**INSTITUTO OSWALDO CRUZ**

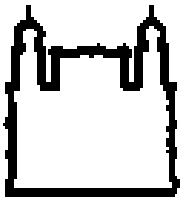
**DOUTORADO EM MEDICINA TROPICAL**

# Aspectos epidemiológicos, virológicos e patológicos de casos fatais suspeitos de dengue ocorridos entre 1986 e 2015

**PRISCILA CONRADO GUERRA NUNES**

**Rio de Janeiro**

**2018**



**MINISTÉRIO DA SAÚDE**

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**CURSO DE PÓS-GRADUAÇÃO EM MEDICINA TROPICAL**

Priscila Conrado Guerra Nunes

# Aspectos epidemiológicos, virológicos e patológicos de casos fatais suspeitos de dengue ocorridos entre 1986 e 2015

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

Orientadoras:

Drª. Flávia Barreto dos Santos

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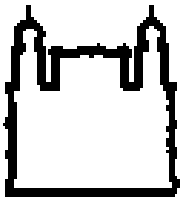

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Profª. Drª. Amanda Torrentes de Carvalho (Suplente)

Prof. Dr. Filipe Anibal Carvalho Costa (Revisor)

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Feliz aquele que transfere o que sabe e aprende
o que ensina (Cora Carolina)

Que os vossos esforços desafiem as
impossibilidades, lembrai-vos de que as grandes
coisas do homem foram conquistadas do que
parecia impossível.
(Charles Chaplin)

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coração; Quando te chamei, tu me respondeste;
deste-me força e coragem. (Salmos 138:1,3)

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# ASPECTOS EPIDEMIOLÓGICOS, VIROLÓGICOS E PATOLÓGICOS DE CASOS FATAIS SUSPEITOS DE DENGUE OCORRIDOS ENTRE 1986 E 2015

## RESUMO

Nos últimos 32 anos, extensas epidemias de dengue, com emergência e reemergência dos diferentes sorotipos ocorreram no Brasil. A infecção possui um amplo espectro, que varia desde formas assintomáticas até quadros graves que podem resultar no óbito. Nesta tese, visamos analisar casos fatais de dengue ocorridos no Brasil em 30 anos (1986-2015), realizando estudos que englobaram três aspectos importantes para o entendimento deste desfecho: epidemiológico, virológico e patológico. Inicialmente, realizou-se uma revisão sobre os óbitos por dengue ocorridos no Brasil no período, utilizando os dados secundários epidemiológicos obtidos no Sistema de Informação de Agravos de Notificação (SINAN) e o Sistema de Informações sobre Mortalidade (SIM), ambos mantidos pelo Ministério da Saúde. Os casos foram analisados por região, variáveis demográficas, classificação clínica, com base nos dados disponíveis. A análise de 30 anos revelou que a região Sudeste registrou 43% dos óbitos por dengue no país, seguidos pela região Centro-Oeste. Após o ano de 2000, todos os estados reportaram óbitos, com exceção de Santa Catarina e Rio Grande do Sul. Entre 2011 e 2015, Goiás tornou-se o estado com maior taxa de mortalidade de todo o país e o Rio Grande do Sul relatou os primeiros óbitos por dengue. Na casuística, uma distribuição homogênea entre o sexo foi observada e casos fatais por dengue foram mais frequentes em indivíduos acima de 15 anos no período entre 1986 e 2006. No entanto, este cenário mudou em 2007-2008, com taxas de mortalidade mais elevadas em crianças até 14 anos. Um estudo posterior, baseado na vigilância laboratorial realizada pelo Laboratório de Flavivírus (LabFla/FIOCRUZ), Laboratório de Referência Regional no mesmo período corroborou muitas das observações do reportado para o país, principalmente em relação às regiões e faixas etárias mais acometidas. Dos óbitos suspeitos investigados na casuística do LabFla/FIOCRUZ, 34,2% (359/1047) foram confirmados e DENV-1 (11,1%), DENV-2 (43,9%), DENV-3 (32,8%) e DENV-4 (13,7%) foram detectados. Em geral, uma maior frequência dos óbitos foi associada às infecções primárias (59,3%). No entanto, em 2008, os casos fatais foram principalmente associados às infecções secundárias. Além disso, crianças infectadas pelo DENV-2 apresentaram maiores chances de evoluir ao óbito. Considerando os componentes virais, foi demonstrado que casos fatais por dengue apresentaram uma maior antigenemia de NS1 e viremia do que casos não fatais, e estes níveis variaram de acordo com o sorotipo infectante. Independentemente do desfecho, os casos por DENV-1 apresentaram níveis mais elevados de NS1. Análises histopatológicas foram realizadas em placenta, cordão umbilical e órgãos fetais de casos de óbitos materno-fetal, causadas pelo DENV-2 e DENV-4. Foram observados diferentes tipos de anormalidades teciduais que incluíram inflamação, hemorragia, edema, necrose na placenta, bem como desorganização tecidual no feto, como parênquima esponjoso cerebral, inflamação microglial, esteatose, hialinose arteriolar, células inflamatórias nos septos alveolares e desorganização do folículo linfoide esplênico. O aumento de macrófagos, células de Hofbauer e linfócitos TCD8+, bem como a regulação de mediadores inflamatórios como IFN-γ, TNF-α, RANTES/CCL5, MCP1/CCL2 e VEGF/R2 foram detectados no fígado, pulmão, baço, cérebro e placenta, indicando a inflamação dos tecidos periféricos da placenta e do feto. A detecção das proteínas virais NS1 e NS3 em macrófagos e endotélio sugere replicação viral. Embora os componentes virais podem ser fatores que influenciam o desfecho da doença, o estado imunológico do hospedeiro, comorbidades e a qualidade do suporte médico, no entanto, não podem ser descartados como interferentes no desfecho da doença.

**Palavras-Chaves:** Dengue, Casos Fatais, Epidemiologia, Antigenemia de NS1, Viremia, Histopatologia, Mediadores inflamatórios.

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EPIDEMIOLOGICAL, VIROLOGICAL AND PATHOLOGICAL ASPECTS OF SUSPECTED DENGUE FATAL CASES, 1986-2015

## ABSTRACT

In the last 32 years, extensive epidemics of dengue, with emergence and re-emergence of different serotypes have occurred in Brazil. The infection has a broad spectrum, ranging from asymptomatic to severe conditions that can result in death. In this thesis, we aimed to analyze fatal cases of dengue that occurred in Brazil in 30 years (1986-2015), carrying out studies that encompassed three important aspects to understand this outcome: epidemiological, virological and pathological. Initially, a review was carried out on dengue deaths occurring in Brazil in the period, using the secondary epidemiological data obtained in the SINAN and the Mortality Information System (SIM), both maintained by the Ministry of Health. The cases were analyzed by region, demographic variables, clinical classification, based on available data. The analysis of 30 years revealed that the Southeast region reported 43% of dengue deaths in the country, followed by the Midwest region. After 2000, all states reported deaths, with the exception of Santa Catarina and Rio Grande do Sul. Between 2011 and 2015, Goiás became the state with the highest mortality rate in the country, and Rio Grande do Sul reported the first dengue deaths. In this analysis, a homogeneous distribution between sex was observed, and fatal cases due to dengue were more frequent in individuals over 15 years of age in the period between 1986 and 2006. However, this scenario changed in 2007-2008, with higher mortality rates in children up to 14 years. A subsequent study, based on laboratory surveillance performed by the Laboratory of Flavivirus (LabFla / FIOCRUZ), Regional Reference Laboratory, in the same period, corroborated many of the observations reported to the country, especially in relation to the region and most affected age groups. From the fatal cases investigated in the LabFla/FIOCRUZ, 34.2% (359/1,047) were confirmed and, DENV-1 (11.1%), DENV-2 (43.9%), DENV-3 (32.8%) and DENV-4 (13.7%) were detected. In general, a higher frequency of deaths was associated with primary infection (59.3%). However, in 2008, fatal cases were primarily associated with secondary infections. In addition, children infected with DENV-2 had a higher chance of evolving to death. Considering the viral components, it was demonstrated that dengue fatal cases dengue showed a higher NS1 antigenemia and viremia than non-fatal ones, and these levels varied according to the infecting serotype. Regardless of the outcome, cases by DENV-1 showed higher levels of NS1. Histopathological analyzes were performed on placenta, umbilical cord and fetal organs of cases of maternal-fetal deaths, caused by DENV-2 and DENV-4. Different types of tissue abnormalities, including inflammation, hemorrhage, edema, placental necrosis, as well as tissue disorganization in the fetus, such as spongy parenchyma, microglial inflammation, steatosis, arteriolar hyalinosis, inflammatory cells in the alveolar septa and disorganization of the splenic lymphoid follicle. Increased macrophages, Hofbauer cells and CD8 + T lymphocytes, as well as the regulation of inflammatory mediators such as IFN-γ, TNF-α, RANTES / CCL5, MCP1 / CCL2 and VEGF / R2 were detected in the liver, lung, spleen, brain and placenta, indicating inflammation of the peripheral tissues of the placenta and the fetus. Detection of viral NS1 and NS3 proteins in macrophages and endothelium suggest viral replication. Viral components may be factors that influence the outcome of the disease. However, the host's immune status, comorbidities and the quality of medical support can not be ruled out as interfering with the outcome of the disease.

**Key Words**: Dengue, Fatal Cases, Epidemiology, NS1 Antigenemia, Viremia, Histopathology, Inflammatory mediators

**SUMÁRIO**

**LISTA DE FIGURAS:**

**SIGLAS E ABREVIATURAS**

| | |
|---|---|
| °C | Graus Celsius |
| ADE | facilitação dependente de anticorpos (do inglês “, *antibody dependent enhancement*”) |
| *Ae aegypti* | *Aedes aegypti* |
| *Ae. albopictus* | *Aedes albopictus* |
| Anvisa | Agência Nacional de Vigilância Sanitária |
| C | Proteína estrutural do capsídeo ou core viral |
| CCL2/MCP-1 | Proteína quimiotática de monócitos |
| D | Domínio |
| d.C. | depois de Cristo |
| DC | Dengue Clássico |
| DCC | Dengue com complicações |
| DC-SIGN | Molécula de adesão de células dendríticas |
| DENCO | Controle do Dengue (do inglês “*Dengue Control”)* |
| DENV | Vírus dengue |
| DENV-1 | Vírus dengue sorotipo 1 |
| DENV-2 | Vírus dengue sorotipo 2 |
| DENV-3 | Vírus ddengue sorotipo 3 |
| DENV-4 | Vírus dengue sorotipo 4 |
| DF | Febre do Dengue (do inglês *“Dengue fever”*) |
| DR | Receptor de Dopamina |
| DSCA | Dengue com sinais de alerta |
| DSSA | Dengue sem sinais de alerta |
| E | Proteína do Envelope |
| ECP | Efeito Citopático |
| ELISA | Ensaio imunoenzimático |
| EUA | Estados Unidos da América |
| FFPE | Tecido fixado em formalina e embebida em parafina |
| FHD | Febre Hemorrágica do Dengue |
| FIOCRUZ | Fundação Oswaldo Cruz |

| | |
|---|---|
| HCV | Vírus da hepatite C |
| HI | Inibição da Hemaglutinação |
| HIV | Vírus da imunodeficiência adquirida |
| ICAM-1 | Moléculas de adesão intercelular-1 |
| IFI | Imunofluorescência Indireta |
| IgG | Imunoglobulina G |
| IgM | Imunoglobulina M |
| IHQ | Imuno-histoquímica |
| IL | Interleucina |
| IOC | Instituto Oswaldo Cruz |
| JEV | Vírus da encefalite japonesa |
| kb | kilobases |
| kDa | Kilodaltons |
| LABFLA | Laboratório de Flavivírus |
| LPS | Lipopolissacarídeo |
| MIF | Fator inibitório da migração de macrófagos |
| mNS1 | NS1 associada membrana |
| MR | Receptor de manose |
| MS | Ministério da Saúde |
| NC | Não codificante |
| NS | Proteínas não estruturais |
| OMS | Organização Mundial de Saúde |
| ORF | Fita de leitura aberta (do inglês *Open Reading Frame*) |
| PAHO | Organização Pan-americana de Saúde |
| PrM/M | Proteínas estruturais Pré-membrana/Membrana |
| PRNT | Teste de neutralização por redução de placas |
| qRT-PCR | Transcrição reversa seguida da reação em cadeia pela polimerase em tempo real |
| RdRp | RNA polimerase RNA-dependente |
| RNA | Ácido ribonucléico |
| RT-PCR | Transcrição reversa seguida da reação em cadeia pela polimerase |
| SCD | Síndrome de choque por dengue |
| SES | Secretaria de Estado da Sáude |

| | |
|---|---|
| sNS1 | NS1 secretada |
| SVS | Secretaria de Vigilância em Saúde |
| VCAM-1 | Moléculas de adesão vascular-1 |
| VEGF | Fator de crescimento endotelial vascular |
| WNV | Vírus do Nilo Ocidental (do inglês *West Nile Virus*) |
| YFV | Vírus da febre amarela (do inglês *Yellow Fever Virus*) |
| ZIKV | Vírus da Zika (do inglês *Zika Virus*) |

# 1 INTRODUÇÃO

## 1.1 HISTÓRIA NATURAL

Apesar da dengue ter sido clinicamente caracterizada no século XVIII, relatos de uma doença semelhante foram descritos em uma enciclopédia médica chinesa na Dinastia Chin (265-420 dC), formalmente editada nas dinastias Tang (610 dC) e Norte-Sung (992 dC) (NOBUCHI, 1979).

As primeiras epidemias de uma doença clinicamente compatível com a dengue, ocorreram em 1779 na Ilha de Java, na Indonésia (REZENDE, 2004) e em 1780 na Filadélfia, nos Estados Unidos da América (REZENDE, 2004). Assim, a doença teve uma ampla distribuição geográfica antes do século XVIII (GUBLER et al., 2014).

Muito se discute sobre a origem e a evolução do vírus da dengue (DENV). A migração de pessoas e o comércio, levaram os vírus para as cidades da Ásia tropical, onde foram provavelmente transmitidos por mosquito *Aedes albopictus* (*Ae. albopictus)*. Por outro lado, o tráfico de escravos, entre a África Ocidental e as Américas, foi responsável pela introdução e distribuição geográfica do mosquito africano *Aedes aegypti* (*Ae. aegypti)* no Novo Mundo, durante os séculos XVII, XVIII e XIX. Esta espécie tornou-se altamente adaptada aos seres humanos e ambientes urbanos, dispersando pelos trópicos do mundo por veleiros. Foi neste cenário que grandes epidemias de dengue foram reportadas, à medida que a indústria marítima se desenvolvia e as cidades portuárias eram urbanizadas (GUBLER, 2006; GUBLER et al., 2014).

A origem do nome dengue é incerta e controversa e a doença teve muitos nomes ao longo do tempo. O primeiro uso do nome "dengue" ocorreu durante a epidemia de 1828 em Cuba (SOLER; PASCUAL; PETINTO, 1949), no entanto, a origem mais provável, provém da palavra *Ki-Dinga pepo*, do dialeto Swahili (CHRISTIE, 1972, 1991) que significava: "uma doença causada por um espírito mal", reportada em epidemias ocorridas em Zanzibar e na costa da África Oriental em 1823 e 1870. Na epidemia de Cuba (1828), a doença era conhecida como Dunga, sendo mais tarde chamada de dengue (MUÑOZ, 1828). A doença também foi conhecida como "Veneno da Água", por estar associada com o vôo de mosquitos próximo à água (GUBLER, 1997; GUZMAN et al., 2010b).

Em 1906, Bancroft sugere que a transmissão da doença é por mosquitos *Ae. aegypti*, observações comprovadas em 1918. Simmons (1931) descreveu que *Ae. albopictus* seria outro possível vetor da doença (GUBLER, 1997; GUBLER et al., 2014).

*Ae. aegypti* e *Ae. albopictus*, são provenientes da família *Culicidae*, subfamília *Culicinae*, subgênero Stegomyia, são os vetores mais importantes na transmissão do DENV entre humanos. *Ae. aegypti* é freqüentemente encontrado em ambientes urbanos. No entanto, *Ae. albopictus* adapta-se facilmente ao ambiente rural, urbano e peri-urbano, presumindo-se que este pode servir como ponte entre os ciclos urbano e silvestre (VALLE; PIMENTA; CUNHA, 2015).

No ciclo de transmissão viral, o artrópode é infectado ao ingerir sangue do hospedeiro virêmico. O período de incubação extrínseco compreende o tempo entre a ingestão do sangue infectado, pelo mosquito susceptível, e a presença de partículas virais infecciosas na secreção salivar. Esse perído dura de 7 á 14 dias. Após esse período, o mosquito torna-se capaz de transmitir o vírus para um novo hospedeiro, permanecendo infectado durante toda a sua vida e transmitindo a infecção, de forma vertical, para sua prole (HARDY et al., 1983; SALAZAR et al., 2007; TABACHNICK, 2013).

Na segunda metade do século XX, a transmissão do DENV acompanhou a dispersão do seu principal vetor, *Ae. Aegypti* (MOUSSON et al., 2005), e foi provavelmente acelerada pela urbanização e globalização (GUBLER, 2011; WEAVER, 2013). O colapso da campanha de erradicação *Ae. aegypti* nas Américas, nos anos 70, também foi importante para marcar o início da disseminação da infecção da Ásia para as Américas, seguida pela rápida re-introdução do vetor em ambos os continentes (GUBLER; CLARK, 1995).

Diferentes tipos de agentes causadores da doença foram sugeridos, porém Ashburn e Craig sugeriram a etiologia viral em um paciente doente em 1907, quando observaram que o agente era filtrável e de tamanho ultramicroscópico (ASHBURN; CRAIG, 2004). Porém, somente em 1943 o vírus foi isolado, durante a Segunda Guerra Mundial. Os japoneses e os militares americanos estabeleceram comissões para estudar a doença e ambos os grupos foram bem-sucedidos em isolar o vírus. Hotta e Kimura foram os primeiros a isolar o vírus, por inoculação do soro de paciente em cérebro de camundongo (HOTTA, 1952; KIMURA; HOTTA, 1944). Esta cepa foi, posteriormente, identificada como vírus dengue tipo 1 (DENV-1, cepa Mochizuki). Em 1944, Sabin e colaboradores isolaram os sorotipos 1 e 2 de soldados americanos, no Havaí e na Nova Guiné (SABIN, 1952; SABIN; SCHLESINGER, 1945). A cepa viral do Havaí foi designada de DENV-1 (protótipo Haw-DEN-1) e os isolados de Nova Guiné, como vírus dengue tipo 2 DENV-2 (protótipo NG C-DEN-2), por serem

antigenicamente distintas (GUBLER et al., 2014). Os sorotipos 3 (DENV-3, cepa H87) e 4 (DENV-4, cepa H241), foram isolados durante uma epidemia nas Filipinas, em 1956, por Hammon e colaboradores (HAMMON; RUDNICK; SATHER, 1960).

## 1.2 CLASSIFICAÇÃO, MORFOLOGIA E CARACTERIZAÇÃO GENÔMICA DOS VÍRUS DENGUE

O último relatório do Comitê Internacional sobre Taxonomia de Vírus (ICTV) reportou que a família *Flaviviridae* é dividida em quatro gêneros: *Flavivirus, Hepacivirus, Pestivirus e Pegivirus* (SIMMONDS et al., 2017). *Flavivirus* é o principal gênero, contendo 53 espécies, incluindo 34 transmitidas por mosquitos e cerca de 13 transmitidas por carrapatos (ICTV, 2018).

Aproximadamente 40 espécies desse gênero estão associadas a doenças em humanos (MOUREAU et al., 2015), tais como DENV, vírus do Nilo Ocidental (WNV), vírus da encefalite japonesa (JEV), vírus da febre amarela (YFV) e o vírus da Zika (ZIKV) (CHEN et al., 2018).

Os DENV são classificados como arbovírus, ou seja, vírus mantidos na natureza através de um ciclo de transmissão envolvendo hopesdeiros vertebrados, como primatas não humanos e humanos, e artrópodes hematófagos (GUBLER, 2002; ROUNDY et al., 2017). Possuem quatro sorotipos relacionados, mas antigenicamente distintos (DENV-1 a -4) (CALISHER et al., 1989) e todos podem causar doença grave e fatal em humanos (GUBLER, 2006).

A partícula viral é esférica e tem aproximadamente 50 nanômetros (nm) de diâmetro. O genoma é constituído por uma única fita de RNA, de polaridade positiva, com aproximadamente 11 kilobases (kb), uma única fase de leitura aberta (ORF, do inglês *open reading frame*) e que é flanqueado por duas regiões não codificantes (5'e 3'NC) de 96-450 nucleotídeos, respectivamente. O RNA viral funciona como RNA mensageiro e é traduzido, a partir da maquinaria da célula hospedeira, em uma poliproteína única que é, posteriormente, clivada em três proteínas estruturais (capsídeo [C], pré-membrana [prM] e envelope [E]) e sete não-estruturais (NS1, NS2A, NS2B, NS3 , NS4A , NS4B e NS5) (KUHN et al., 2002; VASILAKIS et al., 2011), como observados na Figura 1. As proteínas estruturais formam a

partícula viral, enquanto as não estruturais possuem um importante papel na replicação viral, montagem e modulação da resposta imune inata (ZEIDLER et al., 2017).

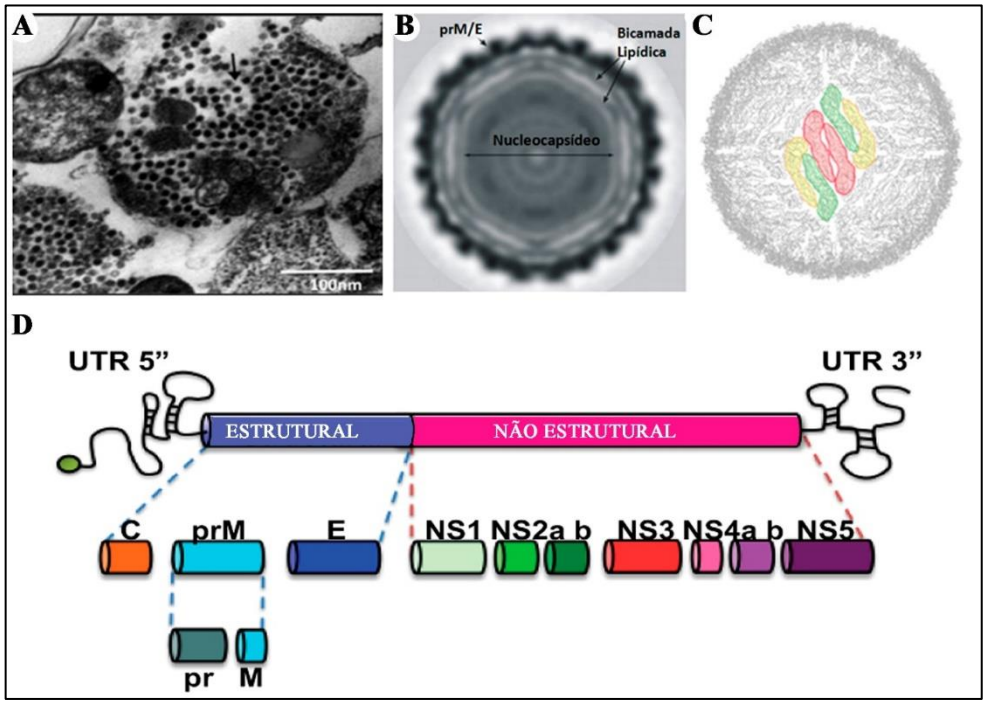


Figura 1: (A) Micrografia eletrônica partículas virais (seta) no interior de células de mosquito Ae. albopictus (C6/36) infectada. (B e C) Micrografia eletrônica e tridimensional dos DENV. (D) Diagrama esquemático da poliproteína dos DENV e as proteínas após clivagem (ANGEL; VALLE, 2013; BARRETO-VIEIRA; BARTH-SCHATZMAYR; SCHATZMAYR, 2010; YU et al., 2009).

A proteína E possui 53 kDa e está ancorada no envelope viral através de duas hélices transmembranares antiparalelas localizadas na extremidade carboxi-terminal da proteína e possui resíduos de asparagina glicosilada (N67 e N153) que estão envolvidos na ligação com a superfície celular (JOHNSON; GUIRAKHOO; ROEHRIG, 1994; POKIDYSHEVA et al., 2006). Possui três domínios (D) distintos: I, II e III (MODIS et al., 2005; ZHANG et al., 2003a, 2003b). DI é uma estrutura central, enquanto DII é o segmento diretamente envolvido na fusão de membranas virais e celulares durante a entrada do vírus. DIII está exposto na superfície do DENV e contém epitopos de ligação à células (MONDOTTE et al., 2007; ZHANG et al., 2004). É o principal alvo para os anticorpos neutralizantes, sendo de grande importância na virulência (CLYDE; KYLE; HARRIS, 2006; YACOUB; MONGKOLSAPAYA; SCREATON, 2016).

A proteína M é sintetizada como uma proteína precursora (prM, aproximadamente 21 kDa) e está presente na partícula viral imatura. A maturação do vírus ocorre na rede transGolgi, onde uma protease celular, provavelmente furina, cliva o prM, gerando a proteína M (8 kDa) e o peptídeo "pr" (ZHANG et al., 2003b). A prM interage e estabiliza o DII da proteína E, impedindo mudanças conformacionais que poderiam ativar sua atividade fusogênica durante a via secretora da rede transGolgi (LI et al., 2008; YU et al., 2008; ZHANG et al., 2003b; ZHENG; UMASHANKAR; KIELIAN, 2010).

A proteína C é composta por 100 resíduos de aminoácidos, é alcalina e atua na penetração celular (FREIRE et al., 2013). O segmento contendo os primeiros 20 resíduos de aminoácidos interagem com gotículas lipídicas celulares (MARTINS et al., 2012), organelas às quais a proteína C se associa durante o ciclo replicativo (SAMSA et al., 2009).

O processo de replicação do genoma viral dentro da célula hospedeira é impulsionado, principalmente, pelas proteínas não estruturais (NS).

A NS1 ancora o complexo de replicação à membrana do retículo endoplasmático e interage fisicamente com NS4B (MULLER; YOUNG, 2013).

A NS2A, uma proteína de 22kDa, é responsável pela síntese do RNA viral e montagem do virion (XIE et al., 2015) e, possivelmente, antagonista de interferon (JONES et al., 2005).

