrogen). Cells were incubated at 28°C for 5 to 7 days and observed for cytopathic effects. Isolates were identified by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies [35] and infected supernatant was clarified by centrifugation and virus stocks stored in 1-mL aliquots at –70°C.

RNA extraction

Viral RNA was extracted by using QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's instructions and stored at -70^{0}C for DENV typing and sequencing. To avoid mutations introduced by *in vitro* passages of the virus in cell cultures, we used DENV-3 strains extracted directly from serum and mosquitoes macerate previously detected by RT-PCR for characterizing the viral 3'UTR and the first passage in cell culture for the full genome sequencing of the DENV-3 strain (BR77754/01).

RT –PCR (Reverse transcriptase- polymerase chain reaction)

RT—PCR for detecting and typing DENV was performed as described previously [36]. Briefly, consensus primers were used to anneal to any of the four DENV types and amplify a 511-bp product in a reverse transcriptase-polymerase reaction. A cDNA copy of a portion of the viral genome was produced in a reverse transcriptase reaction. After a second round of amplification (nested PCR) with type-specific primers, DNA products of unique size for DENV-3 (290 bp) were generated.

Sequencing

The sequencing reaction was performed by reverse transcription using 5 µL of extracted RNA in 25 µL of AccessQuick™ RT-PCR System (Promega Corporation) and specific oligonucleotides primers, which sequences can be provided upon request, to amplify approximately 900bp amplicons with overlapping fragments of 200bp spanning the complete DENV-3 genome. Thermocycling conditions consisted of a single step of 42°C for 60 minutes and 40 cycles of desnaturation at 94°C (30 seconds), annealing at 56°or 63°C (60 seconds) depending on the set of primers, extension at 72° C (2 minutes) and a final extension at 72° C (10 minutes). Amplification was conducted using a Model 9700 thermal cycler (Applied Biosystems). PCR products were purified from 1.0% agarose gels using QIAquick Gel extraction Kit or QIAquick PCR purification Kit (Qiagen) and used as template for cycle sequencing. Sequencing reactions were performed as recommended in the BigDye Dideoxy Terminator sequencing kit (Applied Biosystems) and the products were analyzed using an automated 3130 DNA Sequencer (Applied Biosystems). The mosquitoes DENV-3 full-length genome sequence and 3´UTR sequences obtained in this study were deposited in GenBank (www.ncbi.nlm.nih.gov) and are described on Table 1.

Sequences and phylogenetic analysis

Sequence and similarity identity analysis was performed using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.htmL). The multiple alignments were performed using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and the phylogenetic analysis by MEGA 5 software (www.megasoftware.net), using the Neighbor-joining method, according to the Tamura-Nei model, with a bootstrap of 1,000 replications for the analysis of the complete genome. For the 3´UTR analysis, the Maximum likelihood method, according to the Kimura-2 model was chosen as determined by the best-fit substitution model. Strains representative from the five genotypes available in GenBank (www.ncbi.nlm.nih.gov) were used for the comparison, DENV-1, DENV-2 and DENV-4 strains were used as outgroup to root the tree.

Secondary structure analysis

The predicted secondary structures were generated by MFOLD web server ((http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) with default folding parameters and folding predictions at 37° C.

RESULTS

DENV-3 entomological surveillance

The entomological surveillance performed in the first semester of 2001 in Nova Iguaçu, RJ, resulted in the isolation of three DENV-3 strains from the districts of Santa Efigênia (BR73354/01),California (BR73356/01) and Morro Agudo (BR73636/01) isolated from three pools (9 mosquitoes/pool) of naturally infected *Ae aegypti* females [8]. In January 2006, one DENV-3 strain (BR81200/06) was isolated from one *Ae. aegypti* pool composed of three females , collected indoors in the Vargem Pequena neighborhood, west region of RJ.

Molecular characterization of a full-length Brazilian DENV-3 isolated from naturally infected *Ae. aegypti*

In order to access the differences among the Brazilian DENV-3, we sequenced the entire genome of one virus isolated from naturally infected *Ae. aegypti* (BR 73354/01) and compared to the a Brazilian DENV-3 isolated from a human fatal case during the 2002 epidemic [33] and sequences available on GenBank. The complete genome sequence obtained was deposited on Genbank under accession number FJ177308. The comparison of complete genome sequences showed that the *Ae. aegypti* strain BR73354/01 is more closely related to the Brazilian strain 74886/02 (AY679147), isolated from the liver of a fatal case during the DENV-3 epidemic occurred in 2002 with a nucleotide similarity of 99.3% and the phylogeny based on the analysis of the complete genome (coding region) characterized the Brazilian strain as belonging to genotype III (Indian Subcontinent), Figure 1. The nucleotide and amino acid similarities among the Brazilian DENV-3 strains and strains representative from all four genotypes

are shown on Table 2. Furthermore, amino acid substitutions were observed throughout the entire coding region, when the Brazilian DENV-3 strains were compared to the prototype PHIL/H87/1956 and strains representative of the other genotypes. Some substitutions were exclusive to the Brazilian DENV-3 strains analyzed (Table 3, gray-shadowed cells) and some were shared among the Brazilian strains and the strain

NV-3 Brazilian strains analyzed in

M→I) and C$_{\text{xxx}}$ (T→A) on the prM

e positions E$_{81}$ (I→V), E$_{169}$ (A→T), E$_{301}$ I→V). On the NS1 gene only NS1$_{339}$ (N→S) $_{37}$ (L→F), NS2A$_{158}$ (I→M) and NS2A$_{195}$ d NS2B$_{109}$, (V→I) and (I→V), respectively. as exclusive to the Brazilian DENV-3. On bserved on positions NS4B$_{21}$ and NS4B$_{138}$. ten substitutions exclusive to the Brazilian N), NS5$_{371}$ (K→R), NS5$_{374}$ (E→G), NS5$_{422}$ T), NS5$_{639}$ (L→P) and NS5$_{835}$ (D→N), an DENV-3 isolated from *Ae. agypti* nsecutively, on NS5$_{97}$ and NS5$_{98}$, (V→L) bstitution resulted in a stop codon and other →T), NS5$_{112}$ (E→A), NS5$_{114}$ (V→R), NS5$_{117}$ NS5$_{133}$ (F→D), NS5$_{135}$ (L→P) and NS5$_{233}$

shadowed cells). Exclusive substitutions to the DE

this study were observed on the capsid gene, C$_{\text{xxx}}$ (M

gene, prM$_{86}$ (H→R), and on the envelop (L→T), E$_{380}$ (I→T), E$_{383}$ (K→N) and E$_{452}$ (was observed, on NS2A positions NS2A (T→A) and on NS2B positions NS2B$_{60}$ an On the NS3 gene, only NS3$_{115}$ (I→T) wa NS4B, the same substitution (V→I) was o However, on the NS5 gene, besides the DENV-3 on NS5$_{229}$ (S→A), NS5$_{288}$ (S→N (R→K), NS5$_{429}$ (E→D), NS5$_{585}$ (K→T substitutions exclusive to the Brazili (BR73354/2001) were observed almost co and (T→S), respectively. On NS5$_{101}$, the su substitutions were observed on NS5$_{105}$ (K→ (S→P), NS5$_{127}$ (M→R), NS5$_{131}$ (D→E), (M→R).

Interestingly, the analysis of the 3´UTR of the strain BR73354/01 genome isolated from mosquitoes showed an eight nucleotides deletion within the eleven nucleotides insertion on the VR, previously observed for the Brazilian DENV-3 strain isolated from humans (Miagostovich et al., 2006) and common to genotype III DENV-3 strains from the Latin America/Caribbean and Sri Lanka regions. Nucleotides substitutions exclusive to the strain isolated from the mosquitoes BR73354/01 were observed on positions 10,383 and 10,391 from the 3´UTR. One substitution on the RCS2 and one on the CS2 were shared by all Brazilian DENV-3 when compared to the prototype (Figure 2).

Molecular characterization and phylogeny based on the 3´UTR from DENV-3 isolated from naturally infected *Aedes aegypti* and human host

We additionally sequenced the 3´UTR from other three DENV-3 strains isolated from naturally infected *Ae. aegypti* isolated in 2001 and 2006 in RJ (BR73356/01, BR73636/01 and BR81200/06) and from ten DENV-3 isolated from humans from 2001 to 2008. All 3´UTR sequences were deposited on GenBank. The strain BR73356/01 presented the same 8-nucleotides deletion observed for the strain BR73354/01. However, the other two strains also isolated from mosquitoes in RJ (BR73636/01 and BR81200/06) presented the 11-nucleotides insertion common to the human strains. The analysis of the 3´UTR from DENV-3 isolates from humans showed that nine out of ten strains also presented the 11-nucleotides previously described. However, one of the strains isolated in Rio de Janeiro in 2002 (BR74792/02) showed the same 8-nucleotides deletion observed on the mosquitoes strains (Figure 3). Despite those observations, it was also shown a high conservation on the 3´UTR RCS2, CS1 and CS2 conserved regions (Figure 3, gray-shadowed areas) among all the Brazilian strains analyzed.

Therefore, we focused on the VR of the 3´UTR, aiming to better understand the impact of those mutations on the predicted structures of that region. All three sequences with the 8 nucleotide deletion (BR73354mosq/2001, BR73356mosq/2001 and BR74792/2002) presented similar secondary structures. However, not all strains presenting the 11 nucleotide insertion were similar in structure. In fact, despite the 11 nucleotide insertion, the strain BR72/2008 presented a unique secondary structure (Figure 4). The only difference from the latter is a nucleotide substitution (G →A) on the 11 nucleotide insertion region, when compared to all other Brazilian sequences analyzed (Figure 3). The strain BR80996/2006, also showed a unique secondary structure, probably due to the six nucleotide substitution presented in the VR, despite the presence of the insertion shared with the other strains. The slight difference presented by the strain BR74947/2002, may be due to a substitution (C→T) exclusive to this sequence.

The Maximum-Likelihood phylogenetic tree of Brazilian DENV-3 strains isolates from naturally infected *Ae. aegypti* mosquitoes and humans based on the 3´UTR sequence analysis places those strains as belonging to genotype III, corroborating the findings of the full-length genome analysis (Figure 5).

Discussion

The DENV-3 was re-introduced in Latin America in 1994, after an absence of 17 years, being initially isolated in Panama and spreading throughout Central America [37, 38] to Caribbean [39-41] and South America [6, 28, 42-44]. However, some phylogenetic studies point to its introduction through Mexico [45] a few years earlier [13]. This introduction caused by the genotype III of DENV-3, originally from the Southeast Asia and characterized by an increased virulence, coincided with the occurrence of a higher number of severe and DHF cases [38, 46-48].

In Brazil, the first DENV-3 was isolated in December of 2000 in Nova Iguaçu, RJ [5] when the *Ae aegypti* infestation level was 8.1% and, 58% of those mosquitoes were resistant to temephos at that time [8, 49]. Due to the role of the city of Nova Iguaçu in dengue epidemiology, after the DENV-1 introduction in 1986 [4], field studies were conducted for detection of DENV in field-caught vectors [8, 50]. The entomological surveillance performed in the first semester of 2001 in Nova Iguaçu resulted in the isolation of DENV-3 strains from pools of naturally infected *Ae aegypti* females [8]. After a co-circulation with DENV-1 and DENV-2 [51], the newly introduced serotype caused one of the most severe epidemic in RJ in 2002, in terms of morbidity and mortality [6]. During an entomological surveillance performed during the interepidemic year of 2006, another DENV-3 strain was isolated from an *Ae. aegypti*, pool collected in the west region of RJ (strain BR 81200/06).

The phylogeny based on the complete genome sequencing of the DENV-3 isolated from naturally infected *Ae. aegypti* classified the Brazilian strain isolated during this serotype

355 introduction in 2001as GIII, corroborating previous studies of DENV-3 isolated from
356 DF and DHF cases [7, 12, 33].
357
358 The potential emergence of strains associated with severe disease highlights the need for
359 the surveillance of DENV in human host and vectors, as the detection of DENV in
360 infected field-caught vectors is considered a useful tool for the early prediction of
361 epidemics and detection of new serotypes/genotypes introductions [55, 56]. Moreover,
362 due to the evolutionary constrain that mosquitoes may impose on arthropod borne
363 viruses such as DENV, the analysis of viruses isolated from invertebrate host are
364 extremely important and have been addressed [57].
365
366 Despite the use of the E gene for phylogenetic and evolutionary studies of DENV [9,
367 12, 28, 54, 57-65] due to its biological properties and selective pressure imposed by the
368 host immune response, the role of sequences heterogeneity in other genomic regions
369 which includes the non-structural genes and the genome UTRs cannot be excluded [58].
370 Therefore, aiming to contribute for the DENV phylogenetic and evolutionary studies,
371 we sequenced the entire genome of a DENV-3 isolated from naturally infected *Ae.*
372 *Aegypti* from RJ. It was shown the mosquitoes strain BR73354/01 was more closely
373 related to the Brazilian strain 74886/02 (AY679147) isolated from a fatal case occurred
374 in 2002 [33] than to any other isolates from humans and mosquitoes. Amino acid
375 substitutions were observed throughout the entire coding region, when the Brazilian
376 DENV-3 strains were compared to the prototype PHIL/H87/1956 and strains
377 representative of the other genotypes. Some substitutions were exclusive to the
378 Brazilian DENV-3 analyzed and some were shared among the Brazilian strains and the
379 strain isolated from *Ae. aegypti* in Taiwan in 1998 (TAIWAN/TWmosq/1998).

Besides the exclusive substitutions to the Brazilian DENV-3 strains analyzed, those observed on C_{108} (M→I), C_{112} (T→A), E_{81} (I→V), E_{169} (A→T), E_{301} (L→T), E_{383} (K→N), E_{452} (I→V), $NS1_{339}$ (N→S), $NS2A_{37}$ (L→F), $NS2A_{158}$ (I→M), $NS2A_{195}$ (T→A), $NS5_{209}$ (S→N), $NS5_{423}$ (R→K), $NS5_{585}$ (K→T) and $NS5_{825}$ (D→N) were also shared by other genotype III strains previously analyzed [11], and presente

ed changes in the polarity, charges or hydrophobicity. Also on the NS5 gene, substituti

ons exclusive to the DENV-3 isolated from the *Ae. aegypti* (BR73354/2001) were obser

ved on $NS5_{97}$ (V→L), $NS5_{98}$ (T→S), $NS5_{105}$ (K→T), $NS5_{112}$ (E→A), $NS5_{114}$ (V→R), N

$S5_{117}$ (S→P), $NS5_{101}$, a substitution resulted in a stop c

(M→P). Or DENV-3 containing either stop codons or del

odon. The presence of genome-defective [31].

etions *in vivo* has been reported previously

ence and secondary structures of the 5´and Previous studies have suggested that the sequ

in viral replication and differences in these 3´UTR of flaviviruses play an important role

26, 27, 66]. Moreover, mutations and regions may influence viral virulence [24,

nfectivity and reduce efficiency of viral deletions within these regions may alter i

replication [22, 23].

ization of the genome through interactions The 3´UTR, which is involved in the circular

tinct domains [18]. The domain I from the with the 5´UTR [67], is divided into three dis

the NS5 stop codon. It is considered the DENV 3´UTR is located immediately after

18] and can serve as a good marker for the most variable region (VR) from the 3´UTR [

of the 3´UTR of the strain BR73354/01 evolution of DENV [19-21]. The analysis

an 8 nucleotides deletion within the 11 genome isolated from mosquitoes showed

nucleotides insertion on the VR, previously observed for the Brazilian DENV-3 strain isolated from humans [33] and common to genotype III DENV-3 strains from the Latin America/Caribbean and Sri Lanka regions [27, 68].

Aiming to compare the extent of sequence variation in the 3´UTR from Brazilian DENV-3 from mosquitoes and humans, we additionally sequenced other strains isolated from naturally infected *Ae. aegypti* and humans, isolated from epidemics occurred just after this serotype introduction in RJ. The strain BR73356/01 presented the same 8-nucleotides deletion observed for the strain BR73354/01. However, the other two strains also isolated from mosquitoes presented the 11-nucleotides insertion common to the human strains. The analysis of the 3´UTR from DENV-3 isolates from humans showed that nine out of ten strains also presented the 11-nucleotides previously described, however one of the strains isolated in Rio de Janeiro in 2002 (BR74792/02) showed the same 8-nucleotides deletion observed on the mosquitoes strains. Previous studies have shown deletions and nucleotide variations in the VR within a same serotype [28, 29,

despite these observations, high conservation near the 3´

nalyzed. This was quite conserved regions was detected among all the Brazilian strains a

III, the most conserved expected as domain II is of moderate conservation and domain region of all [18].

tions, we analyzed the To better understand the conformational impact of those muta

s with the 8 nucleotides predicted structures of that particular region. All three sequence

ll strains presenting the deletion presented similar secondary structures. However, not a

in structure. In fact, despite the 11 nucleotide 11 nucleotides insertion were similar

nted a unique secondary structure. The strain insertion, the strain BR72/2008 prese

BR80996/2006, also showed a unique secondary structure, probably due to the six nucleotide substitution presented in the VR, despite the presence of the insertion shared with the other strains. The slight difference presented by the strain BR74947/2002, may be due to a substitution (C→T) exclusive to this sequence.

Phylogenetic studies based on the 5`and 3`UTR have shown to be very useful for molecular epidemiological studies [19, 21, 27, 28, 70] and the analysis carried out using the 3´UTR of the Brazilian DENV-3 isolates from naturally infected mosquitoes and humans placed those strains as belonging to GIII, corroborating the sam the full-length genome analysis.

It has been shown that due to the non-proofreading nature of the viral RNA p many RNA viruses may show high degree of sequence variation, not o isolates from different individuals but also among viruses within a same indiv Moreover, the co-circulation of different serotypes/genotypes in a particular the feeding behavior of *Ae. aegypti* that frequently takes blood multipl different hosts [71], may result in the co-infection of genetically distinct DE in both mosquito and human hosts [54].

Conclusions

Here, we analyzed the coding region and the 3´UTR of DENV-3 from both h and mosquitoes and described insertions, deletions and a substitution leadi codon formation. The majority of DENV-3 in this study was characterized nucleotide insertion in the 3´UTR, despite the observation of strains carry nucleotide deletion. In spite the presence of distinct viral variants, it is sug

the major variant is transmitted [32]. However, how those distinct viral populations are maintained or transmitted is not fully understood, therefore the availability of viruses isolated from both hosts are crucial for the better comprehension of the vector-virus-human host interactions and for quasispecies investigations. Furthermore, the analysis of those distinct viral populations in experimentally infected mosquitoes may help to elucidate those observations.

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The authors have no conflict of interest.

Author´s contributions

ıed the experiments,

FBS, RMRN and RLO designed the study. MGC, FNB perform
MGC, FBS and RLO wrote the paper.

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722 **FIGURE LEGENDS**

723

724 Figure 1

725 Neighbor-joining phylogenetic analysis of the complete genome sequence from DENV-
726 3 isolated from naturally infected mosquitoes in Brazil, 2001. Black circle represent
727 DENV-3 sequence generated in this study. Strains representative from the four
728 genotypes available in Genbank (www.ncbi.nlm.nih.gov) were used for the comparison,
729 DENV-1, DENV-2 and DENV-4 strains were used as outgroup to root the tree. The
730 percentage of replicate trees in which the associated taxa clustered together in the
731 bootstrap test (1000 replicates) is shown next to the branches. DENV strains used were
732 named as follows: GenBank accession number/country/year.

733

734 Figure 2

735 Nucleotide sequence alignment of the 3´UTR from Brazilian DENV-3. The Brazilian
736 DENV-3 strain isolated from naturally infected *Ae. aegypti* mosquitoes was compared
737 to other Brazilian DENV-3 strains isolated from human cases and to the prototype strain
738 H87 (GenBank accession number M93130). Dots (.) indicate identity among strains
739 based on the H87 prototype, dashes (-) indicate gaps in the alignment. The 11
740 nucleotides insertion common to Genotype III DENV-3 strains from the Latin
741 America/Caribbean and Sri Lanka regions and the 8 nucleotides deletion characteristic
742 to the mosquitoes isolate are shown. Two nucleotides substitutions specific to the
743 mosquitoes isolate are shown by the black arrows and nucleotide positions are indicated
e regions (RCS2, CS2 and 744 according to the prototype sequence. Conserved sequenc
745 CS1) are marked by squares.
746

Figure 3

Multiple nucleotide sequence alignment of the Brazilian DENV-3 3´UTR from additional strains isolated from mosquitoes *Ae. aegypti* (*n*=4) and humans (*n*=10) from 2001 to 2008. Dots (.) indicate identity among strains based on the Brazilian strain BR74886/02, characterized by the 11 nucleotides insertion on the variable region (VR). Dashes (-) indicate gaps in the alignment. The 8 nucleotides deletion characteristic to strains isolated from mosquitoes BR73354mosq/2001, BR73356mosq/2001 and from a strain isolated in human (BR74792/2002) are marked by black squares. Eleven nucleotide insertion and conserved sequence regions (RCS2, CS2 and CS1) are gray-shadowed.

Figure 4

Predicted secondary structure of the variable region (VR) from the Brazilian DENV-3 strains isolated from humans and mosquitoes *Ae. aegypti* and compared to the prototype PHIL/87/1956. Structures on black bold-line squares are those with the 8 nucleotide deletions and on dashed-line squares are predicted structures differing from the majority of the Brazilian strains.

Figure 5

Maximum-Likelihood phylogenetic tree (Kimura 2-parameter model) of Brazilian DENV-3 strains isolates from naturally infected *Aedes aegypti* mosquitoes and humans based on the 3´UTR sequence analysis. Black circle represent DENV-3 sequenc viruses isolated from mosquitoes (*n*=4) and white circles, strains isolated from (*n*=10) generated in this study. Strains representative from the four genotypes a

ces from humans available

in Genbank (www.ncbi.nlm.nih.gov) were used for the comparison. DENV strains used were named as follows: GenBank accession number/country/year.

797 Table 1: Brazilian DENV-3 isolated from naturally infected vectors and human cases
798 analyzed in this study.

Strain	Origin State*	Year of isolation	Source	GenBank accession number	Sequence analyzed	Reference
BR73354/01	RJ	2001	Mosquitoes	FJ177308	Complete genome	This study
BR73356/01	RJ	2001	Mosquitoes	JN383345	3´UTR	This study
BR73636/01	RJ	2001	Mosquitoes	JN383346	3´UTR	This study
BR81200/06	RJ	2006	Mosquitoes	JN383344	3´UTR	This study
BR70562/01	RJ	2002	Human serum	JN380902	3´UTR	This study
BR74792/02	RJ	2002	Human serum	JN380899	3´UTR	This study
BR74916/02	RJ	2002	Human serum	JN380901	3´UTR	This study
BR74947/02	RJ	2002	Human serum	JN380904	3´UTR	This study
BR77515/03	RJ	2003	Human serum	JN380900	3´UTR	This study
BR78969/04	RJ	2004	Human serum	JN380905	3´UTR	This study
BR80740/05	RJ	2005	Human serum	JN380906	3´UTR	This study
BR80996/06	RJ	2006	Human serum	JN380903	3´UTR	This study
BR83904/07	RJ	2007	Human serum	JN380898	3´UTR	This study
BR072/08	RJ	2008	Human serum	JN380907	3´UTR	This study
BR74886/02	RJ	2002	Human liver	AY679147	Complete genome	Miagostovich et al., 2006

799 *RJ: Rio de Janeiro

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Table 2: Sequences identity among Brazilian DENV-3 and distinct genotypes based on the complete coding region analysis.

	BR/74886/ 2002 (GIII)	BR73354mosq/ 2001 (GIII)	PHIL/H87/ 1956 (GV)	INDO/PH86/ 2004 (GI)	TAIWAN/TWmosq/ 1998 (GII)	TAHITI/136116/ 1994 (GIV)
BR/74886/ 2002 (GIII)	-	99.3[a]	94.7	93.3	93.8	93.5
BR73354mosq/ 2001 (GIII)	**99.4**[b]	-	94.6	93.1	93.6	93.3
PHIL/H87/ 1956 (GV)	98.1	97.8	-	94.9	94.9	95.
INDO/PH86/ 2004 (GI)	**97.8**	**97.4**	**98.0**	-	93.3	96.
TAIWAN/TWmosq/ 1998 (GII)	**98.1**	**97.8**	**98.1**	**98.0**	-	93.
TAHITI/136116 /1994 (GIV)	**97.9**	**97.5**	**98.1**	**98.5**	**98.0**	-

*Taxons name were shortened to fit in table. [a]percentage of nucleotide identity as determined by BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.htmL); [b]: percentage of amino acid identity (bold). GI to GV: Genotypes I to V, respectively

...o acid differences among selected Brazilian DENV-3 and strains representative of the DENV-3 genotypes, compared to the

825 Table 3: Amir

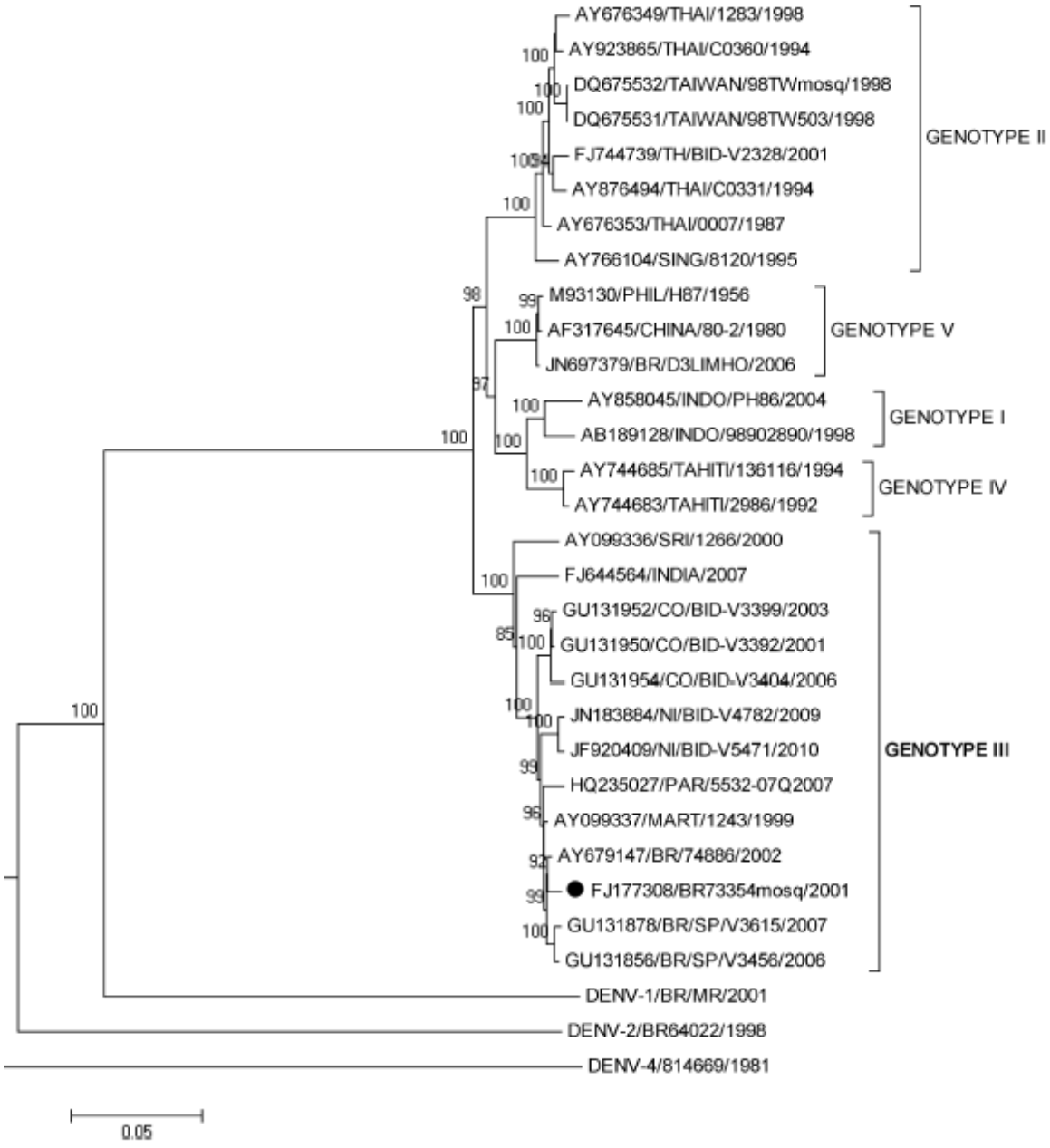
826 prototype H87

Genotypes		GV	GIII				GI	GII	GIV
Position	Strain	PHIL/H87/ 1956 human	BR/74886/ 2002 human	BR/73354/ 2001 mosquitoes	BR/SP/V345 6/2006 human	BR/SP/V361 5/2007 human	INDO/PG86/ 2004 human	TAIWAN/TW mosq/ 1998 mosquitoes	TAHITI/136 116/1994 human
C_{108}		M	I	I	I	**I**			
C_{112}		T	A	A	A	**A**			
prM_{86}		H	R	R	R	**R**			
E_{81}		I	V	V	V	**V**			
E_{124}		S	**P**	**P**	L	L	L	**P**	
E_{132}		H	**Y**	**Y**	**Y**	**Y**		**Y**	
E_{169}		A	T	T	T	**T**	V	V	V
E_{270}		T	**N**	**N**	**N**	**N**		**N**	
E_{301}		L	T	T	T	**T**	S		
E_{380}		I			T	**T**			
E_{383}		K		N	N	**N**			
E_{452}		I	V	V	V	**V**			
$NS1_{84}$		I			V				
$NS1_{94}$		T	I		I	I	I	I	I
$NS1_{139}$		S	N		N	N	N	N	N
$NS1_{339}$		N	S	S	S	**S**			
$NS2A_{37}$		L	F	F	F	**F**			
$NS2A_{158}$		I	M	M	M	**M**			
$NS2A_{175}$		I	**V**	**V**	**V**	**V**		**V**	
$NS2A_{195}$		T	A	A	A	**A**			
$NS2B_{60}$		V	I	I	I	**I**			
$NS2B_{109}$		I	V	V	V	**V**			

NS3	$NS3_{115}$	I	**T**	**T**	**T**	**T**			
	$NS3_{452}$	V	**A**	**A**	**A**	**A**		**A**	
NS4A	$NS4A_{99}$	D	**E**	**E**	**E**	**A**			
NS4B	$NS4B_{21}$	V	**I**	**I**	**I**	**I**			
	$NS4B_{138}$	V	**I**	**I**	**I**	**I**			
NS5	$NS5_{97}$	V		**L**					
	$NS5_{98}$	T		**S**					
	$NS5_{101}$	R		***STOP**					
	$NS5_{105}$	K		**T**					
	$NS5_{112}$	E		**A**					
	$NS5_{114}$	V		**R**					
	$NS5_{117}$	S		**P**					
	$NS5_{127}$	M		**R**					
	$NS5_{131}$	D		**E**					
	$NS5_{133}$	F		**D**					
	$NS5_{135}$	L		**P**					
	$NS5_{229}$	S	**A**	**A**	**A**	**A**			
	$NS5_{233}$	M		**R**					
	$NS5_{288}$	S	**N**	**N**	**N**	**N**			
	$NS5_{365}$	P	**S**	**S**	**S**	**S**			
	$NS5_{371}$	K	**R**	**R**	**R**	**R**			
	$NS5_{374}$	E	**G**	**G**	**G**	**G**			
	$NS5_{389}$	R		**K**	**K**	**K**		**K**	
	$NS5_{422}$	R	**K**	**K**	**K**	**K**			
	$NS5_{429}$	E	**D**	**D**	**D**	**D**			
	$NS5_{585}$	K	**T**	**T**	**T**	**T**			
	$NS5_{639}$	L	**P**	**P**	**P**	**P**			
	$NS5_{763}$	T	**S**	**S**	**S**	**S**	A	**S**	A
	$NS5_{835}$	D	**N**	**N**	**N**	**N**			

827 In gray shadow amino acid substitutions exclusive to all four Brazilian DENV-3 strains. Black-shadowed cell, amino acid substitutions shared only by the Brazilian DENV-3
828 and the strain TAIWAN/TWmosq/ 1998, isolated from *Ae. aegypti* mosquitoes in Taiwan in 1998. Blank cells indicate amino acid similarities to the prototype strain H87
829 (GenBank accession number M93130). *STOP: stop codon. GI, GII, GIII, GIV and GV: Genotypes I to V, respective

830


AY676349/THAI/1283/1998
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DQ675531/TAIWAN/98TW503/1998
FJ744739/TH/BID-V2328/2001
AY876494/THAI/C0331/1994
AY676353/THAI/0007/1987
AY766104/SING/8120/1995
GENOTYPE II
M93130/PHIL/H87/1956
AF317645/CHINA/80-2/1980
JN697379/BR/D3LIMHO/2006
GENOTYPE V
AY858045/INDO/PH86/2004
AB189128/INDO/98902890/1998
GENOTYPE I
AY744685/TAHITI/136116/1994
AY744683/TAHITI/2986/1992
GENOTYPE IV
AY099336/SRI/1266/2000
FJ644564/INDIA/2007
GU131952/CO/BID-V3399/2003
GU131950/CO/BID-V3392/2001
GU131954/CO/BID-V3404/2006
JN183884/NI/BID-V4782/2009
JF920409/NI/BID-V5471/2010
HQ235027/PAR/5532-07Q2007
AY099337/MART/1243/1999
AY679147/BR/74886/2002
FJ177308/BR73354mosq/2001
GU131878/BR/SP/V3615/2007
GU131856/BR/SP/V3456/2006
GENOTYPE III
DENV-1/BR/MR/2001
DENV-2/BR64022/1998
DENV-4/814669/1981
0.05

Figure 1

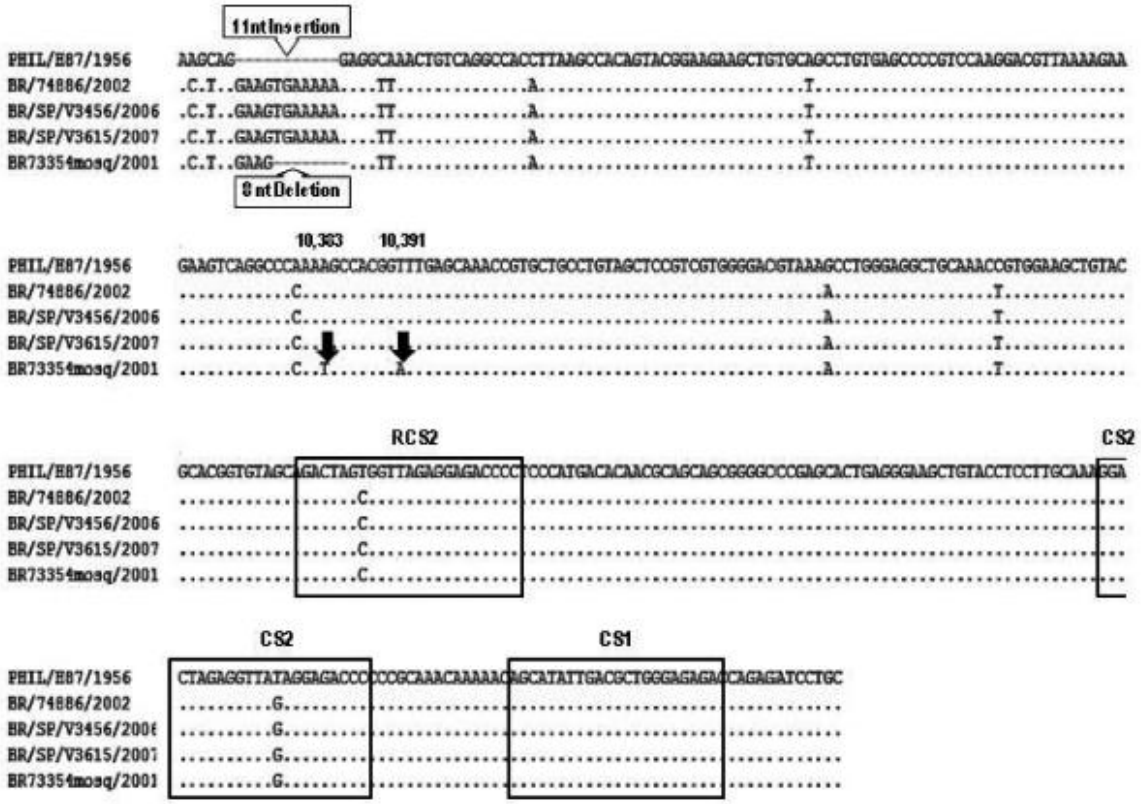

11ntInsertion
PHIL/E87/1956
BR/74886/2002
BR/SP/V3456/2006
BR/SP/V3615/2007
BR7335¶mosq/2001
8 nt Deletion
10,383
10,391
RCS2
CS2
CS1

Figure 2

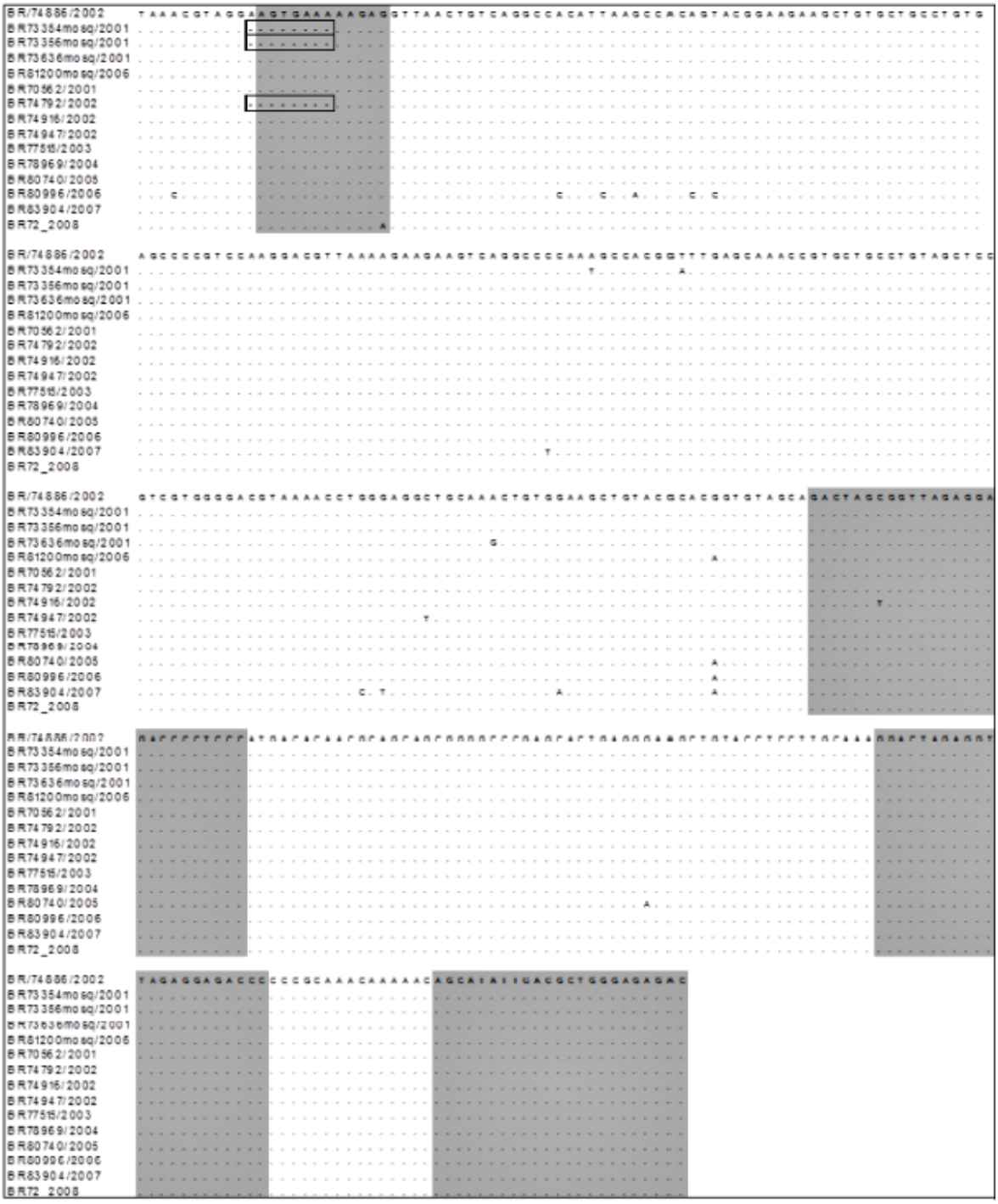


Figure 3

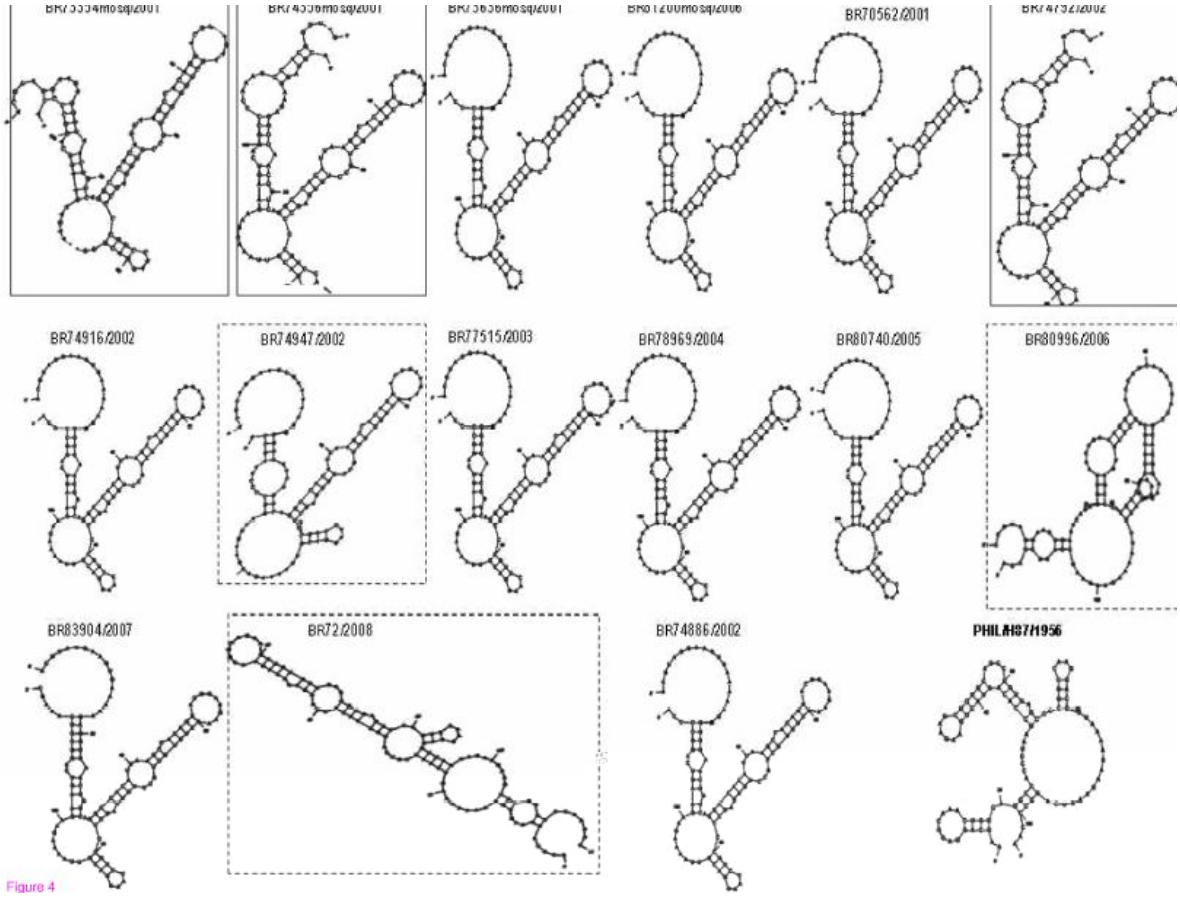
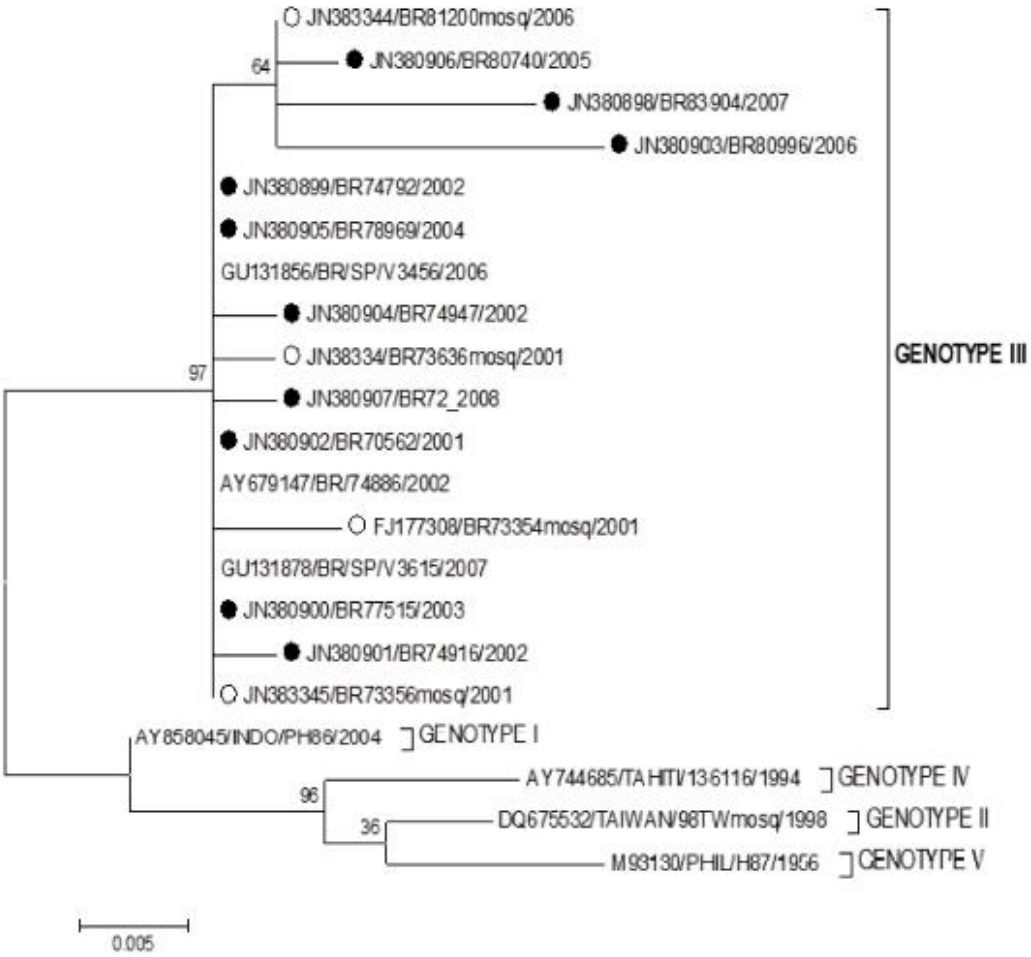

BR70562/2001
BR74916/2002
BR74947/2002
BR77515/2003
BR78969/2004
BR80740/2005
BR80996/2006
BR83904/2007
BR72/2008
BR74886/2002
PHILAI87/1956

Figure 4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64
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JN380905/BR78969/2004
GU131856/BR/SP/V3456/2006
JN380904/BR74947/2002
JN38334/BR73636mosq/2001
97
JN380907/BR72_2008
JN380902/BR70562/2001
AY679147/BR/74886/2002
FJ177308/BR73354mosq/2001
GU131878/BR/SP/V3615/2007
JN380900/BR77515/2003
JN380901/BR74916/2002
JN383345/BR73356mosq/2001
GENOTYPE III
AY858045/INDO/PH86/2004
GENOTYPE I
AY744685/TAHITI/36116/1994
GENOTYPE IV
96
36
DQ675532/TAIWAN/98TWmosq/1998
GENOTYPE II
M93130/PHIL/H87/1956
GENOTYPE V
0.005

Figure 5

4.3- Artigo 4. Dengue virus type 4 (DENV-4) in Niteroi, Rio de Janeiro: The role of molecular techniques in the laboratorial diagnosis and entomological surveillance.