A NS3 é uma proteína de 70 kDa multifuncional e atua como serina protease no domínio N-terminal (LI et al., 1999), helicase no domínio C-terminal e possui atividade nucleosídeo-trifosfatase estimulada por RNA (NTPase) e RNA 5'-trifosfatase (RTPase) (CUI et al., 1998; XU et al., 2005). Está envolvida no processamento da poliproteína viral, bem como na replicação do RNA (GORBALENYA et al., 1989; LI et al., 1999) e sua atividade é dependente da NS2B (FALGOUT et al., 1991). NS4A e NS4B, são proteínas pequenas, de 16 e 27 kDa, respectivamente.

A NS4A induz o rearranjo da membrana dentro da célula hospedeira, auxiliando assim na formação de vesículas de replicação (MILLER et al., 2007). Sugere-se que NS4B funcione como um inibidor de sinalização de interferon (JONES et al., 2005; MUÑOZ-JORDAN et al., 2003).

A NS5 é uma proteína multifuncional com um domínio metiltransferase (superfamília MTase) e um domínio RNA polimerase dependente de RNA, é altamente conservada entre os *Flavivirus*(ACOSTA; KUMAR; BARTENSCHLAGER, 2014; LINDENBACH; RICE, 1999).

### 1.2.1 PROTEÍNA NS1

A proteína NS1 compartilha um elevado grau de homologia entre *Flavivirus*, com 1.056 nucleotídeos que codificam um polipeptídeo de 352 aminoácidos (MACKOW et al., 1987; MANDL et al., 1989) e, dentre os sorotipos dos DENV, sua similaridade é superior a 70% (CHAMBERS; MCCOURT; RICE, 1990).

É uma glicoproteína de 43-48 kDa, expressa em células de mamíferos como monômeros solúveis, que dimerizam no lúmen do retículo endoplasmático (FLAMAND et al., 1999; YOUNG et al., 2000; ZHOU et al., 2006). Subsequentemente a NS1 é transportada para a superfície celular, onde permanece associada à membrana (mNS1) ou é secretada (sNS1) para o meio extracelular, na forma hexamérica (FLAMAND et al., 1999; MASON, 1989).

O monômero consiste em três domínios estruturais, um domínio de dimerização de *β-roll*, um domínio em asa e um domínio *β-ladder* (AKEY et al., 2014; SCATURRO et al., 2015), como demostrado na figura 2. Dímeros de NS1 são formados quando dois domínios de β-roll dimerizam no centro, estes dímeros tendem a trimerizar resultando num hexamero (FLAMAND et al., 1999; GUTSCHE et al., 2011; MULLER et al., 2012; SCATURRO et al., 2015; YAP et al., 2017).

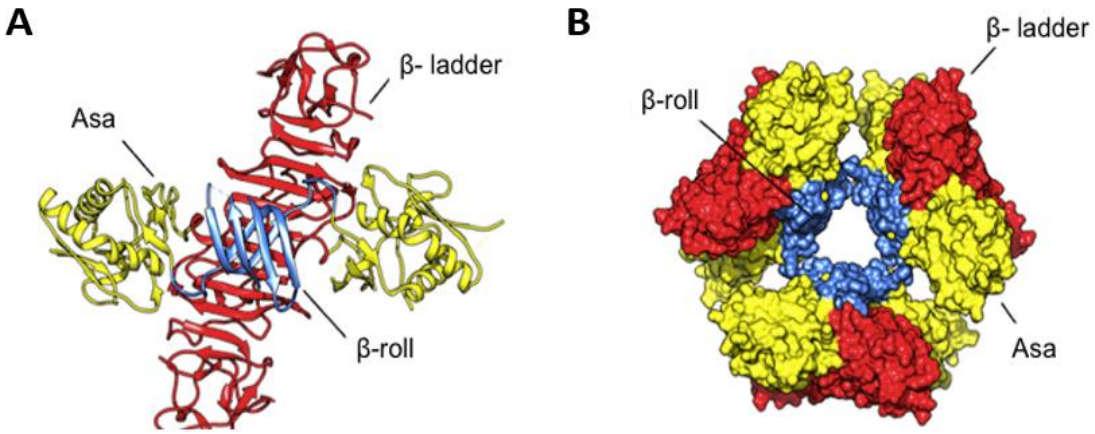


Figura 2: Organização tridimensional do dímero (A) e hexâmero (B) da proteína NS1 do DENV. Os domínios β-roll, em asa e β-ladder estão apresentados em azul, amarelo e vermelho, respectivamente (adaptado de SCATURRO et al., 2015).

A sNS1 hexamérica possui uma forma de barril com um canal aberto central, com três dímeros dispostos simetricamente voltados para o interior, que é hidrofóbico (AKEY et al., 2014). Esta característica permite que o hexâmero NS1 seja secretado como uma lipoproteína (GUTSCHE et al., 2011).

Embora as funções da NS1 ainda não tenham sido completamente elucidadas, evidências experimentais indicam que esta proteína está envolvida na replicação do RNA (LINDENBACH; RICE, 1999; SAMPATH; PADMANABHAN, 2009; SCATURRO et al., 2015). As funções da forma secretada também não são claras, embora o envolvimento na patogênese e em mecanismos de evasão imune foram propostos (AKEY et al., 2014; MULLER; YOUNG, 2013; RASTOGI; SHARMA; SINGH, 2016).

Durante a infecção pelo DENV, a sNS1 pode acumular-se em níveis muito elevados, podendo ser detectados até 50µL/mL em soros de alguns pacientes, com consequente produção de anticorpos contra esta proteína (ALCON-LEPODER et al., 2006; CHUNG; DIAMOND, 2008; LIBRATY et al., 2002a; YOUNG et al., 2000). Algumas proteínas virais estão envolvidas na patogênese das infecções pelos DENV e a NS1 tem sido proposta como um marcador de gravidade da doença (ALLONSO et al., 2014; AVIRUTNAN et al., 2006; DE LA CRUZ HERNÁNDEZ et al., 2013; PARANAVITANE et al., 2014; WATTERSON; MODHIRAN; YOUNG, 2016).

A NS1 interage com o sistema complemento (AVIRUTNAN et al., 2006), está envolvida com a produção de citocinas inflamatórias e imunossupressoras pela indução de células imunes (ADIKARI et al., 2016; MODHIRAN et al., 2015), induz a hiperpermeabilidade endothelial *in vitro* (BEATTY et al., 2015) e extravasamento vascular *in vivo* (MALAVIGE; OGG, 2017; PUERTA-GUARDO; GLASNER; HARRIS, 2016).

## 1.3 CICLO REPLICATIVO VIRAL

O reconhecimento do vírus pelas células-alvo depende da interação entre as proteínas da superfície viral e os componentes da membrana plasmática celular. A susceptibilidade dos tecidos do hospedeiro ao vírus é intimamente dependente da abundância e distribuição dos receptores celulares, tornando os receptores alvos valiosos para o desenvolvimento de drogas antivirais (GROVE; MARSH, 2011).

Apesar dos esforços para determinar moléculas de reconhecimento de DENV pelas células-alvo, um receptor específico ainda não foi definitivamente identificado (HIDARI; SUZUKI, 2011). No entanto, diferentes receptores foram descrito, incluindo os glicosaminoglicanos, como sulfato de heparano (CHEN et al., 1997) e lectinas (NAVARRO-SANCHEZ et al., 2003), a molécula de adesão de células dendríticas (DC-SIGN) (TASSANEETRITHEP et al., 2003), o receptor de manose (MR) de macrófagos (MILLER et al., 2008), o lipopolissacarídeo (LPS), o receptor CD14 (CHEN; WANG; KING, 1999), as proteínas induzidas por estresse, como Hsp70 e Hsp90 (REYES-DEL VALLE et al., 2005) e a chaperonina GRP78/BiP (JINDADAMRONGWECH; THEPPARIT; SMITH, 2004). Esta diversidade de receptores resulta em uma ampla disseminação viral e, consequentemente, amplo espectro de manifestações clínicas (CRUZ-OLIVEIRA et al., 2015).

O ciclo replicativo do DENV envolve a adsorção viral, tradução e processamento da poliproteína, replicação do RNA, montagem, maturação e liberação da partícula viral (LINDENBACH et al., 2013), Figura 3.

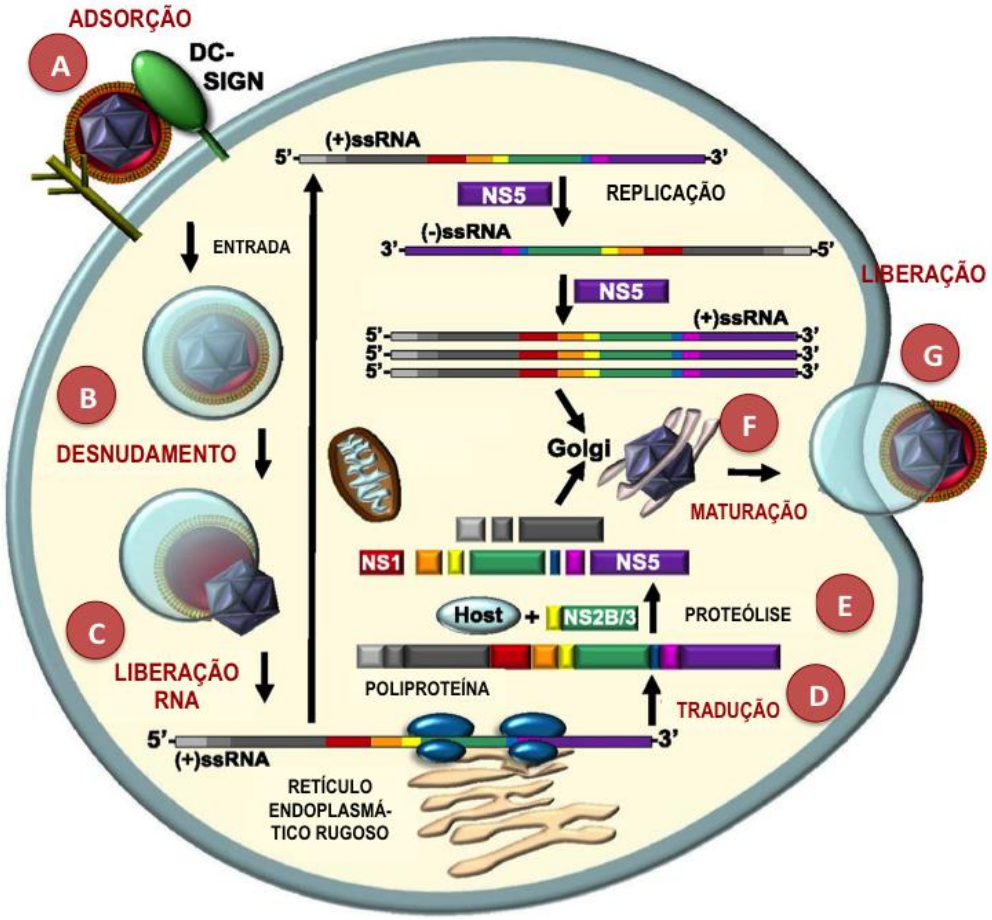

Figura 3: Esquema representativo do ciclo replicativo do DENV. (A) O vírus se liga a receptores na membrana plasmática e entra na célula do hospedeiro por meio da endocitose. (B) Dentro do endossoma, a proteína E sofre uma mudança conformacional para mediar a fusão do envelope viral com a membrana do endossoma, ocorrendo então o desnudamento. (C) O RNA viral é liberado no citoplasma e (D) traduzido em uma poliproteína. (E) A poliproteína é clivada em proteínas individuais nas membranas do retículo endoplasmático por proteases virais e celulares. (F) Partículas virais imaturas são transportadas através da via secretória e, um pH mais baixo na rede trans-Golgi ativa a protease hospedeira, a furina, para produzir partículas maduras, que serão (G) liberadas para o meio extracelular (Adaptado de SIMON; SUTHERLAND; PRYZDIAL, 2015).

A proteína E viral liga-se a receptores específicos encontrados na superfície celular e a interação ligante-receptor resulta na endocitose viral. Dentro do endossomo ácido ocorre a trimerização irreversível da proteína E, expondo o domínio III que, em seguida, media a fusão do envelope viral com a membrana celular. O nucleocapsídeo é desencapsulado permitindo a liberação do RNA genômico no citoplasma, que é traduzido no retículo endoplasmático, dando origem a um poliproteína de aproximadamente 3.400 aminoácidos. Esta poliproteína é clivada co- e pós-traducionalmente por uma combinação de proteases celulares e a protease viral NS2B/NS3, em proteínas estruturais e não estruturais (ACOSTA; KUMAR; BARTENSCHLAGER, 2014; ALLISON et al., 1995; MODIS et al., 2004; SIMON; SUTHERLAND; PRYZDIAL, 2015).

Após a tradução e processamento da poliproteína, um complexo replicativo é montado, composto pela NS5, que é uma RNA polimerase RNA-dependente (RdRp), proteínas não estruturais virais, RNA viral de polaridade positiva e, provavelmente, fatores da célula hospedeira. A replicação começa com a síntese da sequência complementar do genoma (-), que serve então como molde para a produção de novas fitas de RNA (+). O RNA (+) recém-sintetizado pode ser utilizado para iniciar uma nova tradução nos ribossomos ou para a montagem de novos virions, que provavelmente se formam no retículo endoplasmático. Durante a montagem das novas partículas virais, o RNA viral é empacotado pelas proteínas C para formar o nucleocapsídeo. O vírus imaturo é formado quando as proteínas E e prM encontram o nucleocapsídeo. As proteínas prM e E formam heterodímeros e migram para dentro do lúmen do retículo endoplasmático. A partícula imatura migra pela rede trans-Golgi que, em pH 5,8-6,0, provoca a dissociação das proteínas prM/E e clivagem via protease celular (furina), gerando um vírus maduro, que é liberado no ambiente extracelular através da via

secretora (ACOSTA; KUMAR; BARTENSCHLAGER, 2014; SIMON; SUTHERLAND; PRYZDIAL, 2015; STADLER et al., 1997; YU et al., 2008).

## 1.4 DIVERSIDADE GENÉTICA

Durante o cliclo replicativo viral, a falta do mecanismo de correção da RdRp (DRAKE, 1993; STEINHAUER; DOMINGO; HOLLAND, 1992; TAO; YE, 2010), associada às rápidas taxas de replicação, o grande tamanho populacional e a pressão imunológica do hospedeiro podem levar à mutações do vírus, de forma que variantes genéticas são observadas para os quatro sorotipos de DENV (HOLMES; TWIDDY, 2003).

O sequenciamento do genoma viral permitiu a caracterização das diferentes variantes de DENV e o estabelecimento de relações evolutivas entre os quatro sorotipos. O termo "genótipo" foi definido como o agrupamento de DENV com divergência nucleotídica $\geq 6\%$ para uma determinada região do genoma (CHEN; VASILAKIS, 2011; RICO-HESSE, 1990).

Estudos baseados no sequenciamento parcial ou completo do genoma caracterizaram cinco genótipos para o DENV-1 (GI a V), seis genótipos para o DENV-2 (Asiático I, Asiático II, Cosmopolitano, Americano, Sudeste Asiático/Americano e Selvagem), cinco genótipos para o DENV-3 (GI a V) e quatro genótipos para o DENV-4 (GI a IV) (CHEN; VASILAKIS, 2011; WEAVER; VASILAKIS, 2009).

No Brasil atualmente circula o genótipo V (Américas/África) de DENV-1, porém linhagens distintas dentro deste genótipo já foram descritas (CARNEIRO et al., 2012; DE BRUYCKER-NOGUEIRA et al., 2015; DOS SANTOS et al., 2011; DRUMOND et al., 2012; DUTRA et al., 2017). Linhagens distintas também foram descritas para o genótipo do Sudeste Asiático/Americano de DENV-2 (DRUMOND et al., 2013; FARIA et al., 2013; MIR et al., 2014; OLIVEIRA et al., 2010) e do genótipo III de DENV-3 circulantes no Brasil (ARAÚJO et al., 2009; MIAGOSTOVICH et al., 2006). No entanto, o genótipo V foi identificado em Porto Velho e Belo Horizonte (AQUINO et al., 2009) e a cocirculação dos genótipos III e V, foi reportada em Rondônia (NOGUEIRA et al., 2008).

Mesmo com a recente introdução do DENV-4 no país, dois genótipos (I e II) já foram identificados em circulação no país (CAMPOS et al., 2013; DE SOUZA et al., 2011; DUTRA et al., 2017; FARES et al., 2015; NUNES et al., 2012; RAMOS-CASTAÑEDA et al., 2017).

## 1.5 EPIDEMIOLOGIA

A emergência da dengue e outras arboviroses, como chikungunya (CHIKV), zika e febre amarela, tem sido sem precedentes e resultante da urbanização, globalização e mobilidade internacional, tríade do mundo moderno (WILDER-SMITH et al., 2017). Além disso, a dispersão destas arboviroses tem sido impulsionada pelas mudanças climáticas, evolução do vírus e programas de controle de vetores insuficientes (KATZELNICK; COLOMA; HARRIS, 2017; MURRAY; QUAM; WILDER-SMITH, 2013).

A Organização Mundial da Saúde (OMS) considera a dengue a doença viral de transmissão vetorial mais importante na atualidade, evoluindo de um cenário de ocorrência esporádica, com epidemias em intervalos longos, a um grave problema de saúde pública, impactando o mundo social e economicamente (GUZMAN; HARRIS, 2015; WHO, 2016). Nos últimos 50 anos a incidência global da doença aumentou 30 vezes (GUZMAN; HARRIS, 2015; KATZELNICK; COLOMA; HARRIS, 2017), com as regiões tropicais e sub-tropicais tornando-se hiperendêmicas, com a cocirculação dos quatro sorotipos de DENV na maioria dos centros urbanos (GYAWALI; BRADBURY; TAYLOR-ROBINSON, 2016; MESSINA et al., 2014; SHARP et al., 2017) (Figura 4).

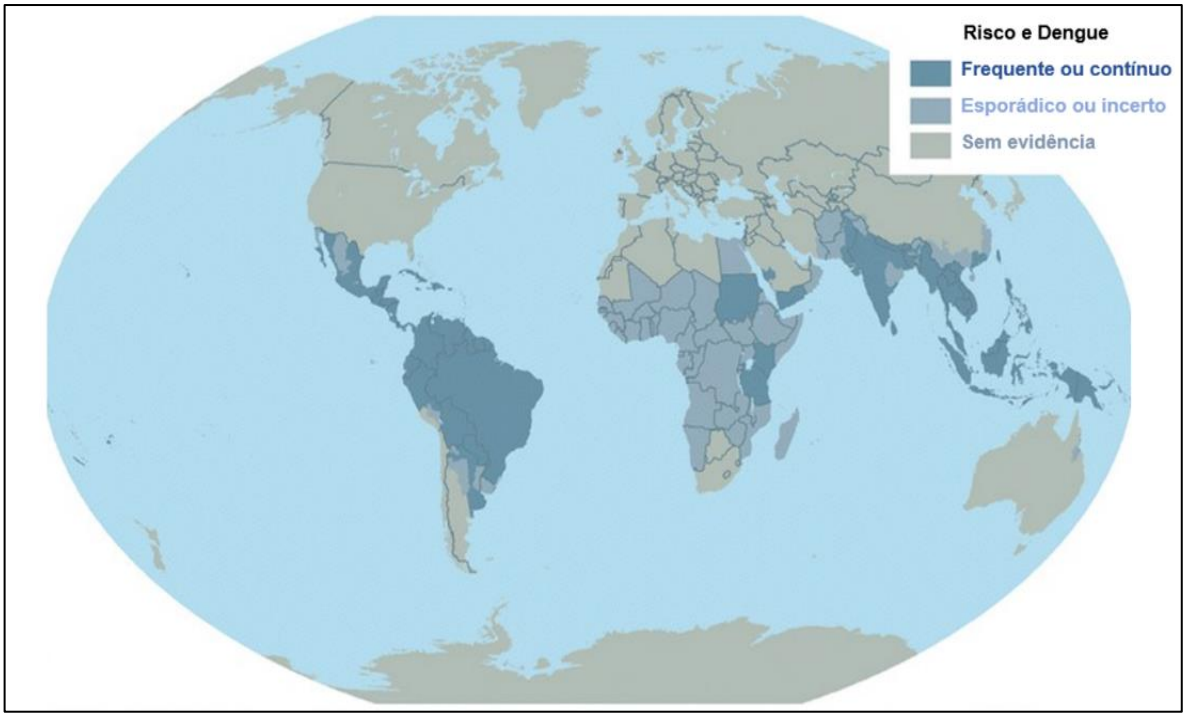


Figura 4: Distribuição mundial atual das infecções de dengue (Adaptado de SHARP et al., 2017).

As estimativas mais recentes são de que 390 milhões de casos de dengue ocorram anualmente e de que 96 milhões apresentem-se clinicamente com alguma gravidade (BHATT et al., 2013; MESSINA et al., 2015). Os registros anuais são de cerca de 500.000 pessoas hospitalizadas e nas Américas, um aumento no número de casos tem sido relatado durante as últimas décadas, com surtos que ocorrem a cada 3 e 5 anos (BRATHWAITE DICK et al., 2012; WHO, 2016).

### 1.5.1 DENGUE NAS AMÉRICAS

A dengue provavelmente foi introduzida nas Américas no século XVII, com relatos de doença compatível em 1635 na Martinica e Guadalupe, e em 1699, no Panamá. No final do século XIX e início do século XX, uma ampla distribuição de doença semelhante a dengue foi descrita em países do norte, como o Estados Unidos e do sul, como Chile e Argentina (BRATHWAITE DICK et al., 2012).

Um programa de erradicação *Ae. aegypti* iniciado pela Organização Pan Americana de Saúde (OPAS), nas décadas de 1940 e 1950, para prevenir epidemias urbanas de febre amarela, resultou na diminuição significativa das epidemias de dengue. No entanto, a descontinuação do programa no início de 1970, resultou na re-infestação do vetor. Em 1995, o mosquito já apresentava uma distribuição similar àquela da década de 1940 (GUBLER, 1997).

O primeiro sorotipo isolado nas Américas foi o DENV-2, em 1953, em Trinidad e Tobago (BRATHWAITE DICK et al., 2012; CUCUNAWANGSIH; LUGITO, 2017; MESSINA et al., 2014). O DENV-1 foi introduzido nas Américas, primeiramente na Jamaica, em 1977. Contudo, sua rápida dispersão no continente americano causou epidemias em diversos países do Caribe e da América Central e, até 1980, mais de 700.000 casos da doença foram notificados (BRATHWAITE DICK et al., 2012).

Os primeiros registros do DENV-3 nas Américas ocorreram em 1963, em Porto Rico e, em 1994, uma nova variante deste sorotipo foi introduzido na Nicarágua e no Panamá e foi associada à epidemia de febre hemorrágica do dengue/síndrome do choque por dengue (FHD/SCD). Este sorotipo dispersou para os países da América Central e México em 1995, Porto Rico em 1998 e outras ilhas do Caribe, além de países da América do Sul, como Brasil e Colômbia (CUCUNAWANGSIH; LUGITO, 2017; MESSINA et al., 2014).

O DENV-4 foi introduzido nas Américas em 1981, na Ilha de Saint Bartolomeu, no entanto, naquele mesmo ano, a circulação de uma nova variante de DENV-2 foi evidenciada em Cuba, resultando na primeira epidemia de FHD (CUCUNAWANGSIH; LUGITO, 2017; GUBLER, 2006; MESSINA et al., 2014).

Entre 1989 e 1990, a segunda maior epidemia de FHD foi reportada nas Américas, com cerca de 20 mil casos, na Venezuela, causada pela cocirculação dos DENV-1, DENV-2 e DENV-4 (BRATHWAITE DICK et al., 2012; MALAVIGE et al., 2004; MESSINA et al., 2014).

Na epidemia causada pelo DENV-3 (2002), um número recorde de 1.015.420 casos foi registrado, incluindo 255 óbitos (BRATHWAITE DICK et al., 2012). Naquele ano, o Brasil foi responsável por mais de 75% do número total de casos (NOGUEIRA et al., 2002). Em 2008, a re-emergência do DENV-2 no Brasil, resultou em uma epidemia com mais de 700 mil casos suspeitos e 223 óbitos (SVS/MS, 2008) e em 2009, várias epidemias foram registradas, incluindo na Bolívia, Argentina, México e Nicarágua (BRATHWAITE DICK et al., 2012).

Em 2010, aproximadamente 1,7 milhões de casos de dengue e 1.185 óbitos foram registrados, com uma incidência >200 casos/100.000 habitantes. Vários países sofreram surtos de dengue, com um total de casos que excederam os dados históricos conhecidos, incluindo a introdução da doença em Key West na Flórida, EUA. No Brasil, o número de pessoas infectadas excedeu 1 milhão de casos e, 678 casos fatais foram reportados (SIQUEIRA JR et al., 2011). Honduras, Caribe, Guadalupe, Martinica, República Dominicana e Porto Rico também registraram epidemias importantes (BRATHWAITE DICK et al., 2012).

Em 2011, foram notificados mais de 1 milhão de casos de dengue, dos quais mais de 15.000 apresentavam formas graves, necessitando de hospitalização e aproximadamente, 700 óbitos foram reportados. A introdução do DENV-4 foi observada no Panamá e em alguns estados do Brasil. Durante 2012, assim como o ano anterior, mais de 1 milhão de casos foram registrados e a maior taxa de incidência foi no Cone Sul (242,54/100.000 habitantes), onde 58,1% dos óbitos foram concentrados (PAHO, 2017).

Entre 2013 e 2016, aproximadamente 5,5 milhões de casos de dengue, 3.200 óbitos foram reportados nas Américas, além do Brasil, o México, Colômbia, Venezuela, El Salvador, Honduras e Nicarágua foram os países mais acometidos (PAHO, 2017). O Chile e Uruguai eram os únicos países sem transmissão autóctone de qualquer sorotipo e, desde a confirmação

do primeiro caso em fevereiro de 2016, o Uruguai registrou 570 casos suspeitos da doença (GYAWALI; BRADBURY; TAYLOR-ROBINSON, 2016). Em 2017, uma redução no número de casos de dengue e óbitos foi registrado nas Américas, quando aproximadamente 500 mil casos e 308 óbitos foram notificados (PAHO, 2018). Até a 22ª semana epidemiológica de 2018, um total de 191.524 casos e 73 óbitos de dengue foram registrados (PAHO, 2018).

### 1.5.2 DENGUE NO BRASIL

A referência mais antiga à dengue no Brasil ocorreu no período colonial. Uma doença, clinicamente compatível com a dengue foi descrita em Recife, em 1685. Em 1692, óbitos por esta mesma doença foram relatados em Salvador. Em 1846, um surto ocorreu nos estados do Rio de Janeiro, Bahia e Pernambuco (MARIANO, 1917; SALLES et al., 2018).

Embora *Ae. aegypti* tenha sido erradicado em 1958 (MAGALHÃES, 2016), o vetor foi re-introduzido no país o que resultou, em 1981-1982, na primeira descrição de um surto de dengue no Brasil, que ficou restrito à cidade de Boa Vista, em Roraima. Estimou-se, naquela ocasião, a ocorrência de 7.000 casos causados pela cocirculação de DENV-1 e DENV-4 (OSANAI et al., 1983).

Em 1986 ocorreu, no estado do Rio de Janeiro, uma epidemia causada pela introdução do DENV-1 e que, posteriormente, dispersou-se para os demais estados da federação, (SCHATZMAYR; NOGUEIRA; TRAVASSOS DA ROSA, 1986) com altos números de notificações, principalmente no Nordeste (FIGUEIREDO, 2000; MIAGOSTOVICH et al., 1993, 1999). Entre 1986 e 1989, 175.608 casos prováveis de dengue foram notificados (FUNASA, 2002a).

O DENV-2 foi detectado pela primeira vez também no estado do Rio de Janeiro, em 1990. A cocirculação com DENV-1, levou à ocorrência dos primeiros casos de FHD/SCD (NOGUEIRA et al., 1990). Epidemias explosivas por estes sorotipos também foram reportadas em 1997 e 1998, quando 249.239 e 528.388 casos foram notificados, respectivamente (FUNASA, 2002a).

DENV-1 e DENV-2 co-circularam até dezembro de 2000 quando DENV-3 foi detectado inicialmente no Rio de Janeiro e uma nova epidemia se estabeleceu em 2002, com um elevado número de hospitalizações e óbitos, sendo essa a epidemia mais grave vivenciada no país até então (ARAÚJO et al., 2009; NOGUEIRA et al., 2005).

Dezessete anos depois da sua introdução, DENV-2 reemergiu em 2007, causando uma grande epidemia em 2008, caracterizada por uma mudança no perfil epidemiológico com ocorrência de casos graves em menores de 15 anos (TEIXEIRA et al., 2008).

Em 2009, a re-emergência do DENV-1 ocorreu e levou à possibilidade de uma nova epidemia, tendo em vista a baixa circulação deste sorotipo desde o início da década e consequentemente, o elevado número de suscetíveis (SALLES et al., 2018; SVS/MS, 2009). A epidemia de 2010 apresentou um padrão espacial bastante distinto das epidemias de 2002 e 2008 (SVS, 2010), com uma alta incidência, exceto na região Nordeste (SALLES et al., 2018).

Em julho de 2010 ocorreram os primeiros casos de DENV-4 em Roraima e Amazonas, cerca de 30 anos após a primeira detecção deste sorotipo no país (1981-1982). Em março de 2011 foram identificados os primeiros casos de DENV-4 no estado do Rio de Janeiro, introduzidos a partir do município de Niterói (NOGUEIRA; EPPINGHAUS, 2011; SVS/MS, [s.d.]).

Nos últimos anos, extensas epidemias de dengue ocorreram no Brasil, caracterizadas por emergências e re-emergências dos diferentes sorotipos, mudança no perfil epidemiológico e aumento no número de casos graves e fatais. A introdução consecutiva e a cocirculação dos quatro sorotipos resultaram em um cenário hiperendêmico. levando a epidemias com grandes números de casos, principalmente nos anos de 2013 e 2015, nos quais foram registrados mais de 1 milhão de casos suspeitos da doença (SVS, 2017; SVS/MS, 2011, 2012, 2014). O perfil histórico dos casos de dengue podem ser visualizados na Figura 5.

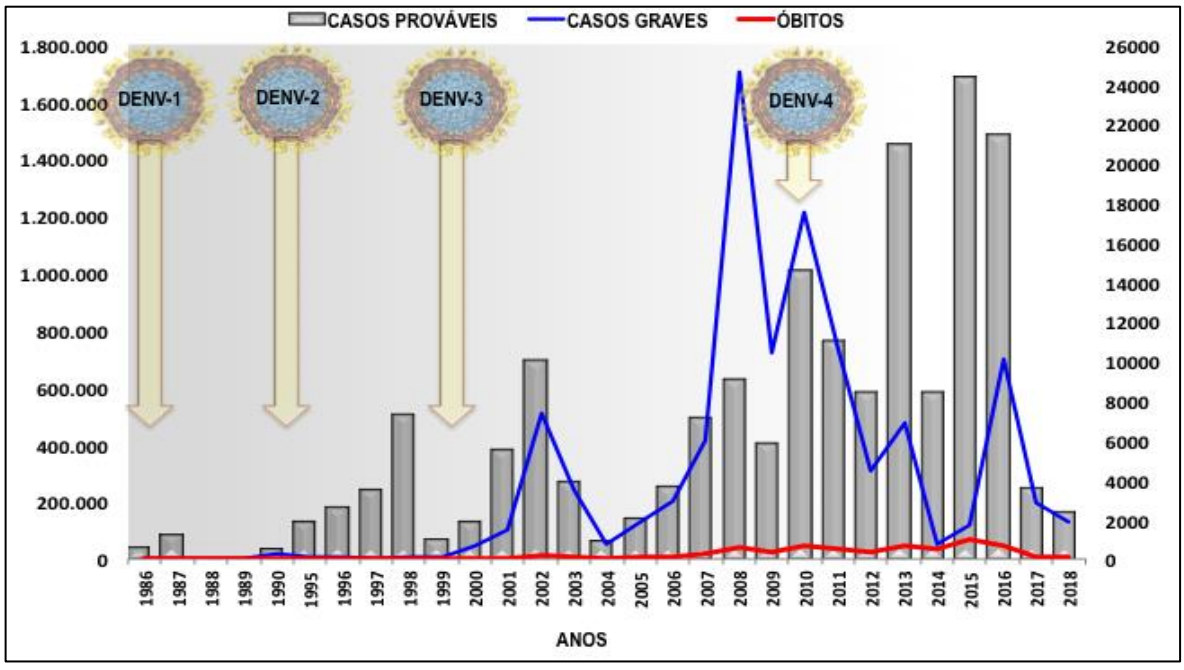

Figura 5: Casos prováveis, casos graves e óbitos por dengue, Brasil, 1986-2018.

Casos autóctones de ZIKV foram registrados pela primeira vez no Brasil a partir de abril de 2015. Naquele mesmo ano, o país já era acometido por surtos de CHIKV (SVS, 2017). A cocirculação desses outros arborvírus resultou na redução dos casos de dengue nos anos 2017 e 2018. Até a 25º semana epidemiológica (SE) de 2018 (25/06/2018), 171.582 casos prováveis e 77 óbitos por dengue foram notificados no país (SVS/MS, 2018).

## 1.6 MANIFESTAÇÃO CLÍNICA

A doença possui um amplo espectro clínico, variando de formas oligo-sintomáticas, hemorragias, choque e óbito. Três fases clínicas podem ocorrer: febril, crítica e de recuperação (MS, 2016a).

### 1.6.1 FASE FEBRIL

Após a picada do mosquito infectado, o vírus se dissemina e infecta vários tecidos. A carga viral acumula-se, levando ao aparecimento dos sintomas clínicos como febre, cefaléia, mialgia, presumivelmente secundários a um estado antiviral do hospedeiro no qual a expressão de interferon é abundante. A viremia atinge o pico logo após o início da febre (dia 0) e depois atinge o platô por 1-2 dias antes de diminuir gradualmente, impulsionada pela resposta imune adaptativa do hospedeiro (SIMMONS et al., 2015). Esta fase é caracterizada por febre, em torno de 39º-40ºC, com duração de 2 a 7 dias, de início rápido, podendo estar associada à cefaleia, astenia, mialgia, artralgia e dor retro-orbitária. Em torno de 50% dos casos apresentam exantema do tipo máculo-papular, atingindo face, tronco, plantas dos pés e palmas de mãos, com ou sem prurido, associado ao decréscimo da febre. Manifestações hemorrágicas leves como petéquias e sangramento de nariz e gengivas são observados (WHO, 2009). Anorexia, náuseas, vômitos e diarreia (fezes pastosas com frequência de 3 à 4 evacuação por dia) podem ocorrer. A maioria dos pacientes recuperam-se gradualmente no estado geral e retorno do apetite. Essa fase é indistinguível entre os casos graves e não graves. Desta forma, sinais de alerta e outros parâmetros clínicos devem ser monitorados para identificar sua progressão para a fase crítica da doença (EDELMAN, 2007; MS, 2016a).

### 1.6.2 FASE CRÍTICA

Entre o 4-6º dia de doença, a febre cessa na maioria dos pacientes e os sintomas diminuem de intensidade. Por volta de 10 à 15% evoluem para esta fase (VERDEAL et al., 2011). O aumento da permeabilidade capilar, muitas vezes subclínico, é mensurável em muitos pacientes durante a fase crítica, provavelmente com início durante a fase febril. O aumento da permeabilidade, coagulopatia, trombocitopenia e outros distúrbios laboratoriais são mais pronunciados na defervescência, e este é o ponto no tempo em que a maioria das complicações se manifesta (SIMMONS et al., 2015).

Surgem sinais de alerta, como dor abdominal intensa, vômitos persistentes, acúmulo de líquidos (ascite, derrame pleural e derrame pericárdico), hipotensão postural e/ou lipotimia, hepatomegalia, sangramento de mucosas, letargia e/ou irritabilidade, e aumento do hematócrito (Figura 6). Casos com sinais de alerta se recuperam com reidratação intravenosa, porém alguns casos evoluem para doença grave (MS, 2016a; WHO, 2009). Sinais de gravidade da doença podem manifestar-se com extravasamento de plasma, levando ao choque ou ao acúmulo de líquidos, com desconforto respiratório, sangramento grave ou sinais de disfunção no coração, nos pulmões, nos rins, no fígado e no sistema nervoso central, até o óbito (MS, 2016a).

### 1.6.3 FASE DE RECUPERAÇÃO

Durante a fase de recuperação há uma gradual normalização das características clínicas e laboratoriais (SIMMONS et al., 2015). O estado geral melhora, o apetite retorna, os sintomas gastroentestinais diminuem e o estado hemodinâmico se estabiliza. O hematócrito se estabiliza ou pode ser menor devido ao efeito dilucional do líquido reabsorvido. A contagem de glóbulos brancos geralmente começa a aumentar logo após a defervescência, mas a recuperação da contagem de plaquetas é tipicamente mais tardia do que a contagem de leucócitos. Durante as fases críticas e/ou de recuperação, a fluidoterapia excessiva pode levar ao edema pulmonar ou insuficiência cardíaca congestiva. Infecções bacterianas poderão ocorrer nesta fase, o que podem contribuir para o óbito (MS, 2016a; WHO, 2009).

## 1.7 CLASSIFICAÇÃO DOS CASOS DE DENGUE

A Organização Mundial de Saúde (OMS), por consenso de especialistas, estabeleceu em 1975, uma classificação para os casos de dengue, baseada em estudos realizados em crianças na Tailândia, nos anos de 1950 e 1960, com modificações em 1986 e 1997 (HORSTICK et al., 2014). Desta forma, os sinais e sintomas da dengue foram agrupados em febre do dengue (DF), febre hemorrágica do dengue (FHD) e síndrome do choque por dengue (SCD) (WHO, 1997). No Brasil, a classificação da OMS de 1997 foi usada entre 1986 e 2013. Em 2000, no entanto, o Ministério da Saúde propôs a inclusão da classificação de dengue com complicações (DCC), para definir casos graves de dengue que não atendiam aos critérios da OMS para FHD/SCD (MS, 2009, 2011).

A DF ou dengue clássica (DC) é uma doença incapacitante, com febre alta (39° a 40°C), de início abrupto, podendo estar acompanhada de cefaléia, lombalgia, dor retro-orbitária, mialgia, artralgia, náuseas, vômitos, exantema e prurido. O aparecimento de petéquias é comum e, a leucopenia e trombocitopenia podem ser observados. Pode ser acompanhado por complicações hemorrágicas, como epistaxe, sangramento gengival, sangramento gastro-intestinal, hematúria e menorragia (WHO, 1997). O curso da doença tem duração de 5 a 7 dias, mas o período de convalescença pode ser acompanhado de grande debilidade física, podendo prolongar-se por semanas (MS, 2010). A forma clássica geralmente resulta numa recuperação rápida (NISHIURA; HALSTEAD, 2007).

As manifestações clínicas iniciais da FHD são as mesmas descritas no DC. No entanto, entre o terceiro e o sétimo dia do início da doença, com a defervescência, surgem sinais e sintomas como vômitos, dor abdominal intensa, hepatomegalia dolorosa, desconforto respiratório, letargia e derrames cavitários (pleural, pericárdico e ascite) (FUNASA, 2002b; MS, 2007).

De acordo com o seu grau de gravidade, a FHD foi classificada em Grau I – febre acompanhada de sintomas inespecíficos, em que a única manifestação hemorrágica é a prova do laço positiva; Grau II – além das manifestações do grau I, hemorragias espontâneas leves (sangramento de pele, epistaxe, gengivorragia e outros); Grau III – colapso circulatório com pulso fraco e rápido, estreitamento da pressão arterial ou hipotensão, pele pegajosa e fria e inquietação e Grau IV – SCD, ou seja, choque profundo, com ausência de pressão arterial e de pulso imperceptível (WHO, 1997). Trombocitopenia ($\leq$100.000/mm$^3$), sinais hemorrágicos como prova do laço positiva, petéquias, equimoses ou púrpuras, sangramentos de mucosas,

extravasamento do plasma devido ao aumento de permeabilidade capilar, manifestado por um aumento de 20% do hematócrito ou queda de 20% após o tratamento adequado, presença de derrame pleural, ascite e hipoproteinemia, serviam de alerta para a identificação da FHD (FUNASA, 2002b; MS, 2007).

Os pacientes que evoluem para SCD pioram rapidamente após o 2º até o 7º dia de febre. Essa piora ocorre junto com a queda de temperatura. É observada a presença de sinais típicos de insuficiência circulatória, como a pele fria, manchada e congestionada, cianose circumoral e o pulso torna-se rápido. Os pacientes podem inicialmente ser letárgicos, ficar inquietos e entrar rapidamente em um estágio crítico de choque. A dor abdominal aguda é queixa frequente pouco antes do início do choque. O choque é caracterizado por um pulso rápido e fraco ou hipotensão com pele fria, úmida e inquietação. A duração do choque é curta: normalmente, o paciente evolui para o óbito em até 24 horas, ou recupera-se após a terapia adequada de reposição volêmica. Mesmo em casos de choque profundo, uma vez que o choque é superado, os pacientes se recuperam dentro de 2 à 3 dias. Não tratado, o choque resulta em acidose metabólica, sangramento grave do trato gastro-intestinal e em outros órgãos. Pacientes com hemorragias intracranianas podem convulsionar e entrar em coma. A encefalopatia pode ocorrer em associação com distúrbios metabólicos e eletrolíticos, ou sangramento intracraniano (MS, 2010; WHO, 1997).

O DCC era caracterizado por pelo menos um dos achados: anormalidades neurológicas, insuficiência hepática, disfunção cardio-respiratória, sangramento gastrointestinal, plaquetopenia igual ou inferior a 50.000/$mm^3$, leucometria global igual ou inferior a 1.000/$mm^3$, derrame pleural e pericárdico, ascite e óbito (MS, 2007). Alterações do sistema nervoso podem surgir no inicio da doença ou mais tardiamente e são caracterizados por delírio, sonolência, coma, depressão, irritabilidade, psicose, demência, amnésia, sinais meníngeos, paresias, paralisias, polineuropatias, síndrome de Reye, síndrome de Guillain-Barré e encefalite (MS, 2007).

### 1.7.1 CLASSIFICAÇÃO ATUAL DOS CASOS DE DENGUE

Com base nos resultados obtidos em um estudo multicêntrico (DENCO, do inglês *Dengue Control*) realizado no Sudeste Asiático e na América Latina para avaliar as limitações da classificação de 1997, um novo critério de classificação foi proposto em 2009,

caracterizando infecções pelo DENV em: Dengue sem Sinais de Alerta, Dengue com Sinais de Alerta e Dengue Grave (HADINEGORO, 2012, p. 201; WHO, 2009), conforme demonstrado na Figura 7. O Brasil adotou essa nova classificação, à partir de Janeiro de 2014 (MS, 2016a).


Figura 6: Classificação dos casos de dengue de acordo com os critérios da WHO (2009).

A classificação revista apresentou uma maior sensibilidade em detectar a gravidade da doença (CAVALCANTI et al., 2014; HORSTICK et al., 2014; KHURSHEED et al., 2013; VIEIRA MACHADO et al., 2014). Sua especificidade, no entanto, é muito menor (73,0%) em comparação com a classificação de 1997 (93,4%). A maior sensibilidade, apesar de permitir o melhor manejo do paciente grave e diminuir a mortalidade (ALEXANDER et al., 2011; BASUKI et al., 2010), pode resultar, por outro lado, na classificação superestimada de alguns casos graves (MACEDO et al., 2014). De fato, atribui-se a menor especificidade desta nova classificação em parte, à falta de critérios claros para a definição dos sinais de gravidade (MORRA et al., 2018).

Um estudo recente demonstrou que a classificação de casos de dengue de 2009 pode não ter efeito sobre a taxa de letalidade, embora os resultados indiquem uma taxa de mortalidade mais baixa (LOW et al., 2018a).

### 1.7.2 ÓBITO POR DENGUE

Em 2006, o Ministério da Saúde criou a Portaria nº 1.405 com o objetivo de instituir a Rede Nacional de Serviços de Verificação de Óbito e Esclarecimento da Causa Mortis (SVO), integrante do Sistema Nacional de Vigilância em Saúde, que visam o esclarecimento da causa do óbito, além da detecção e investigação de qualquer agravo suspeito ou confirmado de doença de notificação compulsória atendido no hospital. Para tal, exames anátomo-patológico, histopatológico, hematológico, bioquímico, sorológico e microbiológicos são aplicados para o desfecho dos casos fatais (MS, 2006).

A notificação do óbito suspeito de dengue deve ser realizada em até 24 horas e seu encerramento no Sistema de Informação de Agravos de Notificação (SINAN) em até 60 dias. Sua investigação consiste na coleta de dados clínicos e epidemiológicos em fontes secundárias e entrevistas com os familiares. Após a investigação, os casos são discutidos em um comitê, a fim de classificar adequadamente o caso suspeito e identificar situações que possam ter contribuído com a ocorrência do óbito (MS, 2016b).

De acordo com o Ministério da Saúde (2016) considera-se óbito por dengue, todo paciente que cumpra os critérios da definição de caso suspeito ou confirmado que tenha evoluído ao óbito como consequência da dengue. A confirmação do caso pode ser realizada através da detecção de anticorpos IgM anti-dengue, NS1, isolamento viral, RT-PCR e pela técnica de imuno-histoquímica (MS, 2016b).

Os dados de mortalidade por dengue podem ser consultados no SINAN, no Sistema de Informações sobre Mortalidade (SIM) e no Sistema de Informação Hospitalar do Sistema Único de Saúde (SIH-SUS). Além disso, as Secretarias Estaduais de Saúde (SES) repassam os dados de casos e óbitos da dengue para o Programa Nacional de Controle da Dengue (PNCD). Esses sistemas de informação, são utilizados para a vigilância e controle da doença, mas também como fontes de dados para pesquisas. Porém, os dados disponíveis nesses sistemas podem apresentar discordância (MORAES; DUARTE, 2009; RIBEIRO, 2017). Além disso, é sugerido que o número de óbitos relacionados à dengue ainda seja subestimado (CAVALCANTI et al., 2016; MELO et al., 2018).

Embora, ao longo dos anos, advieram avanços consideráveis no conhecimento da dengue, os comitês de vigilância epidemiológica e de investigação de óbitos enfrentam desafios

para determinar se um óbito ocorreu devido ao vírus ou por devido uma disfunção fisiológica próprio paciente infectado. Essa questão reflete na dificuldade em estabelecer se a doença contribuiu para a morte ou, de fato, foi a causa básica da morte. (CAVALCANTI et al., 2017). É importante ressaltar que esta é uma doença infecciosa aguda na qual a maioria dos óbitos geralmente ocorrem durante a fase aguda da doença (CAMPOS et al., 2015; LEE et al., 2018a; NUNES et al., 2018; WOON et al., 2016).

Em pacientes com dengue e comorbidades que evoluírem para óbito durante o curso da doença, a causa básica do óbito dever ser considerada a dengue (MS, 2016a). A presença de comorbidades e outras doenças crônicas pode contribuir para o aumento da mortalidade da doença na população adulta (GUBLER, 2012; RIGAU-PÉREZ; LAUFER, 2006; THEIN et al., 2013). Choque refratário grave, coagulação intravascular disseminada, síndrome do desconforto respiratório do adulto, insuficiências hepática e cardíaca, encefalite, meningite e síndrome da disfunção múltipla de órgãos, podem levar ao óbito por dengue (MS, 2016).

As manifestações muitas vezes são relativamente inespecíficas na autópsia em casos fatais. É evidenciado coagulação intravascular disseminada, levando a hemorragias interna e subcutânea, falência de múltiplos órgãos com extravasamento vascular, cuminado na efusão pericárdica, pleural e intraperitoneal com edema visceral. Além disso, insuficiência hepática, hepatite, miocardite, desconforto respiratório, insuficiência renal aguda, hemorragia subaracnóidea e ruptura esplênica podem ser causadas pelo DENV. Uma superinfecção bacteriana associada pode complicar a doença e ser fatal (ARAÚJO et al., 2010; BYARD, 2016; DE FILIPPIS et al., 2016; DE MOURA MENDONÇA et al., 2011; LEONG et al., 2007; WICHMANN et al., 2007).

A letalidade de pacientes com FHD pode ser acima de 20%, porém o tratamento oportuno, pode reduzir o número de óbitos, chegando a menos de 1% dos casos (MONATH, 1994; MSF, 2014). Com a utilização da nova classificação de casos sugerida pela OMS em 2009, foi observada uma dimunuição na taxa de letalidade, principalmente devido à detecção precoce dos sinais de alerta, que, geralmente, resulta na internação do paciente para o monitoramento cuidadoso e administração de fluidoterapia. No entanto, a internação precoce pode, por vezes,, aumentar a taxa de letalidade resultante de tratamento com fluidos intravenosos e consequentemente, com a sobrecarga de fluidos dos pacientes que podem evoluir ao óbito devido ao desconforto respiratório como conseqüência de derrame pleural maciço (LOW et al., 2018b).

Adicionalmente, o aumento da taxa de letalidade pode ser resultante do aumento na admissão de pacientes, que sobrecarrega o sistema de saúde, afetando a qualidade do atendimento devido à limitação de recursos e mão de obra necessários ao correto manejo dos pacientes (LOW et al., 2018b). No entanto, apesar do manejo adequado, mortes por dengue podem ocorrem (WOON et al., 2016).

## 1.8 GRUPOS VULNERÁVEIS NAS INFECÇÕES POR DENGUE: CRIANÇAS E GESTANTES

Conforme descrito previamente, o padrão epidemiológico de dengue no Brasil tem sofrido mudanças da distribuição etária, com aumento da incidência e gravidade dos casos em crianças e adolescentes (CAVALCANTI et al., 2011; TEIXEIRA et al., 2008).

Outro grupo de grande preocupação, é constituído por gestantes e neonatos, uma vez que são também considerados grupos de risco ao desenvolvimento de formas graves da doença (BRASIL; LUPI, 2017; PAIXAO et al., 2018; WAKIMOTO et al., 2015).

O risco de dengue com sinais de alerta e dengue grave varia com a idade, sendo os quadros graves mais frequentes em lactentes quando comparados a adolescentes e adultos. De fato, casos graves têm sido registrados em lactentes no primeiro ano de vida e nascidos de mães imunes ao DENV, e em menores de 15 anos apresentando infecção secundária (CHAU et al., 2008; HALSTEAD, 2002; HARRIS et al., 2000; JAIN; CHATURVEDI, 2010). A letalidade nas crianças mais jovens com dengue grave é mais elevada do que em adultos (HAMMOND et al., 2005).

Ainda não há um consenso a respeito dos efeitos da doença em gestantes e/ou neonatos, porém alguns estudos apontam que a transmissão vertical pode ocorrer e apresentar consequências graves, como o parto prematuro e morte fetal (BASURKO et al., 2009; BRASIL; LUPI, 2017; CARLES, 2016; CHANSAMOUTH et al., 2016; SHARMA; JAIN; RAJARAM, 2016; TIEN DAT et al., 2018).

Estudos prévios realizados no mundo (ADAM et al., 2010; CHAU et al., 2009; KARIYAWASAM; SENANAYAKE, 2010; LIBRATY et al., 2009; PENGSAA et al., 2006; TIEN DAT et al., 2018) e no Brasil (ARGOLO et al., 2013; BRAGA et al., 2016, 2016; CASTANHA et al., 2016, 2016; FEITOZA et al., 2017; LEITE et al., 2014; MACHADO et al.,

2013; NASCIMENTO et al., 2017; PAIXAO et al., 2018; RIBEIRO et al., 2016), já avaliaram os impactos das infecções pelos DENV nestes grupos. De fato, foi observado um risco de morte materna três vezes maior em casos de dengue (NASCIMENTO et al., 2017) e de 450 vezes, quando a gestante apresentava FHD (PAIXAO et al., 2018).

## 1.9 PATOGÊNESE

Diversas teorias são propostas (Figura 8) tentando explicar os fatores e/ou mecanismos relacionados ao desenvolvimento de um quadro mais grave de dengue (HALSTEAD; COHEN, 2015), no entanto, estes ainda não foram totalmente elucidados, principalmente devido à falta de modelos animais adequados que reproduzam a infecção de maneira similar ao observado em humanos (THEIN et al., 1997; ZUBAIR et al., 2016).

A teoria da infecção sequencial ou da facilitação dependente de anticorpos (do inglês *"ADE, antibody dependent enhancement"*, considera que há um aumento da replicação viral em macrófagos via anticorpos heterólogos. Em infecções secundárias, anticorpos reagem de forma cruzada com o novo sorotipo, sem neutralizá-los. Estes complexos, ao serem reconhecidos e internalizados por fagócitos mononucleares, resultam na infecção celular e replicação viral (HALSTEAD, 1988). A reação cruzada de linfócitos T de memória pode culminar na ativação exacerbada de vias pró-inflamatórias (SCHMID; DIAMOND; HARRIS, 2014; YACOUB; MONGKOLSAPAYA; SCREATON, 2016) e no aumento da ativação de macrófagos (OLIVEIRA et al., 2016).