Referência Bibliográfica: Márcia Gonçalves de Castro, Rita Maria Ribeiro Nogueira, Ana Maria Bispo de Filippis, Anielly Alves Ferreira, Monique da Rocha Queiroz Lima, Nieli Rodrigues da Costa Faria, Fernanda de Bruycker Nogueira, Jaqueline Bastos Santos Simões, Priscila Conrado Guerra Nunes, Simone Alves Sampaio, Ricardo Lourenço-de-Oliveira, Flávia Barreto dos Santos.

Situação do manuscrito. Submetido ao periódico "Memórias do Instituto Oswaldo Cruz".

Este artigo atende ao objetivo específico 4.

Resumo: Foi detectado casos de DENV-4 em humanos e no mosquito *Ae. aegypti*, com o auxílio de várias técnicas moleculares, onde se observou que a utilização dessa ferramenta pode ser considerado um importante avanço para a vigilância entomológica e virologica.

Dengue virus type 4 (DENV-4) in Niteroi, Rio de Janeiro: The role of molecular techniques in the laboratorial diagnosis and entomological surveillance

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Running Title: DENV-4 epidemiological and entomological surveillance in Rio de Janeiro

KEY WORDS: Dengue virus type 4, *Aedes aegypti,* RT-PCR, Real Time RT-PCR, Simplexa™ Dengue

ABSTRACT

In Rio de Janeiro (RJ), Southeast region of Brazil, DENV-4 was first isolated in March of 2011 in the city Niteroi. Here, we analyzed the laboratorial findings on the first DENV-4 cases occurred in Niteroi and evaluated the use of molecular techniques for detecting DENV-4 in field-caught *Aedes aegypti* collected during an explosive DENV-1 epidemic in 2011. The MAC-ELISA confirmed 22.2% (2/9) of the investigated DENV-4 acute cases analyzed, virus isolation was possible in 55.5% (5/9) cases and both NS1 ELISA and NS1 Ag STRIP confirmed 44.4% (4/9). On the other hand, both Conventional RT-PCR and Simplexa™ Dengue Real Time RT-PCR confirmed 100% of the human cases analyzed. DENV-4 was also identified by conventional RT-PCR and Simplexa™ Dengue Real Time RT-PCR *Ae. aegypti* female (1/72; 1.4%) captured in captured in a household from the district São Domingos, Niteroi. From our knowledge, this is the first time the Simplexa™ Dengue Real Time RT-PCR is used for human cases investigation and entomological surveillance. No virus was recovered from any of the 72 mosquitoes by virus isolation. However the quantitive Real Time RT-PCR detected 1.08 x10^3 copies/mL of DENV-4 in the macerate of a single *Ae. aegypti* female naturally infected, also positive to DENV by both NS1 capture tests. NS1 was also detected in an *Ae. aegypti* male. The use of molecular techniques, such as conventional RT-PCR and Real-Time RT-PCR showed to be important approaches for the surveillance of DENV from field-caught vectors, even when a new serotype is introduced during a distinct serotype epidemic scenario.

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

DENV-4 was reintroduced in the country in 2010 in the municipalities of Boa Vista and Canta, Roraima State (Temporão et al. 2011). The virus spread to different regions of Brazil, with cases of infection registered in the North, Northeast and Southeast (SVS 2011) and genome sequencing characterized the DENV-4 Brazilian strains as belonging to genotype II (de Sousa et al. 2011). In RJ, the first DENV-4 cases detected occurred in the Cafubá neighborhood, in the oceanic region of the municipality of Niterói, which is located in the metropolitan region of RJ (Nogueira & Eppinghaus 2011).

The entomological surveillance of DENV in adult as well as in immature mosquito stages constitutes an important tool for early prediction of dengue epidemics. Moreover, virological surveillance screening field-caught dengue vectors by using molecular techniques such as RT-PCR has been useful to early detect dengue outbreaks in endemic regions and/or for the detection of new DENV introductions (Chow et al. 1998, Pinheiro et al. 2005, Mendez et al. 2006, Chen et al. 2010, Guedes et al. 2010).

Here, we aimed to laboratorial characterize the first DENV-4 cases and show the role of rapid molecular techniques, such as conventional RT-PCR and Real Time RT-PCR in the entomological surveillance of the newly introduced DENV-4 in vector populations from Niteroi, RJ, Brazil, just after its first isolation in humans. Furthermore, here we evaluated a newly available Real Time PCR commercial kit for detecting and typing DENV in serum samples and mosquitoes macerates.

MATERIAL AND METHODS

Human sera were obtained during a surveillance system performed by the Laboratory of Flavivirus, IOC/FIOCRUZ, Rio de Janeiro, Brazil 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.

DENV-4 cases investigation was performed during the DENV-1 epidemic RJ in 2011.The first two cases confirmed were from two sisters living the Cafuba neighborhood, Niteroi, both, with onset of symptoms on March 6th 2011 (Nogueira & Eppinghaus 2011). We further investigated nine other suspected cases from individuals living Cafuba, São Francisco, São Domingos, and Engenho do Mato, with onset of symptoms from March 23rd 2011 to April 11th 2011 (Figure 1A, Supplementary data).

Ae. aegypti adult mosquitoes were collected in nine residential and non-residential sites at (São Domingos) where DENV-4 human cases were confirmed. Collections were performed on four sites (Figure 1B, Supplementary data) on May 4th, 2011 by using battery-operated aspirators. Mosquitoes were anaesthetized at 4^{0}C, identified, sexed and stored in liquid nitrogen at the same day of collection. A total of 72 *Ae. aegypti* (33 females and 39 males) adult mosquitoes were collected. From these, 47 (18 females and

29 males) were collected in a single site village-like residential area (site #1), composed by six houses (Supplementary data). *Ae.aegypti* were individually macerated in 1 ml of Leibovitz L-15 medium (Sigma) plus antibiotics (penicillin-streptomycin, 10,000 units – Invitrogen) and centrifuged (6,000 rpm at 4 ° C for 30 min). Supernatant was transferred to an Eppendorf tube containing 100 mL of streptomycin / fungizone and penicillin, kept in an ice bath for 1 h and centrifuged (3,000 rpm at 4°C, 15 min). Supernatant was transferred to an Eppendorf tube containing 0.3ml of fetal calf serum (Invitrogen) and frozen (-70 ° C).

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 1984). Briefly, patients´ sera or vector macerates were inoculated into C6/36 *Aedes albopictus* cell monolayers in Leibovitz L-15 medium (Sigma) supplemented with 2% fetal calf serum (Invitrogen) and 0.2 mM of nonessential amino acids (Invitrogen). Cells were incubated at 28°C for 5 to 7 days and observed for cytopathic effects. Infected supernatant was clarified by centrifugation and virus stocks stored in 1-mL aliquots at –70°C until use.

Viral RNA was extracted directly from mosquitoes macerates using QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's instructions and stored at -70^{0}C for DENV detection and typing.

RT—PCR for detecting and typing DENV was performed as described previously (Lanciotti et al. 1992). Briefly, consensus primers (D1, forward and D2, reverse) were used to anneal to the capsid (C) and pre-membrane (prM) regions of any of the four DENV types and amplify a 511-bp product in a reverse transcriptase-polymerase

reaction. After a second round of amplification (nested PCR) with type-specific primers (TS1 to TS4), DNA products of unique size for DENV-4 (392 bp) were generated and analyzed by agarose gel electrophoresis and ethidium bromide staining.

For viral quantification, the RNA from the original *Ae. aegypti* individually macerated was submitted to quantitive Real Time Reverse transcriptase- polymerase chain reaction (qRT-PCR) according to the protocol described by Drosten et al (2002).

Simplexa™ Dengue Real Time RT –PCR

For viral qualitative detection and typing, the RNA from the original *Ae. aegypti* individually macerated was submitted to the Simplexa™ Dengue Real Time RT-PCR (Focus Diagnostics, Cypress, CA) according to the manufacturer´s protocol. The kit is for use on the 3M Integrated Cycler instrument for the *in vitro* detection and typing of DENV-1 to 4. The assay is a real-time RT-PCR that detects DENV-1 and 4 in one reaction, and DENV-2 and 3 in another reaction using bi-functional fluorescent probe-primers and reverse primers in DENV specific regions: DENV-1 (NS5 gene), DENV-2 (NS3 gene), DENV-3 (NS5 gene) and DENV-4 (capsid gene). An RNA internal control is used to monitor the extraction process and to detect RT-PCR inhibition. In a Real Time RT-PCR a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (which exceeds the background level). Ct values are inversely proportional to the amount of target nucleic acid in the sample: lower the Ct value the greater the amount of target nucleic acid in the sample. The Simplexa™ Dengue Real Time RT-PCR undergo 40 cycles of amplification. The Simplexa™ Dengue kits from Focus Diagnostics were kindly provided for evaluation performed for research purposes only and authors have no financial interest.

Anti-dengue IgM antibodies in sera were measured with the commercially available Panbio Dengue IgM capture ELISA and the results are classified as positive, negative and equivocal according to the manufacturer's instructions.

For NS1 antigen capture two commercial kits were used in sera and macerates. The Platelia™ Dengue NS1 Ag ELISA (BioRad Laboratories) is an one-step sandwich format microplate enzyme immunoassay used detect DENV NS1 antigen in human serum or plasma. The test uses murine monoclonal antibody for

capture and revelation.
)-NS1-Mab/peroxidase
)oratories, Marnes-La-
the rapid detection of
f sample in a specimen
ı control line (C) and a
ration time of 15 min
icates a negative result.
)eated.

If NS1 antigen is present in the sample, an immune-complex Mal will be formed. The Dengue NS1 Ag STRIP (Biorad Lab Coquette, France) is an immunochromatographic test (ICT) for NS1 antigen. One drop of migration buffer was added to 50 µL o tube and a strip was placed in the tube. The strip has two lines: a test line (T). The appearance of the T and C lines after a mig indicates a positive result. The appearance of the C line alone indi If the C line is not present, the test is considered invalid and is rep

es were confirmed by
S1 capture ELISA and
ɛm were the first cases
5th, 2011 (Nogueira &
ghborhood of the first
'rancisco and one from
46 years-old (mean ±
cases (up to 4 days of

During March and April 2011, a total of eleven DENV-4 cas using routine laboratorial diagnosis techniques: MAC-ELISA, N rapid test, virus isolation and conventional RT-PCR. Two of the previously analyzed, both with onset of symptoms on March (Eppinghaus 2011). Three other cases were from the same neig cases (Cafuba), three were from São Domingos, two from São F Engenho do Mato (Figure 1A). Patient´s age ranged from 14 to 24.7), where six were males and three females. All were acute

illness), and two out of nine cases (22.2%) were positive by MAC ELISA. Virus isolation was possible in five out of nine cases (55.5%) and all cases were positive by conventional RT-PCR. Four cases (44.4%) were positive when analyzed by both NS1 capture ELISA and NS1 Ag Strip test. We further analyzed all cases b Dengue Real Time RT-PCR and all cases (9/9) were confirmed as DENV values obtained from the DENV-4 human samples ranged from 16.8 to 25.0). Figure 2.

Due to the establishment of a Sentinel network for dengue surveillance city of Niterói, RJ which includes blood collections from febrile c detection, it was possible to first detect early DENV-2 and DENV-4 in 1990 and 2011, respectively (Nogueira et al. 1990 ; Nogueira & Eppi Immediately after DENV-4 isolation, an intensive surveillance was monitor the dispersion of the newly introduced virus. Other nine DENV the neighborhoods of Cafuba, São Francisco, São Domingos and Eng were laboratorially confirmed (Figure 1A).

The development of conventional RT-PCR and Real Time RT-PCR te significantly reduced the processing time and permitted the detection of t early stage of the infection in humans and transmission in vectors. The investigated in this study were initially detected by conventional RT-PC established for all acute dengue suspected cases. Conventional RT-P usually released 24 to 48 after reception in the laboratory. Also as a routi introduced serotype, all DENV-4 cases were re-tested with the typing prir for confirmation. Concomitantly, cases are submitted to MAC-ELISA, N virus isolation. As those constituted acute cases, MAC-ELISA confirme

of nine cases, both with four days of illness. All others cases were on the first and second days of symptoms. The most used technique for dengue serodiagnosis is still based on the detection of anti-DENV IgM by using MAC-ELISA (Huang et al. 2001). However, one of the limitations consists in the variations on the detection rate during the acute phase of the disease. Usually, it takes from 3 -5 days after the onset of the symptoms to detect anti-DENV IgM, depending on whether the patient has primary or secondary infections (Schilling et al. 2004).

A previous study has shown that the NS1 capture ELISA presents a higher detection rate during the first four days after the onset of the symptoms (Lima et al. 2010). Here, both NS1 tests, independently of its format, ELISA or immunocromatographic confirmed four out of nine cases up to the 4^{th} day of symptoms. During the acute phase, the NS1 exists as secreted as well as a membrane-associated protein and both forms are demonstrated to be immunogenic (Mason 1987, Falconar 1997, Young et al. 2000). High NS1 levels was demonstrated to circulate in the acute phase of dengue being found in the sera of patients with primary and secondary DENV infections, up to the ninth day after the onset of the symptoms (Young et al. 2000, Alcon et al. 2002).

Despite the longer time for a final result, virus isolation is still the "gold-standard" technique and, after inoculation into C6/36 cells, DENV-4 could be recovered and typed in five human cases. This is quite important, as viral supernatants can be further used for molecular epidemiologic studies by partially or entirely sequencing the viral genome. In fact, DENV-4 sequencing and phylogeny has characterized the Brazilian DENV-4 as belonging to genotype II (de Sousa et al. 2011).

All individual macerates were initially submitted to conventional RT-PCR, virus isolation and Simplexa™ Dengue Real Time RT-PCR. DENV-4 was identified by

conventional RT-PCR in a single *Ae. aegypti* female (1/72; 1.4%) captured in one of the residences (15.2), where 19 adult mosquitoes (13 males and 6 females) have been collected (Supplementary data). As many unspecific bands were visualized in the agarose gel (Figure 3), due to the nature of the material, all macerates were retested by conventional RT-PCR using all four typing primers (TS1, TS2, TS3 and TS4) separately. Only one macerate was amplified by the primers combination (D1+TS4) specific for DENV-4 amplification (data not shown). The same *Ae aegypti* female, here designated as 15.2.4/11, was also the only positive for DENV-4 when all macerates were submitted to the Simplexa™ Dengue Real Time RT-PCR with a Ct value of 23.5 (Figure 4). No viruses were recovered from any of the 72 mosquitoes´ macerates by virus isolation in C6/36 cells. Real Time RT-PCR detected 1.08 $x10^3$ copies/mL of DENV-4 in the macerate of a single *Ae. aegypti* female naturally infected.

As a positive *Ae aegypti* female was identified in a residence from site #1 by molecular techniques, we further performed both NS1 capture ELISA and NS1 Ag Strip test to all 47 macerates available from that location. The same positive female (15.2.4/11) was positive by both NS1 capture tests. Interestingly, both tests also detected NS1 from a macerate of an *Ae. aegpypti* male (15.2.3). The potential use of NS1 antigen capture kit for detection of the DENV antigen in *Ae, aegypti* has been recently shown (Tan et al. 2011). However, none of the others techniques available confirmed infection nor the infecting serotype. Transovarial transmission of DENV, when the virus is transmitted to the progeny of an infected female has been reported previously (Khin & Khin 1983, Joshi et al 1996, Joshi et al 2002, Le Goff et al 2011).

DENV detection rates on *Aedes* mosquitoes by RT-PCR may vary depending on the geographic settings, epidemiological backgrounds and vector population. In Taiwan, only 0.2% of *Ae. aegypti* females analyzed were positive for DENV (Chen et al. 2010).

However, it has been shown that 16.1% of the *Ae aegypti* females collected in Mexican schools were DENV infected (García-Rejón et al. 2011). In Brazil, previous studies have shown rates of 17% in a DENV-3 surveillance performed during an epidemic in the city of Manaus, North region of Brazil (Pinheiro et al. 2005). On the other hand, only 0.1% of adult mosquitoes was infected by the same serotype when an entomological surveillance was performed in RJ, during the inter-epidemic year of 2006 (unpublished data). In Recife, Northeast Brazil 10% of tested pools tested was infected and, despite the prevalence of DENV-3 in human cases, DENV-2 and DENV-1 were also detected in mosquitoes (Guedes et al. 2010).

by our group in 2001, DENV-1
NV-3 was being investigated.
/-4 surveillance the state of RR
been suggested that a prevalent
ed by a new one (Chow et al.
nd mosquitoes were confirmed
RJ and other Brazilian states,
es reported in 2011 were due to

During an entomological surveillance performed in RJ
was detected in *Ae. aegypti* mosquitoes when DE
Likewise, DENV-1 was also detected during the DENV
in 2010 (Castro et al. submitted for publication). It has
serotype may persist for one or two years until replac
1998). Here, infection by DENV-4 cases in humans a
during an explosive DENV-1 epidemic in the state of
where approximately 87% of the dengue confirmed cas
DENV-1infections (SVS 2011).

is a more rapid and sensitive for
Callahan et al. 2001, Drosten et
/e used the quantitive RealTime
s/mL) from a single *Ae. aegypti*

Real-Time RT-PCR techniques have been established a
detecting and quantifying DENV in clinical samples (
al. 2002, Johnson et al. 2005, Lai et al. 2007). Here, w
RT-PCR to quantify the DENV-4 titer (1.08 x 10^3copie
female naturally infected and individually macerated.

We also evaluated for the first time, the usefulness of the Simplexa™ Dengue Real Time RT-PCR kit for detecting and typing DENV from human cases and *Ae. aegypti* samples. All DENV-4 human cases analyzed in this study were confirmed by the commercial Real Time RT-PCR kit and the Ct values observed ranged from 16.8 to 35.1 (mean ± 25.0). The cycle threshold (Ct) values in a Real Time PCR are inversely proportional to the amount of target nucleic acid in the sample. Lower Ct values are observed on higher viraemia samples. Moreover, Cts < 29 are strong positive reactions indicative of abundant nucleic acid in the sample, Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid while Cts of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid. As all samples were very acute (2 samples with 4 days of symptoms, 3 with 2 days of symptoms and 3 with 1 day of symptoms and 1 without information), high viraemia would be expected, so would lower Ct values (Figure 2). From all *Ae. aegypti* macerates submitted to the Simplexa™ Dengue Real Time RT-PCR, only female 15.2.4/11 was positive for DENV-4 with a low Ct value (23.5), suggesting a high viral load in the single female (Figure 3).

Despite, the confirmation of DENV-4 cases, a major DENV-1 epidemic was established in RJ at that time. In December 2011, eight months after the first cases were confirmed, a new DENV-4 case was identified in Niteroi and confirmed by the laboratory methodologies available. Therefore, our overall results on the laboratorial diagnosis and entomological surveillance using molecular techniques such as conventional RT-PCR and/or Real Time RT-PCR show that those approaches are fast, reliable, sensitive and specific for dengue serotype surveillance, even when a new serotype is introduced or re-emerge during an epidemic caused by another prevalent serotype.

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Competing interest

The authors have no conflict of interest.

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SUPPLEMENTARY DATA

454 *Aedes aegypti* adult mosquitoes collected during an entomological surveillance
455 performed on four sites from the neighborhood of São Domingos, Niteroi, RJ in
456 May2011.

Collection site #	Household code #	Mosquito ID	Gender	Total per Gender	Total per House hold
1	15.1	A	Male	4	6
	15.1	B	Male		
	15.1	C	Male		
	15.1	D	Male		
	15.1	1	Female	2	
	15.1	2	Female		
	15.2	1	Male	13	19
	15.2	2	Male		
	15.2	3	Male		
	15.2	4	Male		
	15.2	5	Male		
	15.2	6	Male		
	15.2	7	Male		
	15.2	8	Male		
	15.2	9	Male		
	15.2	10	Male		
	15.2	11	Male		
	15.2	12	Male		
	15.2	13	Male		
	15.2	1	Female	6	
	15.2	2	Female		
	15.2	3	Female		
	15.2	4	Female		
	15.2	5	Female		
	15.2	6	Female		
	15.3	1	Male	10	12
	15.3	2	Male		
	15.3	3	Male		
	15.3	4	Male		
	15.3	5	Male		
	15.3	6	Male		
	15.3	7	Male		
	15.3	8	Male		
	15.3	9	Male		
	15.3	10	Male		
	15.3	1	Female	2	
	15.3	2	Female		
	15.4	1	Male	3	13
	15.4	2	Male		
	15.4	3	Male		
	15.4	1	Female	10	
	15.4	2	Female		
	15.4	3	Female		
	15.4	4	Female		
	15.4	5	Female		
	15.4	6	Female		
	15.4	7	Female		

	15.4	8	Female		
	15.4	9	Female		
	15.4	10	Female		
	15.5	1	Female	2	2
	15.5	2	Female		
	15.6	1	Female	1	1
2	60.A	1	Male	3	4
	60.A	2	Male		
	60.A	3	Male		
	60.A	1	Female	1	
3	60.1	1	Female	3	3
	60.1	2	Female		
	60.1	3	Female		
4	60	1	Male	6	12
	60	2	Male		
	60	3	Male		
	60	4	Male		
	60	5	Male		
	60	6	Male		
	60	1	Female	6	
	60	2	Female		
	60	3	Female		
	60	4	Female		
	60	5	Female		
	60	6	Female		
TOTAL					**72**

Table: Human dengue virus type 4 (DENV-4) cases laboratorial investigation in Niteroi, RJ, Brazil.

Dengue Cases	Routine Laboratorial Diagnosis Methodologies Positive/Tested					Newly Available Diagnosis Methodology Positive/Tested
	MAC-ELISA	Virus Isolation	NS1 ELISA	NS1 Ag STRIP	Conventional RT-PCR	Simplexa™ Dengue Real Time RT-PCR
Males (*n*=6)	2/6	4/6	3/6	2/6	6/6	6/6
Females (*n*=3)	0/3	1/3	1/3	2/3	3/3	3/3
TOTAL (%)	2/9 (22.2)	5/9 (55.5)	4/9 (44.4)	4/9 (44.4)	9/9 (100)	9/9 (100)

FIGURE LEGENDS

FIGURE 1

Dengue virus type 4 (DENV-4) introduction in Niteroi, Rio de Janeiro, Brazil, 2011. (A) Niteroi neighborhoods with DENV-4 confirmed cases. Dates shown are DENV-4 confirmed cases in the different neighborhoods. (B) Entomological surveillance in the São Domingos neighborhood, Niteroi. White crosses represent the four collection sites within an area with confirmed DENV-4.

FIGURE 2

Simplexa™ Dengue Real Time RT-PCR amplification on dengue type 4 cases (n=9) from Niteroi, RJ, according to the number of days after the onset of the symptoms. Cts values are shown. NA: not available.

FIGURE 3

Conventional RT-PCR agarose gel electrophoresis analysis from *Aedes aegypti* adult mosquitoes, individually macerated from the entomological surveillance performed in nine residential and non-residential locations in the São Domingos neighborhood, Niteroi, Rio de Janeiro, Brazil in 2011. Lanes 1, 14, 15 and 25: 100bp molecular weight (Invitrogen), lanes 2 to 12 and 16 to 19: *Aedes aegytpi* macerates, lane 5: DENV-4 positive Aedes aegypti individually macerated, lane 13: DENV-1 to 4 positive controls mix, lane 20: negative control (water), lanes 21 to 24: DENV-1 to 4 positive controls, respectively.

FIGURE 4

Simplexa™ Dengue Real Time RT-PCR amplification on *Aedes aegypti* mosquitoes collected in the neighborhood of São Domingos and individually macerated. (A) Experiment report sheet after reaction. Samples 5A to E, 5G and 5H: negative *Aedes aegypti* macerates. Sample 5F: *Aedes aegypti* female # 15.2.4/11 positive for DENV-4 at a cycle threshold (Ct) of 23.5. Red line: DENV-4 probe fluorophore CFR610. Purple line: Internal control (IC) probe fluorophore Q670.

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537 Figure 1

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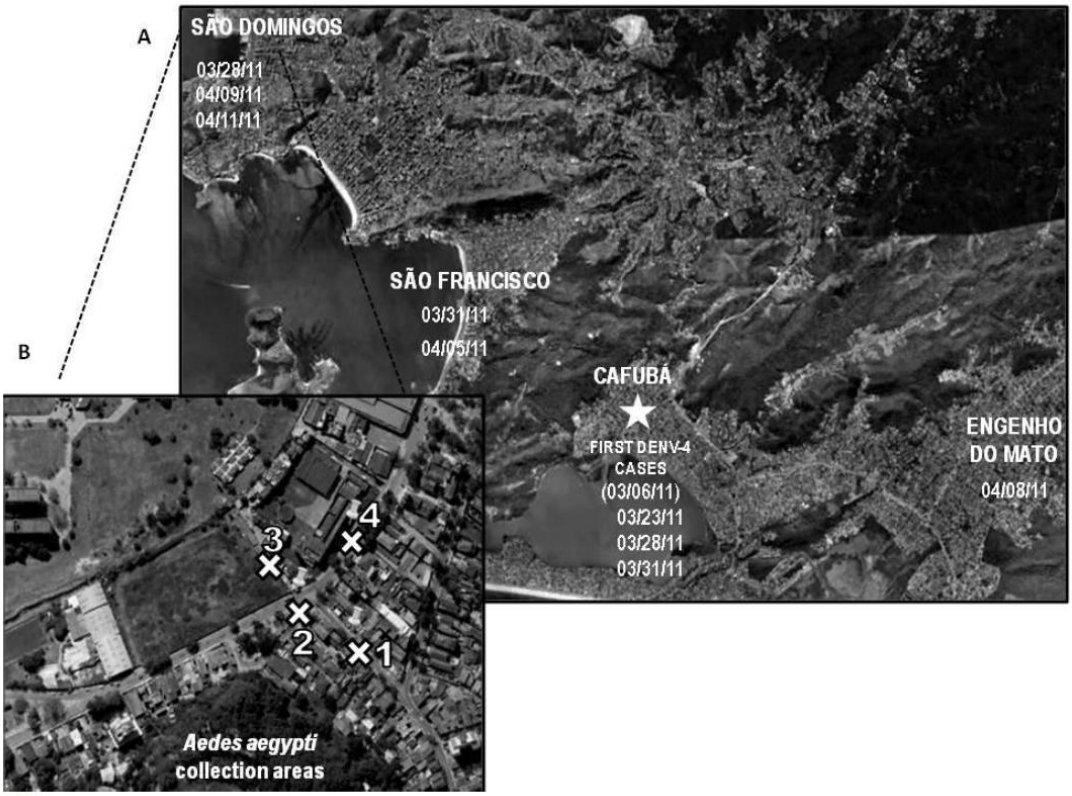

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548 Figure 2

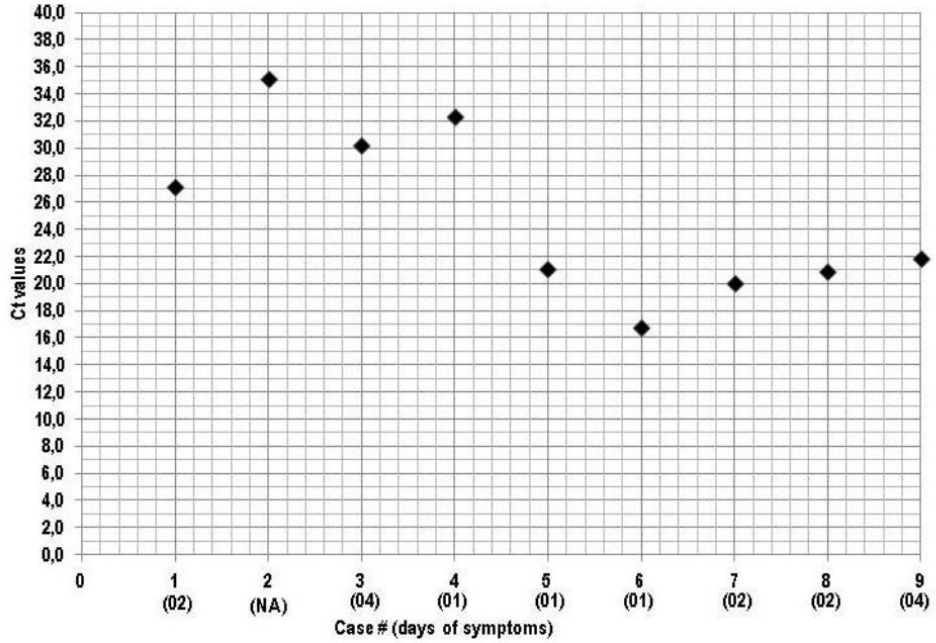
Ct values
40,0
38,0
36,0
34,0
32,0
30,0
28,0
26,0
24,0
22,0
20,0
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
0
1 (02)
2 (NA)
3 (04)
4 (01)
5 (01)
6 (01)
7 (02)
8 (02)
9 (04)
Case # (days of symptoms)

Figure 3

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570 Figure 4

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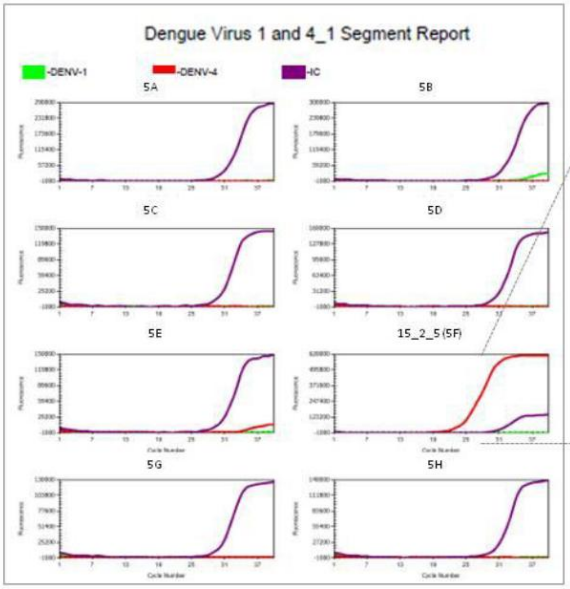
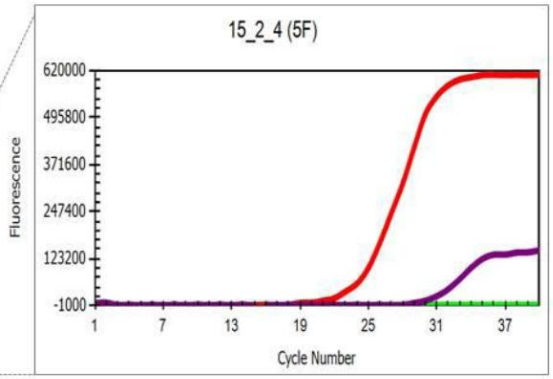
A



B



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5. Discussão

Nos últimos 25 anos têm sido descrito um aumento na distribuição global do *Ae. aegypti* e de epidemias de dengue (Mackenzie et al 2004, Jansen e Beebe 2010). No Brasil, a doença tornou-se um problema de saúde pública na maioria das cidades, sobretudo no Rio de Janeiro, que é considerado uma das principais portas de entrada dos DENV no país. A dispersão dos vírus dentro da cidade, bem como para outros estados da federação é favorecida pela alta densidade populacional e pela movimentação frequente de residentes e entrada de turistas (Lourenço-de-Oliveira et al 2004, Urdaneta-Marquez e Failloux 2011).

O estado do Rio de Janeiro, situado na região Sudeste do país, tem sido importante para a epidemiologia do dengue, com a introdução do DENV-1 em 1986, do DENV-2 em 1990 e do DENV-3 em 2000 (Nogueira et al 2007). O DENV-3 predominou na maior parte dos estados do Brasil entre 2002 e 2006. Em 2007, o agravamento nos casos da doença foi observado pela a re-emergência do DENV-2, que causou intensa epidemia no ano seguinte.

Durante os períodos inter-epidêmico e epidêmico de 2007 e 2008, foi coletado no município do Rio de Janeiro um total de 10.627 larvas de *Ae. aegypti* (*n*=7.499) e *Ae. albopictus* (*n*=3.128) como parte da vigilância entomológica mantida pelo Laboratório de Transmissores de Hematozoários, IOC/FIOCRUZ. As larvas maceradas foram inicialmente testadas por RT-PCR e submetidas ao isolamento viral em cultura de células. Por RT-PCR foi possível detectar precocemente o DENV-2 em um *pool* de larvas de *Ae. aegypti* coletadas no primeiro semestre de 2007. A posterior re-emergência deste sorotipo foi responsável pela epidemia de dengue que grassou no estado do Rio de Janeiro em 2008. Entretanto, no nosso estudo, a propagação do vírus em cultura celular para posterior sequenciamento e caracterização foi inviabilizada pelo baixo título viral da amostra original. Desde o período epidêmico de 2008 até a presente data, apesar da contínua vigilância entomológica para o isolamento de novas cepas virais em diversas localidades do estado, não foi possível o isolamento de novos DENV-2 em *Ae. aegypti* para caracterização molecular e genotipagem.

O monitoramento dos sorotipos circulantes em 2009 apontou para uma nova mudança no sorotipo predominante, com a re-emergência do DENV-1. Assim, tendo em vista a baixa circulação deste sorotipo desde o início da década, em 2010 este foi responsável pela epidemia mais explosiva de dengue registrada no país em termos de notificações, com 1.4 milhões de casos (SVS 2009, 2010). O risco da introdução do DENV-4 no país era iminente, uma vez que este sorotipo circula em países vizinhos, como a Venezuela e Colômbia (Guzman e Kouri 2002). Após o isolamento deste sorotipo em Roraima, em 2010, o vírus dispersou para outros estados da federação e, no Rio de Janeiro, os primeiros casos de DENV-4 foram isolados em Niterói, em 2011 (Nogueira e Eppinghaus 2011).

Desde 1986 um programa de vigilância virológica e entomológica foi estabelecido no Rio de Janeiro para o monitoramento dos DENV em soros humanos e vetores (Nogueira et al 1988, 1990, 1999, Lourenço-de-Oliveira et al 2002). Apesar da presença do *Ae. albopictus*, o *Ae. aegypti* tem sido o vetor responsável pela transmissão dos DENV no Brasil (Lourenço-de-Oliveira et al 2004). A vigilância entomológica destes vírus no mosquito adulto e em larvas constitui uma ferramenta importante para a detecção precoce de epidemias de dengue.

O aumento global da dispersão e co-circulação de múltiplos sorotipos de DENV, associados à falta de fidelidade da RNA polimerase e RNA dependente, levaram a um aumento na diversidade destes vírus e na evolução dos genótipos dentro de cada sorotipo (Holmes e Burch 2000, Uzcategui et al 2001, Foster et al 2004, Bennett et al 2006, Chen et al 2008, Weaver e Vasilakis 2009, Kumar et al 2010). Além disso, sabe-se que a evolução dos DENV é determinada por complexas interações, que vão do nível celular ao populacional em humanos e mosquitos e mutações que ocorrem ao longo do genoma, assim como mutações nos genes virais estruturais e não estruturais e na região 3´NC já foram implicados como marcadores moleculares de virulência (Hurrellbrink e Mc Minn 2003). Porém, as formas que as variações genéticas no vírus atuam durante a transmissão entre humanos e mosquitos permanecem desconhecidos.

Estudos de caracterização molecular e análise filogenética podem revelar a origem geográfica dos vírus e determinantes moleculares de virulência em potencial. Dado que a

introdução de novos sorotipos/genótipos pode acarretar na ocorrência de epidemias, estudos filogenéticos são de grande relevância para avaliar o impacto na população. A caracterização molecular de amostras de DENV isoladas no Brasil de áreas geograficamente e temporalmente distintas é uma ferramenta fundamental para a análise epidemiológica molecular, que por sua vez pode fornecer informações importantes sobre a introdução, dispersão e circulação de distintas linhagens destes vírus.

Portanto, visando contribuir para um melhor conhecimento dos DENV e sua interação com o mosquito vetor, realizamos neste trabalho, a caracterização molecular e filogenética de cepas de DENV isoladas de mosquitos naturalmente infectados, provenientes de epidemias ocorridas de 1986 a 2011 no Brasil.

Realizar a análise filogenética baseada no gene E de cepa de DENV-1 isoladas durante o período estudado (1986 a 2011) – Trabalhos 1 e 2

Nos trabalhos intitulados "First report of multiple lineages of dengue viruses type 1 in Rio de Janeiro, Brazil" e "Dengue Virus Type 1 from Field-Caught Vectors and Humans in Brazil: Phylogeny Reveals Different Lineages of the American African Genotype in 25 Years" foram estudadas cepas de DENV-1 isoladas a partir de casos humanos e mosquitos *Ae. aegypti* desde a sua introdução em 1986, após a emergência deste sorotipo em 2009, e provenientes das epidemias ocorridas em 2010 e 2011.

A utilização da técnica de RT-PCR para a triagem de vetores coletados no campo tem se mostrado útil para a vigilância virológica e detecção de novas introduções dos DENV (Chow et al 1998, Kow et al 2001, Pinheiro et al, 2005, Méndez et al 2006, Costa et al 2009, Guedes et al 2010). Esta permitiu detectar DENV-1 em mosquitos coletados em 2001 e 2010, assim como fornecer uma ferramenta para o estudo retrospectivo, pela detecção do RNA viral de um macerado de mosquito coletado em 1986 e congelado a -70^0 C por 24 anos.

As taxas de detecção de DENV em mosquitos *Aedes* por RT-PCR podem variar dependendo da área geográfica, perfil epidemiológico e população vetorial. Neste estudo, as taxas de detecção dos DENV-1 por RT-PCR variaram de 0,78% a 25%, sendo que o último pode não ser representativo, pois é o resultado de uma análise de apenas quatro

pools. No entanto, taxas de 0,2 % foram observadas em Taiwan (Chen et al 2010) e de 16,1% em escolas mexicanas (García-Rejón et al 2011). No Brasil, estudos prévios demonstraram taxas de detecção de 17% durante uma vigilância realizada em Manaus (Pinheiro et al 2005), de 10% em estudo realizado em Recife (Guedes et al 2010) e de 0,1% em uma vigilância realizada no Rio de Janeiro durante o ano inter-epidêmico de 2006 (dados não publicados). Apesar do RT-PCR ter mostrado ser uma ferramenta útil e eficaz para a vigilância dos DENV em mosquitos e formas imaturas coletados no campo, as altas taxas de detecção e até mesmo co-infecções devem ser consideradas cuidadosamente e preferencialmente confirmadas por outras técnicas, tais como o isolamento viral e o sequenciamento genômico.

As técnicas de RT-PCR em Tempo Real têm sido estabelecidas e descritas como mais rápidas e sensíveis para a detecção e quantificação dos DENV de amostras clínicas (Callahan et al 2001, Drosten et al 2002, Johnson et al 2005), de vetores infectados experimentalmente em laboratório (Richards et al 2007) e coletados no campo (Tan et al 2011). Neste estudo, o RT-PCR em Tempo Real foi utilizado para quantificar o título de DENV-1 ($1,6 \times 10^4$ cópias/mL) proveniente de uma única fêmea naturalmente infectada, que se caracteriza como observação relevante, uma vez que a quantidade de vírus em humanos necessária para infectar mosquitos ainda não foi precisamente determinada (Halstead 2008).

O DENV-1 é caracterizado por possuir cinco genótipos distintos, genótipo I (Sudeste Asiático/China/Oeste Africano), genótipo II (Tailândia), genótipo III (Malásia), genótipo IV (Pacífico Sul) e genótipo V (Américas/África), como também pela existência de linhagens com relações geográficas e temporais diferenciadas descritas (Myat Thu et al 2005, Kukreti et al 2009, Mendez et al 2010). Substituições e alternância de linhagens têm aparecido com mais frequência em estudos filogenéticos e o termo “linhagem” tem sido utilizado, não oficialmente, para caracterizar os vírus agrupados em clades em um nível taxonômico abaixo de genótipo (Mendez et al 2010).

A análise de sequências do gene E de vírus isolados de mosquitos adultos, larvas e casos humanos demonstrou que os DENV-1, representantes de seis estados das regiões Sudeste, Centro-Oeste, Nordeste e Norte do país, pertencem ao genótipo V

(Américas/África), porém a existência de três linhagens distintas foi caracterizada. Cepas de DENV-1 isolados de vetores em 1986 e 2001 agruparam-se na linhagem I com cepas isoladas de humanos em 1986, 1990, 1997 e 2001. Cepas de DENV-1 isoladas em 2010 e 2011 agruparam-se formando um clade distinto (linhagem II), sendo este mais intimamente relacionado com as cepas isoladas em Cingapura, em 1990 e 2005, sugerindo uma origem asiática provável para esta linhagem. No entanto, a cepa de DENV-1 isolada da fêmea de *Ae. aegypti* em 2010, em Roraima, agrupou-se em uma outra linhagem (linhagem III), também caracterizada durante a reemergência do DENV-1 em 2009 e 2010. As cepas caracterizadas como pertencentes à linhagem III agruparam-se com cepas isoladas em 2007 e 2008 na Colômbia, Venezuela e México, sugerindo uma origem latino-americana para as mesmas.

A comparação entre as sequências das diferentes linhagens demonstrou uma grande divergência entre as linhagens II e III, e que a maioria das substituições de aminoácidos foi observada no domínio III da proteína E. Além disso, foi demonstrado que alguns resíduos são exclusivos de algumas linhagens, e podem ser os responsáveis pela diferenciação entre elas.

Apesar de reduzida, porém contínua, a circulação de DENV-1 no país apresenta um baixo percentual de identidade dos vírus recentemente isolados, tanto de vetores quanto de casos humanos, com os vírus inicialmente introduzidos na década de 1980, sugere que o DENV-1 reemergente não evoluiu localmente, mas é resultante da introdução de novas linhagens no país. A circulação de mais de uma linhagem de DENV-1 já foi descrita na Ásia (Kukreti et al 2005) e nas Américas (Mendez et al 2010). A introdução de múltiplas linhagens dos diferentes sorotipos de DENV, com frequente alternância de linhagens, foi descrita no México (Carrillo-Valenzo et al 2010). De fato, a alternância de linhagens parece ser um fenômeno mais comum do que a longa persistência das mesmas (Bennett et al 2003).

Realizar o sequenciamento completo do genoma de DENV-3 isolado de mosquitos naturalmente infectados durante a epidemia ocorrida em 2001 no Rio de Janeiro e caracterizar a região 3'-não codificante (3'NC) de DENV-3 isolados de mosquitos *Ae. aegypti* e de casos humanos – Trabalho 3

A dispersão global dos quarto sorotipos de DENV nos últimos 50 anos resultou na dispersão de genótipos associados com uma doença mais grave (Kyle e Harrris 2008). No Brasil, o genótipo III, de DENV-3, tem sido associado com epidemias graves desde sua introdução em 2000 no Rio de Janeiro (Nogueira et al 2001, 2005, Miagostovich et al 2003, 2006).

Dos cinco genótipos descritos para os DENV-3, os genótipos I, II e III (GI, GII e G III) têm sido responsáveis pela maioria das infecções em humanos e associados a casos de dengue clássico e casos graves no Sudeste da Ásia, Subcontinente Indiano, Pacífico Sul, Leste Africano e Américas, enquanto que os genótipos IV e V (GIV e GV) não têm sido associados a casos graves e são representados por vírus mais recentes circulantes nas Américas, Pacífico Sul e Ásia (Lanciotti et al 1994, Wittke et al 2002, King et al 2008, Araújo et al 2009). Neste contexto, a potencial emergência de cepas associadas com um quadro mais grave ressalta a necessidade da vigilância destes vírus nos hospedeiros humanos e vetores.

Apesar da utilização do gene E para estudos filogenéticos e evolutivos (Lanciotti et al 1994, Aquino et al 2006, Kochel et al, 2008, Amarillia et al 2009, Araujo et al 2009, Chen et al 2011), devido a suas propriedades biológicas e pressão seletiva imposta pela resposta imune do hospedeiro, a importância da heterogeneidade em outras regiões genômicas, como os genes não-estruturais e regiões não-codificantes, não deve ser excluída (Chao et al 2005).

Assim, visando contribuir para os estudos de interação vetor-vírus-hospedeiro humano e estudos filogenéticos, realizamos no manuscrito "Molecular differences of the 3´untranslated region from Brazilian Dengue Virus Type 3 isolated from Naturally Infected Mosquitoes and Humans" a caracterização molecular do genoma completo de uma cepa de DENV-3 isolada a partir de *Ae. aegypti* naturalmente infectado no estado do Rio de Janeiro, assim como a caracterização da região 3´NC dos vírus isolados de mosquitos em comparação com os vírus isolados em humanos.

Na análise do genoma completo do DENV-3 foram observadas substituições de aminoácidos ao longo de toda região codificante, quando as cepas brasileiras foram comparadas à cepa protótipo PHIL/H87/1956 e cepas representantes dos demais genótipos. Algumas destas substituições foram observadas exclusivamente nas cepas brasileiras

analisadas. Além disso, substituições exclusivas à cepa de DENV-3 isolada em mosquito (BR73354/2001) foram observadas no gene NS5, incluindo a substituição que resultou na formação de um códon de terminação. Na região 3´NC de genoma foi demonstrada uma deleção de 8 nucleotídeos dentro da inserção de 11 nucleotídeos previamente descrita na região variável (VR), característica de cepas do GIII de DENV-3 das regiões Latino-Americanas/Caribenhas e do Sri Lanka (Peyrefitte et al 2003, Miagostovich et al 2003, Silva et al 2008). A presença de DENV-3 com genoma defectivo contendo tanto códon de terminação ou deleções *in vivo* já foi demonstrada (Wang et al 2002).

Portanto, com o objetivo de comparar a extensão das variações nas sequências da região 3´NC, foi realizado o sequenciamento e caracterização de novas cepas de DENV-3 brasileiras isoladas em mosquitos e humanos. Neste estudo, na análise desta região de cepas adicionais de ambos hospedeiros, pode-se detectar vírus caracterizados pela inserção de 11 nucleotídeos e com a deleção de 8 nucleotídeos. Apesar da presença de variantes virais distintas, sugere-se que a variante prevalente seja a transmitida (Lin et al 2004). Os resultados obtidos sugerem que as cepas com a inserção de 11 nucleotídeos sejam as mais prevalentes, uma vez que estas foram as mais frequentemente detectadas. Contudo, a análise de uma amostragem maior destes vírus se faz necessária para a confirmação destas observações.

As sequências e estruturas secundárias das regiões 5´e 3´NC dos flavivvírus possuem um papel importante na replicação viral e diferenças nestas regiões podem influenciar na virulência (Leitmeyer et al 1999, Cologna e Rico-Hesse 2005, Clyde e Harris 2006, Silva et al 2008). Além disso, mutações e deleções dentro destas regiões podem alterar a infectividade e reduzir a eficiência da replicação viral (Men et al 1996, Mandl et al 1998). Todas as sequências com a deleção de 8 nucleotídeos apresentaram estruturas secundárias similares quando a RV da 3´NC foi analisada. No entanto, nem todas as cepas que apresentaram a inserção de 11 nucleotídeos formaram estrutura similar.

As análises filogenéticas baseadas no sequenciamento completo tanto do genoma quanto da região 3´NC caracterizou os DENV-3 deste estudo como pertencentes ao GIII, corroborando com estudos prévios realizados sobre o tema (Miagostovich et al 2003, 2006, Araújo et al 2009). Ainda não está bem elucidado como populações virais distintas são mantidas ou transmitidas e, neste contexto, a disponibilidade de vírus isolados em ambos

hospedeiros se torna de grande importância para o melhor entendimento das interações vetor-vírus-hospedeiro humano e estudos de quasispécies.

Realizar a vigilância entomológica do DENV-4 pelo meio da utilização de metodologias moleculares – Trabalho 4

No Brasil, um surto causado pelos DENV-1 e DENV-4 foi registrado na cidade de Boa Vista, Roraima, região Norte do país, em 1983 (Osanai et al 1983). Porém, foi após 28 anos de seu primeiro isolamento que o DENV-4 foi reintroduzido nesta mesma cidade (Temporão et al 2011) e dispersou para as demais regiões do país (SVS 2011). O sequenciamento das cepas de DENV-4 recém-introduzidas caracterizou estes vírus como pertencentes ao genótipo II (de Sousa et al 2011).

No Rio de Janeiro, os primeiros dois casos de DENV-4, após esta reintrodução no país, ocorreram no bairro de Cafubá, Niterói, em março de 2011 (Nogueira e Eppinghaus 2011). Historicamente, o Rio de Janeiro apresenta condições altamente favoráveis para a transmissão dos DENV (Nogueira et al 2005, Lourenço-de-Oliveira et al 2008). Sua dispersão dentro da cidade, bem como para outros estados, é favorecida pelas altas densidades populacionais e pela frequente movimentação de residentes e turistas (Lourenço-de-Oliveira et al 2004, Urdaneta-Marquez e Failloux 2011).