A teoria do "Pecado Original" descreve a ativação de células T $CD4^+$ e T $CD8^+$ durante a infecção por DENV como importante mecanismo na patogêse da doença (MENTOR; KURANE, 1997), uma vez que a liberação de citocinas pró-inflamatórias podem agir diretamente sobre o endotélio vascular e resultar no extravasamento de plasma, característico das infecções graves por DENV (PANG; CARDOSA; GUZMAN, 2007).

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

Os níveis elevados de NS1 podem estar associados à patogênese do dengue (CHUANG et al., 2013; SRIKIATKHACHORN; KELLEY, 2014), uma vez que ligada à superfície da célula hospedeira pode causar dano tecidual (BEATTY et al., 2015), além de induzir a produção de citocinas, capaz de interferir na integridade das células endoteliais dos vasos sanguíneos (MODHIRAN et al., 2015). Anticorpos anti-NS1 de DENV podem promover a ativação da célula endotelial, levando ao aumento da expressão e secreção da IL-6 e IL-8, que são associados a casos de FHD (AVIRUTNAN et al., 1998; CHATURVEDI et al., 2007).

A teoria da virulência viral sugere que a gravidade da doença esteja relacionada às variações genéticas e antigênicas das diferentes cepas de vírus (MAMMEN et al., 2014; RICO-HESSE, 1990; UBOL et al., 2008) e genótipos de DENV de origem asiática estão relacionados a casos mais graves da doença (COLOGNA; ARMSTRONG; RICO-HESSE, 2005; LEITMEYER et al., 1999).

Apesar das várias teorias, considera-se que a ocorrência de casos graves seja multifatorial, incluindo fatores nutricionais e genéticos do hospedeiro, idade, sexo, estado imunológico, sorotipo e genótipo viral infectantes atuem no desenvolvimento do quadro (GUZMÁN; KOURÍ, 2002a; MALAVIGE et al., 2004).

O RNA subgenômico do vírus também parece ter implicações na patogênese da doença. O RNA pode acumular nas células infectadas e suprimir a resposta imune antiviral do hospedeiro (MANOKARAN et al., 2015), além de facilitar a replicação viral pela alteração da estabilidade do RNA mensageiro do hospedeiro (PANG et al., 2017; SCHNETTLER et al., 2012).

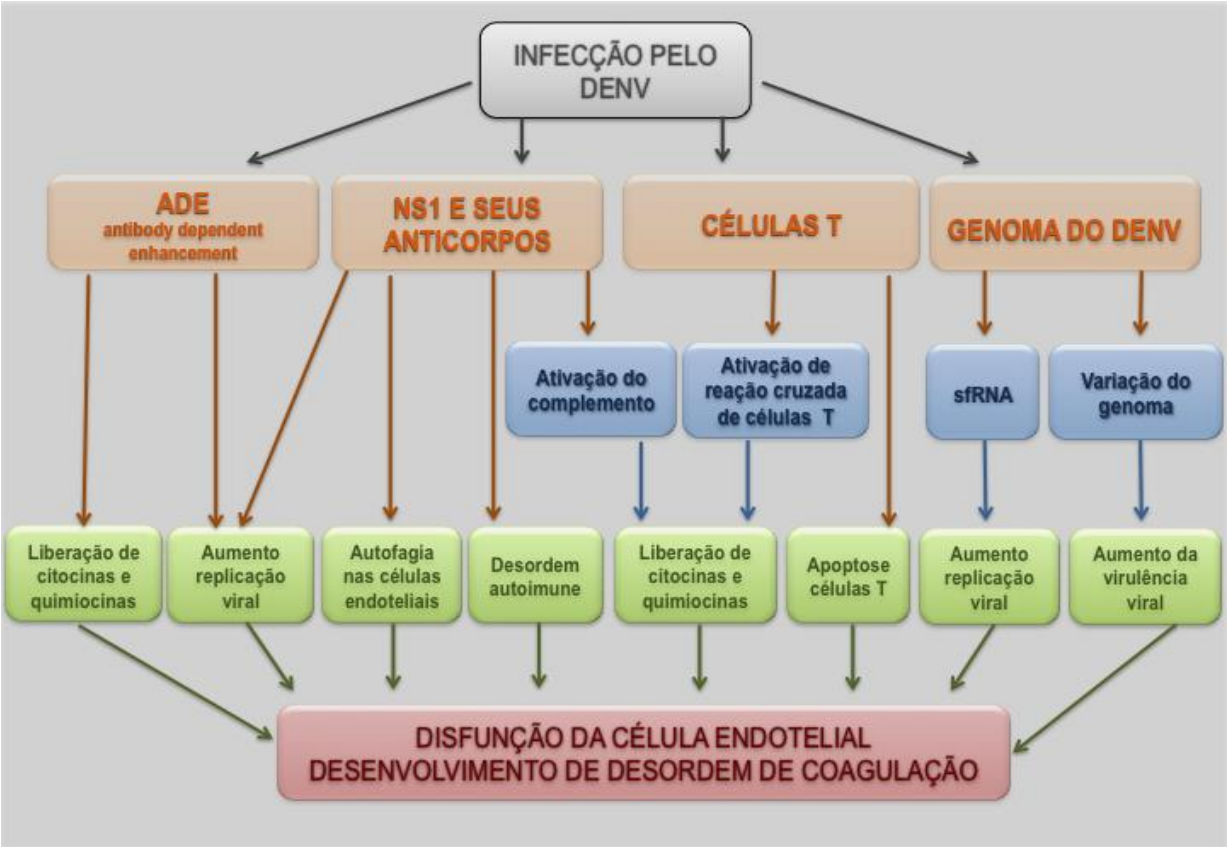


Figura 7: Mecanismos propostos envolvidos na patogênese das infecções pelos DENV (Adaptado de PANG et al., 2017).

### 1.9.1 CITOCINAS E QUIMIOCINAS

Após a picada do mosquito vetor e inoculação do DENV na derme e epiderme (MARTINA, 2014), os primeiros alvos de infecção são as células de Langerhans (células dendríticas epidérmicas) e queratinócitos (LIMON-FLORES et al., 2005; WU et al., 2000).

As células infectadas migram do local de infecção para os linfonodos, onde os monócitos e macrófagos são recrutados e tornam-se alvos da infecção. Consequentemente, a infecção é amplificada, e o vírus é disseminado através do sistema linfático. Como resultado da viremia primária, o vírus infecta macrófagos teciduais de vários órgãos, particularmente baço e fígado. A replicação de DENV em vários tecidos resulta em viremia secundária, que é detectável em média 12 horas antes do início da febre (BLACKLEY et al., 2007; DURBIN et al., 2008; JESSIE et al., 2004; KOU et al., 2008; LAFLEUR et al., 2002; LIBRATY et al., 2001).

Após a infecção, as células mononucleares morrem, predominantemente, por apoptose (ESPINA et al., 2003; PALMER et al., 2005), enquanto as células dentríticas infectadas são estimuladas a produzir a maior parte dos mediadores envolvidos nas inflamações (BOSCH et al., 2002; HO et al., 2004; LIBRATY et al., 2001; LUPLERTLOP et al., 2006) e na hemostase (CHAN et al., 2006; HUERTA-ZEPEDA et al., 2008; KRISHNAMURTI; ALVING, 1989).

As citocinas são proteínas secretadas que desempenham um papel na sinalização celular, nas fases de indução, inibição e efetoras das respostas imune e inflamatória. As quimiocinas são um subconjunto de pequenas citocinas que recrutam e exercem a migração quimiotáxica de outras células, para uma área localizada, para exercer uma variedade de efeitos biológicos, incluindo inflamação e homeostase (LEE; LEONG; WILDER-SMITH, 2016).

Perfis de proteínas imuno-moduladoras mudam com o curso clínico da dengue, diferem entre a gravidade (KUMAR et al., 2012; RATHAKRISHNAN et al., 2012), e acredita-se que tenham impacto direto no aumento da permeabilidade vascular, derrame plasmático e trombocitopenia (GREEN; ROTHMAN, 2006; MURPHY; WHITEHEAD, 2011).

A interleucina (IL) -10 é uma importante citocina anti-inflamatória e supressora geral de reações imunes, inibindo a ação das IL-1, IL-6, IL-12, IL-18, CSF e TNFα, bem como a síntese das citocinas IL-2, IL-3, GM-CSF, TNFα e IFN-γ ((D'ANDREA et al., 1993). É secretada por uma variedade de tipos de células, incluindo células T CD4 + e CD8 +, células B, macrófagos, monócitos, eosinófilos e mastócitos (O'GARRA; VIEIRA, 2007). O momento da produção de IL-10 é dinâmico e varia ao longo do curso da doença, exemplificado por níveis de IL-10 com pico de defervescência precoce em pacientes graves, mas menos em pacientes não graves (ADIKARI et al., 2016; KUMAR et al., 2012; LIBRATY et al., 2002a).

A IL-10 pode contribuir para a gravidade da doença, induzida por NS1 nos monócitos, o que, por sua vez, suprime as respostas das células T específicas da dengue (ADIKARI et al., 2016; MALAVIGE et al., 2013). No entanto, a IL-10 foi relatada como um mediador protetor contra o extravasamento do plasma e a disfunção vascular (CHENG; SHARMA, 2015).

As células T CD8+ e células NK de pacientes com dengue exibem marcadores de ativação como CD69, HLA-DR, CD38 e TIA-1 de grânulos citotóxicos e moléculas de adesão celular CD44 e CD11a durante a fase aguda (AZEREDO et al., 2006). A ativação de células T resulta em cascatas de citocinas inflamatórias e outros mediadores químicos, que desencadeiam a morte de células-alvo por apoptose (FINK; GU; VASUDEVAN, 2006).

As células dendríticas ativadas podem contribuir para o extravasamento vascular através da produção de TNF-α, IFN-γ e metaloproteases-2, 3 e 9 (LIBRATY et al., 2001;

LUPLERTLOP et al., 2006). Foi demonstrado que as células T CD4 + e CD8+ específicas para DENV proliferam, produzem IFN-γ e lisam as células alvo infectadas (GAGNON; ENNIS; ROTHMAN, 1999; KURANE et al., 1989), controlando as infecções através do *clearence* viral (COSTA et al., 2012; PAL et al., 2014; SHRESTA et al., 2004).

As células dendríticas infectadas com DENV produzem respostas de quimiocinas e citocinas, que ativam ou recrutam células imunológicas para o endotélio (AVIRUTNAN et al., 1998; HUANG et al., 2000) e, a ativação descontrolada e persistente do endotélio, leva à permeabilidade vascular, trombose microvascular e inflamação (LEE; LILES, 2011; PAGE; LILES, 2013).

A proteína quimiotática de monócitos (CCL2/MCP-1) é uma quimiocina produzida por macrófagos, linfócitos T, fibroblastos, queratinócitos e células endoteliais e causa a abertura de junções estreitas de células endoteliais infectadas *in vitro* pelo DENV (STAMATOVIC et al., 2003). Sua expressão induzida pelo fator de crescimento endotelial vascular (VEGF), em células endoteliais vasculares eleva as alterações de permeabilidade endotelial i*n vivo* (YAMADA et al., 2003). Os níveis plasmáticos de VEGF e VEGFR-1 foram maiores em FHD e em SCD, enquanto os níveis de VEGFR-2 foram menores nos casos FHD/SCD (FURUTA et al., 2012; VAN DE WEG et al., 2014).

Casos graves de dengue apresentam uma "tempestade de citocinas", com altos níveis de citocinas e quimiocinas circulantes e, células T, células NK, monócitos, macrófagos, hepatócitos e células endoteliais contribuem para o aumento da produção de citocinas e quimiocinas. Níveis aumentados de IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-15, IL-17, IL-18, o fator inibitório da migração de macrófagos (MIF) e as quimiocinas CCL2 (MCP-1), CCL4, CCL5 (RANTES) e CXCL10 (IP-10) estão presentes em pacientes com FHD (GREEN et al., 1999; MUSTAFA et al., 2001; VAN DE WEG et al., 2013) e podem ser usados como potenciais preditores da gravidade da doença (ASSUNÇÃO-MIRANDA et al., 2010; BETHELL et al., 1998; BOZZA et al., 2008; CHEN et al., 2006; DE-OLIVEIRA-PINTO et al., 2012).

### 1.9.2 PATOGÊNESE SISTÊMICA

Os DENV podem infectar diversos tipos celulares em diferentes órgãos. Estudos realizados em autópsias e biópsias de pacientes demonstraram presença viral em monócitos e macrófagos no fígado, pulmão, baço, cérebro, rim, medula óssea e coração (BALSITIS et al.,

2009; DE ARAÚJO et al., 2009a; HUERRE et al., 2001; JESSIE et al., 2004; LIMA et al., 2011; MIRANDA et al., 2013a, 2013b; PAGLIARI et al., 2016; PÓVOA et al., 2014, 2016).

O acometimento viral no fígado, um dos órgãos-alvo da doença (BASÍLIO-DE-OLIVEIRA et al., 2005; PÓVOA et al., 2014; TRUNG et al., 2010), leva à evidência de esteatose, tumefação seguida de degeneração vacuolar baloniforme, necrose hepatocitária, hemorragia, edema, hiperplasia de célula de Kupffer e infiltrados no espaço porta (BASÍLIO-DE-OLIVEIRA et al., 2005; KULARATNE et al., 2014; LEONG et al., 2007; LIMONTA et al., 2012; PÓVOA et al., 2014; SENEVIRATNE; MALAVIGE; DE SILVA, 2006).

No tecido pulmonar, as descrições das alterações morfológicas incluem danos em áreas extensas de hemorragia e edema, congestão de septo alveolar, ruptura focal da parede do septo e seu espessamento, presença de infiltrados mononucleares, hiperplasia de macrófagos alveolares e hipertrofia de pneumócitos tipo II (BASÍLIO-DE-OLIVEIRA et al., 2005; BHAMARAPRAVATI; TUCHINDA; BOONYAPAKNAVIK, 1967; PÓVOA et al., 2014; RODRIGUES et al., 2014; WANG et al., 2007).

Em exames de tomografia computadorizada e ressonância magnética demonstraram: edema cerebral, lesões focais, hematoma subdural e cerebelar e hemorragia intracraniana nos casos graves de dengue (BHOI et al., 2014; CAM et al., 2001; MISRA et al., 2006). Estas manifestações podem ocorrer como hemorragia intracraniana, hiponatremia, hipoxia cerebral, edema cerebral (CAM et al., 2001; PUCCIONI-SOHLER et al., 2009; VARATHARAJ, 2010). Além disso, alterações histopatológicas, como edema, congestão macroscópica e microscópica cerebelar, focos hemorrágicos e infiltrados linfocitários perivasculares, micro-abscessos no parênquima foram observados nos casos fatais de dengue (AHSAN; AHMAD; RAFI, 2018a; CAROD-ARTAL et al., 2013).

O comprometimento cardíaco pelo vírus promove principalmente miocardite (LEE; TEO; LOW, 2009; SALGADO et al., 2010; WEERAKOON et al., 2011). Dano tecidual grave com hemorragia, edema intersticial com infiltração de células inflamatórias e necrose de fibras miocárdicas foi observado. Tanto miocardite focal como difusa foram demonstradas em pacientes que morreram de dengue, com inflamação e infiltração por linfócitos, neutrófilos e eosinófilos, com raras descrições de pericardite (LEE; TEO; LOW, 2009; PÓVOA et al., 2014; TORRES et al., 2013; WEERAKOON et al., 2011).

Tecidos esplênicos de relatos de casos apresentaram congestão, hematomas subcapsulares, (BHAMARAPRAVATI; TUCHINDA; BOONYAPAKNAVIK, 1967), grande quantidade de sangue coagulado na cavidade abdominal, atrofia dos folículos linfoides (região rica em células T e B), associados à ruptura esplênica fatal e não fatal causada pelo DENV (DE MOURA MENDONÇA et al., 2011; DE SOUZA et al., 2017; PUNGJITPRAPAI; TANTAWICHIEN, 2008).

Fatores patogênicos renais incluem efeitos virais direto sobre o tecido renal, instabilidade hemodinâmica, rabdomiólise, hemólise, lesão glomerular aguda e síndrome da resposta inflamatória sistêmica (KHUNCHAI et al., 2015; MISHRA; SINGH; NANDA, 2015; MOHSIN et al., 2009; OLIVEIRA; BURDMANN, 2015; PAGLIARI et al., 2016; PÓVOA et al., 2014; REPIZO et al., 2014; UPADHAYA et al., 2010). A análise histológica mostrou danos circulatórios e parênquima, apresentando necrose tubular aguda, caracterizada por descamação de células necróticas e perda da membrana basal principalmente em túbulos contorcidos proximais, microangiopatia trombótica e glomerulopatia (MOHSIN et al., 2009; PÓVOA et al., 2014; REPIZO et al., 2014; UPADHAYA et al., 2010; WIERSINGA et al., 2006).

A avaliação histopatológica em placentas evidenciou alterações como hipóxia (causando edema do estroma das vilosidades, formação excessiva de nós sincicial e corioangiose), deciduites, coriodeciduites, intervilosites e villosites focais e multifocais, vilosidades necrosantes, vilosite proliferativa e vilosite necrosante multifocal, além de modificações inflamatórias (RIBEIRO et al., 2017).

## 1.10 DIAGNÓSTICO LABORATORIAL

O diagnóstico laboratorial é uma importante ferramenta para a confirmação da infecção viral, para a vigilância epidemiológica (principalmente em períodos inter-epidêmicos), estudos de patogênese, desenvolvimento de vacinas e medicamentos (GUZMÁN; KOURÍ, 2004; PEELING; SMITH; BOSSUYT, 2010; VORNDAM; KUNO, 1997). Além disso, é essencial para o diagnóstico diferencial de outras doenças clinicamente semelhantes ao dengue (MS, 2016a). A rapidez e precisão são imperativas no diagnóstico viral, tanto para o manejo clínico quanto para a vigilância e, para garantir que o tratamento precoce e as medidas de controle sejam realizados (SEKARAN; SOE, 2017).

O período da doença em que o paciente se encontra é importante para escolha do método mais adequado a ser utilizado e para a correta interpretação dos resultados obtidos (SIMMONS et al., 2012). O período de viremia ocorre antes mesmo do aparecimento dos primeiros sintomas, que permanece detectável durante a fase febril aguda, geralmente por três a cinco dias (VORNDAM; KUNO, 1997).

Na infecção primária, há uma resposta lenta e com baixo título de anticorpos IgM, que na maioria dos pacientes os níveis são detectáveis no sexto dia após o aparecimento dos sintomas. O pico ocorre por volta de duas semanas, mantendo-se detectáveis por 2 a 3 meses. Anticorpos IgG começam a ser detectados a partir do quinto dia de doença, na infecção primária e continuam detectáveis por toda a vida. Na infecção secundária, um alto título de IgG pode ser detectado na fase aguda. Por outro lado, os níveis de IgM, na resposta secundária, tendem a ser mais baixos do que na resposta primária (GUZMAN et al., 2010b).

O diagnóstico laboratorial das infecções pelos DENV pode ser realizado através do isolamento viral e/ou detecção do ácido nucléico viral, de técnicas sorológicas para detecção de anticorpos específicos (IgM/IgG), de antígeno (NS1) e pela detecção de antígenos virais em tecidos (FATIMA; WANG, 2015; GUZMAN et al., 2010b). A disponibilidade de métodos de detecção do ácido nucléico viral e sorológicos para a investigação de amostras coletadas em diferentes fases da doença é de suma importância (SEKARAN; SOE, 2017).

### 1.10.1 ISOLAMENTO VIRAL

O isolamento viral, considerado como “padrão ouro” na análise virológica, é capaz de evidenciar a infecção ativa (SEKARAN; SOE, 2017), pode ser realizado através da inoculação de amostras clínicas em uma variedade de linhagens de células de mosquito (AP61, TRA-284, AP64 e C6/36), células de mamíferos (LLCMK2, Vero e BHK-21), por inoculação intra-torácica em mosquitos adultos vivos e inoculação intra-cerebral em camundongos recém-nascidos (GUZMÁN; KOURÍ, 2002a; SHU; HUANG, 2004). O uso da linhagem celular clone C6/36 de *Aedes albopictus* tem sido o método de escolha, por sua facilidade de manutenção e sensibilidade (IGARASHI, 1978; KUNO; GÓMEZ; GUBLER, 1987). A identificação viral geralmente é observada pela presença do efeito citopático (ECP) e a confirmação do sorotipo

infectante é realizada por imunofluorescência utilizando anticorpos monoclonais específicos (GUBLER et al., 1984).

Amostras de sangue retiradas de pacientes infectados com até 5 dias após o início da doença produzem os resultados mais bem-sucedidos (TELES; PRAZERES; LIMA-FILHO, 2005). Embora a detecção de DENV pelo isolamento do vírus seja definitiva, não é particularmente prática, pois o isolamento pode levar de dias a semanas para ser finalizado (LANCIOTTI et al., 1992).

Apesar desta técnica detectar a partícula infectiva, ela foi gradualmente substituída pela transcrição reversa seguida da reação em cadeia pela polimerase (RT-PCR) e por ensaios imunoenzimáticos (ELISA) de captura do antígeno NS1 (SHU et al., 2004).

### 1.10.2 MÉTODOS SOROLÓGICOS

#### 1.10.2.1 TESTES IMUNOENZIMÁTICOS

Os testes imunoenzimáticos (ELISA) são os mais utilizados para confirmação laboratorial de rotina. Detectam imunoglobulinas da classe M (IgM, MAC-ELISA) e G (IgG, IgG-ELISA) produzidas após a infecção, e detecção de antígeno NS1 (NS1-ELISA) (MULLER; DEPELSENAIRE; YOUNG, 2017). Devido às respostas diferenciais de anticorpos desencadeadas pelo estado imunológico do hospedeiro, testes baseados na detecção de IgG e IgM específicos para dengue podem ser realizados utilizando soros pareados, coletados em um intervalo de tempo específico, para identificar a soroconversão entre amostras agudas e convalescentes (MARTINS et al., 2014), bem como para diferenciar entre infecções primárias e secundárias (SEKARAN; SOE, 2017).

Kits comercialmente disponíveis para detecção de IgM e IgG são muito utilizados, porém a sensibilidade e especificidade podem variar e, resultados falso-positivos podem ocorrer em pacientes com infecções prévias de dengue ou outros flavivírus, devido a epitopos de reação cruzada compartilhados na proteína E destes vírus (MULLER; DEPELSENAIRE; YOUNG, 2017; PEELING; SMITH; BOSSUYT, 2010).

A sNS1 pode ser encontrada circulando no soro de pacientes do primeiro ao nono dia após o início da febre (ALCON et al., 2002; FLAMAND et al., 1999; XU et al., 2006; YOUNG et al., 2000) e, aproveitando-se destas características, ensaios imunoenzimáticos para a detecção específica desta proteína, já foram desenvolvidos para a confirmação de casos de dengue (DUONG et al., 2011; DUSSART et al., 2006; KUMAR et al., 2018; LAPPHRA et al., 2008; NAWAZ et al., 2018; SÁNCHEZ-VARGAS; SÁNCHEZ-MARCE; VIVANCO-CID, 2014).

A NS1 pode ser detectada ao mesmo tempo que o RNA viral e antes que uma resposta de anticorpos em infecções primárias (ALCON-LEPODER et al., 2006; YOUNG et al., 2000).

O desenvolvimento comercial da NS1 como uma ferramenta de diagnóstico revolucionou o diagnóstico da dengue, uma vez que forneceu ensaios simples e de baixa tecnologia que têm alta sensibilidade e especificidade (BESSOFF et al., 2008; BLACKSELL et al., 2012; CHAIYARATANA et al., 2009; LAPPHRA et al., 2008; LIBRATY et al., 2002b; PHUONG et al., 2009), permitindo o diagnóstico precoce e o gerenciamento mais eficaz do paciente.

Testes de ELISA e imunocromatográficos rápidos para a detecção específica da proteína NS1 de DENV foram desenvolvidos e avaliados para a confirmação de casos (AMORIM et al., 2014; BLACKSELL et al., 2012; CHAIYARATANA et al., 2009; DUSSART et al., 2006a; LIMA et al., 2014; PHUONG et al., 2009). A sensibilidade pode exceder a 90% em amostras de pacientes com infecções primárias (CHATERJI et al., 2011; DUSSART et al., 2006b; TRICOU et al., 2011). No entanto, em infecções secundárias a antigenemia mostrou ser mais curta e com sensibilidade de 60-80% (GUZMAN et al., 2010a).

Nas infecções por DENV-4, o teste de captura de NS1 pode apresentar limitações devido à formação de imuno-complexos que podem não ser facilmente detectados. A utilização de Métodos de dissociação através do calor ou reagentes ácidos levam ao aumento da sensibilidade na detecção desse antígeno (LIMA et al., 2014)

#### 1.10.2.2 INIBIÇÃO DA HEMAGLUTINAÇÃO (IH)

O teste IH descrito por Clarke e Casals (1958) é considerado “padrão ouro” para a quantificação de anticorpos IgM e IgG, permitindo a caracterização do tipo de infecção em primária ou secundária. O aparecimento, aumento ou diminuição de quatro vezes no título de

anticorpos anti-DENV entre amostras pareadas de um mesmo paciente, confirma a soroconversão sorológica e, portanto, a infecção. No entanto, consiste em um método trabalhoso, além da necessidade de coletas pareadas (CLARKE; CASALS, 1958; NISALAK, 2015; NOGUEIRA; DOS SANTOS, 2015).

#### 1.10.2.3 TESTE DE NEUTRALIZAÇÃO POR REDUÇÃO DE PLACAS (PRNT)

O PRNT é considerado "padrão ouro" para a determinação de imunidade sorotipo específica aos DENV (CALISHER et al., 1989), sendo o teste recomendado pela OMS para os estudos de eficácia das vacinas em desenvolvimento (ROEHRIG; HOMBACH; BARRETT, 2008). No entanto, diante das características laboriosas desses testes, atualmente, na grande maioria dos casos, sua utilização está restrita a poucos laboratórios (CHATCHEN; SABCHAREON; SIRIVICHAYAKUL, 2017).