Conforme previamente descrito neste trabalho, as vigilâncias virológica e entomológica utilizando técnicas moleculares, como RT-PCR, têm se mostrado útil para a detecção precoce de surtos e/ou para a deteção de novas introduções de DENV (Chow et al 1998, Kow et al 2001, Pinheiro et al 2005, Mendez et al 2006, Costa et al 2009, Chen et al 2010, Guedes et al 2010). Neste estudo, visamos caracterizar laboratorialmente os primeiros casos de DENV-4 detectados em humanos e demonstrar o papel de métodos moleculares, como o RT-PCR e o RT-PCR em tempo real, na vigilância entomológica desse vírus, recentemente introduzido nas populações de vetores de Niterói. Ressalta-se que estes casos foram confirmados durante uma grave epidemia de DENV-1 no Rio de Janeiro e no país, onde aproximadamente 87% dos casos confirmados de dengue eram por DENV-1 (SVS 2011).

Todos os casos humanos de DENV-4 investigados neste estudo (*n*=9) foram inicialmente confirmados por RT-PCR e, concomitantemente, submetidos ao MAC-ELISA, captura de antígeno NS1 e isolamento viral. O MAC-ELISA confirmou 22,2% (2/9) dos casos agudos de DENV-4, o isolamento viral foi possível em 55,5% (5/9) dos casos e ambos os testes de NS1 (ELISA e teste rápido) confirmaram 44,4% (4/9) dos casos.

Com o objetivo de detectar DENV-4 em populações de *Ae. aegypti* provenientes de áreas com história pregressa de casos positivos para este sorotipo, coletas de vetores adultos foram realizadas no bairro de São Domingos, Niterói, em 2011. Apesar da circulação do DENV-1, identificada por RT-PCR, foi possível detectar DENV-4 em uma fêmea naturalmente infectada, capturada em uma das residências onde 19 mosquitos adultos foram coletados.

Durante a vigilância entomológica realizada em 2001 por nosso grupo, um DENV-1 foi detectado em mosquitos *Ae. aegypti* quando o DENV-3 estava circulando. Já em 2010, durante a investigação do DENV-4 em Roraima, o DENV-1 foi de novo detectado (Castro et al submetido para publicação). O RT-PCR em tempo real determinou 1,08 x 10^3 cópias/mL de DENV-4 em uma única fêmea de *Ae. aegypti* naturalmente infectada.

Nesta investigação, utilizamos pela primeira vez, tanto para a vigilância virológica quanto para a entomológica, um teste comercial de RT-PCR em tempo real (Simplexa™ Dengue Real Time RT-PCR) para a detecção dos DENV. O teste foi desenhado para detectar os DENV-1 e DENV-4 em uma reação e os DENV-2 e DENV-3 em outra reação, onde foram utilizados iniciadores e sondas fluorescentes que hibridizam em regiões específicas do genoma: nos DENV-1 e DENV-3, gene NS5, no DENV-2, gene NS3, e no DENV-4, gene do capsídeo. Um controle interno foi utilizado para monitorar a extração do RNA viral e inibição da reação.

Neste estudo, o Simplexa™ Dengue Real Time RT-PCR, disponível recentemente, confirmou todos os casos humanos testados e também o DENV-4 como o sorotipo infectante na fêmea previamente positiva por RT-PCR convencional, com um Ct (*cycle thresold*) de 23.5. Os valores de Ct no PCR em tempo real são inversamente proporcionais à quantidade de ácido nucleico da amostra. Valores de Ct< 29 indicam

reações fortemente positivas de ácido nucleico alvo abundante nas amostras, valores de Ct entre 30-37 indicam reações positivas com quantidades moderadas do ácido nucleico alvo, enquanto que valores de Ct entre 38 e 40, indicam reações com quantidades mínimas do ácido nucleico alvo. Os valores de Ct obtidos para os casos humanos variaram de 16.8 a 35.1 (média ± 25.0).

Os resultados obtidos no diagnóstico laboratorial e vigilância entomológica utilizando técnicas moleculares, como o RT-PCR convencional e/ou RT-PCR em tempo real, apontam estas abordagens como eficientes e específicas para a vigilância dos DENV, mesmo quando um novo sorotipo é introduzido ou reemerge durante uma epidemia causada por outro sorotipo prevalente.

6- Conclusões

- A vigilância entomológica realizada no período de 1986 a 2011 permitiu o isolamento de DENV em mosquitos naturalmente infectados possibilitando a caracterização molecular de DENV-1, 3 e 4 e a análise filogenética destes, assim como de DENV provenientes de casos humanos.

- A RT-PCR para a triagem de DENV em vetores se mostrou uma ferramenta útil para a vigilância virológica e estudo retrospectivo, com taxas de detecção que variaram de 0,78% a 25%.

- A análise filogenética de vírus isolados em vetores e de casos humanos caracterizou os DENV-1 como pertencentes ao genótipo V (Américas/África), com a descrição de três linhagens distintas.

- A análise filogenética de vírus isolados em vetores e de casos humanos caracterizou os DENV-3 como pertencentes ao GIII e a caracterização molecular destes vírus demonstrou a presença de inserções e deleções na região 3´NC do genoma.

- As deleções observadas na região 3´NC resultaram em estruturas secundárias similares, porém nem todas as cepas com inserções nesta região apresentaram estrutura similar.

- O RT-PCR em Tempo Real possibilitou, pela primeira vez, a quantificação de DENV em fêmeas individuais naturalmente infectadas.

- O teste comercial Simplexa™ Dengue Real Time RT-PCR, disponível recentemente, foi utilizado pela primeira vez para detecção dos DENV e se mostrou um método molecular alternativo para as vigilâncias entomológica e virológica.

- Os resultados neste trabalho, utilizando técnicas moleculares, apontam estas abordagens como rápidas, sensíveis e específicas para a vigilância dos DENV, mesmo quando um novo sorotipo é introduzido ou reemerge durante uma epidemia causada por outro sorotipo prevalente.

7. Pespectivas

Pretende-se investigar os aspectos virológicos e entomológicos de interação mosquito vetor-DENV-3, pela região 3'NC de infecções experimentais das cepas com as delecões e inserções para possíveis variações nucleotidicas na pós-infecção do mosquito.

Como perspectivas futuras, pretendemos dar continuidade as vigilâncias epidemiológicas que envolve tanto a entomológicas como a virológicas, principalmente nos intervalos interepidêmicos, que possibilite a detecção da circulação viral, e genotipagem dos vírus isolados dos vetores para obter informações relevantes na compreensão da interação genótipo/sorotipo-mosquito.

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9- Apêndice

Neste item incluímos a relação dos artigos publicados durante a vigência da tese de doutorado (2008 – 2011) referentes à infecção experimental com o vírus dengue e a biologia do *Ae*. *aegypti*, desenvolvidos em Roraima (Região Norte) e Rio de Janeiro (Região Sudeste).

Aguiar DB, Fontão A, Rufino P, Macedo VA, Ríos-Velasquez CM, Castro MG, Honório NA. First record of *Aedes albopictus* (*Diptera*: *Culicidae*) in the state of Roraima, Brazil. Acta Amazonica. 2008; 38: 357-60.

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ACTA
AMAZONICA

Primeiro registro de *Aedes albopictus* (Diptera: Culicidae) em Roraima, Brasil

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RESUMO

Aedes albopictus é registrado pela primeira vez no estado de Roraima, Brasil. Entre junho de 2006 e maio de 2007 foram coletadas três pupas e dez larvas, duas das quais chegaram à fase adulta, durante atividades de vigilância rotineiras em três bairros urbanos da cidade de Boa Vista. Embora essa espécie não seja incriminada como vetor primário do dengue, a sua presença pode favorecer a ligação entre os ciclos silvestre e urbano da febre amarela e de outras arboviroses no Brasil.

PALAVRAS-CHAVE: *Aedes albopictus*, Dengue, Roraima

First record of *Aedes albopictus* (Diptera: Culicidae) in the state of Roraima, Brazil

ABSTRACT

Aedes albopictus is registered for the first time in Roraima, Brazil. From June 2006 to May 2007, three pupae and ten larvae of *Ae. albopictus* were collected, during routine surveillance work in three urban neighborhoods in the city of Boa Vista. Two larvae reached adulthood as females. Although *Ae. albopictus* is not presently considered of primary importance in dengue transmission, its occurrence could favor a linkage between urban and forest cycles of yellow fever and other arboviruses in Brazil.

KEYWORDS: *Aedes albopictus*, Dengue, Roraima

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Aedes albopictus (Skuse 1894) é uma espécie oriunda do sudeste da Ásia, onde é considerado o vetor primário do vírus dengue (Hawley, 1988). No Brasil, foi registrado primeiramente em 1986 no Rio de Janeiro e em Minas Gerais. Posteriormente, foi invadindo estados vizinhos, como São Paulo e Espírito Santo (Consoli & Lourenço-de-Oliveira, 1994). Até a presente comunicação, apenas 6 dos 27 estados brasileiros ainda não registraram a ocorrência dessa espécie: Amapá, Acre, Tocantins, Piauí, Sergipe e Roraima (Santos 2003; Martins *et al.*, 2006).

A presença de *Ae. albopictus* é um sério problema para a saúde pública. Apesar de *Ae. albopictus* ainda não ter sido incriminado como vetor natural do dengue no Brasil (Schatzmayr, 2000), foi comprovado que em condições de laboratório e de campo, populações brasileiras desta espécie têm a capacidade de se infectar com o vírus do dengue e transmiti-lo (Lourenço-de-Oliveira *et al.*, 2003; Castro *et al.*, 2004).

Ao contrário do *Ae. aegypti*, *Ae. albopictus* se espalha nos ambientes urbano, suburbano e rural, não dependendo de locais de grande concentração humana (Consoli & Lourenço-de-Oliveira, 1994). Além disso, apresenta ampla plasticidade ecológica evidenciada pela capacidade de colonizar os mais variados tipos de recipientes, naturais e artificiais (Hawley, 1988). O presente estudo teve o objetivo de registrar a primeira ocorrência de *Ae. albopictus* no estado de Roraima, Brasil.

Os primeiros exemplares de *Ae. albopictus* foram coletados durante as atividades de rotina do Programa de Vigilância e Controle do Dengue na cidade de Boa Vista. Agentes de endemias realizam visitas domiciliares bimestralmente em busca de criadouros contendo formas imaturas do *Aedes aegypti*, além de implantarem armadilhas de oviposição em cinco bairros da cidade para obtenção dos índices de infestação. Durante uma dessas visitas, em junho de 2006, foram coletadas duas pupas de *Ae. albopictus* (1 macho e 1 fêmea) no bairro Araceli Souto Maior (02° 46' 44.4" N; 60° 43' 10.8" W), em um depósito abandonado no quintal de uma residência. Em novembro de 2006 foi coletada mais uma pupa fêmea de *Ae. albopictus,* em um depósito artificial no peridomicílio de uma residência localizada no bairro Cinturão Verde (02° 48' 00.7" N; 60° 41' 56.9" W), que fica a 50 m do Igarapé Pricumã. Em maio de 2007, foram coletadas 10 larvas de *Ae. albopictus,* em uma ovitrampa, localizada no bairro Pricumã (02° 48' 21,9" N; 60° 41' 50,5" W), situado na zona oeste da cidade de Boa Vista. Destas, apenas 2 chegaram à fase adulta. Os bairros onde as 3 pupas e 10 larvas de *Ae. albopictus* foram coletadas apresentam ampla cobertura vegetal, acompanhados por extensa mata aluvial e cortados pelos Igarapés Uai Grande e Pricumã (Figura 1). As larvas, as pupas e os adultos do mosquito coletados nesse trabalho foram identificados seguindo a chave dicotômica de Consoli & Lourenço-de-Oliveira (994). As larvas e pupas foram mantidas em álcool a 70% e os adultos montados em alfinetes entomológicos. Os espécimes foram depositados na coleção entomológica do Núcleo Avançado de Vetores do Departamento de Medicina da Universidade Federal de Roraima - UFRR.

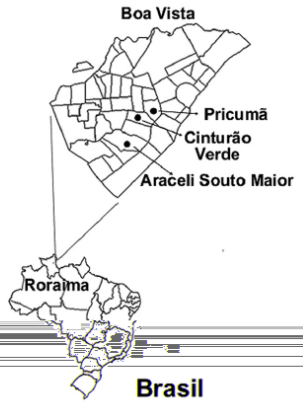

Figura 1 - Mapa da ocorrência de *Aedes albopictus* nos bairros Pricumã, Cinturão Verde e Araceli Souto Maior, Boa Vista, Roraima.

O estado de Roraima é considerado uma porta de entrada de patógenos em decorrência da sua posição geográfica (acesso ao Brasil a partir do Caribe, Venezuela e América Central), características climáticas e alta incidência de dengue (Rosa-Freitas *et al.*, 2003). A rodovia BR-174, que liga Manaus à Caracas, proporciona um intenso fluxo de pessoas vindas da região do Caribe (onde a dengue é endêmica) e de Manaus (onde doenças como a dengue e a febre amarela são endêmicas), tornando as cidades que margeiam a rodovia, como é o caso de Boa Vista, particularmente suscetíveis à epidemias. Além disso, foi em Roraima que houve o primeiro registro clínico e laboratorial de dengue em 1981-1982 (Osanai *et al.*, 1983).

Embora o *Ae. albopictus* seja considerado vetor potencial de dengue (Schatzmayr, 2000), seus aspectos ecológicos tais como: eclético quanto a alimentação sangüínea (Consoli & Lourenço-de-Oliveira, 1994), capacidade de dispersão (Honório *et al.*, 2003) segregação espacial, competição interespecífica (Braks *et al.*, 2003, 2004) e adaptabilidade ecológica, podem favorecer a ligação entre os ciclos silvestre e urbano da febre amarela e de outras arboviroses no Brasil (Braks *et al.*, 2003, Lourenço-de-Oliveira *et al.*, 2003).

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Seasonal dynamics of *Aedes aegypti* (Diptera: Culicidae) in the northernmost state of Brazil: a likely port-of-entry for dengue virus 4

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Roraima is the northernmost state of Brazil, bordering both Venezuela and Guyana. Appropriate climate and vector conditions for dengue transmission together with its proximity to countries where all four dengue serotypes circulate make this state, particularly the capital Boa Vista, strategically important for dengue surveillance in Brazil. Nonetheless, few studies have addressed the population dynamics of Aedes aegypti *in Boa Vista. In this study, we report temporal and spatial variations in* Ae. aegypti *population density using ovitraps in two highly populated neighbourhoods; Centro and Tancredo Neves. In three out of six surveys,* Ae. aegypti *was present in more than 80% of the sites visited. High presence levels of this mosquito suggest ubiquitous human exposure to the vector, at least during part of the year. The highest infestation rates occurred during the peak of the rainy seasons, but a large presence was also observed during the early dry season (although with more variation among years). Spatial distribution of positive houses changed from a sparse and local pattern to a very dense pattern during the dry-wet season transition. These results suggest that the risk of dengue transmission and the potential for the new serotype invasions are high for most of the year.*

Key words: *Aedes aegypti* - dengue fever - ovitraps - seasonality - Amazon

Separated from the rest of country by the Amazonian Forest, Roraima (RR) is the most isolated state in Brazil. Boa Vista, its capital, is a medium-sized city (249,853 inhabitants) (IBGE 2008a) located in the middle of a sparsely populated savannah. A highway is the only paved interstate route, connecting Boa Vista to the city of Manaus 805 km to the south and the Venezuelan border 220 km to the north. Despite its isolation, Boa Vista (and RR as a whole) has received - and continues to receive - a large inflow of visitors and migrants from Venezuela, Guyana and the Caribbean countries, as well as migrants from other regions of Brazil. As a consequence, RR's population increased from 79,400 inhabitants in 1980 to 395,725 inhabitants in 2008, a 5-fold increase in fewer than 30 years. Today, 53.7% of RR's population are immigrants, the highest proportion observed in Brazil (IBGEb 2008).

The first dengue fever epidemic in Brazil after the re-invasion by *Aedes aegypti* occurred in Boa Vista in 1981-1982 with 11,000 reported cases (Osanai et al. 1983, Travassos-da-Rosa et al. 1998). DENV-1 and DENV-4 were isolated. For four years prior to this, DENV-1 was spreading throughout the Caribbean Islands, Central America and Northern South America (Moros et al. 2003, Uzcategui et al. 2003). The year 1981 also marked the arrival of DENV-4 in the Americas (Lanciotti et al. 1997). In Boa Vista, this first invasion was interrupted through vector control programs and the country was spared any new dengue invasions until 1986, when DENV-1 arrived in Rio de Janeiro, triggering nationwide epidemics (Nogueira et al. 2007). Dengue fever has become an endemic disease in Brazil, with the co-circulation of DENV-1, DENV-2 and DENV-3 in most of the 27 Brazilian states. DENV-4 did not persist in Brazil after RR's 1981-1982 outbreak (Nogueira et al. 2007). In 2008, Figueiredo et al. reported that three patients had been infected with DENV-4 in Manaus, Amazonas. This result has been contested by the Ministry of Health and so far no consensus exists on the presence of DENV-4 in the Brazilian Amazon (de Melo et al. 2009).

For several years now, RR has consistently had one of the highest dengue incidence rates among Brazilian states. In 2007, RR registered an incidence rate of 594.8 cases per 10,000 inhabitants. This was the highest rate after Amapá (877.7), Tocantins (TO) (1415.3) and Mato Grosso do Sul (MS) (3213). In 2006, the incidence rate in RR was 657.8, highest after MS (688.3) and TO (665.7) (MS 2007).

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Its proximity to Venezuela, where all four dengue serotypes are found, and appropriate vector and climate conditions for dengue transmission, make Boa Vista a strategic point for dengue surveillance in Brazil. Despite this fact, studies on *Ae. aegypti* population dynamics in this area are still scarce (Zeidler et al. 2008). In this paper, we report spatial and temporal variations in the number of *Ae. aegypti* eggs collected in Boa Vista. The characteristics of the locations with high egg numbers are also noted.

MATERIALS AND METHODS

The city and the studied neighbourhoods - RR is located in Northern Brazil (Fig. 1). Its capital, Boa Vista (02°49'11"N 60°40'24"W 85 m), has an area of 5,687 km^2 and 249,853 inhabitants (IBGE 2008a). Boa Vista is located in a savannah ecoregion (Rosa-Freitas et al. 2007). The climate is tropical wet and dry, with an average temperature of 27.8°C (10-year average) (NASA 2001) and very low intra-annual variability. Average yearly rainfall amounts to approximately 429 mm (4-year average) (NASA 2001). Schmidt (1942) described the regional climate as having two distinct seasons: a rainy season between April-November, with high rainfall indices during the months of June and July and a dry season, from December-March. A three-year study of Boa Vista showed a dry season between October-March and a wet season between April-September, with especially high rainfall indices in June and July (Rosa-Freitas et al. 2006).

Centro and Tancredo Neves were the two neighbourhoods chosen for this study. Centro is the downtown area, situated along the Branco River. It is the oldest part of the town. The resident population is ca. 6,000 individuals (Rosa-Freitas et al. 2003), with an average residence time of 27.5 years (estimated from this study sample). Commercial activity is intense and most of the Boa Vista population commutes through this area. Residents in Centro earned, on average, US$ 799/month (equivalent to 3 current Brazilian minimum salaries) (Rosa-Freitas et al. 2003).

Tancredo Neves is located in a more recently settled area in the west part of Boa Vista, 6 km from Centro. Tancredo Neves is characterised by a more precarious infrastructure, a lower average income (US$ 265/month, equivalent to 1 Brazilian minimum wage), 17% illiteracy and an average residence time of 10 years.

Ovitrap setting and sampling - Fifty coordinate pairs were randomly sampled from street maps of each area (with a restriction that distance between coordinate pairs should be > 50 m). A field team visited the area with a GPS to locate the premise closest to each coordinate pair. The resident was invited to participate in the study and was interviewed upon signed consent.

Six mosquito surveys were carried out. To sample during the rainy season, collections were performed in July 2005 and June 2007. To sample during the dry season, collections were performed in November 2005, November 2006 and November 2007 (early dry season) and in March 2007 (peak of the dry season). In each survey, one ovitrap was placed in the peridomicile of 50 premises per neighbourhood, usually in the garden or in a shady area. An ovitrap is a black plastic container filled with a mixture of water (270 mL) and an infusion of diluted hay (30 mL), with a wooden stick (15 cm x 2 cm) glued to the container wall to collect eggs (Reiter et al.

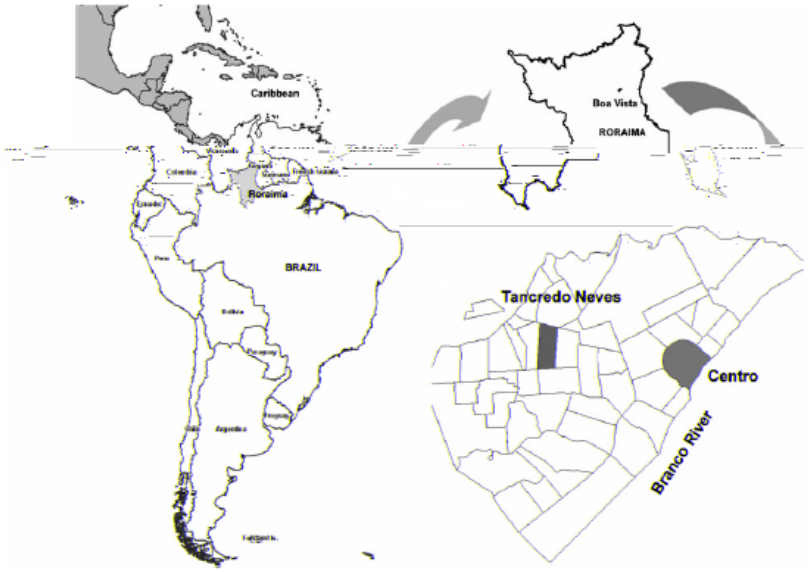

Fig. 1: geographical location of Boa Vista, Roraima, Brazil. Centro and Tancredo Neves are the two study sites.

1991, Honório et al. 2003). One week after being set, the ovitrap was removed and taken to the laboratory, where wooden sticks positive for eggs were submerged in water. Ecloded larvae were bred to their 4th larval instar, identified [using taxonomic keys from Consoli and Lourenço-de-Oliveira (1994)] and counted.

Temporal and spatial indices - Trap positivity index (TP) and egg density index (MED) were calculated. TP is the proportion of positive traps; MED is the ratio between the total number of eggs collected and the total number of traps (Regis et al. 2008). Differences in TP and MED between seasons per neighbourhood were analysed (χ^2 and Wilcoxon tests respectively calculated using the software R 2.6.0, (R Development Core Team 2006). Infestation maps were made using ArcGis 9.1 (ESRI, Redlands, CA, US). For each area and season we calculated a smooth kernel with a 250 m radius.

Building characteristics - Premises were classified as residential, commercial or both. Yards, when present, were classified according to the amount of shade (no shade, partially shaded, completely shaded). After inspection, all containers that could potentially become breeding sites (either currently filled with water or not) were identified and recorded. By inquiring with the householder, we obtained information regarding access to public services (water, sewage) and socio-demographic characteristics of the residents (income, schooling, time of residence in Boa Vista and in the neighbourhood). Associations between infestation and premise characteristics were tested (Kruskall-Wallis and χ^2 tests).

RESULTS

Study area, city and the neighbourhoods - In Centro, 65% of the premises included in the survey were residential and 35% were commercial. All but one premise had a yard and 68% of the yards had shady areas. Containers that could potentially be colonised by *Aedes* were found on 14 premises (28%). Most of the containers were small flower vases and discarded bottles. All premises (but one) were linked to the city water system. Five premises also stored water for consumption in large cisterns (1 school and 4 residences). Most premises (95%) were made of brick.

In Tancredo Neves, all premises in the survey were residential (4% with double function, residential and commercial). All but one premise had yards and 98% of the yards had shady areas. Containers that could potentially be colonised by *Aedes* were found on 21 premises (42%). Most of the containers were flower vases and discarded bottles. A total of 82% premises were linked to the city water system and 40% were also storing water for consumption in large cisterns. Most premises were made of brick (90%).