#### 1.10.2.4 IMUNO-HISTOQUÍMICA (IHQ)

Na investigação de casos fatais, o diagnóstico de dengue pode ser dificultado pela falta de soro ou de amostras de tecidos fresco congelados. As amostras de tecidos obtidas por autópsia são rotineiramente fixadas em formalina e embebida em parafina (FFPE), devendo, por tanto, serem analisadas por IHQ (BHATNAGAR et al., 2012; JESSIE et al., 2004). Esta técnica baseia-se, na conjugação de marcadores, como anticorpos, que com auxílio de um substrato específico localiza o antígeno tecidual. A positividade pode ser observada em vários órgãos, porém o fígado é o que tem apresentado maior percentual de confirmação e o órgão de escolha para identificação do DENV (NOGUEIRA; DOS SANTOS, 2015)

### 1.10.3 TRANSCRIÇÃO REVERSA SEGUIDA DA REAÇÃO EM CADEIA PELA POLIMERASE (RT-PCR)

Diversos protocolos de amplificação genômica utilizando a RT-PCR, importantes na identificação do sorotipo infectante, têm sido utilizados no diagnóstico rápido das infecções pelos DENV (DE PAULA et al., 2002; HARRIS et al., 1998; LANCIOTTI et al., 1992).

O protocolo mais utilizado é o descrito por Lanciotti *et al.* (1992), sugerido pela OMS, detecta os quatro sorotipos de DENV simultaneamente em um procedimento "*semi-nested"*, gerando produtos amplificados com tamanhos específicos em pares de base para cada sorotipo.

Avanços no diagnóstico molecular permitiram o desenvolvimento RT-PCR em tempo real (qRT-PCR) fundamentada no uso de corantes e sondas (SYBR green e TaqMan), que é realizado em uma única etapa e é capaz de fornecer dados quantitativos (HOLLAND et al., 1991; MULLER; DEPELSENAIRE; YOUNG, 2017). Além da quantificação, as vantagens do qRT-PCR sobre a RT-PCR convencional incluem rapidez, maior sensibilidade e especificidade (SEKARAN; SOE, 2017). Diversos protocolos para o diagnóstico ou para a quantificação dos DENV já foram descritos (DROSTEN et al., 2002; GO et al., 2016; GURUKUMAR et al., 2009; HUE et al., 2011; JOHNSON; RUSSELL; LANCIOTTI, 2005; KONG et al., 2006; WAGGONER et al., 2013).

As técnicas de RT-PCR e qRT-PCR podem ser aplicadas em amostras de soro, plasma, células e mosquitos infectados, tecidos frescos e FFPE. Porém, são limitadas a amostras coletadas durante a fase aguda da infecção, o que é útil apenas para diagnosticar pacientes de 0 a 5 dias após o início da infecção (MS, 2016a). Além disso, estes ensaios requerem infra-estrutura, aparelhos, reagentes caros e treinamento especializado (NISALAK, 2015).

## 1.11 MANEJO CLÍNICO E TRATAMENTO

A Secretaria de Vigilância em Saúde do Ministério da Saúde no Brasil revisou e atualizou o protocolo para o manejo clínico dos pacientes com dengue durante o ano de 2015, e as novas recomendações foram publicadas em 2016. Nesta, foi enfatizada a nova classificação de dengue, revisado os volumes de hidratação oral dos pacientes, a reposição volêmica e o monitoramento dos casos graves. Neste cenário, apenas o tratamento sintomático e os cuidados de suporte em um ambiente hospitalar estão disponíveis para os pacientes (MS, 2016a) e, apesar

dos esforços globais, não há uma terapia antiviral clinicamente aprovada, contra infecções por DENV

Desde o início dos anos 2000, vários compostos foram testados, mas nenhum foi capaz de demonstrar eficácia clínica (LOW et al., 2018c). Com a ocorrência de casos graves da doença é imperativo o desenvolvimento de modalidades terapêuticas eficazes frente às infecções por DENV e, neste contexto, diferentes classes de fármacos-candidatos têm sido aplicadas na última década (BOTTA et al., 2018).

## 1.12 PREVENÇÃO E CONTROLE

Diante da problemática da falta de uma vacina eficaz e segura, estratégias preventivas mais eficazes foram desenvolvidas sob a forma de controle vetores (DEROECK; DEEN; CLEMENS, 2003; RATHER et al., 2017), que englobam a vigilância, programas comunitários e a eliminação de potenciais fontes de criadouro (MACHADO; OLIVEIRA; SOUZA-SANTOS, 2009).

A vigilância possibilita a compreensão da distribuição espaço-temporal dos casos de dengue e fornece vínculos entomológicos e epidemiológicos para um melhor planejamento (SCARPINO; MEYERS; JOHANSSON, 2017; WHO, 2012). Programas de controle baseados na comunidade são desenvolvidos com o objetivo de fornecer informações sobre as medidas de eliminação dos criadouros de mosquitos e dependem do conhecimento, educação e comportamento dos envolvidos (VU et al., 2005).

Atualmente, o controle genético de *Ae. aegypti* tem crescido como um conjunto de técnicas promissoras, dentre as quais, a paratransgênese é um método popular (ARAÚJO et al., 2015; OGAUGWU; DURVASULA, 2017). Essa abordagem utiliza bactérias simbióticas geneticamente modificadas que são re-introduzidas no vetor, para colonizar a população vetorial, limitando assim a transmissão de doenças (ARAÚJO et al., 2015; WILKE; MARRELLI, 2015). O agente bacteriano mais eficaz utilizado é *Wolbachia* (JEFFERY et al., 2009; SALDAÑA; HEGDE; HUGHES, 2017), que é um parasita que interfere nos mecanismos celulares e reprodutivos de espécies de vetores (ARAÚJO et al., 2015; KAMTCHUM-TATUENE et al., 2017). A liberação de espécies de mosquitos geneticamente modificados, no Brasil, mostrou um declínio de 85% na população de *Ae. aegypti* (PAHO, 2014), indicando que

espécies vetoriais geneticamente modificadas são métodos inovadores e viáveis, usados para bloquear a transmissão de doenças transmitidas por mosquitos (FAVIA, 2015; FRASER, 2012).

Outra forma de controle é o uso de técnica de insetos estéreis, que se referem à liberação de vetores masculinos esterilizados, em laboratório, na população alvo. Uma vez liberados, esses mosquitos machos ajudam a suprimir a taxa de fecundidade em mosquitos fêmeas e, consequentemente, controlam a densidade vetorial em ambientes urbanos (DUMONT; CHIROLEU, 2010; YAKOB et al., 2017).

Inseticidas têm sido utilizados para controle de mosquitos por muitas décadas, no entanto, seu uso contínuo resultou em resistência na população-alvo e impactos negativos ao ambiente (ARAÚJO et al., 2015).

### 1.12.1 VACINAS

A existência de quatro sorotipos virais e a falta de um modelo animal adequado que reproduza a doença têm sido fatores limitantes e desafiantes para o desenvolvimento de uma vacina eficaz para as infecções pelos DENV (BEAUMIER et al., 2013; KATZELNICK; COLOMA; HARRIS, 2017; ROBINSON; DURBIN, 2017).

A vacina tetravalente Dengvaxia®, fabricada pela Sanofi Pasteur (Lyon, França) foi aprovada e licenciada em diversos países endêmicos. É uma quimera da dengue incorporada na cepa vacinal do vírus da febre amarela-17D (CYD-TDV) (GUIRAKHOO et al., 2001), para aplicação em indivíduos entre 9 e 45 anos (GUY et al., 2010).

Estudos com crianças no Sudeste da Ásia e América Latina demonstraram uma eficácia de 60% contra a doença sintomática e, eficácias ainda menores contra DENV-1 e DENV-2 (CAPEDING et al., 2014; DAYAN et al., 2013; FERGUSON et al., 2016; TORRESI; EBERT; PELLEGRINI, 2017; VILLAR et al., 2015). Porém, estudos nestas mesmas regiões reportaram um aumento inesperado na incidência de hospitalizações por dengue grave (HADINEGORO et al., 2016; WHO, 2018a), indicando que novas avaliações relacionadas a esta vacina, são necessárias (HALSTEAD, 2018; LIU; LIU; CHENG, 2016; TORRESI; EBERT; PELLEGRINI, 2017).

Estudos de fases II e III conduzidos no Brasil, demonstraram um aumento de casos de dengue grave e hospitalização nos indivíduos previamente expostos ao DENV. Diante destas

observações, em 28/11/2017, a Agência Nacional de Vigilância Sanitária (Anvisa) recomendou que a vacina Dengvaxia® não seja administrada em indivíduos sem exposição prévia ao DENV e que novos estudos sejam realizados (ANVISA, 2017).

Em abril de 2018, o Grupo Estratégico de Especialistas da OMS recomendou que em países considerando a introdução desta vacina, uma triagem sorológica pré-vacinal seja realizada e somente pessoas soropositivas para dengue devem ser vacinadas (ARIËN; WILDER-SMITH, 2018).

O Instituto Nacional de Alergia e Doenças Infecciosas (do inglês "National Institute of Allergy and Infectious Diseases" - NIAID), dos Estados Unidos, projetou candidatos recombinantes atenuados de DENV recombinantes, ao introduzir mutações na região não traduzida do genoma da dengue, ou recombinação de proteínas estruturais em uma cepa vacinal atenuada (BLANEY et al., 2006). Monovalente ou tetravalente (TV003) induziram, com segurança, uma resposta imunológica para todos os sorotipos em participantes não expostos aos Flavivirus (DURBIN et al., 2013, 2016). Participantes previamente expostos aos Flavivirus, após uma dose da vacina candidata tetravalente, 76% apresentaram viremia e 87% tinham anticorpos responsivos a todos os quatro sorotipos (WHITEHEAD et al., 2017). Uma segunda dose de vacina teve pouco impacto adicional e a vacina foi bem tolerada. O NIAID transferiu a tecnologia para o Instituto Butantan, para desenvolver a vacina e realizar testes clínicos no Brasil. A vacina produzida pelo Butantan está sendo testada, desde 2016, em 14 instituições de pesquisas, em um estudo randomizado fase III, patrocinado pelo governo federal do Brasil (ANVISA, 2016; GODÓI et al., 2017; NIH, 2019; SES/SP, 2016).

No entanto, diversas outras abordagens para o desenvolvimento de uma vacina eficaz, encontram-se em andamento e em avaliação, como vírus inativados, vacinas recombinantes, quiméricas e de DNA (GUY; JACKSON, 2016; KIRKPATRICK et al., 2015; OSORIO et al., 2015; SCHMITZ et al., 2011).

## 2 JUSTIFICATIVA

Globalmente, estima-se que uma média de 9 mil óbitos por dengue tenha ocorrido entre 1990 e 2013 (STANAWAY et al., 2016). Nas Américas, a dengue tem um padrão endêmico-epidêmico, com surtos que ocorrem a cada 3 a 5 anos (BRATHWAITE DICK et al., 2012) e, de 1995 a 2015, mais de 18 milhões de casos foram registrados em todo o continente americano, com cerca de 14 milhões registrados apenas em países da América do Sul. Nesse cenário, o Brasil contribuiu com 55% dos casos registrados nas Américas. Um total de 8.788 casos fatais foi confirmado nas Américas, sendo o Brasil responsável por 48% destes casos (PAHO, 2018).

A dispersão dos DENV pelo território brasileiro, com um aumento nas notificações de casos graves e óbitos ressalta a importância da vigilância destes agentes no país. Neste cenário, o laboratório possui um papel fundamental atuando constantemente no monitoramento deste agravo. O Laboratório de Flavivírus (LABFLA) IOC/ FIOCRUZ, estabelecido desde 1986, é Centro de Referência Regional de Dengue, Febre Amarela, Zika e Chikungunya e têm recebido casos suspeitos e óbitos por dengue ao longo dos últimos 32 anos (DOS SANTOS et al., 2013).

Diante do crescente número de óbitos, estudos que investiguem marcadores precoces de evolução de gravidade têm sido realizados para a melhor compreensão da patogênese. Vários fatores de risco para a doença grave foram determinados, incluindo a exposição a um sorotipo heterólogo de DENV, infecção por certos sorotipos e/ou genótipos, viremia, antigenemia de NS1, idade, sexo e algumas variantes genéticas do hospedeiro (ALLONSO et al., 2014; AVIRUTNAN et al., 2006; BEATTY et al., 2015; DE ARAÚJO et al., 2009b; GLASNER et al., 2017; KATZELNICK; HARRIS, 2018; LIBRATY et al., 2002a; NUNES et al., 2016; PUERTA-GUARDO; GLASNER; HARRIS, 2016; TRICOU et al., 2011).

Além disso, estudos in vivo sobre a infecção por DENV são limitados pela falta de um modelo animal experimental adequado, capaz de mimetizar todo o espectro da doença como observado em humanos (YAUCH; SHRESTA, 2008). Desta forma, estudos histopatológicos post-mortem são extremamente importantes e podem auxiliar na identificação de preditores precoces da gravidade da doença e, consequentemente, em intervenções clínicas adequadas (AHSAN; AHMAD; RAFI, 2018a). Diante do exposto, este estudo visa analisar os dados epidemiológicos, laboratoriais, virológicos e histopatológicos dos casos fatais suspeitos de dengue, ocorridos no Brasil entre 1986 e 2015.

# 3 OBJETIVOS

## 3.1 OBJETIVO GERAL:

Analisar os aspectos epidemiológicos, virológicos e histopatológicos de casos fatais por dengue ocorridos no Brasil em 30 anos (1986 e 2015).

## 3.2 OBJETIVOS ESPECÍFICOS:

- Descrever os aspectos epidemiológicos dos casos fatais de dengue disponíveis na base de dados do Ministério da Saúde, ocorridos no Brasil, no período de 1986 a 2015;
- Descrever os aspectos epidemiológicos, clínicos e laboratoriais dos casos fatais suspeitos de dengue recebidos no LABFLA, IOC/FIOCRUZ no período de 1986 a 2015;
- Analisar a viremia e antigenemia da NS1 como potenciais marcadores de evolução ao óbito;
- Investigar as alterações histopatológicas, presença de marcadores virais e mediadores inflamatórios em tecidos de casos de óbitos materno e fetal.

# 4 RESULTADOS

Os resultados obtidos serão apresentados sob a forma de artigos científicos publicados e/ou fase de submissão, e serão listados na ordem em que serão apresentados e discutidos.

## 4.1 ARTIGO 1: 30 ANOS DE CASOS FATAIS DE DENGUE NO BRASIL: UMA VISÃO RETROSPECTIVA

**Revista:** BMC Public Health

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**Resumo:** Nos últimos 30 anos, epidemias extensas de dengue ocorreram no Brasil, caracterizado por emergências e re-emergências de diferentes sorotipos, mudança no perfil epidemiológico e aumento do número de casos graves e fatais. Desta forma, descrevemos uma revisão sobre os óbitos por dengue ocorridos no país entre 1986 a 2015. Foram utilizados os dados secundários epidemiológicos sobre casos fatais de dengue obtidos no Sistema de Informação de Agravos de Notificação (SINAN) e o Sistema de Informações sobre Mortalidade (SIM), ambos mantidos pelo Ministério da Saúde. Os casos foram analisados por região, variáveis demográficas, classificação clínica, com base nos dados disponíveis. Durante os 30 anos (1986-2015), a região Sudeste registrou 43% (n = 2.225) de todos os óbitos por dengue no país, seguida pela região Centro-Oeste, responsável por 18% dos casos fatais. Após o ano 2000, os óbitos ocorreram em todos os estados, com exceção de Santa Catarina e Rio Grande do Sul. De 2006 à 2010, um aumento no número de óbtitos foi reportado, com maior mortalidade, especialmente em Goiás e Mato Grosso. Entre 2011 e 2015, Goiás tornou-se o estado com maior taxa de mortalidade de todo o país, e o Rio Grande do Sul relatou os primeiros óbitos por dengue. Uma distribuição homogênea entre o sexo foi observada, com um total de 2.682 óbitos provenientes do sexo masculino e 2.455 do sexo feminino. Os casos fatais por dengue foram mais frequentes nos indivíduos acima de 15 anos (1986 a 2006), mas este cenário mudou em 2007-2008, com taxas de mortalidade mais elevadas em crianças até 14 anos. Grande parte dos óbitos relacionadas à dengue são sub-notificados, mesmo após a experiência de 30 anos no país. Atualmente, o Brasil vive num cenário hiperendêmico, com a cocirculação dos quatro sorotipos, e com a crescente ocorrência de casos graves e fatais. Neste contexto, estudos de vigilância da doença constituem ferramentas importantes para a compreensão dos fatores envolvidos no desfecho do óbito e desta forma, no manejo mais adequado e eficaz para a redução da gravidade da doença.

BMC Public Health

# 30 years of fatal dengue cases in Brazil: a review

--Manuscript Draft--

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| Abstract: | Background: Over the last 30 years, extensive dengue epidemics have occurred in Brazil, characterized by emergencies and re-emergencies of different serotypes, a change in the epidemiological profile and an increase in the number of severe and fatal cases. Here, we present a review on the dengue fatal cases occurred in Brazil in 30 years (1986-2015).<br>Methods: We performed an ecological study by using secondary data from the epidemiological data on dengue fatal cases obtained in the National System of Reported Diseases (Sistema de Informação de Agravos de Notificação -SINAN) and the Mortality Information System (SIM), both maintained by the Ministry of Health. Cases were analyzed by region, demographic variables, clinical classification and complications based on the data available.<br>Results: In 30 years (1986-2015), the Southeast region reported 43% (n=2,225) of all dengue deaths in the country. The Midwest region was responsible for 18% of the fatal cases. After 2000, deaths occurred in almost all states, with the exception of Santa Catarina and Rio Grande do Sul, South region. From 2006 to 2010, the number of deaths increased, with higher rates mortality, especially in Goiás and Mato Grosso. From 2011-2015, Goiás became the state with the highest mortality rate in the country, and Rio Grande do Sul reported its first dengue deaths. In 30 years, a total of 2,682 dengue deaths were of males and 2,455 females and a homogeneous distribution between the sex was observed. From 1986 to 2006, dengue deaths occurred predominantly in individuals over 15 years old, but this scenario changed in 2007-2008. After 2009, fatal cases on individuals above 15 years old became more frequent, with peaks in the years 2010, 2013 and 2015.<br>Conclusions: There are many dengue-related deaths underestimated in many health services, even after 30 years of dengue surveillance in Brazil. Currently, the country is experiencing a hyperendemic scenario, with the co-circulation of the four DENV serotypes and with the increasing occurrence of severe and fatal cases. The disease surveillance and studies characterizing what has been reported over time, are still important tools to better understand the factors involved in disease outcome. |
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# 30 years of fatal dengue cases in Brazil: a review

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## Abstract

Background: Over the last 30 years, extensive dengue epidemics have occurred in Brazil, characterized by emergences and re-emergences of different serotypes, a change in the epidemiological profile and an increase in the number of severe and fatal cases. Here, we present a review on the dengue fatal cases that occurred in Brazil in 30 years (1986-2015).

Methods: We performed an ecological study by using secondary data on dengue fatal cases obtained in the National System of Reported Diseases (Sistema de Informação de Agravos de Notificação -SINAN) and in the Mortality Information System (SIM), both maintained by the Brazilian Ministry of Health. Cases were analyzed by region, demographic variables, clinical classification and complications based on the data available.

Results: In 30 years (1986-2015), the Southeast region reported 43% (n=2,225) of all dengue deaths in the country. The Midwest region was responsible for 18% of the fatal cases. After 2000, deaths occurred in almost all states, with the exception of Santa Catarina and Rio Grande do Sul, South region. From 2006 to 2010, the number of deaths increased, with higher rates of mortality, especially in Goiás and Mato Grosso. From 2011-2015, Goiás became the state with the highest mortality rate in the country, and Rio Grande do Sul reported its first dengue deaths. In 30 years, a total of 2,682 dengue deaths occurred in males and 2,455 in females, and an equal distribution between the sexes was observed. From 1986 to 2006, dengue deaths occurred predominantly in individuals over 15 years old, but this scenario changed in 2007-2008.

After 2009, fatal cases on individuals above 15 years old became more frequent, with peaks in the years of 2010, 2013 and 2015.

Conclusions: The Brazil is experiencing a hyperendemic scenario, which has resulted in the co-circulation of the four DENV serotypes and with the increasing occurrence of severe and fatal cases. The disease surveillance and studies characterizing what has been reported overtime, are still important tools to better understand the factors involved in the disease outcome.

## Background

Dengue viruses (DENV) are arboviruses belonging to the *Flaviviridae* family and the genus *Flavivirus*, and are represented by four antigenically distinct serotypes (DENV-1 to 4) causing a mild self-limiting illness or more severe forms of the disease and death [1]. According to WHO [1], currently dengue cases can be classified as dengue without warning signs, dengue with warning signs and severe dengue. A severe dengue case is characterized by severe bleeding, severe organ involvement and severe plasma leakage. The viruses are responsible for high rates of disease and mortality [2] Dengue is a mosquito-borne viral disease endemic in several tropical and sub-tropical countries worldwide and, in recent decades the disease has grown drastically throughout the world [3]. Globally, it is estimated an average of 9 thousand dengue deaths per year from 1990 to 2013 have occurred [4]. In the Americas, dengue has an endemo-epidemic pattern with outbreaks occurring every 3 to 5 years [5].

From 1995 to 2015, more than 18 million cases of dengue were reported throughout the American continent and, about 14 million cases were reported only in South American countries. Brazil contributed 55% of the cases reported in the Americas over this period.

A total of 8,788 fatal cases were confirmed in the Americas, and Brazil accounted for 48% of those cases [6]. Despite that, dengue cases are still underreported and many cases are incorrectly classified, with one notification for every twenty cases of dengue fever (95%) [7, 8].

Over the last 30 years, extensive dengue epidemics have occurred in Brazil, characterized by the emergence and re-emergence of different serotypes, a change in the epidemiological profile and an increase in the number of severe and fatal cases. Here, our goal is to present a review on the fatal dengue cases that occurred in Brazil over 30 years (1986-2015) based on the Brazilian Dengue Surveillance Systems, as understanding the patterns of case fatalities, may be critical for dengue case management in the country.

## Methods

We performed an ecological study by using secondary data from the dengue epidemics available in Brazil. Official data on dengue fatal cases occurred from 1986 to 2013, from TabNet (DATASUS) from the National System of Reported Diseases (Sistema de Informação de Agravos de Notificação -SINAN) and from the Mortality Information System (SIM), both maintained by the Brazilian Ministry of Health (MoH), were obtained. Cases occurring in 2014 and 2015 were obtained from epidemiological reports available at http://portalsaude.saude.gov.br/index.php/situacao-epidemiologica-dados-dengue.

Dengue severity was considered according to the final classification of the Brazilian MoH and to the epidemiological reports available, as follows: Dengue with

complications (DCC), Dengue Hemorrhagic Fever (DHF), Dengue Shock Syndrome (DSS) and Severe Dengue (SD). In this study, the 1997 World Health Organization (WHO) dengue case classification (DHF and DSS) was used from 1986 to 2000. From 2000 to 2013, the Brazilian MoH DCC classification was used to define severe dengue cases that did not meet the WHO criteria for DHF/DSS and, from 2014 and on, the 2009 WHO dengue case classification, Dengue with warning signs (DwWS) and SD were employed [1–6, 9]. Here, we considered dengue deaths to be reported in the SINAN database filled out as "death due to dengue," or from the SIM database where cause of death was with the code "A90" or "A91," according to the 10th International Classification of Diseases (ICD-10).

The case fatality rate of each classification was calculated using number of deaths from DHF/DSS, DCC, DwWS or SD per number of confirmed cases from each classification x 100. The overall fatality rate was calculated by the sum of each classification per number of dengue confirmed cases x 100. The mortality rate was calculated using the number of deaths per dengue per total number of the locality's population, per year x 100,000 inhabitants. The population data of each year and by region were obtained from Instituto Brasileiro de Geografia e Estatística (IBGE) available at https://www.ibge.gov.br. Cases were analyzed by regions, demographic variables and clinical classification based on the data available on the reporting and investigation forms using a database in Excel Software.

Odds ratio (OR) of dengue fatal cases occurred in Brazil from 1987-2015 was calculated with a 95% confidence interval (CI) and *p*-values for each year, with 1986 as the reference year. The analysis was performed by GraphPad Prism software version 6.

We used 1986 as the reference year as it was the first year of dengue introduction in Brazil and the first year of data availability on the Brazilian MoH database.

## Results

### Overview on dengue epidemics in Brazil

The first reports of a disease with signs and symptoms compatible with dengue fever in Brazil, date back to 1846 [10]. Late 1981 and early 1982, a first dengue outbreak, caused by DENV-1 and DENV-4 was characterized in Brazil, which was restricted to the city of Boa Vista, Roraima (RR) in the north region [11]. In1986, after 4 years without dengue cases confirmation, an epidemic occurred due to the DENV-1 introduction in the state of Rio de Janeiro (RJ), which spread to other states [12]. Five fatal cases were confirmed in 1986. DENV-2 was detected, for the first time in RJ, in 1990 [13], when the first DHF/SCD cases occurred (n=8). In the following years, DENV-1 and DENV-2 co-circulated and caused epidemics throughout the country [14] . Through 1999, a total of 75 fatal cases were reported (1991-1999), Figure 1.

In December 2000, a newly introduced serotype, DENV-3, was initially detected in RJ [15] and quickly spread to other states of the country. The 2002 epidemic, caused mainly by DENV-3, was the largest and most severe epidemic experienced in the country so far, with increased hospitalizations and 150 deaths confirmed. It was suggested that, the introduction of a new serotype of Asian origin (Genotype III), would have been an explanation for the severity of the epidemic [16]. Despite the prevalence of Genotype III in Brazil, the co-circulation of Genotypes III and V was detected in Rondônia [17]. In 2002, the number of DHF deaths exceeded malaria deaths for the first time in the country [14].

Seventeen years after its introduction, DENV-2 reemerged in 2007 causing a major epidemic in 2008, with a higher proportion of DHF, more than double the number of cases reported in previous years [18]. A total of 561 deaths, mainly caused by this serotype, were reported only in that year (Figure 1A). Oliveira et al. [19] observed that the DENV-2 emerged in the 2008 epidemic was genetically different from the strain introduced in 1990, and despite belonging to the same genotype, those viruses were considered a new lineage (Lineage II). Studies by Faria et al. [20] concluded that there were no nucleotide changes between the two strains that led to an increase in the severity of Lineage II viruses. On the other hand, Nunes et al. [21] demonstrated that the viremia of the DENV- 2 Lineage I cases was lower than that observed by Lineage II cases. Furthermore, severe cases caused by Lineage II, had 1,000 times more circulating virus than those from Lineage I. The factors that led to the severity of this particular epidemic are still unclear. However, one cannot exclude the difficulties experienced in public health systems in controlling the epidemic, and the situation caused panic and insecurity throughout the Brazilian society [22, 23].