Species captured - More than 99% of the larvae that hatched from collected eggs were identified as *Ae. aegypti*. Less than 1% of larvae were *Culex nigripalpus* and *Cx nigripalpus* was only found in Tancredo Neves. All further analyses considered total eggs counted as a measure of the degree of *Ae. aegypti* infestation.

Comparison between neighbourhoods. Ovitraps results - A total of 21,399 eggs were collected during the whole study, with two thirds of eggs collected in Tancredo Neves ($\chi^2 = 2565$, df = 1, $p < 0.001$). Considering each survey separately, Tancredo Neves always yielded the highest number of eggs (Fig. 2B). TP positivity, on the other hand, was similar in both Centro and Tancredo Neves in all surveys but the last one, performed in November 2007 ($\chi^2 = 11.5$, df = 1, $p < 0.001$).

Temporal pattern - TP peaked during the rainy season (July 2005 and June 2007), with TP > 80% in both neighbourhoods. This was also the period with the highest egg density in Tancredo Neves (with MED = 49.5 and 116 eggs/trap during the rainy seasons of 2005 and 2007, respectively). Centro showed lower egg densities and its only survey with high density values (MED = 69.2 eggs/trap) also occurred during a rainy season (Fig. 2).

TP in the early dry season presented high variability between years. High infestation (> 80%) was observed in 2005, intermediary infestation (60-75%) in 2006 and relatively low infestation (35-70%) in 2007. November of 2007 was an unusually dry month, registering only 4.8 mm of rainfall (in November 2005 and 2006, rainfall averaged 85 mm).

Only during the peak of the dry season (March 2007) were infestation indices low, with TP < 25% and MED < 10 eggs/trap.

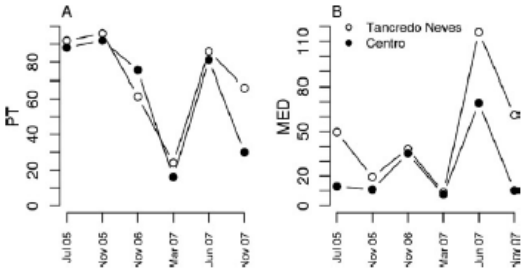

Fig. 2: *Aedes aegypti* infestation measured in Centro and Tancredo Neves, Boa Vista, Roraima, Brazil. A: percentage of positive ovitraps (PT); B: mean egg density (MED).

Egg distribution among premises - The number of eggs per premise was highly skewed (Centro: average of 30 eggs/premise, median of 1 egg/premise; Tancredo Neves: average of 56 eggs/premise, median of 13 eggs/premise). A small number of premises contributed most of the eggs captured (Fig. 3). During the infestation peaks (July 2005, November 2006 and June 2007), 10-20% of the premises yielded half of the total *Ae. aegypti* eggs in Centro and 12-18% yielded the same proportion in Tancredo Neves. In Centro, during the lowest infestation period (March 2007, in the dry season), two premises alone were responsible for > 50% of eggs collected. These premises were a large commercial site and a

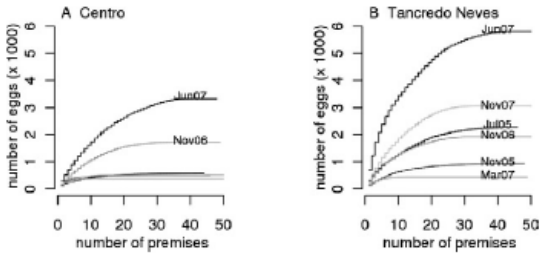

Fig. 3: cumulative distribution of *Aedes aegypti* eggs collected from the most productive premises, during the rainy and dry seasons, in Centro and Tancredo Neves neighborhoods, Boa Vista, Roraima, Brazil.

residence. In Tancredo Neves, three premises produced > 50% of all eggs collected in the same period of time. These three premises were a car-wash and two residences. Infestation maps for each season and neighbourhood are shown in Figs 4 and 5 with the most productive premises marked as black circles (dark grey colour indicates highly infested areas).

No association was found between the following measured premise traits and *Ae. aegypti* egg density: (i) be a residence (not commercial) (Centro: Wilcoxson's W = 4052, p = 0.24; Tancredo Neves: W = 2135, p = 0.8926), (ii) degree of shade in courtyard (Centro: Kruskal-Wallis = 1.0642, df = 2, p = 0.58; Tancredo Neves: Kruskal-Wallis = 0.3008, df = 2, p = 0.86) and (iii) have potential breeding sites in the yard (Downtown: W = 4446.5, p = 0.14; Tancredo Neves: W = 4824.5, p = 0.98). The other characteristics measured (water supply, sewage service etc.) showed low variation among premises and could not be tested.

DISCUSSION

Routine surveillance of *Ae. aegypti* in Boa Vista based on household surveys and the premise index calculation suggests that mosquito abundance in Boa Vista has a strong seasonal profile, with a high peak during the wet season (May-August) (Fig. 6B). In contrast, dengue incidence in Boa Vista has shown a variable year-to-year profile, with peaks occurring in the rainy season in some years and in the dry season in other years (Fig. 6C) (Rosa-Freitas et al. 2003).

Our data show that the period of high mosquito abundance is larger than suggested by the premise index data. In particular, we found high infestation indices during the early dry season (November), when the premise index consistently showed low values. A comparable result was found in another neighbourhood of Boa Vista, where 77.8% TP in November 2006 was measured (Zeidler et al. 2008). The greater sensitivity of ovitrap indices when compared to premise indices may explain the observed discrepancies between premise and ovitrap indices and underscore the value of using ovitraps (or other similar traps) for surveillance in Boa Vista.

The main meteorological determinant of mosquito population dynamics in Boa Vista seems to be the amount of rainfall (Zeidler et al. 2008). This result

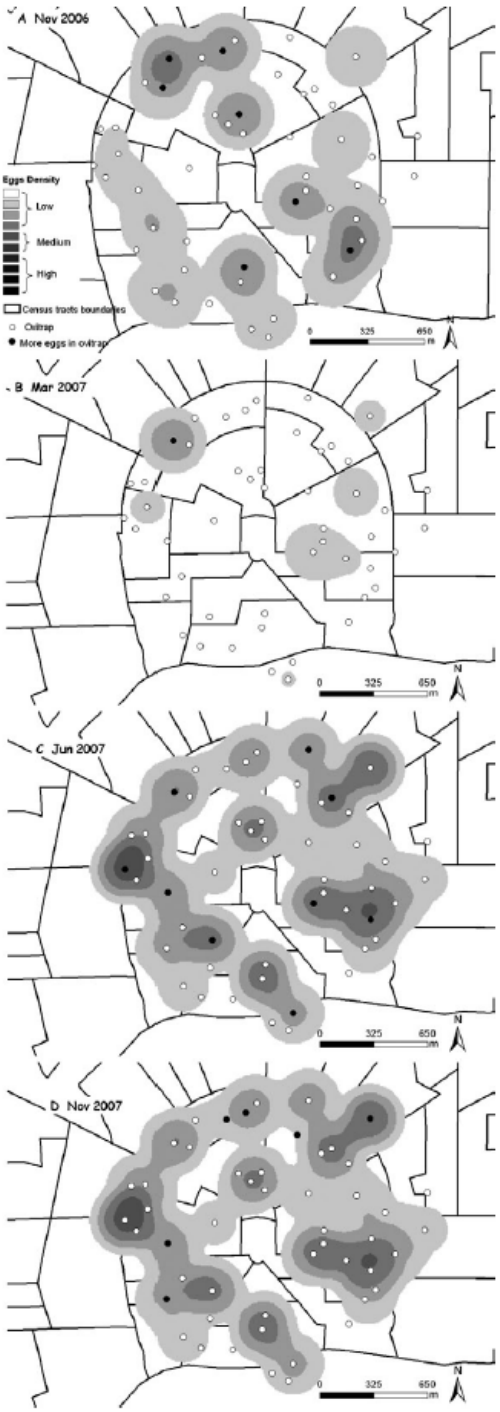

Fig. 4: *Aedes aegypti* infestation maps in Centro, Boa Vista, Roraima, Brazil during the (A) early dry season (B) dry season, (C) rainy season and (D) early dry season. Lines represent the streets. Circles indicate the 50 premises where ovitraps were installed. Black circles indicate the most productive premises at each season (they together produced ≥ 50% of the total number of collected eggs). Grey intensity indicates the estimated egg density.

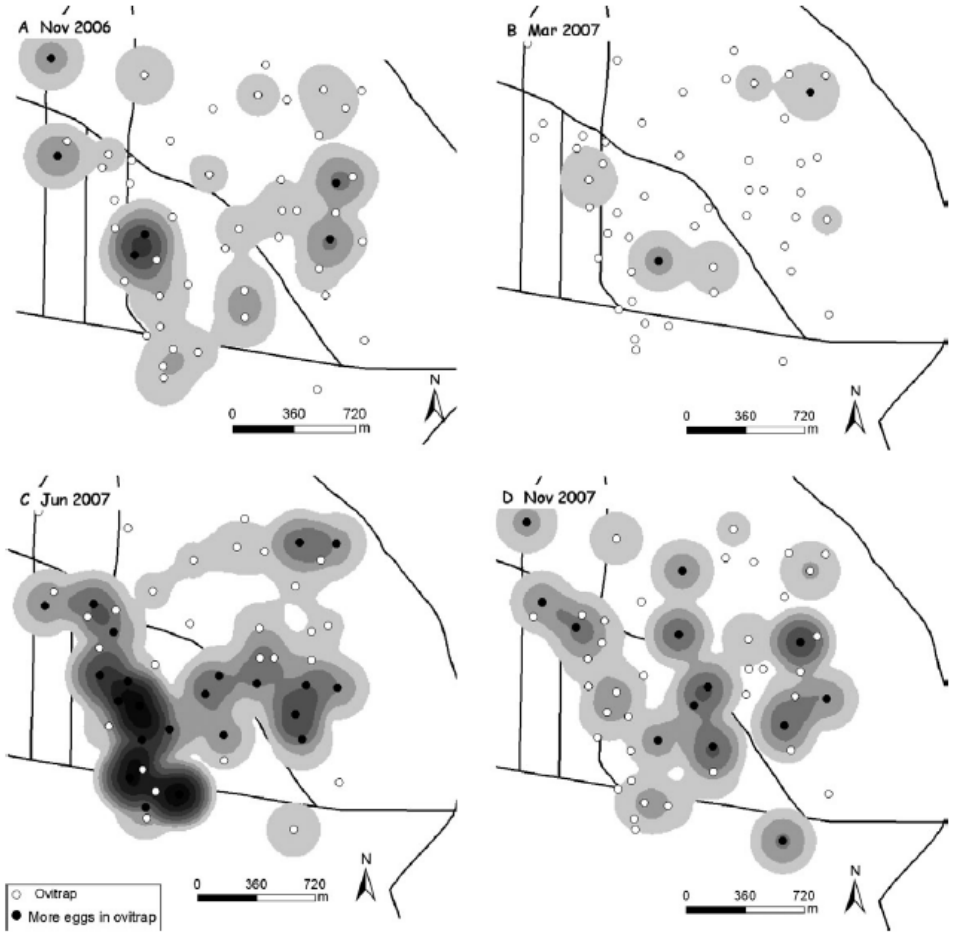

Fig. 5: *Aedes aegypti* infestation maps in Tancredo Neves, Boa Vista, Roraima, Brazil during: (A) early dry season (B) dry season, (C) rainy season and (D) early dry season. Lines and colors are explained in Fig. 4.

contrasts with the southern part of the country, where temperature seems to play the major role (NA Honório et al., unpublished observations). Rainfall is a "noisier" external force than temperature and is also characterised by higher variation between years. In Fig. 6A, a 10 year time series of rainfall in Boa Vista is depicted. This plot shows the occurrence of wetter and drier rainy seasons, depending on the year. Moreover, a shorter rainfall season at the end of the year (November-December) is noticeable, varying in magnitude from year to year. This short wet season may be responsible for the highly variable egg counts observed in November in our study.

Human behaviour may also change throughout the year in response to rainfall. It is interesting to note that mosquito infestation was always higher in Tancredo Neves, the neighbourhood, with twice as many households reporting that they store drinking water on their property. This difference between Tancredo Neves and Centro is consistent with previous observations based on premise indices (Fig. 8 in Rosa-Freitas et al. 2003), which placed Tancredo Neves high on the list of infested neighbourhoods and Centro somewhere in the middle. This year-to-year consistency at the neighbourhood scale contrasts with the apparently random risk of infestation at the household level. None of the investigated variables at household level (house type, presence and type of yard, number of households, access to piped water, presence of potential breeding sites, drinking water storage) was found to be significantly associated with the presence of eggs or the number of eggs collected. For some variables, a lack of association at the household level can be attributable to a lack of variation among houses (access to piped water, type of yard, for example). For other variables - such as the presence of potential breeding sites - a lack of association may be attributable to the type of trap used, which can attract mosquito females from the

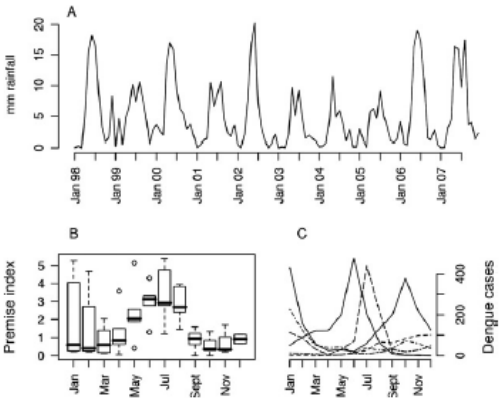

Fig. 6: A: log-transformed average monthly rainfall in Boa Vista, Roraima, Brazil between 1998-2007; B: box-plot of *Aedes aegypti* premise index per month, data from 1997-2003; C: monthly notification of dengue fever in Boa Vista, 2001-2006 (Instituto Nacional de Pesquisas Espaciais - INPE, Ministério da Saúde - MS).

vicinity of the house itself. The infestation maps show that during the high infestation periods, *Ae. aegypti* is present through the entire neighbourhoods.

In Boa Vista, the lack of strong external forces (such as temperature) may increase the role of stochasticity in infection demographics. Without a strong seasonal force, small oscillations in environmental variables (such as rainfall) may trigger large oscillations in disease incidence, as has been shown by mathematical models (Alonso et al. 2007). Poor predictability points to the need for continuous surveillance of vector density and dengue cases, especially during the rainy and transition seasons. Persistent differences between neighbourhoods in terms of *Ae. aegypti* density should be investigated further by integrated research approaches, combining anthropological and epidemiological methods to reveal the interplay between irregular water supply, urban environment and *Ae. aegypti* infestation (Caprara et al. 2009).

As within-country travel to and from Boa Vista increases, the risk of a DENV 4 introduction in Brazil increases. The particular features of dengue dynamics in Boa Vista show the need for customized vector and dengue surveillance and control measures for this city.

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The spatial distribution of *Aedes aegypti* and *Aedes albopictus* in a transition zone, Rio de Janeiro, Brazil

Padrões da distribuição espacial do *Aedes aegypti* e *Aedes albopictus* em uma zona de transição no Rio de Janeiro, Brasil

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Abstract

Dengue fever has become the most important vector-borne viral disease in Brazil. Human facilitated transport of desiccation-resistant eggs has led to its two most important vectors, Aedes aegypti *and* Ae. albopictus, *becoming widespread. In this paper, we report seasonal and spatial variation in larval abundances of* Ae. aegypti *and* Ae. albopictus *across a small-scale transition zone between an urban area and an urban wooded/forested area within Rio de Janeiro, Brazil. We installed 400 ovitraps across 10 sites with different human population densities and vegetation coverage. Eggs and larvae were collected for three weeks during the wet and dry seasons of 2002 and 2003.* Ae. albopictus *was predominantly found in the forested areas of the study site whereas in the urbanized area* Ae. aegypti *was more abundant. Both species peaked during the wet season. This distribution pattern, which may reflect adult flight range, may favor the co-occurrence of larvae of these species in a small-scale urban/urban forest transition zone.*

Aedes*; Dengue; Spatial Analysis*

Introduction

The reemergence of dengue fever in Brazil began in 1986, a few years after the recolonization of the country by its main vector *Aedes aegypti*. The other potential dengue vector, *Ae. albopictus,* was discovered in Brazil in 1986. Factors such as human-facilitated transportation of desiccation-resistant eggs have led to *Ae. aegypti* and *Ae. albopictus* becoming widespread. In addition, the Brazilian climate offers favorable conditions for the invasion and expansion of dengue vectors [1]. The spread of dengue vectors is helped by the intensity, frequency and speed at which people and cargo are transported within the country [2]. A recent study, conducted in Nova Iguaçu in the metropolitan area of Rio, showed that densely populated neighborhoods located close to highways with intense traffic can facilitate the introduction and circulation of dengue viruses [3,4]. In these areas, strategic points such as gasoline stations, tire shops and scrap metal yards are strategically important for the surveillance of dengue vectors [3].

Ae. aegypti and *Ae. albopictus* are sympatric species that tend to breed in similar sites, most commonly in artificial containers [5,6]. Inter-specific competition between these species has been documented. Early work suggested *Ae. aegypti* to be the superior competitor [7]. More recent works, however, have shown that *Ae. albopictus* may be superior under laboratory [8,9] and

field conditions [10,11], which probably explains displacements of *Ae. aegypti* by *Ae. albopictus* in some areas [10,12]. In North America and Brazil, the introduction of *Ae. albopictus* has been associated with a decrease in the abundance of *Ae. aegypti* [13,14], with an impact on the geographical distribution of this species [15,16,17].

Urbanization and vegetation coverage seem to have significant but opposite effects on the occurrences of *Ae. aegypti* and *Ae. albopictus* [18]. Studies conducted in the Tijuca Forest in Rio de Janeiro [19,20], showed that *Ae. aegypti* and *Ae. albopictus* were more abundant near houses than deeper in the forest [19]. The dispersal of *Ae. aegypti* and *Ae. albopictus*, estimated using mark-release-recapture techniques, showed that *Ae. albopictus* can fly between sylvatic and peridomestic environments while *Ae. aegypti* had a lower tendency to disperse into the forest [20], suggesting that the urban forest is a refuge for *Ae. albopictus*.

Several authors have shown that *Ae. aegypti* is more prevalent in highly urbanized and densely populated neighborhoods, whereas *Ae. albopictus* is more prevalent in rural, suburban, and forested urban areas [17,18,19,20,21,22]. However, most of these studies have analyzed the distribution of *Ae. aegypti* and *Ae. albopictus* on a large (city) scale. The goal of the present study was therefore to add further analysis to this process by looking at *Ae. aegypti* and *Ae. albopictus* distribution patterns at a local scale. In this paper, we report seasonal fluctuations in the larval occurrences of *Ae. aegypti* and *Ae. albopictus* across a spatially heterogeneous landscape, characterized by two distinct habitats – a highly populated low income urban area, and a vegetated, more sparsely populated area – separated by a sharp transition zone (the border of the Oswaldo Cruz Foundation – FIOCRUZ – campus), in the city of Rio de Janeiro. By characterizing the seasonal and spatial variation in the larval density of both species, we attempted to address the factors contributing to the competitive advantages for each species.

Material and methods

Study site

The area encompasses the campus of FIOCRUZ, Rio de Janeiro, Brazil (22°52'30"S, 43°14'53"W; 697,000m²), with large vegetation patches, and the surrounding area, which are densely populated by low income residents and known as slums (*comunidades* or *favelas*) (Figure 1). This forested area within the campus is a refuge for a variety of mammals and birds. Ten collecting sites were defined, each representing different levels of anthropic modifications, types of urbanization and land use. Table 1 lists the main characteristics of these sites.

The climate in Rio is tropical, with a drier winter (May-September) and a rainy summer (November-March). Air temperature and precipitation data were obtained from the nearest meteorological station, located 10km from the study site.

Classification of land coverage and infestation maps

A visual review of an aerial photograph of the area allowed us to classify the study area into four zones: (1) densely built urban – an area composed mainly of residential and commercial buildings; (2) low vegetation coverage – an area composed of short vegetation, mostly grass; (3) medium vegetation coverage – an area composed of average size vegetation, shrubs or small trees that were spatially scattered; and (4) high vegetation coverage – an area composed mainly of tall vegetation, in patches (Figure 1). Maps showing the spatial distribution of *Ae. aegypti* and *Ae. albopictus* larval density in wet and dry seasons were created using a kernel density estimator (Gaussian function and smooth kernel of 100m radius. ArcGis 9.1, ESRI; http://www.esri.com/software/arcgis/index.html). The kernel density estimator that was chosen is an interpolation and smoothing technique for generalizing point location to an entire area and consists of a bi-dimensional function of the events, forming a surface whose value is proportional to the intensity of samples for the area [23].

Entomological survey

Four entomological surveys were carried out during the wet and dry seasons of 2002 and 2003. In each of the ten study sites (Figure 1), we installed 40 ovitraps (a plastic vase with a wooden oviposition paddle and hay infusion as described previously) [16]. After seven days, eggs and larvae were collected. This procedure was repeated during three consecutive weeks. Larvae were identified and counted in the laboratory. Paddles were stored at 25-28°C and > 80% humidity and immersed in water after two weeks; hatching larvae were reared up until the fourth instar, and identified using key by Consoli & Lourenço-de-Oliveira [24].

Statistical analysis

Analysis of variance (ANOVA) was used for testing seasonal and spatial trends of the log-trans-

Figure 1

The study site was an urban/urban forest in a small-scale transition zone.

Areas
1 - Arthur Neiva Pavilion
2 - Carlos Chagas Pavilion
3 - Garden
4 - Day Care Center/INCQS
5 - IPEC
6 - ENSP/FarManguinhos
7 - Faria Timbó River
8 - Amorim Community
9 - Varginha Community
10 - Parque João Goulart Community

Land coverage
Densely built urban
Low vegetation coverage
Medium vegetation coverage
High vegetation coverage

FIOCRUZ limits
Faria Timbó River

N
W E
S

0 560
m

Table 1

Main characteristics of the descriptors of each site within and outside of FIOCRUZ campus, Rio de Janeiro, Brazil.

Sites	Distance from the border (m)	Area (m²)	Exposed human population * (approximate)	Land coverage			
				% urban	% low vegetation	% medium vegetation	% high vegetation
C1 *	52	12,071.63	65 *	21.06	0.00	15.81	63.13
C2 *	109	49,895.80	119 *	7.27	0.00	0.00	85.21
C3 *	66	38,381.01	30 *	16.67	0.00	11.94	71.04
C4 *	340	48,975.61	460 *	20.69	21.97	54.32	3.01
C5 *	254	39,688.21	285 *	17.60	26.96	55.45	0.00
C6 *	88	38,631.98	756 *	19.58	0.85	68.81	0.00
C7 *	10	50,756.49	10 *	27.01	22.64	34.34	0.00
P8 **	-82	18,545.67	3,199 *	73.33	0.00	0.00	12.30
P9 **	-50	19,348.76	1,175 *	82.34	6.49	0.04	0.00
P10 **	-43	25,364.43	4,923 *	89.77	0.00	0.00	0.00

* Exposed population inside forested area (FIOCRUZ Campus) was obtained from the Human Resources Department of FIOCRUZ;

** Data of respected population in slums were obtained from the Instituto Brasileiro de Geografia e Estatística (IBGE).

formed occurrences of immature specimens. Simple Pearson correlations were used to test for associations between species densities. Least squares regression was used for fitting exponential models for infestation vs. distance. The slope of the regression line for each season were compared as described by Zar [25].

Results

Specimens

A total of 57,779 immature mosquito specimens were collected, 41% of which were *Ae. aegypti* and 59% *Ae. albopictus*.