In 2009, DENV-1 reemerged with the possibility of a new epidemic, considering the low circulation of this serotype since the beginning of the decade. The 2010 epidemic presented a pattern quite different from the 2002 and 2008 epidemics, with the highest number of deaths (n= 656), reported. The DENV-1 isolated in Brazil between 2009 and 2010, belonged to Genotype V (American /African) and grouped in a clade (Lineage II) distinct from that of the previous isolates (Lineage I). Moreover, strains isolated in 2011 grouped in another distinct clade (Lineage III) [24]. The introduction of new strains resulted in the substitution of the circulating lineage and the increase in the genetic diversity of DENV-1, probably as a result of local evolution, or introduction of exogenous viruses during the same period or at different times [25].

In 2010, the risk of DENV-4 reintroduction into the country was imminent, as this serotype circulated in neighboring countries such as Venezuela and Colombia [26]. However, only in July of 2010, the first DENV-4 cases were identified in RR and Amazonas (AM), about 30 years after its first detection in the country. Less than 20 cases of DENV-4 were confirmed during the second half of 2010, and the first cases resulting from the spread of the virus, were detected only in January 2011, isolated in Amazonas and Pará. In March of 2011, the first DENV-4 cases were reported in RJ, introduced by the municipality of Niteroi [27, 28].

An increase in deaths was evidenced, especially in 2015, with an explosive epidemic of 1,649,008 dengue cases reported and 986 fatal cases confirmed. In 30 years a total of 11,084,755 suspected dengue cases were reported, with the confirmation of 5,399 deaths nationwide (Figure 1). The years that had the greatest chances of death were 2007-2009 (CI 95% 2.23-19.8), mainly due to the DENV-2 epidemic. The years of 2014-2015 had OR of 3.08 - 17.97 and, despite the co-circulation of the four serotypes, DENV-4 was predominant. In 2010-2011 OR were (CI 95% 2.492 - 14.48), when DENV-1 reemerged and DENV-4 was introduced (Table 1).

## Fatal dengue and regions

In 30 years, the Southeast region reported 43% (n=2,225) of all dengue deaths in the country. São Paulo (SP) confirmed 945 fatal cases, RJ, 738, Minas Gerais (MS) and Espírito Santo (ES) registered 430 and 196 deaths, respectively. In the Northeast, the states with the highest number of fatal cases were Ceará (CE) with 506, Pernambuco (PE) with 277, Bahia (BA) with 228 and Maranhão (MA) with 166. The Midwest region was responsible for 18% of the fatal cases, where the state of Goiás (GO)

reported 600 deaths, Mato Grosso (MT), 187, Mato Grosso do Sul (MS), 128 and Distrito Federal (DF), 66. In the North region, only 7% of the deaths were confirmed. Pará (PA) was the state that reported the highest number of dengue deaths (n=141) in the period. The South region, historically less affected by dengue cases, reported consequently the lowest number of dengue fatal cases (2%). Only Paraná (PR) (n=108) and Rio Grande do Sul (RS) (n = 4) reported dengue deaths.

During 30 years of epidemics, we have observed that RJ historically contributed to the introduction and dissemination of DENV-1, 2 and 3, and since then, has constantly reported dengue fatal cases (Figure 2). After 2000, deaths occurred in almost all states, with the exception of Santa Catarina (SC) and RS. From 2006 to 2010, possibly due to the introduction of DENV-3 and DENV-4, and re-emergence of DENV-1 and DENV-2, the number of deaths increased, with higher mortality rates in the states of RJ, Sergipe (SE), MS, Rondônia (RO) and RR. Rates were even higher in GO, but the state of MT had the highest mortality rate in this period. From 2011-2015, GO became the state with the highest mortality rate in the country, and RS reported the first dengue fatal outcomes.

The analysis of the mortality rates by municipality showed an increase of dengue fatal cases and distribution by the Brazilian territory over the years. In 2008, the North, Northeast and Southeast regions had higher mortality rates. In 2009, dengue deaths were distributed in the North and Midwest regions. From 2010 to 2012, dengue deaths occurred throughout the Brazilian regions (Figure 2).

## Dengue classification

According to the WHO [9] dengue case criteria, infections were classified as dengue fever (DF), DHF and DSS. However, in 2000, the Brazilian MoH proposed the DCC classification, to define severe dengue cases that did not meet the WHO criteria for DHF/DSS. From January 2014 and on, Brazil adopted the new WHO 2009 classification. Therefore, in this analysis, DHF/DSS, DCC, DwWS and SD denominations were used, considering the epidemic year analyzed. The timeline and the characteristics of each classification are available in table 2.

DHF cases fatality rates were high in 1994, 1997, 1998, 2006, 2012, and 2013. By DCC, deaths were more frequently reported in 2003, 2006, 2007, with increasing numbers from 2008 to 2013 and the latter, being the highest peak of DCC mortality. Considering the new classification, 3% of DwWS patients died in 2014. In 2014 and 2015, 8% and 7% of SD cases died, respectively (Figure 3A).

The five-year case fatality rate of each state is shown in Figure 4. From 1986 to 1990 the DHF fatality rate was up to 10% in RJ and Alagoas (AL). From 1991 to 1995, only RJ reported a DHF fatality rate up to 10%. That rate was five times higher (up to 50%) in CE, from 1996 to 2001. On the following years (2001 to 2005), fatality rates were up to 50% in MS, followed by GO (up to 40%), PB (up to 20%), and RJ, ES and PE with up to 10% of DHF cases evolving to death. From 2006 to 2010, this scenario changed and case fatality rate increased in almost all states, being higher in DF (up to 50%), PR (up to 40%), PA and RR (up to 30%) and TO (up to 20%). From 2011-2013, the states of PR, SP, MG, TO, PI, AP had up to 20% DHF fatality rate, however those were higher in CE with 21-30% and DF with 31-40% (Figure 4A). In the years of 1999-2003,

11 to 20% of the cases with DCC died in RR, and in MT, MS, PR, SP, RJ, BA, SE, AL, CE, MA and AP, up to 10% (Figure 4B).

From 2004 to 2008, increased numbers of fatal cases by DCC were also reported in other Brazilian states, and AC, MS, PR reported up to 20%, RJ up to 30% and DF up to 40% of fatality rates, Figure 3B. In 2009 and 2013 the number of deaths was lower, and the states of MA, CE, MS, MG, RJ, ES and PR had up to 20% of case fatality rate. Only the state of SC did not report fatal cases by DCC (Figure 4B).

Considering the new WHO [1] criteria, case fatality rate by DwWS in PA, AC, RO, MT, MS, PR, SP, MG, RJ, ES, BA, CE RN and PE was around 5%, however in PB and AM, it reached 15% in 2014-2015 (Figure 4C). In the same period, deaths from SD occurred in all states, except in SC, with high case fatality rates in the states of AC and RS (100%), AM, PB and DF with 81 to 99%, MG, SP, MS, MT, MA, PE, PB, CE, PA, RO with 51 to 80%, RJ, ES, BA, GO, TO, RR, SE, AL with 21 to 51% and in PR, PE and AP, with up to 20%, Figure 4D.

## Demographic variables associated to dengue fatal cases: sex and age

In the 30 years period (1986-2015), a total of 2,682 dengue deaths occurred on males and 2,455 on females and, during the years, an equal distribution between the sexes, was observed.

From 1986 to 2006, dengue deaths occurred more often in individuals over 15 years old, Figure 4B. This changed in 2007-2008, with the DENV-2 re-emergence, as more than

53% of the dengue deaths cases occurred in children 15 years old and under. In 2008 alone, 190 fatal cases on children from that age group were reported (Figure 3C). After 2009, there was a decrease in fatal cases in children 15 years old and under, while fatal cases on individuals above 15 years old became more frequent, with peaks in the years 2010, 2013 and 2015.

## Discussion

The consecutive introduction of distinct DENV serotypes overtime, resulted in a hyperendemic scenario, with the co-circulation of all serotypes and, an increase in deaths, was evidenced, especially in 2015. However, the years that had the greatest chances of death were between 2007 and 2009, mainly due to the DENV-2 epidemic.

As Brazil is the second largest and most populated country in the Americas, it is important to understand the contribution of the distinct regions in the occurrence of dengue deaths. Historically, the regions in the country with highest dengue incidences and fatal cases have been the Southeast, followed by the Northeast region. During 30 years of epidemics, RJ, in the Southeast region, has historically contributed to the introduction and dissemination of three of the four DENV serotypes (DENV-1 to 3), and since then, has constantly reported dengue fatal cases.

One well-characterized study by Paixão [29] analyzed the trends and factors associated with dengue mortality and fatality in Brazil from 2001 to 2011, and reported the results on the analysis of 3,156 deaths. It was shown that the Southeast and Northeast regions accounted for more than 70% of fatal cases. Moreover, mortality rates increased during the period and that the factors associated with mortality were inequality, high income per capita and higher populations inhabiting urban areas [29].

According to the WHO [9] dengue case criteria, infections were classified as dengue fever (DF), DHF and DSS. However, due to difficulties in using this classification [30], mostly due to changes in the disease epidemiology, a new classification was needed. In 2000, the Brazilian MoH proposed the DCC classification, to define severe dengue cases that did not meet the WHO criteria for DHF/DSS [31, 32]. DCC was characterized when the dengue patient presented at least one of the following: neurological abnormalities, liver failure, cardiorespiratory dysfunction, gastrointestinal bleeding, low platelet count (leukocyte count ≤ 1,000 cells/ml), pleural and pericardial effusion and ascites or death. It was a mandatory classification after 2007 [33].

Based on the results of a multicenter study (Dengue Control, DENCO) to assess the limitations of the 1997 WHO classification, experts from dengue endemic regions agreed on a binary classification represented by two clear entities, severe dengue and dengue and, the term “non-severe dengue” should be avoided, as any dengue case can become severe. Moreover, it was shown that patients exhibiting warning signs are at increased risk of severe disease progression and deserve careful observation [34]. This new classification proposed in 2009, characterized dengue infections in dengue without signs (DWoWS), DwWS and SD [1, 9, 35]. From January 2014 and on, Brazil adopted this new proposed classification. Therefore, in this analysis, DHF/DSS, DCC, DwWS and SD denominations were used, considering the epidemic year analyzed.

A higher sensitivity to detect increased disease severity has been shown by the new WHO 2009 dengue classification [36–39]. Its specificity, however, is much lower (73.0%) compared to the 1997 classification (93.4%). The higher sensitivity allows better patients' management, reducing mortality [40, 41], on the other hand, may also result in the misclassification of some severe cases [42]. In fact, the lower specificity of

this new classification is attributed, partly, to the lack of clear criteria for the definition of the warning signs [43].

DHF cases fatality rates were high in 1994, 1997, 1998, 2006, 2012, and 2013. By DCC, deaths were more frequently reported in 2003, 2006, 2007, with increasing numbers from 2008 to 2013. In 2014, 3% of DwWS patients died, while in 2014 and 2015, 8% and 7% of SD cases died, respectively. From 1986 to 1990 the DHF fatality rate was up to 10% in RJ and AL, but was five times higher in CE, from 1996 to 2001, however, from 2006 to 2010, case fatality rates increased in almost all states.

Sex has also been considered by some authors, as risk factor for the disease severity. Studies in Asia and the Americas, show that women are more likely to have the disease and are at greater risk of developing more severe forms than men [44–47]. In the 30 years period, an equal distribution of dengue fatal cases was observed between the sexes. Previous studies on dengue incidence have sometimes found equal attack rates between the sexes [48–51], and sometimes found uneven distribution of cases, with no clear tendency for males or females to be more affected [45, 52–55].

From 1986 to 2006, dengue deaths occurred more often in individuals over 15 years old. This changed in 2007-2008, with the DENV-2 re-emergence, as more than 53% of the dengue deaths cases occurred in children 15 years old and under [16]. Likewise, the study by Paixão et al. [29] analyzing dengue mortality from 2001 to 2011 in Brazil, showed the highest DHF case fatality rates on individuals over 15 years old and especially on those 80 years old and over. However, children under 1 year old experienced increased fatality rates.

After 2009, there was a decrease in fatal cases in children 15 years old and under, while fatal cases on individuals above 15 years old became more frequent, especially in the years of 2010, 2013 and 2015. The increased risk of death in the older age group may be associated with the difficulty in managing the disease in a population with a high frequency of comorbidities [56]. Cases coincident with sickle cell anemia, autoimmune diseases, asthma, hypertension, uremia and diabetes mellitus have been described in more severe outcomes of dengue [56–60].

## Conclusions

Currently, Brazil is experiencing a hyperendemic scenario, with the co-circulation of the four DENV serotypes and occurrence of severe and fatal cases and, more recently, the co-circulation with other arboviruses such as Zika, Yellow Fever and Chikungunya, Therefore, the possibility of misdiagnosis and even co-infections in a same individual and the its impact in the disease outcome, can not be neglected and need further investigation.

One point to be addressed here and pointed out in a previous study, is the challenge in determining whether a death occurs *due to* DENV infection or in a patient *with* DENV infection, meaning the disease is the cause of death or is the underlying cause of it [61]. Either way, the disease surveillance and studies characterizing what has been reported overtime, are still important tools to better understand the factors involved on the disease outcome.

It is a fact that, there are many dengue-related deaths underestimated in many health services, even after 30 years of dengue surveillance in Brazil and it has been shown that

the structuring and organization of surveillance, autopsy and laboratory teams, may significantly improve this scenario [61–63].

Despite that, the use of secondary data as those analyzed here, imposes some limitations to the study and those include the lack of some clinical and/or demographic information, description of disease course during hospitalization and until death, delay in diagnosis and low adherence to notification by health professionals. Dengue cases are under-reported in Brazil and improvements are needed in the proper filing of report forms [7, 64–66].

## Declarations

### Ethical approval and consent to participate

This is an anonymous secondary data-based study from National available databases and it is part of a goal from an ongoing Project approved by the Oswaldo Cruz Institute Ethical Committee (CAAE 57221416.0.1001.5248). Consent to participate not applicable.

### Consent for publication

No applicable.

### Availability of data and material

All the data generated or analyzed in this study are included in this manuscript.

### Competing interests

The authors declare no conflict of interest exists.

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## Author's contributions

PCGN, RPD and FBS designed the study. PCGN, RDP, MAPH and JCSA performed the analysis. PCGN, RDP and FBS wrote the paper. All authors read and approved the final manuscript.

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## Table titles

Table 1: Odds Ratio of dengue fatal cases occurred in Brazil from 1987-2015, considering the first epidemic year (1986).

Table 2: Timeline and characteristics of dengue classifications used over 30 years of dengue fatal cases investigation in Brazil.

## Figure legends

Figure 1: Dengue cases and dengue fatal cases reported in Brazil in 30 years (1986 to 2015). The bars show the number of dengue cases reportedThe numbers of deaths are shown in lines and *y* axis to the right. The colored squares demonstrate the introduction and re-emergence of the distinct dengue serotypes.

Figure 2: Five-year dengue mortality rate per state, Brazil, 1986-2015. Mortality rate per 100,000 populations.

State abbreviations: Acre (AC); Alagoas (AL); Amapá (AP); Amazonas (AM); Bahia (BA); Ceará (CE); Distrito Federal (DF); Espírito Santo (ES); Goiás (GO); Maranhão (MA); Mato Grosso (MT); Mato Grosso do Sul (MS); Minas Gerais (MG); Pará (PA); Paraíba (PB); Paraná (PR); Pernambuco (PE); Piauí (PI); Roraima (RR); Rondônia (RO); Rio de Janeiro (RJ); Rio Grande do Norte (RN); Rio Grande do Sul (RS); Santa Catarina (SC); São Paulo (SP); Sergipe (SE); Tocantins (TO).

Figure 3: (A) 30-year dengue cases fatality rate by DHF, DCC, DwWS and SD and (B) distribution of mortality rates (per 100,000 populations) by age and year of occurrence, Brazil, 1986-2015.

In figure A: Dengue case fatality rate is demonstrated in percentage (%). The bars show the fatality rate by the Dengue Hemorrhagic Fever (DHF) and Severe Dengue (SD) classifications. Dengue cases fatality rate with Dengue with Complications (DCC) and Dengue with Warning Signs (DwWS) are shown in lines. The axes *y* left are of the rates

by the classification of DHF and DCC, whereas the axis *y* right are the values of the rates classified with SD and DwWs.

Figure 4: Five-year dengue case fatality rate per state from 1986 to 2015, Brazil. Case fatality rate by (A) DHF; (B) by DCC; (C) by DwWS and (D) by SD.

Dengue case fatality rate is demonstrated in percentage (%). DHF: Dengue Hemorrhagic Fever; DCC: Dengue with Complications; DwWS: Dengue with Warning Signs; SD: Severe Dengue

State abbreviations: Acre (AC); Alagoas (AL); Amapá (AP); Amazonas (AM); Bahia (BA); Ceará (CE); Distrito Federal (DF); Espírito Santo (ES); Goiás (GO); Maranhão (MA); Mato Grosso (MT); Mato Grosso do Sul (MS); Minas Gerais (MG); Pará (PA); Paraíba (PB); Paraná (PR); Pernambuco (PE); Piauí (PI); Roraima (RR); Rondônia (RO); Rio de Janeiro (RJ); Rio Grande do Norte (RN); Rio Grande do Sul (RS); Santa Catarina (SC); São Paulo (SP); Sergipe (SE); Tocantins (TO).

**Table 1: Odds Ratio of dengue fatal cases occurred in Brazil from 1987-2015, considering the first epidemic year (1986).**

| Year | Reported cases | Deaths | OR over 1986 | Confidence interval | *P* value |
|---|---|---|---|---|---|
| 1986 | 46309 | 5 | - | - | - |
| 1987 | 88407 | 4 | 0.419 | 0.11 - 1.56 | *0.2903* |
| 1988 | 1570 | 0 | 0.000 | 0 - 32.02 | *>0.999* |
| 1989 | 5367 | 0 | 0.000 | 0 - 9.36 | *>0.999* |
| 1990 | 40279 | 8 | 1.840 | 0.60 - 5 | *0.4054* |
| 1991 | 104399 | 0 | 0.040 | 0 - 0.73 | *0.0027* |
| 1992 | 1696 | 0 | 0.000 | 0 - 29.64 | *>0.999* |
| 1993 | 7374 | 0 | 0.000 | 0 - 6.81 | *>0.999* |
| 1994 | 56691 | 11 | 1.797 | 0.62 - 5.17 | *0.3219* |
| 1995 | 137308 | 2 | 0.135 | 0.02 - 0.69 | *0.0134* |
| 1996 | 183762 | 1 | 0.050 | 0 - 0.43 | *0.0017* |
| 1997 | 249239 | 9 | 0.334 | 0.11 - 0.99 | *0.055* |
| 1998 | 507715 | 10 | 0.182 | 0.06 - 0.53 | *0.006* |
| **1999** | **74670** | **42** | **5.210** | **2.06 - 13.17** | ***<0.0001*** |
| 2000 | 135228 | 4 | 0.274 | 0.07 - 1.02 | *0.053* |
| **2001** | **385783** | **44** | **1.056** | **0.42 - 2.66** | ***>0.999*** |
| 2002 | 696472 | 150 | 1.995 | 0.82 - 4.86 | *0.1352* |
| **2003** | **274975** | **88** | **2.964** | **1.20 - 7.29** | ***0.0111*** |
| **2004** | **70174** | **18** | **2.376** | **0.88 - 6.39** | ***0.0896*** |
| **2005** | **147039** | **69** | **4.346** | **1.75 - 10.77** | ***0.0002*** |
| **2006** | **258680** | **142** | **5.084** | **2.08 - 12.40** | ***<0.0001*** |
| **2007** | **496923** | **290** | **5.405** | **2.23 - 13.08** | ***<0.0001*** |
| **2008** | **632680** | **561** | **8.212** | **3.40 - 19.81** | ***<0.0001*** |
| **2009** | **406269** | **341** | **7.774** | **3.21 - 18.80** | ***<0.0001*** |
| **2010** | **1011548** | **656** | **6.006** | **2.49 - 14.48** | ***<0.0001*** |
| **2011** | **764032** | **482** | **5.843** | **2.42 - 14.10** | ***<0.0001*** |
| **2012** | **589591** | **327** | **5.137** | **2.12 - 12.42** | ***<0.0001*** |
| **2013** | **1470487** | **674** | **4.245** | **1.76 - 10.23** | ***0.0007*** |
| **2014** | **591080** | **475** | **7.443** | **3.08 - 17.97** | ***<0.0001*** |
| **2015** | **1649008** | **986** | **5.538** | **2.30 - 13.34** | ***<0.0001*** |

Footnote: To compare the Odds Ratio of deaths occurred from 1987 to 2015, we calculated OR values, confidence intervals and P-values, setting the year of 1986 as the comparison year. Values were calculated using GraphPad Prism version 6 software.

**Table 2: Timeline and characteristics of dengue classifications used over 30 years of dengue fatal cases investigation in Brazil.**

| Dengue classification | Source | Classifications | Years of use in Brazil |
|---|---|---|---|
| **World Health Organization (WHO), 1997** | World Health Organization (WHO), after a study based on dengue on children in Thailand in the 1950s and 1960s, with modifications in 1986 and 1997 [34]. | DHF and DSS | From 1986 to 2000 |
| **Ministry of Health of Brazil, 2000** | Brazilian Ministry of Health, used to define dengue severe cases that did not meet the WHO criteria for DHF / DSS. Used only in Brazil. | DCC | From 2000 to 2013 |
| **WHO, 2009** | World Health Organization (WHO), based on the results of a multicenter study (DENCO) conducted in Southeast Asia and Latin America to assess the limitations of the 1997 classification. | DwWS and SD | From 2014 to present |

DHF: Dengue haemorraghic fever, DSS: Dengue shock syndrome, DCC: Dengue with complications, DwWS: Dengue with warning signs, SD: Severe dengue.

Click here to access/download;Figure;figure 1 R1.tif

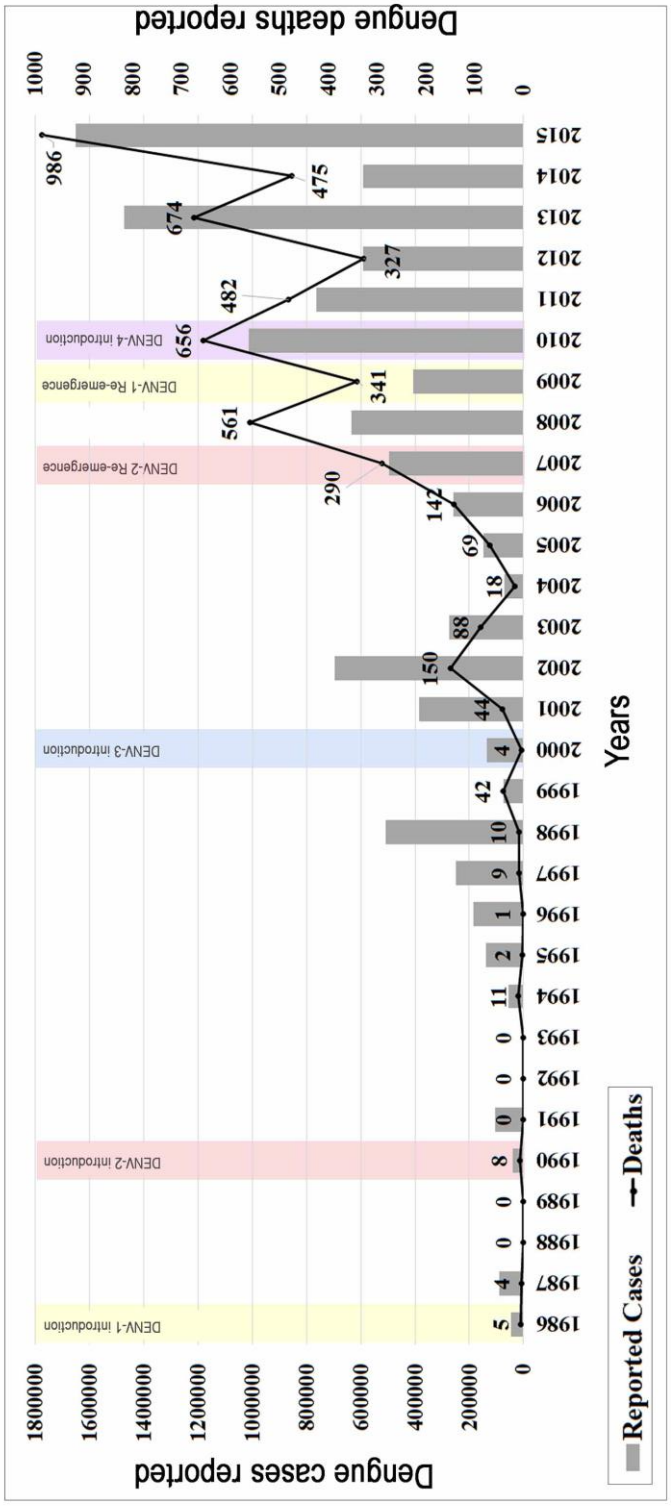


Figure 1

Figure 2

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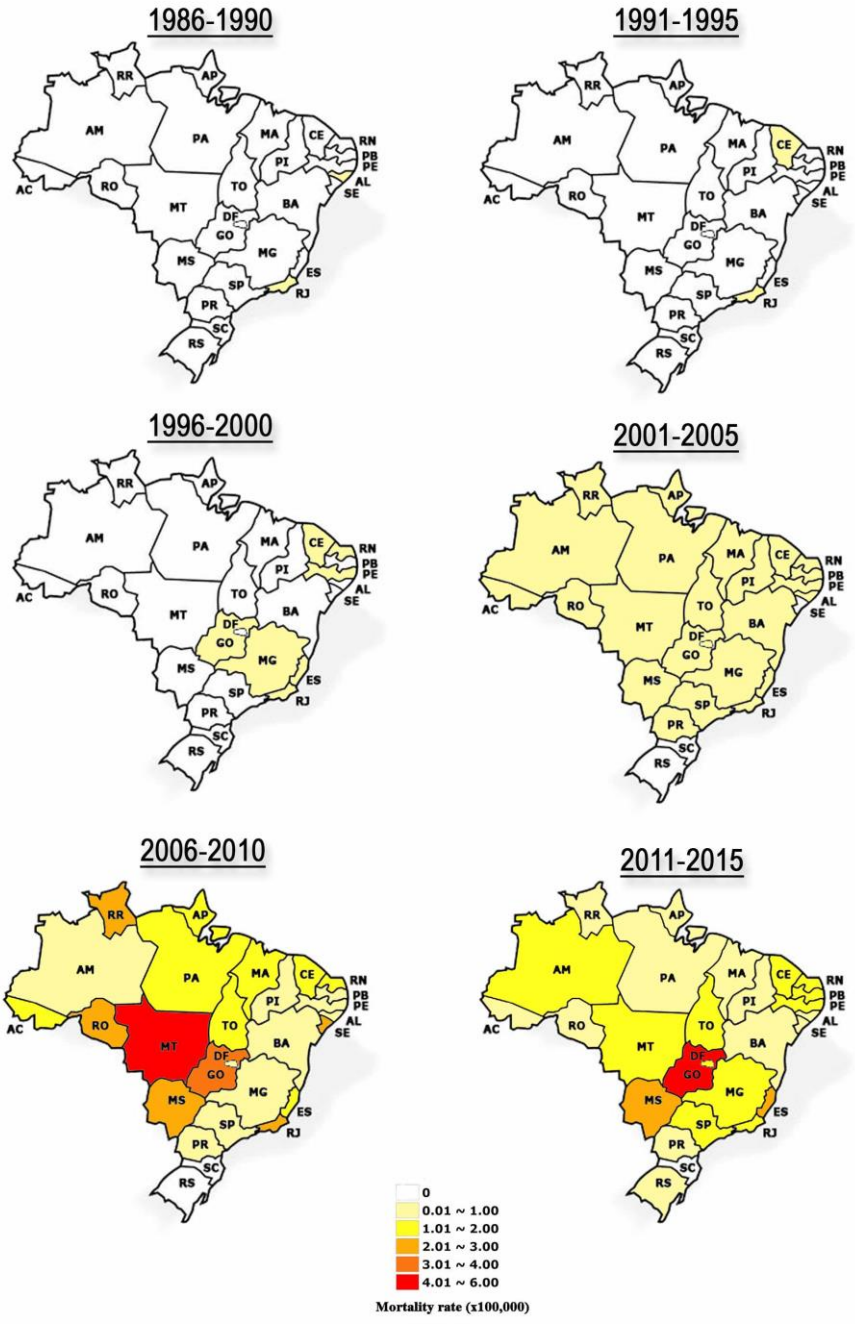

Figure 3

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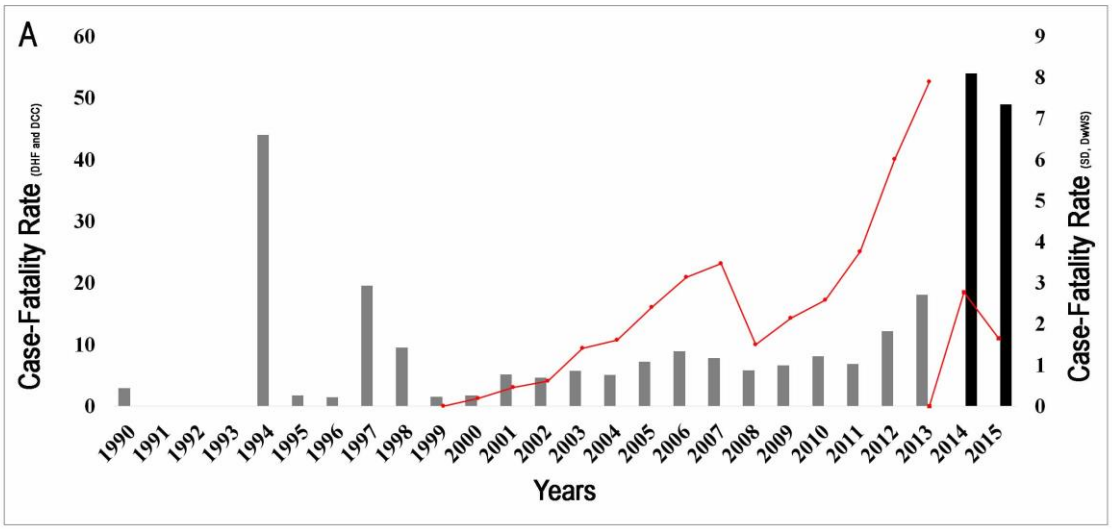


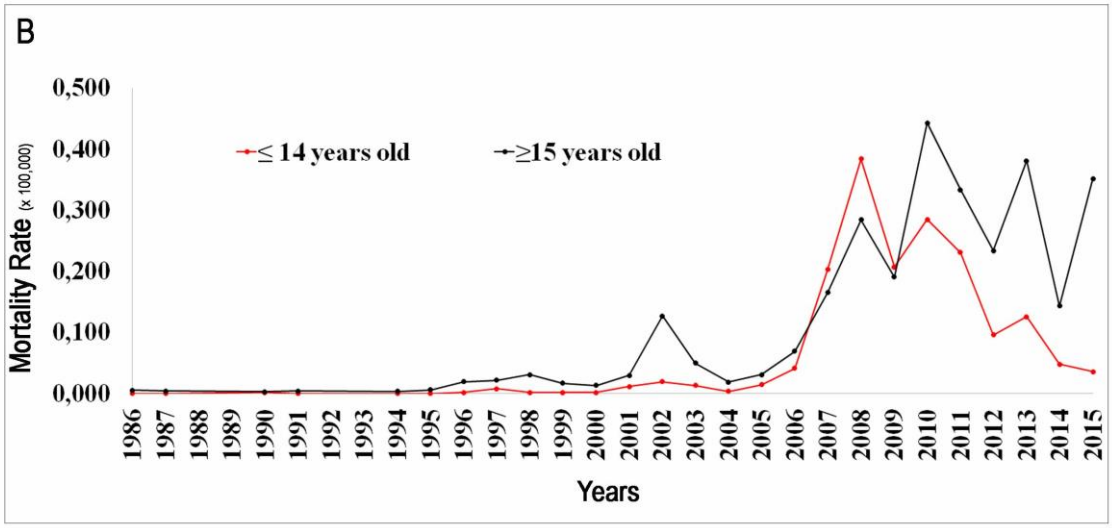

Figure 4

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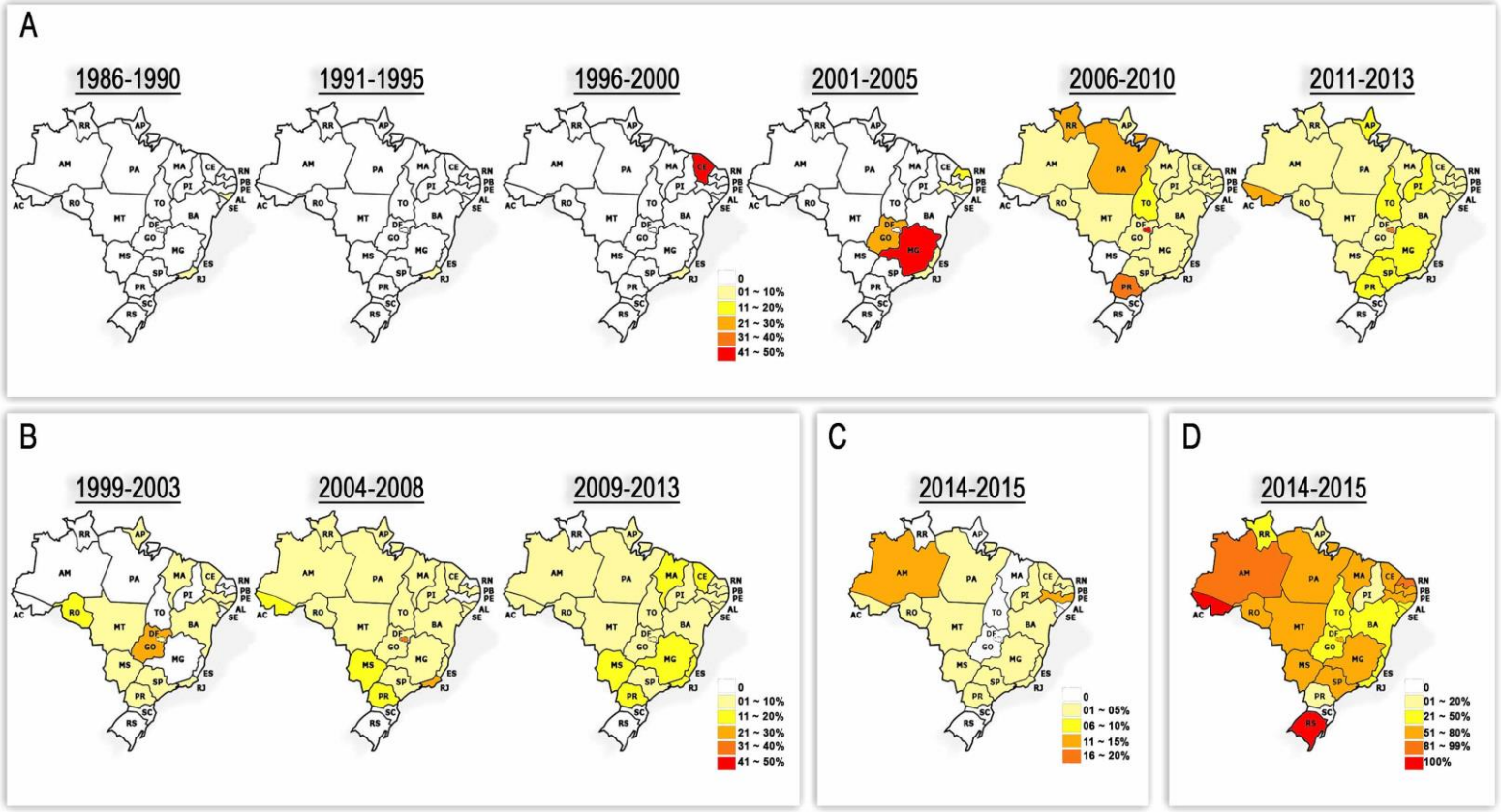

## 4.2 ARTIGO 2: 30 ANOS DE CASOS FATAIS DE DENGUE NO BRASIL: UMA INVESTIGAÇÃO LABORATORIAL DE 1.047 CASOS FATAIS

**Revista:** BMC Infectious Diseases

**Classificação Medicina II:** B1

**Fator de Impacto:** 2.62

**Resumo:** Os vírus dengue (DENV) emergiram e re-emergiram no Brasil nos últimos 30 anos, causando epidemias explosivas. A doença pode variar de infecções assintomáticas, desfechos graves e fatais. O objetivo deste estudo foi descrever os aspectos epidemiológicos, clínicos e laboratoriais dos óbitos de dengue recebidos no Laboratório de Referência Regional, do Instituto Oswaldo Cruz, IOC, FIOCCRUZ durante 30 anos. Um total de 1.047 casos fatais suspeitos de dengue foram recebidos de 1986 a 2015 e analisados no Laboratório de Flavivirus (LABFLA/FIOCRUZ). Os casos fatais foram submetidos a métodos virológicos, sorológicos e moleculares. Após a confirmação laboratorial, foram analisadas as influências do sexo, idade, sorotipo e tipo de infecção (primária / secundária) no desfecho do óbito, assim como as interações entre estas variáveis. Um total de 359 casos (34,2%) foi confirmado e DENV-1 (11,1%), DENV-2 (43,9%), DENV-3 (32,8%) e DENV-4 (13,7%) foram detectados. Em geral, uma maior frequência dos óbitos foi de infecções primárias (59,3%, p = 0,001). No entanto, em 2008, os casos fatais foram principalmente associados às infecções secundárias (65,0%, p = 0,003). Além disso, crianças infectadas pelo DENV-2 apresentaram maiores chances de evoluir ao óbito, assim como àquelas com infecções secundárias, que exibem quatro vezes mais chances de desfecho desfavorável. A dengue é, de fato, uma doença multifatorial e fatores associados à cepa viral, sorotipo infectante, ocorrência de infecções secundárias e comorbidades podem levar a um resultado fatal. Entretanto, a alta incidência e transmissão de dengue durante epidemias, como as observadas no Brasil, podem sobrecarregar e colapsar os serviços de saúde, impactando no aumento da gravidade da doença e na mortalidade.

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RESEARCH ARTICLE Open Access

# 30 years of dengue fatal cases in Brazil: a laboratorial-based investigation of 1047 cases

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## Abstract

**Background:** Dengue viruses (DENV) have emerged and reemerged in Brazil in the past 30 years causing explosive epidemics. The disease may range from clinically asymptomatic infections to severe and fatal outcomes. We aimed to describe the epidemiological, clinical and laboratorial aspects of the dengue fatal cases received by a Regional Reference Laboratory, Brazil in 30 years.

**Methods:** A total of 1047 suspected fatal dengue cases were received from 1986 to 2015 and analyzed in the Laboratory of Flavivirus, FIOCRUZ. Suspected cases were submitted to viral detection, serological and molecular methods for cases confirmation. Influence of gender, age, serotype and type of infection (primary/secondary) on death outcome, as well the interactions between serotype and age or infection and age and type of infection were also studied.

**Results:** A total of 359 cases (34.2%) were confirmed and DENV-1 (11.1%), DENV-2 (43.9%), DENV-3 (32.8%) and DENV-4 (13.7%) were detected. Overall, fatal cases occurred more often in primary infections (59.3%, $p = 0.001$). However, in 2008, fatal cases were mainly associated to secondary infections ($p = 0.003$). In 2008 and 2011, deaths were more frequent on children and those infected by DENV-2 presented a higher risk for fatal outcome. Moreover, children with secondary infections had a 4-fold higher risk for death.

**Conclusions:** Dengue is a multifactorial disease and, factors such as viral strain/serotype, occurrence of secondary infections and co-morbidities may lead to a severe outcome. However, the high dengue incidence and transmission during epidemics, such as those observed in Brazil may overwhelm and collapse the public health services, potentially impacting on increased disease severity and mortality.

**Keywords:** Dengue, Fatal cases, Epidemiology, Laboratorial diagnosis, Brazil

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## Background

Dengue fever is caused by any of the four distinct serotypes (DENV-1 to 4), belonging to the *Flavivirus* family. It is the most important arboviral diseases affecting humans worldwide and its global prevalence has grown dramatically in recent decades. About 100 million people are infected and 500,000 people develop severe dengue leading to about 70,000 deaths annually [1]. It poses a significant public health and economic burden in tropical and subtropical endemic regions [2, 3]. Over the last decades, both the incidence and severity of dengue in Central, the Caribbean and South America have increased significantly [4]. A recent estimate reported that the number of apparent dengue cases more than doubled every decade between 1990 to 2013, from 8.3 million in 1990 to 58.4 million in 2013 and, with an average of 9000 dengue fatal cases occurring per year [5].

Although most DENV infections are asymptomatic, the disease can also present a broad spectrum of clinical signs and symptoms, ranging from an acute undifferentiated febrile illness to severe and fatal outcomes. Fatal cases may occur in over 10% of cases and 90% of deaths occur in children under 15 years old [6]. However, in recent decades, dengue and severe dengue have become more frequent among adults [7]. If dengue is not treated properly, a small proportion of patients may develop life-threatening complications [8], however, with early recognition of the disease severity and intensive care, fatal outcomes can decrease from ~10% to less than 1% among severe cases [9, 10].

Factors such as the occurrence of secondary infections with a heterologous serotype increase the risk of developing a more severe disease, however the infecting and genetic variation of DENV strain, presence of co-morbidities, ethnicity, age and the patient's immune conditions, such as profound thrombocytopenia, may also contribute to a more severe case [7, 11–14]. The early diagnosis and immediate treatment are essential to reduce the mortality caused by DENV [15], however, about 70% of the infected patients may choose not to seek treatment or treat themselves [2].

In Brazil, since dengue introduction in early 80's, more than ten million cases have been reported during successive epidemics, more critically occurred on 2002, 2008, 2010, 2013, 2014 and 2015, and when 150, 561, 656, 674, 475 and 986 fatal cases were confirmed, respectively [16]. Despite the increased mortality, not all cases progressing to a fatal outcome are diagnosed by the health services [17].

The spread of dengue in Brazil resulted in the establishment of a National Network for Dengue Diagnosis in the year of 1989 [18] which aimed to contribute for the disease surveillance in the country, an important tool to predict epidemics [19]. This Network consists of Regional Reference Laboratories responsible for all Brazilian regions [20] and includes the Laboratory of Flavivirus (LABFLA) IOC/FIOCRUZ, established since 1986 and which maintains a surveillance program in the State of Rio de Janeiro.

A review on dengue diagnosis and epidemiology by the Regional Reference Laboratory in 25 years has been published previously [19], however, despite the availability and richness of the fatal cases received in the last 30 years, no review nor detailed report were carried out. Here, we aimed to describe the epidemiological and laboratorial aspects of the dengue fatal cases received between 1986 and 2015.

## Methods

### Suspected dengue cases

Suspected dengue fatal cases ($n = 1047$) were received between March 1986 and December 2015 during an active surveillance program performed by the Laboratory of Flavivirus, IOC/FIOCRUZ, Regional Reference Laboratory for the Brazilian Ministry of Health, located in RJ. As a Regional Reference Laboratory, suspected cases are received as convenience sampling for diagnosis and the cases investigation has been approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05). Suspected cases samples were received accompained by investigation records and questionnaires containing the patient's demographic (age, gender, date of birth, address) and clinical (onset of disease and sign and symptoms) information.

Acute serum samples (up to the 7th day after the onset of the symptoms) were stored at − 70 °C and submitted for virus isolation, molecular methods reverse transcriptase polymerase chain reaction (RT-PCR), Real-time Reverse Transcriptase PCR (TaqMan) assay (qRT-PCR) and NS1 antigen capture ELISA. Convalescent samples (> 7 days of symptoms) were tested by the hemagglutination inhibition (HI) assay and by the anti-DENV IgM and IgG capture ELISA tests. From the 1047 fatal cases, 614 were collected in the acute phase (up to the 7th day of symptoms) and 233 were convalescent cases (> 7 days of symptoms). A paired sampling was available in 43 cases. In 290 cases, the information on the days of illness was not available. Despite this, all dengue suspected cases received in the Regional Reference Laboratory are tested by all methods, when sample volume is available.

### Virus isolation

Virus isolation was performed by inoculation into C6/36 *Aedes albopictus* cell line [21] and isolates were identified by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies [22]. The C6/36 *Aedes albopictus* cell line was kindly provided in many opportunitites during the study by Dr. Pedro Vasconcelos

from the Evandro Chagas Institute, the National Reference Laboratory for Arboviruses for the Brazilian Ministry of Health.

#### Immunoglobulin M (IgM) antibody capture ELISA (MAC-ELISA)

The in-house MAC-ELISA was carried out for dengue cases confirmation as described in Nogueira et al. [23]. Alternatively, the Panbio dengue IgM Capture ELISA (Panbio Diagnostics, Queensland, Australia) was used for the qualitative detection of anti-DENV IgM antibodies in serum for fatal case confirmation.

#### Haemaglutination inhibition (HI) test

HI test was performed to characterize dengue infections as primary or secondary, as described in Clarke and Casals [24].

#### Immunoglobulin G (IgG) antibody detection ELISA (IgG-ELISA)

The IgG-ELISA has been previously described by Miagostovich et al. [25] and was performed for to characterize infections as primary or secondary infections in replacement to the HI test for dengue cases previously confirmed by virus isolation, RT-PCR and/or MAC-ELISA.

#### Dengue NS1 ag detection

The Platelia™ Dengue NS1 Ag-ELISA kit (Biorad Laboratories, Marnes-La-Coquette, France) was peeformed according to the manufacturer's instructions. Additionally, we used the Dengue NS1 Ag STRIP (Bio-Rad Laboratories, Marnes-La-Coquette, França), an immunochromatographic test (ICT), according the manufacturer's instructions.

#### Immunohistochemistry

The immunohistochemistry assay was performed as described elsewhere [26].

#### Viral RNA extraction

The viral RNA was extracted from samples using the QIAamp Viral RNA Mini kit (Qiagen) following the manufacturer's instructions and stored at -70C.

#### Dengue reverse transcriptase-nested polymerase chain reaction (RT-nested-PCR)

RT-PCR for detecting and typing DENV was performed as described previously by Lanciotti et al. [27].

#### Real-time reverse transcriptase PCR (TaqMan) assay –qRT-PCR

The one-step real-time RT-PCR assay was performed as described previously by Johnson et al. [28] in the ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

#### Matrix layout analysis of the laboratorial diagnostic methods

The matrix layout analysis representing all combination of techniques performed for dengue fatal cases investigation was done using UpsetR according to Lex et al. [29].

#### Statistical analysis

The derived data was tabulated in appropriate worksheets using the SPSS 21st version Program and evaluated by t-Test and Anova Test.

We used logistic generalized linear models (GLM) with logit link function to study the influence of gender, age, serotype and type of infection (primary/secondary) on death outcome (death/alive outcome was coded as binary variable).

For this analysis, 4344 confirmed dengue cases that did not evolve to death were included. The inclusion criterion of those cases were non-fatal dengue suspected cases received between 1986 and 2015, confirmed by specific laboratorial diagnosis and with demographical information.

Interactions between serotype and age or infection and age and type of infection were also studied. Odd ratios (OR) were estimated from regression slope coefficients (β) calculating the $OR = e^{\beta}$. Similarly, the 95% confidence interval (95%CI) for each OR was obtained through exponentiation of 95%CI estimated on the GLM. Due to the small number of individuals considered to evaluate the interaction of DENV-4 and the type of infection, the OR was not calculated. This analysis was performed using R statistical environment [30].

## Results

A total of 1047 dengue suspected fatal cases, representative from the North, Northeast, Midwest and Southeast regions of Brazil, were received and analyzed from 1986 to 2015, and 34.3% (359/1047) were confirmed as dengue by using any of the viral, molecular and serological diagnostic laboratory tests available in the routine of the Laboratory. Due to some samples volume restriction, not all samples were tested by all techniques, Table 1. All combinations of laboratorial diagnostic methods performed for the analysis of the dengue fatal cases are show on Fig. 1.

The contribution of each method on the fatal cases confirmed were, as follows: DENV was isolated in 15.2% (46/302) of the confirmed cases after inoculation into C6/36 cells, and nested RT-PCR contributed in 46.5% (153/329) of the confirmed cases. The infecting serotype was more often identified by molecular detection and/or virus isolation in cases presenting 2 to 5 days of illness. The real time RT-PCR contributed confirming the infection on 60.5% (78/129) of the dengue fatal cases. The overall case confirmation by using the NS1 antigen

**Table 1** Laboratorial diagnosis on dengue suspected fatal cases ($n$ = 1047) confirmation in Brazil, 1986–2015

| Diagnostic test | Sample | | Total (%) |
|---|---|---|---|
| | Acute (< 7 days of illness) Positive/Tested (%) | Convalescent (≥7 days of illness) Positive/Tested (%) | |
| Virus isolation | 46/768 (6.0) | Not done | 46/768 (6.0) |
| RT-PCR | 142/774 (18.3) | 11/112 (9.82) | 153/886 (17.3) |
| MAC-ELISA | 120/489 (24.5) | 42/113 (37.2) | 162/602 (26.9) |
| IgG-ELISA | 261/345 (75.6) | 57/73 (78.1) | 318/418 (76.0) |
| NS1-ELISA | 120/415 (28.9) | 24/93 (25.8) | 144/508 (28.3) |

ELISA was 67.2%, (207/308), in cases with up to 7 days of disease, but we observed positivity in convalescent samples, as well. The anti-DENV IgM antibody detection rate was 65.1% (218/335) on the dengue confirmed fatal cases Immunohistochemistry contribution was by confirming 59.9% (18/34) of the fatal cases by analyzing the paraffin embedded tissue samples available.

The highest percentage of confirmed cases were from the Southeast region, predominantly from the state of Rio de Janeiro, with 36.7% (132/359) of the cases, although fatal cases in the states of Espírito Santo, Goiás, Mato Grosso, Mato Grosso do Sul and Rio Grande do Norte were also reported. From the suspected fatal cases of dengue, 43.3% (447/1031) were female and 56.6% (584/1031) was male. The female to male confirmation ratio was 1:1.08 (171:186). We did not find a relationship between gender and the evolution to dengue fatal outcome (Table 2). In 16 cases, gender could not be defined due to the lack of information, dubious names or use of patients' initials.