Spatial distribution

No significant difference was observed between the log transformed mean number of larvae obtained at the first, second, and third weeks of each collecting period. *Ae. albopictus* was more abundant in areas with high to medium dense vegetation coverage ($F_{1,118} = 78.63$, $p < 0.001$), whereas densely populated areas were dominated by *Ae. aegypti* ($F_{1,118} = 94.82$, $p < 0.001$). The species co-occurred at the transition zone between highly populated and highly vegetated areas (Figures 2 and 3). Figure 4 shows the result of fitting a linear regression line to log (larvae) x distance from the campus border ($r^2 = 0.78$, $F_{1,6} = 21.74$ [$p < 0.01$] for the wet season and $r^2 = 0.84$, $F_{1,6} = 32.39$ [$p < 0.01$] for the dry season). In the wet season, the regression curve was: [proportion *Ae. aegypti*] = 1.5115(exp ([distance][-0.0065]). From this model, we estimate that 72% of *Ae. aegypti* larvae were within 50m from the border, approximately 50% at 100m, and only 3.8% beyond 500m. For the dry season, the regression curve was: [proportion *Ae. aegypti*] = 1.6793(exp ([distance][-0.0056]), suggesting slightly wider larval distribution. For example, 57% would fly 100m and 6% would fly 500m. The slope of the regression line for each season did not differ significantly (Student's t-test).

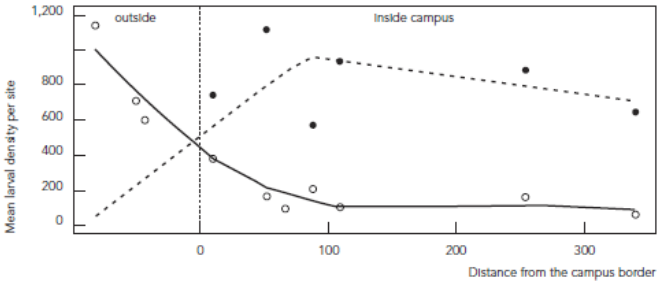
Seasonal variations

Approximately 50% and 28% of all *Ae. albopictus* and *Ae. aegypti* larvae were collected during the wet season and summer, respectively. The percentage of positive ovitraps also varied between seasons. After a period with positive traps typically < 50% during the wet season of 2003, we observed an increase in both *Ae. aegypti* and *Ae. albopictus* frequencies (mean percent of positive ovitrap of ~90%). Seasonal variation in rainfall and temperature is shown in Figure 5. Outside the forest area, both species showed similar temporal patterns, characterized by relatively constant densities during the first year, but with a peak in the wet season of 2003, and a return to the baseline level (Figure 6). Rainfall indices varied

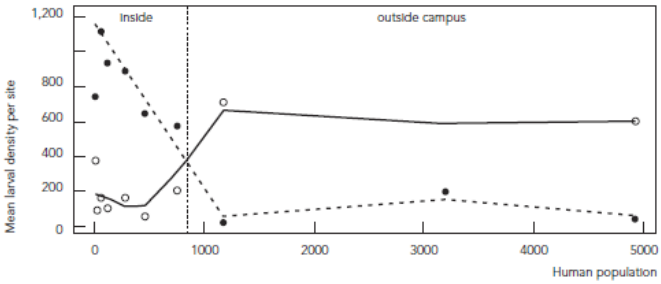
Figure 2

Mean density per site of immatures of *Aedes aegypti* (white cycle) and *Aedes albopictus* (black cycle) as a function of distance from the border of the forest area, human population, and % vegetation coverage.

2a) Distance from the campus border



2b) Human population



2c) % vegetation coverage

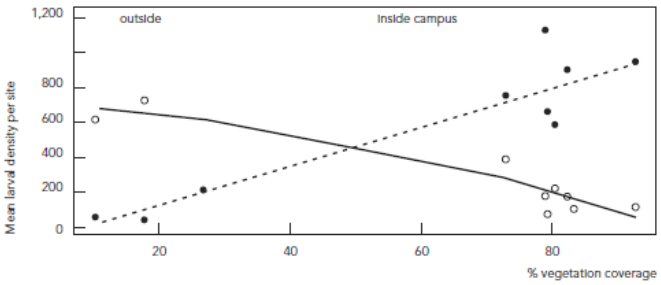


Figure 3

Kernel estimation of density of immatures of *Aedes aegypti* and *Aedes albopictus* inside and outside of forest area during wet and dry seasons, 2002 and 2003. Sizes of the circles indicate abundance of larval density.

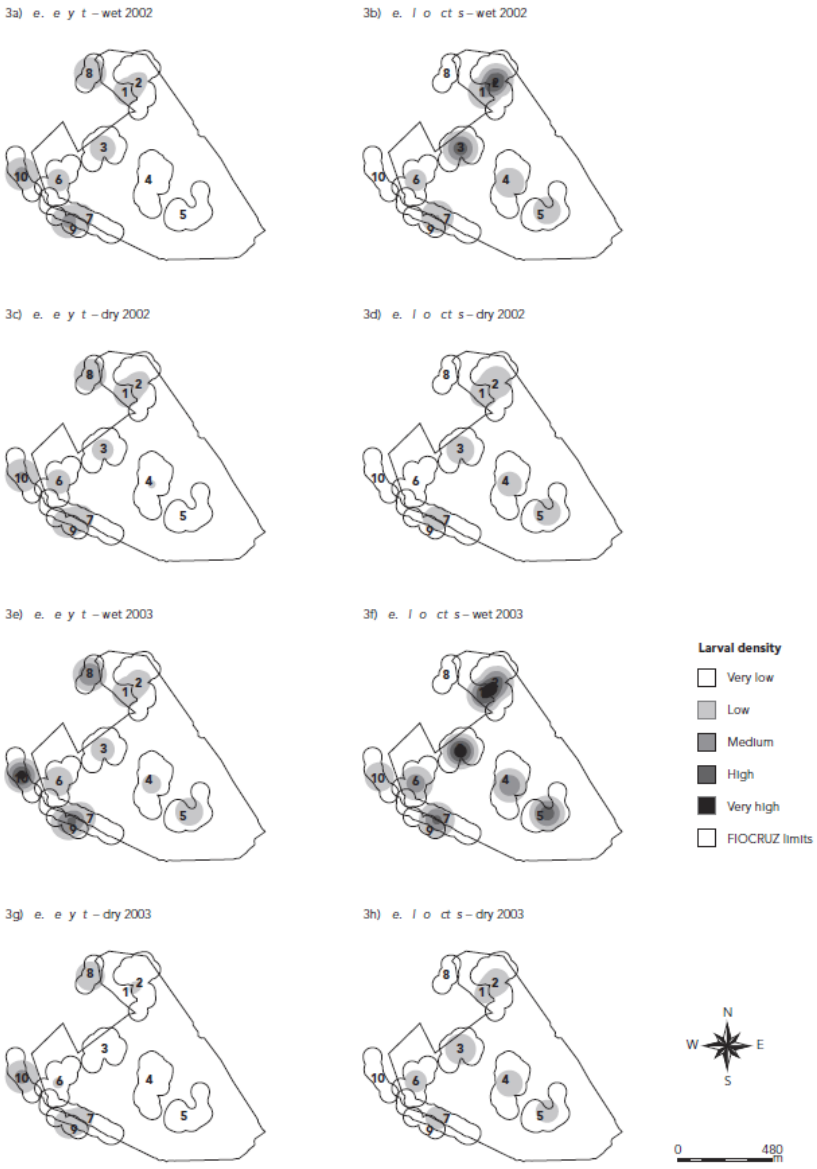


Figure 4

Percentage of *Aedes aegypti* larvae retrieved, as compared to the number obtained in the urbanized area, decreased exponentially with the distance to the wall of the forest area. The lines represent the least squares regression fit of the data for the function y = b*exp(x*a), during the wet season (circles) and dry season (squares).

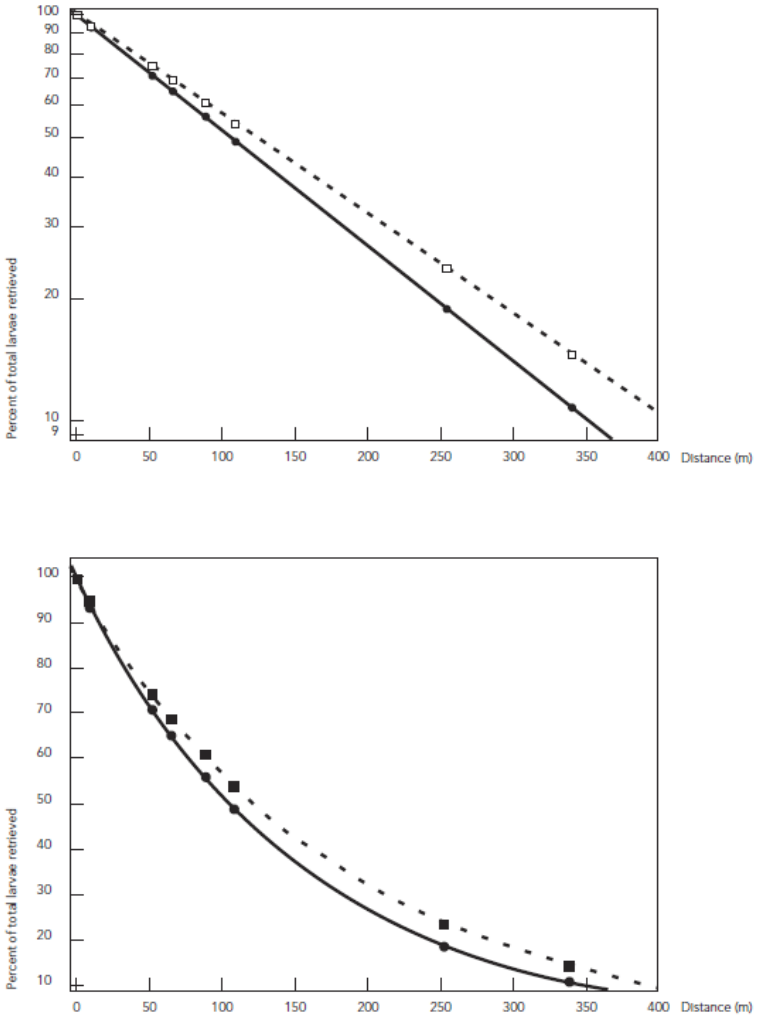
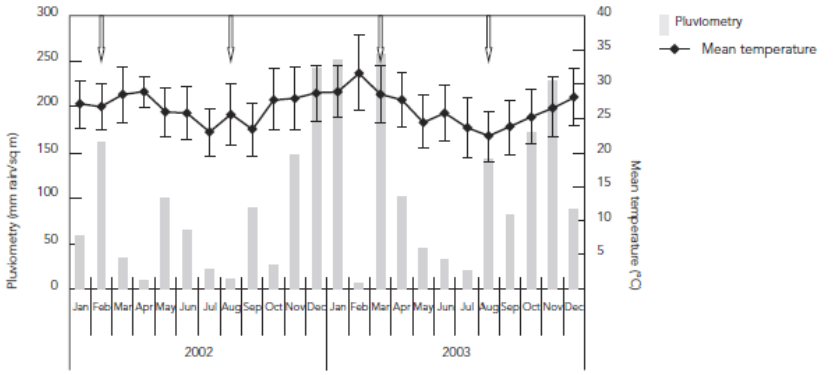


Figure 5

Total rainfall and mean temperature per month, 2002 and 2003. Vertical bars indicate maximum and minimum mean monthly temperature. Arrows indicate periods of data collection.



intra-seasonally depending on the year, particularly for some months of the year. The years 2002 and 2003 showed distinct rainfall regimes: rainfall peaked in February in 2002, and one month later, in 2003; the spring rainfall started later in 2002 (September) than in 2003 (August); and in December, it rained twice as much in 2002 as in 2003. The thermal variation range was bigger in 2003 than 2002 (Figure 5).

In the forested area, *Ae. aegypti* followed the same pattern observed outside the forested area, but always at a lower density than inside (Table 2). Overall, the density of *Ae. albopictus* was twice that of *Ae. aegypti* inside the forested area (Table 3; Figure 6).

Discussion

Our results show *Ae. albopictus* was more abundant in areas with high to medium dense vegetation coverage, whereas the densely populated areas were dominated by *Ae. aegypti*. These results are consistent with distribution patterns of *Ae. aegypti* (in urban areas) and *Ae. albopictus* (in more densely vegetated areas) found in the literature [17,18,19,20,21,22]. *Ae. albopictus* was found associated with vegetated areas mostly inside the forested area of FIOCRUZ Campus (Figures 1 and 3), where vegetation coverage is highest. Coexistance occurred at the transition zone, defined as the area that covers the campus wall and its vicinity (Figure 3). Similarly, co-occurrence of the two species has been reported in suburban areas in Rio de Janeiro [17,22]. We hypothesize that the host-seeking behavior of *Ae. albopictus* may play a role in this distribution. Areas 4 and 5, with low-medium vegetation coverage, in the center of the forest area were systematically less positive to *Ae. albopictus* than other areas. This may be due to low vegetation coverage.

Both species showed seasonal variation in their larval densities. Overall larval densities for both *Ae. aegypti* and *Ae. albopictus* were greater during the wet seasons (Figure 6). Variation was more marked in *Ae. albopictus* (Figures 2, 3, and 6). This is in concordance with previous studies in Rio de Janeiro [6]. In the dry season of 2002, *Ae. albopictus* and *Ae. aegypti* mean densities decreased in the forest area, but remained constant in the densely built urban area. *Ae. aegypti* was more abundant in the wet season of 2003 than it was in the wet season of 2002. The same was true for *Ae. albopictus*. These may have been due to higher rainfall indices in March (Figure 5).

Data suggest that *Ae. aegypti* has a more stable response to climatic variation. The main cause of the shift in distribution of the two species may be due to a greater increase in egg mortality for *Ae. albopictus* under dry conditions, compared to *Ae. aegypti* [26]. Therefore, dry conditions could shift the competitive advantage away from *Ae.*

Table 2

Mean number of *Aedes aegypti* larvae retrieved at each ovitrap area in the wet and dry seasons, 2002 and 2003.

Sites *	2002				2003			
	Wet	SD	Dry	SD	Wet	SD	Dry	SD
C1	119.00	79.67	144.67	190.00	165.00	41.62	25.00	10.58
C2	57.00	46.51	77.00	35.93	103.00	55.03	35.67	48.43
C3	109.00	70.55	71.67	46.82	92.00	19.97	1.00	1.73
C4	28.00	24.27	37.33	28.75	60.67	30.73	12.00	11.14
C5	25.00	12.00	33.33	22.23	161.67	163.84	24.33	28.04
C6	75.33	58.48	98.67	79.86	207.00	49.49	40.00	38.22
C7	123.00	34.60	135.00	43.49	377.67	237.00	108.33	168.66
P8	435.67	184.91	370.33	99.45	1142.67	519.56	470.67	228.82
P9	362.67	243.66	376.33	240.67	711.33	319.31	174.33	95.10
P10	391.33	177.18	183.00	72.92	602.33	295.63	141.67	87.46

Note: standard deviations (SD) for the three weeks are reported.

* Site codes correspond to those in Figure 1.

Table 3

Mean number of *Aedes albopictus* larvae retrieved at each collecting area in the wet and dry seasons, 2002 and 2003.

Sites *	2002				2003			
	Wet	SD	Dry	SD	Wet	SD	Dry	SD
C1	418.00	266.27	165.67	104.85	1,114.33	402.01	66.00	64.44
C2	1,007.67	689.39	298.33	96.73	934.67	295.05	116.33	31.34
C3	888.67	95.71	138.67	202.23	1,276.67	367.22	253.00	112.28
C4	192.67	23.01	97.33	124.42	647.33	266.04	119.00	41.00
C5	140.33	59.54	127.33	132.67	886.67	423.02	80.00	32.79
C6	72.33	53.16	15.33	10.12	572.67	132.88	75.33	51.83
C7	357.00	94.91	94.00	33.41	741.67	234.51	71.33	44.00
P8	31.33	3.06	10.33	10.12	200.33	177.80	30.00	22.61
P9	5.00	6.24	5.67	9.81	27.33	18.58	10.33	8.96
P10	1.00	1.00	15.33	26.56	44.00	3.46	7.67	10.79

Note: standard deviations (SD) for the three weeks are reported.

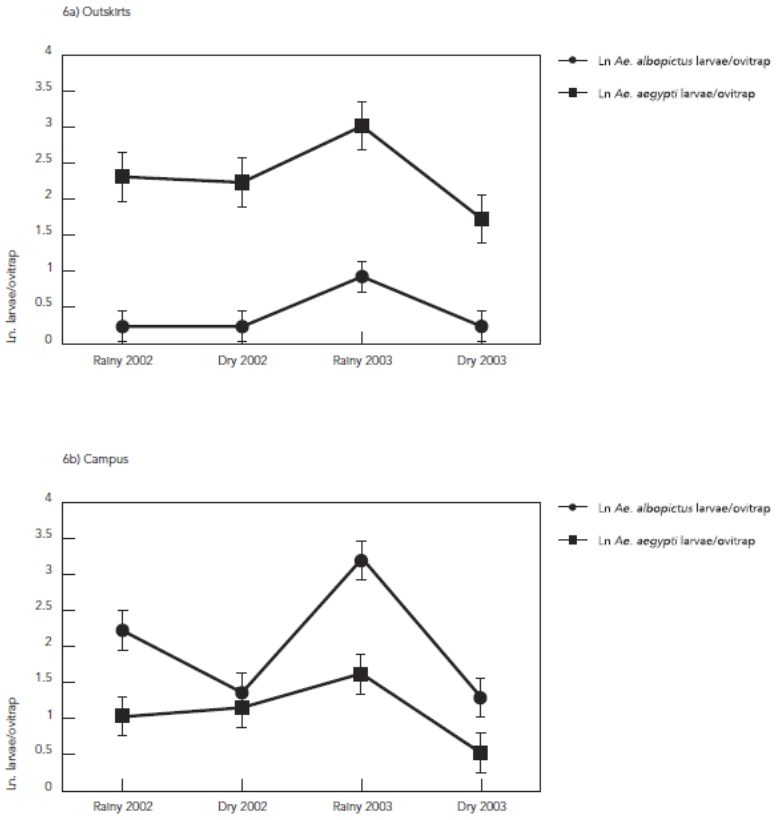
* Site codes correspond to those in Figure 1.

albopictus, favoring *Ae. aegypti*, a pattern documented in controlled laboratory environments [27]. Nevertheless, the greater stability in *Ae. aegypti* numbers in urban areas could also be due to its habitat preference for domestic situations [22]. The relatively protected environment provided by housing may favor stable year-long breeding site availability [20,22].

Our results suggest that the occurrence of both species varies seasonally and spatially (Figures 2, 3 and 6). *Ae. aegypti* and *Ae. albopictus* were more common in the area closer to the border inside FIOCRUZ Campus and *Ae. albopictus* was practically absent inside the densely built urban area. Although there was no statistically significant negative correlation for any of the periods separately, the overall densities of both species in the small transition zones were negatively correlated, despite parallel seasonal fluctuations. Because these species colonize distinct habitats in Rio de Janeiro, *Ae. albopictus* may not replace *Ae. aegypti*, and both species may be common in a single area with their abundances positively correlated [17]. Also, as suggested in studies in Florida, USA, local coexistence of these species may be possible in a seasonal climate because warm,

Figure 6

Mean number of *Aedes aegypti* and *Aedes albopictus* larvae retrieved in ovitraps in the outskirts of and inside the forest area (campus).



Note: vertical bars denote 95% confidence intervals.

dry climates favor *Ae. aegypti* and reduce the impact of competition from *Ae. albopictus* through differential mortality of *Ae. albopictus* eggs [26].

The usual > 1 ratio of *Ae. albopictus* to *Ae. aegypti* larvae was inverted during the dry season of 2002, when *Ae. albopictus* eggs and larvae suffered a decrease in specimen numbers. However, *Ae. aegypti* densities did not increase in collecting sites where *Ae. albopictus* densities remained constant, and this could be a factor related to the distance to the urban area. During the 2003 wet season, when the percentage of positive ovitraps was high, there was a 50% mean decrease in the expected densities of *Ae. aegypti*, as estimated by the flight range equation. This expected density decrease was most marked in densely vegetated areas, where *Ae. albopictus* was most prevalent.

Due to the low proportion of larvae of *Ae. aegypti* that occurred in natural habitats compared to artificial containers [28], we were able to estimate the number of *Ae. aegypti* larvae in the FIOCRUZ campus as a function of the distance

to the urbanized area. The exponential model for *Ae. aegypti* larvae distribution indicates that occurrence of immature forms would be common in the neighboring areas, near the preferred habitat, but would decrease markedly as the distance increased. This distribution pattern, as well as the increased densities of *Ae. albopictus* larvae in the vegetated area, would favor the co-occurrence of larvae of both species.

We have reported seasonal and spatial variations in distribution patterns between *Ae. aegypti* and *Ae. albopictus* within a habitat transition zone. These results suggest that *Ae. aegypti* and *Ae. albopictus* are differentially distributed over habitats and that their numbers may decrease exponentially in relation to the distance traveled [16,18]. This means that a high rate of both species would be present in a small scale transition zones and inter-specific competition could play a role in these urban/urban-forested transition zones, especially when the number of available breeding sites becomes scarce. Longitudinal studies with more frequent sampling may uncover regular seasonal waves of species invasion and retreat in these areas. Further characterization of transition zones may provide a better insight into the interplay of population dynamics of dengue vectors in small-scale habitats.

Resumo

Dengue tornou-se a mais importante doença viral transmitida por vetores no Brasil. O transporte humano de ovos resistentes à dessecação permitiu que seus dois mais importantes vetores, Aedes aegypti *e* Ae. albopictus, *se tornassem cosmopolitas. Neste artigo, nós avaliamos a variação sazonal e espacial da abundância de larvas de* Ae. aegypti *e* Ae. albopictus *por meio de uma pequena escala espacial de zona de transição entre uma área urbana e uma área florestal do Rio de Janeiro, Brasil. Foram instaladas quarenta ovitrampas em cada área dos dez locais caracterizados por diferente densidade populacional humana e cobertura vegetal. Ovos e larvas foram coletados durante três semanas no verão e inverno de 2002 e 2003.* Ae. albopictus *foi predominantemente encontrado nas áreas com maior cobertura vegetal enquanto que nas áreas urbanizadas (comunidades),* Ae. aegypti *foi mais abundante. Ambas as espécies apresentaram pico durante a estação chuvosa. Esse padrão de distribuição das formas imaturas pode refletir no raio de vôo do adulto, favorecer a co-ocorrência de larvas dessas espécies nessa pequena escala espacial da zona de transição entre as áreas urbana e de floresta.*

Aedes*; Dengue; Análise Espacial*

Contributors

N. A. Honório participated in the design, planning, data collection, analysis, interpretation, and drafting of the paper. M. G. Castro participated in the design, data collection and data interpretation. F. S. M. Barros collaborated in analysis, data interpretation and drafting of the paper. M. A. F. M. Magalhães helped in the analysis and data interpretation. P. C. Sabroza contributed to the design, analysis, interpretation data, critical review of the content, and approval of the paper's final version.

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Dengue Infection Increases the Locomotor Activity of *Aedes aegypti* Females

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Abstract

Background: *Aedes aegypti* is the main vector of the virus causing Dengue fever, a disease that has increased dramatically in importance in recent decades, affecting many tropical and sub-tropical areas of the globe. It is known that viruses and other parasites can potentially alter vector behavior. We investigated whether infection with Dengue virus modifies the behavior of *Aedes aegypti* females with respect to their activity level.

Methods/Principal Findings: We carried out intrathoracic Dengue 2 virus (DENV-2) infections in *Aedes aegypti* females and recorded their locomotor activity behavior. We observed an increase of up to ~50% in the activity of infected mosquitoes compared to the uninfected controls.

Conclusions: Dengue infection alters mosquito locomotor activity behavior. We speculate that the higher levels of activity observed in infected *Aedes aegypti* females might involve the circadian clock. Further studies are needed to assess whether this behavioral change could have implications for the dynamics of Dengue virus transmission.

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Introduction

Over the last decades, Dengue outbreaks have been a major [illegible] in tropical countries in many parts of the World. Where Dengue epidemics have been registered with significant [illegible] [1]. There are four antigenically distinct RNA viruses that can cause the disease, and in Brazil three Dengue serotypes (DENV-1, DENV-2 and DENV-3) have co-circulated in several areas and caused some severe Dengue epidemics [2].

Aedes aegypti (Diptera: Culicidae) is the urban vector of Yellow Fever and Dengue viruses. This diurnal mosquito is very anthropophilic and endophilic, being commonly found in urban and suburban areas, especially where house and human densities are high and where it seems to live longer and disperse to short distances (e.g. [3–5]).

It is known that parasites can alter vector behavior (reviewed by [6–8]) and a number of studies have reported behavioral changes in *Ae. aegypti* infected with pathogens and symbionts. For example, Rossignol et al [9] observed that *Ae. aegypti* females experimentally infected with an avian malaria parasite, *Plasmodium gallinaceum*, take more time to locate blood in guinea pigs than the uninfected ones. Rowland and Lindsay [10] studied the flight activity of females of this species infected with the filarial parasite *Brugia pahangi* and observed a decrease in flight capacity in the [illegible]

In the current study we investigated whether Dengue virus causes changes in the locomotor activity behavior of *Ae. aegypti* females under laboratory conditions [illegible]

Methods

Infection of Mosquitoes with the Deng[illegible]

The Paea strain of *Ae. aegypti* was used in experiments. This laboratory strain is highly susceptible to Dengue virus serotype 2 (DEN[illegible]). Mosquito colony was reared according to proc[illegible] [16]. Males and females remained together and [illegible] sucrose solution since emergence.

Five-day-old female mosquitoes were indiv[illegible] intrathoracic inoculation with 0.2 µl of [illegible] Medium containing Dengue virus (DENV-2 [illegible] 66985 [17]) in a concentration of 10^7 PFU [illegible] microinjector (Drummond Scientific). Contro[illegible]

intrathoracically inoculated with 0.21 µl of only L-15 (Leibovitz) Medium.

To verify the success of the experimental infections, the heads of a number of mosquitoes that were inoculated with virus and that were alive by the end of the locomotor activity experiments (around 8–10 days after inoculation), plus negative controls, were tested for Dengue infection via RT-PCR, as described below. The results indicated that over 95% (70/73) of the mosquitoes inoculated with the Dengue virus were infected (data not shown).

Detection of Dengue virus in mosquitoes

Mosquito heads were separated from bodies on a metal plate placed on dry ice and viral RNA was extracted from each head using the QIAmp Viral Mini Kit (Qiagen) according to the manufacturer's protocol. RT-PCR for detecting DENV2 was conducted in a PCR System 9700 GeneAmp (Applied Biosystems) using SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen) and Dengue virus consensus primers D1 and D2 [18], followed by a semi-nested PCR on the resulting product using Go Taq Green Master Mix (Promega) and primers D1 and TS2 [18]. PCR products were electrophoresed on 2.5% agarose gels. A band of 119 pb corresponding to DENV-2 could be seen under UV light in the infected mosquitoes.

Analysis of the locomotor activity behavior

The activity of infected and uninfected control *Ae. aegypti* females was recorded using a larger version of the Drosophila Activity Monitor (TriKinetics) as described in [19]. After inoculation with Dengue virus or L-15 medium, females were individually placed in glass tubes (1 cm×7 cm) with a cotton plug soaked in 15% sucrose solution and these tubes placed in the Activity Monitor inside a Precision Scientific Incubator Model 818 under a constant temperature of 25°C and a photoperiod of 12 hours of light and 12 hours of dark (LD 12:12). For each mosquito, the total locomotor activity of 1 hour-intervals was recorded for about seven days after inoculation. The data of individuals that died during the experiments were excluded, and the data analysis was carried out comparing the activity data of infected and uninfected mosquitoes from the second to sixth day after inoculation.

Results

Table 1 shows the mean hourly locomotor activity of control and Dengue virus infected females in four different experiments. We observed that infected females of *Ae. aegypti* showed more activity than controls in all experiments. The relative increase in activity ranged from ~10% to more than 50%. A two-way ANOVA indicated a significant difference in the activity between infected and uninfected control females ($p<0.01$). Although a significant difference in the overall activity levels was also observed between experiments ($p<0.01$), the interaction between experiments and infection was not significant ($p = 0.82$) indicating that the difference between infected and uninfected females was consistent.

Figure 1 shows the normalized locomotor activity patterns of infected and control females during a full LD 12:12 cycle (Fig. 1A) or during the photophase (Fig. 1B). As previously reported in the literature (reviewed in [20]), *Ae. aegypti* is a diurnal species showing higher activity levels towards the end of the photophase ("late afternoon") and a characteristic startle response to the abrupt light-on/light-off transition [21]. The comparison of the normalized locomotor activity patterns of infected and uninfected females shows that Dengue infection causes an increase in activity throughout the 24 hour period. Although this effect is most dramatic during the light-on/light-off transition (Fig. 1A), an increase in activity is also seen throughout the day and night in infected females, especially during the "natural" activity peak occurring around ZT 9 and in the last hours of the photophase (Fig. 1B). In fact, this increase in activity associated with Dengue infection is still significant ($p = 0.012$) even when we exclude the light-on/light-off transition (Table 1). In summary, our results indicate that the locomotor activity of infected females is consistently increased when compared to that of uninfected females.

Discussion

Very little is known about the effects of viral infection on *Aedes* mosquitoes. Several authors have shown that Dengue virus exhibits

Table 1. Activity increase in Dengue infected *Aedes aegypti* females.

Experiment	Status	N	Mean activity per hour* (± SEM)	Relative increase of activity in infected mosquitoes (%)
1	Control	17	5.06±1.16 (4.73±1.15)	52.6 (48.0)
	Infected	23	7.72±1.27 (7.00±1.26)	
2	Control	53	11.22±1.45 (10.52±1.49)	30.8 (30.5)
	Infected	74	14.68±1.50 (13.73±1.49)	
3	Control	45	9.84±1.19 (8.97±1.15)	43.2 (45.3)
	Infected	66	14.09±1.24 (13.03±1.23)	(13.03±1.23)
4	Control	70	11.88±1.01 (10.95±1.00)	13.3 (10.3)
	Infected	83	13.46±1.13 (12.39±1.06)	

*The numbers in parenthesis refer to the activity excluding the light-on/light-off transition.

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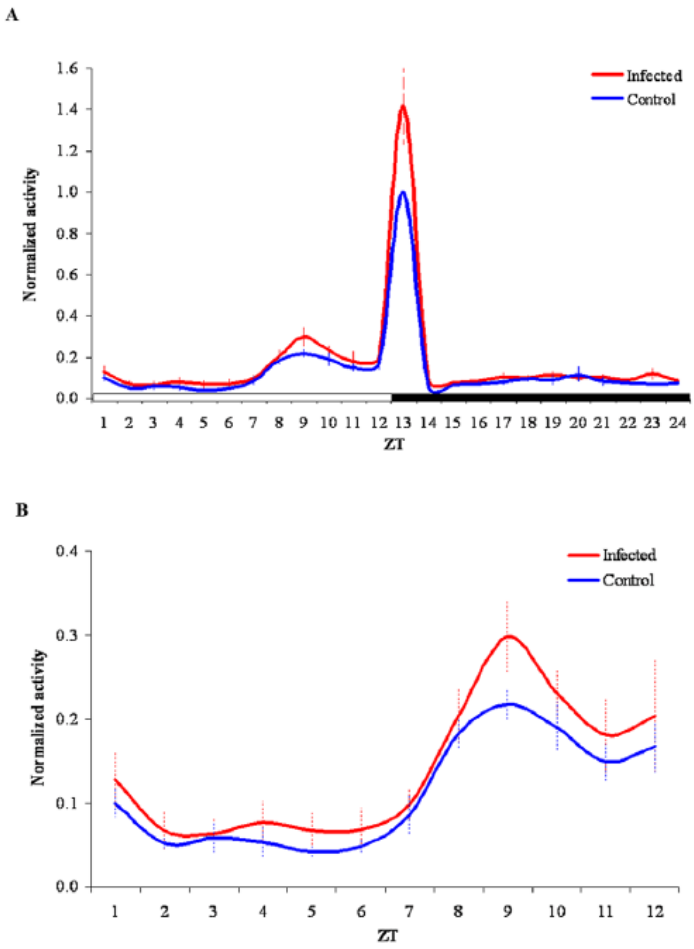

Figure 1. Locomotor activity of control (blue line) and infected (red line) *Ae. aegypti* females under LD 12:12. Lines represent the hourly mean activity (+/− SEM) of control and infected females in the four experiments, normalized to the peak of activity of each respective control. The grey shadow represents the dark phase. ZT is the Zeitgeber Time. Light turns on at ZT 0 and turns off at ZT 12. Panel (A) shows a full LD cycle while panel (B) shows only the photophase.
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a remarkable tropism for the mosquito nervous tissues. Linthicum et al [22] studied the tropism of DENV-3 in parenterally infected female *Aedes aegypti* mosquitoes using immunocytochemical methods and observed that the nervous tissues were among the first tissues to be infected. In fact, these authors suggested that the nervous system is the primary site of virus amplification in mosquitoes infected using this method [22]. Several years later, Salazar et al [23] corroborated these findings by showing that in mosquitoes orally infected with DENV-2, the nervous tissues are among the first to be infected, presenting detectable levels of viral antigens 5 days after an infective blood meal. Interestingly, these authors also showed that heads and salivary glands were the only tissues where viral antigens continued to accumulate throughout the 21 days observed in their study. All other mosquito infected tissues presented a decrease in the infection rate.

This remarkable tropism of Dengue virus for the insect nervous tissues led us to hypothesize that the infection might have some role in modulating the vector locomotor activity behavior, since it is known that activity rhythms in *Drosophila* and other Diptera are regulated by circadian clock neurons in the brain (reviewed in [24],[25]). In fact, our results show that although the daily activity patterns of DENV-2 infected and uninfected mosquitoes are similar, the total level of activity is clearly increased upon infection. This increase is most evident in the light-on/light-off transition (Fig. 1A), an observation that is particularly interesting considering that the visual system is also highly infected [22],[23]. However, it is important to mention that this effect is also clearly detected in the "natural" activity peak occurring during the last hours of the photophase (Fig. 1B), which is under circadian control [20],[21], indicating that a similar effect is likely to occur in nature.

Other authors have already observed alterations in *Aedes* behavior induced by virus infection. Grimstad et al [26] studied the feeding behavior of *Ae. triseriatus* females infected with La Crosse virus and reported that infected mosquitoes tend to probe more and engorge less than uninfected females. These results are in accordance with those obtained by Platt et al [27], who showed that the time required for feeding by DENV-3 infected mosquitoes was significantly longer than that required by uninfected mosquitoes. In contrast, Putnam and Scott [28] observed that DENV-2 infection did not alter *Ae. aegypti* female blood-feeding duration and efficiency in an uninfected host. An explanation for this difference might be that these authors infected mosquitoes with different Dengue virus (3 and 2, respectively) and that Putnam and Scott [28] fed mosquitoes 14 days after an intrathoracic infection while Platt et al [27] only observed significant differences in mosquitoes fed 5, 8 and 11 days after infection. In our study, we observed locomotor activity differences in DENV-2 infected mosquitoes 2 to 6 days after intrathoracic infection.

A considerable amount of information is currently available on the *Aedes aegypti* immune response to Dengue virus infection [29]–[31]. Several authors have shown an association between circadian rhythms and infection/immunity in insects (e.g. [32]–[34]). For example, Shirasu-Hiza et al [32] showed that *Drosophila* infected with bacterium exhibit disrupted circadian activity rhythms and that clock gene mutants are more susceptible to infection than wild-type flies. Also, Lee and Edery [33] showed that *Drosophila*'s ability to fight infections is under circadian control and that flies are significantly more resistant to bacterium when infected in the middle of the night than during the day.

It has been shown that several genes from *Aedes aegypti* are up or down-regulated upon Dengue virus infection, and in DENV-2 infected mosquitoes at least one orthologue (AAEL012562) of a *Drosophila* gene involved in the control of circadian rhythms, *Clock*, has its expression nearly doubled after infection [29]. We believe this variation in a gene probably central to the control of mosquito circadian rhythms could also contribute to the observed changes in activity behavior and we are currently investigating whether Dengue virus infection alters the circadian expression patterns of other clock genes [21].

We are aware that our study suffers from possible caveats. For example, we see a large variation in behavioral effects of Dengue infection between experiments that we cannot explain at the moment. Nevertheless, our study shows that Dengue infection increases mosquito locomotor activity. Changes in vector behavior caused by infection can have potential epidemiological implications. Our results encourage further studies to assess whether increased locomotor activity could have an impact on virus transmission dynamics and Dengue epidemiology.

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Author Contributions

Conceived and designed the experiments: TC RB MS AP. Performed the experiments: TC RB. Analyzed the data: TC RB PL AP. Contributed reagents/materials/analysis tools: MC RO. Wrote the paper: TC RB PL RO MS AP.

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Potential impact of a presumed increase in the biting activity of dengue-virus-infected *Aedes aegypti* (Diptera: Culicidae) females on virus transmission dynamics

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Recently, we showed that infection with dengue virus increases the locomotor activity of Aedes aegypti *females. We speculate that the observed increased locomotor activity could potentially increase the chances of finding a suitable host and, as a consequence, the relative biting rate of infected mosquitoes. We used a mathematical model to investigate the impact of the increased locomotor activity by assuming that this activity translated into an increased biting rate for infected mosquitoes. The results show that the increased biting rate resulted in dengue outbreaks with greater numbers of primary and secondary infections and with more severe biennial epidemics.*

Key words: *Aedes aegypti* - locomotor activity - dengue transmission

Dengue fever is one of the most important mosquito-borne viral diseases in the world and is endemic in approximately 112 countries. It has been estimated that almost 100 million cases of dengue fever and half a million cases of dengue haemorrhagic fever occur worldwide (Gurugama et al. 2010). The main dengue vector, *Aedes aegypti* (Diptera: Culicidae), is a widely distributed species, occurring in many countries in the world. This mosquito is diurnal, very anthropophilic, endophilic and highly abundant in the urban districts and slums of many countries (Braks et al. 2003, Lima-Camara et al. 2006).

The behaviour of insect vectors can be affected by infection with viruses and other parasites (e.g., Grimstad et al. 1980, Rowland & Lindsay 1986) and by symbionts (e.g., Evans et al. 2009, Moreira et al. 2009). There is evidence that the dengue virus can alter the behaviour of *Ae. aegypti*. For example, dengue-infected mosquitoes have been shown to take longer to feed than uninfected mosquitoes (Platt et al. 1997).

Recently, we reported that *Ae. aegypti* females that were experimentally infected with dengue virus showed an increase of up to ~50% in their locomotor activity compared to uninfected controls (Lima-Camara et al. 2011). Although it is yet unknown whether this behavioural change has any effect on the biting activity in the wild, we speculate that the higher levels of locomotor activity observed in dengue-virus-infected *Ae. aegypti* females could potentially increase their chance of finding a suitable host and, as a consequence, their relative biting rate.

In the current study, we used a simplified version of a previously developed model (Luz et al. 2009, 2011) to examine the potential impact of this increased activity level of dengue-virus-infected mosquitoes on dengue transmission dynamics. A complete description of this model is beyond the scope of this short communication and can be found in the above-mentioned references. Briefly, the model describes the vector-human transmission cycle of dengue by incorporating both mosquito and human dynamics (Fig. 1). The model's system of differential equations and the parameters are detailed in the Supplementary data.

For the mosquito population dynamics, six compartments are defined. The first three mosquito life stages, egg, larva and pupa are denoted by E, L and P, respectively. The adult life stage is composed of two compartments: young mosquitoes (Y) (1-5 days of age, which do not yet lay eggs) and old mosquitoes (> 5 days of age, which lay eggs). To simulate dengue transmission, the old adult life stage is further subdivided into uninfected adults (O) and dengue-virus-infected adults (O_I).

The transition rates between life stages are given by t_{ij} (where i is the current life stage and j is the next life stage). Seasonality is built into the transition rates between model compartments. Weather data from Rio de Janeiro were used to parameterise the temporal pattern of the transition rates, which were mathematically defined as sinusoidal functions (Luz et al. 2009). The transition rate from egg to larva (t_{EL}) follows the pattern for precipitation because eggs hatch in response to water. The transition rates from larva to pupa (t_{LP}) and pupa to young adult (t_{Pay}) are functions of temperature because

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temperature accelerates *Ae. aegypti*'s development rate (Scott et al. 2000b). The transition rate from young adult to old adult (t_{YO}) is assumed to be constant.

The death rate of each mosquito compartment (d_i) (where $i = E, L, P, Y, O, O_f$) is the background mortality from natural sources. Field studies have shown that the larval development of *Ae. aegypti* is density dependent (Southwood et al. 1972, Barrera et al. 2006) and this dependence is built into the function of the background death rate of the larvae (d_L). Recently, cohort studies of mosquito populations showed that adult death is age dependent (Styer et al. 2007a). Thus, for the adult life stage, the death rates vary by age group. The rate of production

tibility of humans to secondary infection (σ_2). Thus, $\lambda_2 = [c\sigma_2(\beta_M O_f)]/H$. The majority of humans with I_2 recover at rate γ into a resistant compartment (R_2). Those presenting with dengue haemorrhagic fever die with probability v_2 (which is greater than v_1 because secondary infections are more severe) (Gubler 1998, Guzman & Kouri 2002). Similar to previous analyses (Bartley et al. 2002), we modelled humans as only being susceptible to two dengue infections. Despite this simplification, we incorporated the force of infection for all four serotypes by defining full susceptibility to primary infection and 75% susceptibility to secondary infection resulting from immunity to the primary serotype.

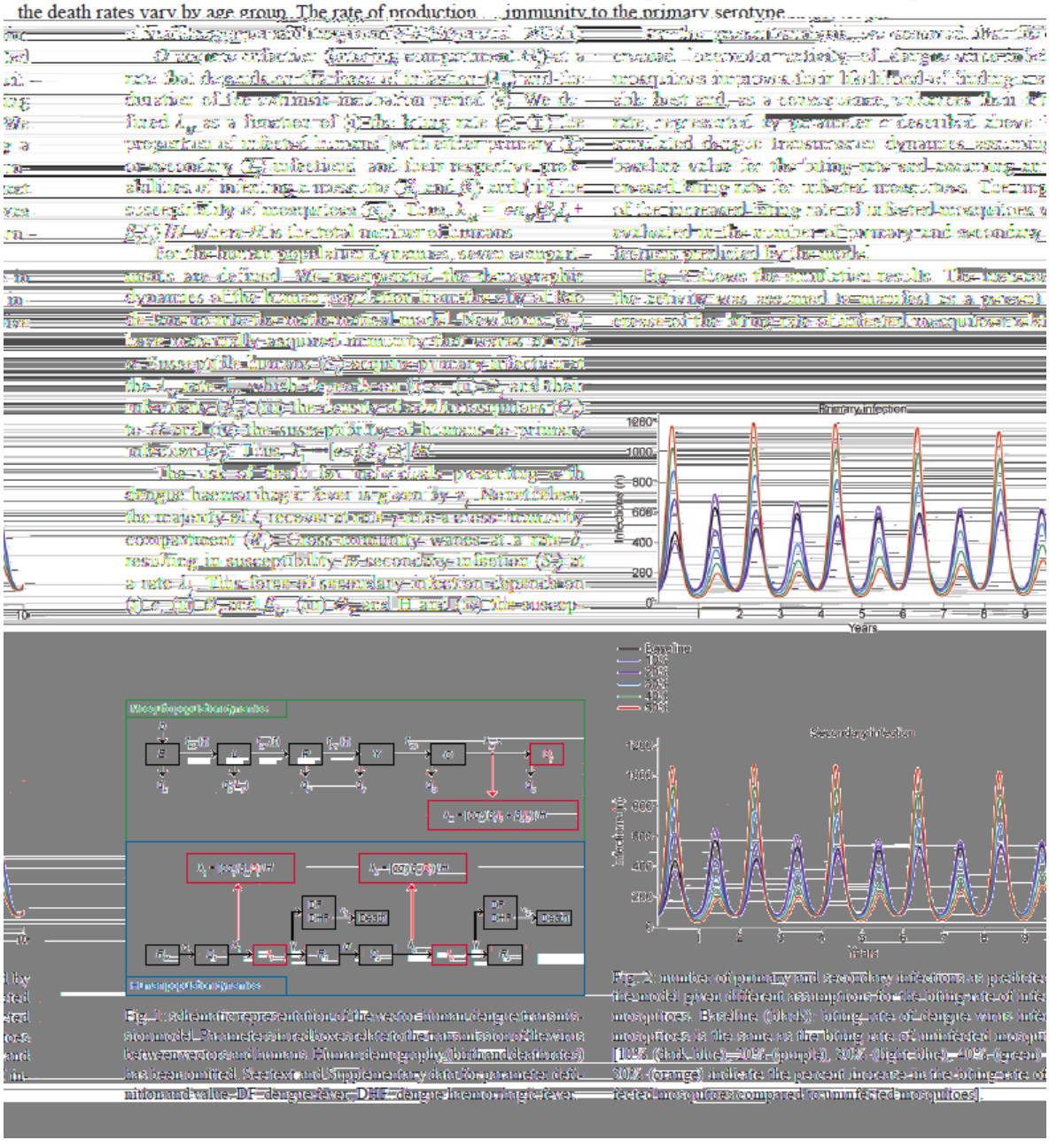

Fig. 1. Schematic representation of the vector-human dengue transmission model. Parameters in red boxes relate to the transmission of the virus between vectors and humans. Human demography (birth and death rates) has been omitted. See text and Supplementary data for parameter definition and value. DF = dengue fever, DHF = dengue haemorrhagic fever.

Fig. 2. number of primary and secondary infections as predicted the model given different assumptions for the biting rate of infe mosquitoes. Baseline (black): biting rate of dengue virus infe mosquitoes is the same as the biting rate of uninfected mosqui [10% (dark blue), 20% (purple), 30% (light blue), 40% (green) 50% (orange) indicate the percent increase in the biting rate of fected mosquitoes compared to uninfected mosquitoes].

to uninfected mosquitoes. Based on our previous experimental findings (Lima-Camara et al. 2011), we explored different degrees of the relative increase in the biting rate of dengue-infected mosquitoes ranging from no increase to a 50% increase in increments of 10%. When no difference was assumed between the biting rates of uninfected and infected mosquitoes (baseline assumption, black), the model predicted annual dengue epidemics of a relatively constant size (i.e., with a relatively constant number of primary and secondary infections per year for 10 years). Overall, when assuming that the biting rate was increased, an increase in the number of infections was observed. For example, when assuming a 50% increase in the biting rate of dengue-infected mosquitoes, the percent increases in the number of primary and secondary infections were 3.8% and 6.5%, respectively, for the 10-year period shown in Fig. 2. In addition, the impact of the increased biting rate of infected mosquitoes on the transmission dynamics of dengue was profound, causing more severe biennial epidemics (colours in Fig. 2).

Transmission dynamics models aim to represent, using mathematical equations, the essential components of biological processes and how these components relate to each other. The simplest model, the SIR model, represents the dynamics of a directly transmitted disease by dividing up the host population on the basis of whether they are susceptible, infectious or recovered/immune. The most important parameter of the SIR model is the rate at which susceptible hosts become infected, usually called the force of infection or the instantaneous incidence rate of infection. For vector-borne diseases such as dengue, this rate depends on parameters such as vector density, vector survival, the extrinsic incubation period of the virus and the biting rate (Nishiura 2006). In our model, these parameters are incorporated into the forces of infection acting on humans and mosquitoes. Uninfected mosquitoes acquire the infection at a rate given by the force of infection (λ_M) (Fig. 1). λ_M is a function of the biting rate (c) which is the average number of bites per mosquito per day. Similarly, acting on susceptible humans are the forces of primary and secondary infection (λ_1 and λ_2) which also depend on the biting rate of the mosquitoes (Fig. 1). In fact, the biting rate is the only parameter entering the formula of all three forces of infection and is thus expected to greatly impact the transmission dynamics.

In our analysis, we found that the increased biting rate modified the dengue transmission dynamics simulated by the model. The absolute number of infections was greater, and most strikingly, the annual pattern of epidemics changed, with more severe biennial epidemics being observed. This pattern of biennial epidemics more closely resembles the observed multi-annual cycle of dengue epidemics in endemic countries, including the pattern observed in Brazil (Nogueira et al. 2007, Nogueira & Eppinghaus 2011). In agreement, other studies have shown the importance of the parameters related to the vector-host interaction, including the biting rate, in determining the seasonal and multi-annual cycle of dengue (Hay et al. 2000, Bartley et al. 2002). A more severe biennial epidemic implies that the virus, during the epidemic, is circulating among a greater proportion of the population, thus causing the number of secondary infections to increase. Indeed, the relative increase in the number of secondary infections was always greater than the relative increase in the number of primary infections. This finding is of clinical relevance because secondary infections have a greater likelihood of resulting in dengue haemorrhagic fever (Guzman & Kouri 2002).

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