Most patients developed systemic symptoms such as fever, myalgia, nausea, headache, and malaise. Hypovolemic shock was present in 137 (39.1%) of the patients and thrombocytopenia in 125 (35.7%). Hypotension was observed in 77 (22.0%) cases and abdominal pain in 93 (26.0%). Hepatomegaly was found in 19 (5.4%) patients and pleural effusion in 24 (6.8%). Coma and splenomegaly were rare (4 and case 1, respectively). The occurrence of hemorrhagic manifestations was also observed. Petechia were observed in 120 (34.3%) cases, epistaxis in 54 (15.4%), gingival bleeding in 46 (13.1%), non-specified bleeding in 43 (12.3%), haematemesis in 23 (6.5%), hematuria in 18 (5.1%) and a positive tourniquet test in 13 (3.7%). Irritability was reported in two cases, profuse

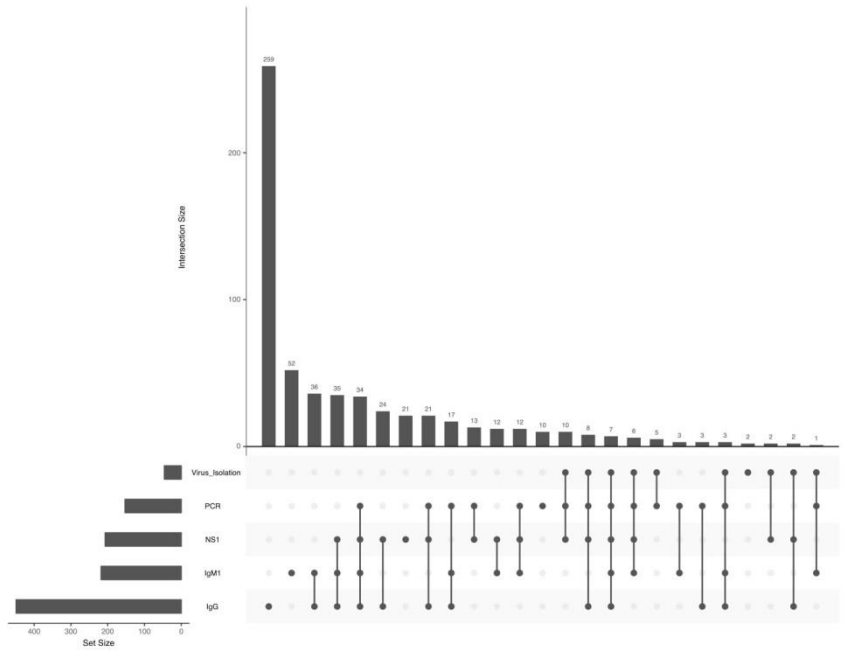


**Fig. 1** Matrix layout representing all combination of laboratorial diagnostic methods performed to study dengue fatal cases. Vertical bars indicate the number of individuals who were tested using each combination of techniques sorted by size. In the bottom of bars, dark circles in the matrix indicate when a combination of techniques was done. The bar chart on the left indicates the frequency of each technique employed

**Table 2** Logistic models with logit links of epidemiological, virological and immunological variables influence on dengue mortality

| Variable (Factor vs.) | Factor | *n* (fatal/non-fatal) | OR (95% CI) | *p* value |
|---|---|---|---|---|
| Gender | **Male** | **2165 (97/2068)** | 1.12 (0.88–1.44) | 0.42 |
| Female vs | | | | |
| *n* = 2279 (91/2188) | | | | |
| Serotypes | **DENV-2** | **1047 (83/964)** | **5.67 (3.823–8.68)** | **< 0.0001** |
| DENV-1 vs. | **DENV-3** | **1279 (62/1217)** | **3.36 (2.23–5.50)** | **< 0.0001** |
| *n* = 1405 (21/1384) | **DENV-4** | **802 (23/779)** | **1.95 (1.17–3.23)** | **0.029** |
| DENV-2 vs. | **DENV-3** | **1279 (62/1217)** | **0.59 (0.42–0.83)** | **0.0024** |
| *n* = 1047 (83/964) | **DENV-4** | **802 (23/779)** | **0.34 (0.21–0.54)** | **< 0.0001** |
| DENV-3 vs. | **DENV-4** | **802 (23/779)** | **0.58 (0.35–0.93)** | **0.0281** |
| *n* = 1279 (62/1217) | | | | |
| Age (years old) | **0–15** | **710 (38/672)** | **1.74 (1.17–2.60)** | **0.021** |
| 16–30 vs. | **31–50** | **1205 (44/1161)** | 1.16 (0.79–1.71) | 0.522 |
| *n* = 1075 (34/1041) | **51–96** | **604 (53/551)** | **2.94 (2.04–4.30)** | **< 0.0001** |
| 0–15 vs. | **31–50** | **1205 (44/1161)** | 0.66 (0.43–1.04) | 0.07 |
| *n* = 710 (38/672) | **51–96** | **604 (53/551)** | **1.68 (1.11–2.61)** | **0.017** |
| 31–50 vs. | **51–96** | **604 (53/551)** | **2.53 (1.68–3.86)** | **< 0.0001** |
| *n* = 1205 (44/1161) | | | | |
| Immune Responses | **Secondary** | **293 (74/219)** | 0.97 (0.71–1.34) | 0.89 |
| Primary | | | | |
| *n* = 265 (67/198) | | | | |

To calculate each logistic GLM, Death/Alive outcome was coded as binary variable. Odd ratios (COR), 95% confidence intervals (95%CI) and *P*-values were calculated using one GLM for each studied variable separately. Values highlighted in bold presented: COR > 1, values of OR contained into the 95%CI range and *p*-values < 0.05

perspiration and tachycardia in three cases; chills, cough, dizziness and neck stiffness in five cases. The clinical manifestations reported in 359 fatal cases with data available, during the period is shown in Fig. 2.

All four DENV serotypes were detected in the period: DENV-1 (11.1%, 21/189), DENV-2 (43.9%, 83/189), DENV-3 (32.8%, 62/189) and DENV-4 (13.7%, 26/189). Analyzing the influence of serotype in mortality, we observed that DENV-2 was more associated with fatal cases, followed by DENV-3 and DENV-4, Table 2.

Dengue infection was confirmed in 47.4% (72/152) cases were 0–15 years old, 44.0% (81/184) cases aged 16–30 years, 49.1% (105/214) cases with 31–50 years and 46.8% (110/235) cases with 51–96 years. Unfortunately, information on the age of 216 cases has not been described. We also observed that compared to 16–30, 0–15 and 31–50 years old group individuals ranging from 51 to 96 years old was more associated with fatal outcomes. Additionally, children age group (0–15 years old) was more associated to fatal outcomes compared to 16–30 years old group, Table 2.

Evaluating the association between dengue serotype and age, we observed that, consistently among the DENV 1 to 4 cases, the 51–96 years old group presented increased odds to present fatal cases compared to other age groups. Additionally, we observed increased odds in 0–15 years old groups compared to 16–30 groups for DENV-2 and DENV-3. Similarly, 31–50 years old groups had increased odds compared to 0–15 years old group for DENV-1 and DENV-3 (Table 3).

The patients' immune response was characterized by IgG-ELISA in 300 fatal cases and, primary infections (59.3%; 178/300) were more often observed than secondary ones (40.6%; 122/300; $p = 0.001$). However, we did not find a relation between the immune response and the evolution to death (Table 2). Except by the year 2008, when most deaths were due to secondary infections, during the most expressive epidemic years 2002, 2010, 2011, 2012 and 2013, fatal cases were mainly due to primary ones ($p = 0.038$), Fig. 3. 46.2% (31/67) of DENV-3 cases were characterized as primary infection while 62.1% (46/74) of DENV-2, as secondary ones. No association was observed among the type of infection (primary or secondary), serotype and the evolution to a fatal outcome, Table 4. Nevertheless, considering the age factor, children 15 years old and under, presenting a secondary infection had almost a 4-fold risk of death, Table 4.

In 2002, an epidemic caused by DENV-3 was reported and 97.7% (43/44) of the fatal cases investigated in that year were due to that infecting serotype. On the other

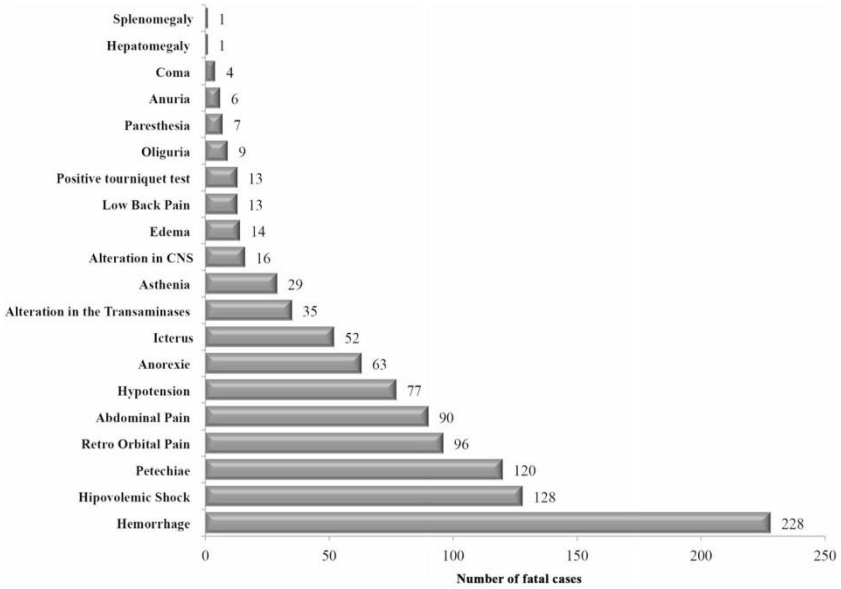


**Fig. 2** Signs and symptoms associated with severe dengue reported in the fatal cases ($n = 359$) analyzed in this study

hand, the 2008 epidemic was mainly caused by DENV-2, the infecting serotype identified in 89.0% (60/67) of the fatal cases investigated in this study. In 2010, DENV-1 (41.7%, 5/12) and DENV-2 (58.3%, 7/12) were the infecting serotypes identified, while the latter was responsible for 82.3% (14/17) of cases studied in 2011. In 2012, two fatal cases were confirmed, one by DENV-2 and one by DENV-3. In 2013, all nine fatal cases confirmed were due to DENV- 4. During the years of 2014 and 2015, three fatal cases were confirmed by DENV-4 and only one case by DENV-1, occurred in 2014, Fig. 4.

The fatal cases' age ranged from 0 to 96 years old, with a predominance of positive cases in children 0–15 years old and between 51 and 96 years old (25.6%, 42/164). Fatal outcome on age groups between 16 to 30 and 31–50 years occurred in 24.4% (40/164) of the cases each. In 195 cases the age was unknown. Despite the prevalence of secondary infections on children 0–15 years old, no significant differences were observed ($p = 0.350$), Fig. 5. In the epidemic years of 2002, 2010, 2012, 2013, 2014 and 2015, the majority of the fatal cases occurred adults, however in 2008 and 2011, increased fatal cases on children 15 years old and under, were observed ($p = 0.045$), Fig. 6. No significant differences were observed when age groups and the patient's immune response were compared, considering the epidemics of 2002, 2008, 2010, 2012, 2013, 2014 and 2015.

## Discussion

Over the past 30 years, more than 5202 deaths from dengue were reported in Brazil, and the disease has become a serious public health problem in several states [31]. During this period, the Laboratory of Flaviviruses (LABFLA) IOC/ FIOCRUZ, a Regional Reference Laboratory for Dengue and Yellow Fever diagnosis based on Rio de Janeiro, Southeast region of Brazil, received suspected dengue cases, meeting the requirements of the Ministry of Health to monitor the disease in the country.

In 1986, with the introduction of DENV-1 in Rio de Janeiro [32], the disease was established causing an explosive epidemic in a naïve population and spread to other Brazilian states. In 1990, DENV-2 was also isolated in Rio de Janeiro and this scenario resulted in the occurrence of the first cases of severe disease [33]. In December 2000, another serotype, DENV-3, was detected in Rio de Janeiro and was responsible until then for the largest and most severe dengue epidemic ever described in the country and in the American continent, not only due to the high number of reports and fatal cases [34–36]. The re-emergence of DENV-2 in 2007 characterized a dramatic increase in the number of severe cases and deaths in children 15 years old and under [37]. In 2009, a new high-transmission cycle of DENV-1 began in Brazil, with more than one million probable cases and 656 deaths reported in 2010 and the occurrence of deaths in patients with comorbidities [38]. In July 2010, DENV-4 was isolated in Roraima [39] and in 2011 this serotype spread to other states.

Since 2014, Brazil has experienced triple epidemics caused simultaneously by DENV, chikungunya virus (CHIKV) and zika virus (ZIKV), hampering the clinical

**Table 3** Logistic models with logit links of association between serotype and age leading to the evolution to a dengue fatal outcome

| Variable Factor (vs.) | Factor | *n* (fatal/non-fatal) | OR (95% CI) | *p value* |
|---|---|---|---|---|
| Serotype (Age group compared) | Age (years old) | | | |
| *DENV-1 (16–30 years old)* | | | | |
| *n* = 309 (03/306) | **0–15** | **169 (05/164)** | 3.13 (0.95–11.59) | 0.122 |
| | **31–50** | **314 (05/309)** | 1.65 (0.50–6.10) | 0.495 |
| | **51–96** | **162 (7/155)** | **4.61 (1.55–16.33)** | **0.028** |
| *DENV-1 (0–15 years old)* | | | | |
| *n* = 169 (05/164) | 31–50 | 314 (05/309) | 0.53 (0.14–1.92) | 0.52 |
| | 51–96 | 162 (7/155) | 1.47 (0.46–5.07) | 0.32 |
| *DENV-1 (31–50 years old)* | | | | |
| *n* = 314 (05/309) | 51–96 | 162 (7/155) | 2.79 (0.88–9.56) | 0.08 |
| *DENV-2 (16–30 years old)* | | | | |
| *n* = 309 (11/306) | **0–15** | **130 (26/104)** | **3.61 (1.97–6.91)** | **0.0006** |
| | **31–50** | **263 (16/247)** | 1.07 (0.56–2.12) | 0.864 |
| | **51–96** | **121 (19/102)** | **3.08 (1.62–6.06)** | **0.005** |
| *DENV-2 (0–15 years old)* | | | | |
| *n* = 130 (26/104) | **31–50** | **263 (16/247)** | **0.3 (0.15–0.57)** | **0.0003** |
| | **51–96** | **121 (19/102)** | 0.85 (0.44–1.62) | 0.62 |
| *DENV-2 (31–50 years old)* | | | | |
| *n* = 263 (16/247) | **51–96** | **121 (19/102)** | **2.88 (1.76–6.65)** | **0.003** |
| *DENV-3 (16–30 years old)* | | | | |
| *n* = 348 (17/331) | **0–15** | **247 (04/243)** | 0.31 (0.11–0.73) | 0.037 |
| | **31–50** | **369 (18/351)** | 0.99 (0.56–1.77) | 0.997 |
| | **51–96** | **174 (17/157)** | **2.11 (1.17–3.80)** | **0.036** |
| *DENV-3 (0–15 years old)* | | | | |
| *n* = 247 (04/243) | **31–50** | **369 (18/351)** | **3.22 (1.18–11.24)** | **0.036** |
| | **51–96** | **174 (17/157)** | **6.79 (2.46–23.94)** | **0.0007** |
| *DENV-3 (31–50 years old)* | | | | |
| *n* = 369 (18/351) | **51–96** | **174 (17/157)** | **2.11 (1.05–4.22)** | **0.036** |
| *DENV-4 (16–30 years old)* | | | | |
| *n* = 225 (03/222) | **0–15** | **136 (03/133)** | 1.66 (0.41–6.75) | 0.54 |
| | **31–50** | **259 (05/254)** | 1.46 (0.44–5.40) | 0.609 |
| | **51–96** | **147 (10/137)** | **5.4 (1.95–18.51)** | **0.011** |
| *DENV-4 (0–15 years old)* | | | | |
| *n* = 136 (03/133) | **31–50** | **259 (05/254)** | 0.88 (0.21–4.34) | 0.86 |
| | **51–96** | **147 (10/137)** | 3.26 (0.97–14.78) | 0.08 |
| *DENV-4 (31–50 years old)* | | | | |
| *n* = 259 (05/254) | **51–96** | **147 (10/137)** | **3.71 (1.29–12.11)** | **0.018** |

To calculate each logistic GLM, Death/Alive outcome was coded as binary variable. Odd ratios (COR), 95% confidence intervals (95%CI) and *p*-values were calculated using one GLM for each studied variable separately. Values highlighted in bold presented: OR > 1, values of OR contained into the 95%CI range and *p*-values < 0.05

differential diagnosis, as those arboviruses share common signs and symptoms. Despite the zika epidemic occurred, in 2015, a total of 1,649,008 probable dengue cases and 863 deaths, mainly caused by DENV-1 and DENV-4, were reported in Brazil [40].

About 75% of dengue infections are known to be clinically unapparent or mildly symptomatic [2]. More severe cases may be characterized by hemorrhagic events, thrombocytopenia and increased leakage of fluid and shock that can evolve and lead to death within 12–36 h [41].

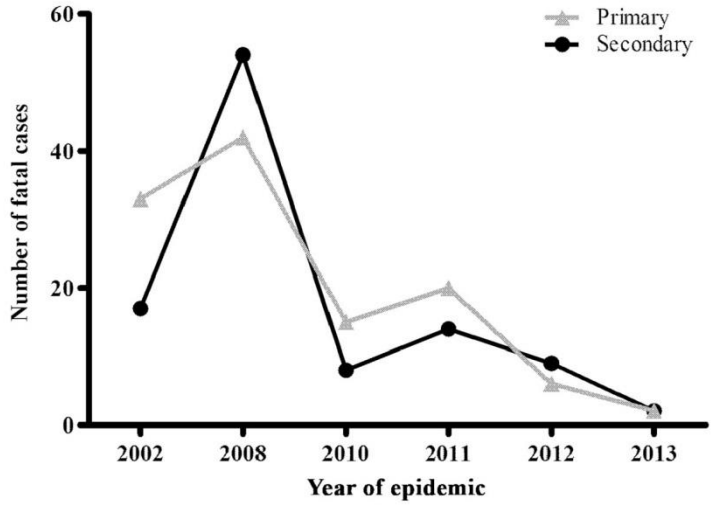


**Fig. 3** Dengue fatal cases ($n = 222$) immune response occurred during epidemics in Brazil

**Table 4** Logistic models with logit links of association of dengue serotype and Age with immune response that lead to the evolution to a dengue fatal outcome

| Variable Factor (vs.) | Factor | *n* (fatal/non-fatal) | OR (95% CI) | *p* value |
|---|---|---|---|---|
| Serotype | **Immune Responses** | | | |
| 'n' Primary Responses | | | | |
| DENV-1 | **Secondary** | **67 (5/62)** | 0.38 (0.14–0.96) | 0.095 |
| *n* = 57 (10/47) | | | | |
| DENV-2 | **Secondary** | **140 (46/94)** | 0.83 (0.49–1.41) | 0.558 |
| *n* = 62 (23/39) | | | | |
| DENV-3 | **Secondary** | **82 (19/63)** | 1.04 (0.60–1.79) | 0.904 |
| *n* = 138 (31/107) | | | | |
| DENV-4 | **Secondary** | **4 (04/00)** | – | – |
| *n* = 8 (03/05) | | | | |
| Age | **Immune Responses** | | | |
| (years old) 'n' Primary Responses | | | | |
| 0–15 | **Secondary** | **46 (24/22)** | **3.93 (1.86–8.62)** | **0.003** |
| *n* = 46 (10/36) | | | | |
| 16–30 | **Secondary** | **63 (10/53)** | 0.6 (0.28–1.26) | 0.267 |
| *n* = 63 (15/48) | | | | |
| 31–50 | **Secondary** | **100 (19/81)** | 0.95 (0.52–1.76) | 0.89 |
| *n* = 86 (17/69) | | | | |
| 51–96 | **Secondary** | **51 (18/33)** | 1.83 (0.89–3.83) | 0.172 |
| *n* = 37 (18/19) | | | | |

To calculate each logistic GLM, Death/Alive outcome was coded as binary variable. Odd ratios (COR), 95% confidence intervals (95%CI) and *p*-values were calculated using one GLM for each studied variable separately. Values highlighted in bold presented: OR > 1, values of OR contained into the 95%CI range and *p*-values < 0.05

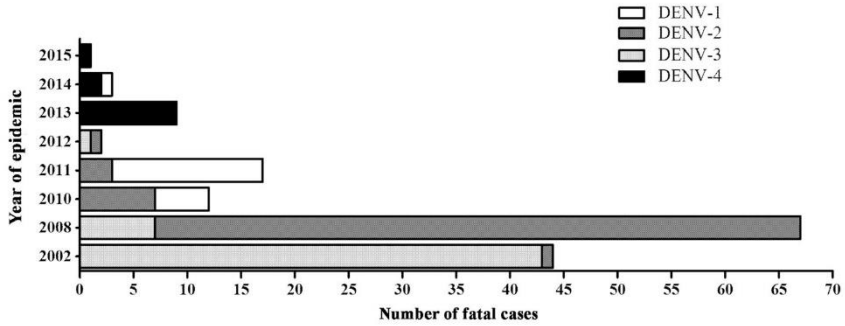


**Fig. 4** Dengue infecting serotypes identified in the fatal cases ($n = 155$) occurred during epidemics in Brazil

The 1997 World Health Organization (WHO) criteria used to classify dengue infections as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [42]. However, due its not always reliable usage to classify patients with a more severe disease, a revised classification (dengue without warning signs, dengue with warning signs, and severe dengue) was suggested and evaluated [43–45], aiming to timely manage the patient and avoid increased severity and fatal outcome.

Among the hypothesis proposed to explain the wide spectrum of dengue clinical manifestations are the samples virulence, sequential infections and multiple causality characteristics represented by individual (age sex, race, nutritional status, co-morbidities), epidemiological (immunity, competence and vector density, hyperendemicity, interval between infections by different serotypes) and viral factors, such as the virulence of the circulating strain and/or strain genotype [46].

In this study, no differences were observed when we analyzed the fatal outcome in relation to the patients gender, corroborating the findings by Wang [47] and Thomas [48]. On the other hand, a study carried out by Anders [49] in Vietnam, associated a greater risk for disease severity in females, with 1.57 higher risk to evolve to death than the males. Similarly, Sam [7] reported fatal cases in 9 out of 10 females analyzed. The study by Araujo [50] in 84 fatal cases reported death in 54% of male. Moreover, Leo [51] reported that 67.9% of men infected with dengue evolved to death in comparison to women.

Fever, myalgia, nausea, headache, malaise, hypovolemic shock, thrombocytopenia, abdominal pain and hypotension were described on the fatal cases analyzed. Less commonly observed were hepatomegaly and pleural effusion. Only four cases went into coma and only one had splenomegaly. Hemorrhagic manifestations more frequently observed were petechiae, epistaxis, gingival bleeding, hematemesis and hematuria (Fig. 2), corroborating observations in Cuba [52, 53], Singapore [54], Malaysia [7], India [55] and Taiwan [56].

Whitehorn & Simmons [57] reported that age is an important factor for severe dengue and death, and vietnamese children up to 5 years old were four times more likely to have a more severe disease than the 11–15 year-old group. On the other hand, García-Rivera & Rigau-Pérez [58] demonstrated that the elderly had 6 times more risk of death than young adults, and almost 2 times more than infants. In our analysis, during the entire study period, we observed the age groups 0–15

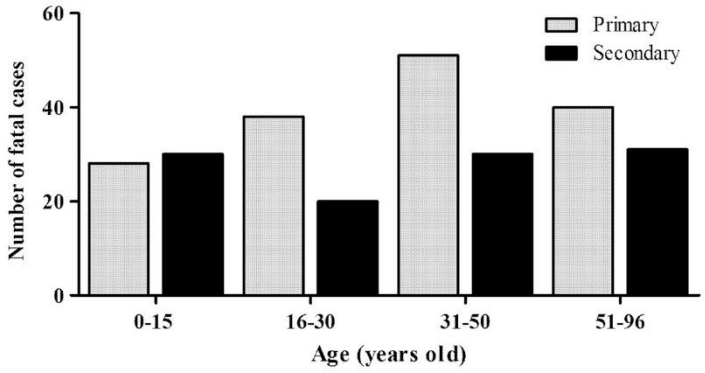


**Fig. 5** The relationship between age and immune response of dengue fatal cases ($n = 205$) analyzed in Brazil, 1986–2015

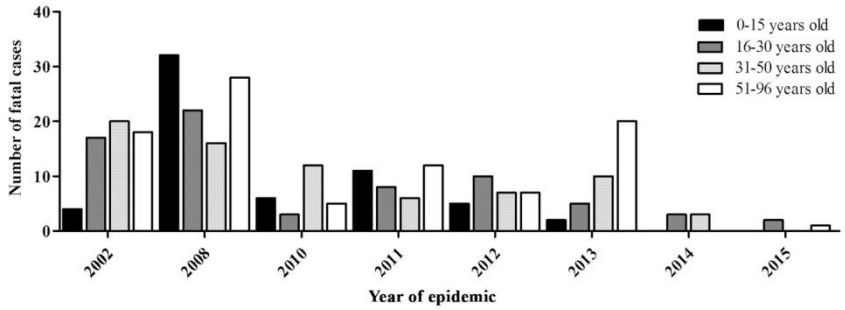


**Fig. 6** Age distribution of dengue fatal cases ($n = 164$) occurred during epidemics in Brazil

and 51–96 years old presented 1744 (95%CI: 1.173–2.600) and 2.945-fold (95%CI: 2.038–4.294) increased risk of death, respectively (Table 2).

When we analyzed epidemic and inter-epidemic periods, it was shown that up to 2006, the highest rates of dengue and severe dengue in Brazil occurred in patients over 15 years old. This same pattern was observed in an epidemic in 2010 in Puerto Rico where adults accounted for 49.7% of severe cases and fatal cases of dengue (92.5%) [59]. In Pakistan, a higher number of severe cases were observed in individuals over 30 years old in 2011 [60].

The initial pattern of severe cases in young adults presented significant changes in recent years in Brazil. In 2007, increased hospitalization rates and severe dengue in children 15 years old and under were reported, similar to the observations on Southeast Asia [61, 62]. These data corroborate those found in our study, since we observed that during the 2008 epidemic caused by DENV-2, fatal cases in children under 15 years old were more frequent and not observed in other epidemics and children aged 0–15 years infected with DENV-2 had increased odds of cases evolve to death (Table 3, Fig. 3-6). As the co-circulation of several DENV serotypes increases in Brazil, adults are less likely to remain susceptible to infection [63].

Several studies have shown that secondary infections were related to increased risk of severe dengue and death [14, 52, 64–66]. A study by Nisalak [67] found that secondary infections had a five-fold increased risk for the occurrence of dengue haemorragic fever than primary infections. In a cohort of 97 pediatric patients in India, the evolution of the disease severity was greater in secondary infections and in approximately one third of primary infections [68]. In our study, fatal cases due to primary infections were more significantly observed than secondary ones ($p = 0.001$). The analysis of fatal cases occurred in 2002 by DENV-3 also reported a higher frequency of primary infections [69]. The highest number of fatal cases due to secondary cases was a characteristic of the DENV-2 epidemic. Furthermore, children 0–15 years old presenting secondary infection showed a 4-fold increased risk (95%CI: 1.863–8.620) to a fatal outcome (Table 4). However, a study in children in Thailand did not point out a relationship between the disease severity and immune response [47]. A recent systematic review on dengue mortality, reported fatal cases to be more common in individuals presenting secondary infections and none of the reports associated deaths to primary infection [70].

It has been postulated that the disease severity may be due to the genetic and antigenic variations of the different DENV strains, as the genetic evolution of the virus within each serotype may give rise to more virulent strains [71, 72]. Despite that, any of the four DENV serotypes may lead to severe and fatal cases and a hyperendemic scenario, with the co-circulation of distinct DENV serotypes may increase the chances of more severe disease [4].

DENV-2 and DENV-3 are the serotypes most commonly associated with fatal cases. In our study, DENV-2 was identified in 43.9% (83/189) of the fatal cases and was associated with a 5-fold increased risk (95% CI: 3.829–8.678) of death when compared to DENV-1. Similarly, DENV-3 caused 32.8% (62/189) of deaths and presented an increase of 3-fold (95% CI: 2.233–5.495) for death (Table 2). Furthermore, previous studies have reported that DENV-2 secondary infections, mainly by the Asian genotype which circulates in Brazil, has led to an increase in severe cases such as hemorrhagic fever and dengue shock syndrome [52, 65, 73]. In fact, the mortality rate was twice higher after the introduction of the new lineage of the Asian DENV-2 (Lineage II) in 2007 in Brazil [61, 74–76]. DENV-3 circulating in Brazil belongs to genotype III, also of Asian origin and it has been associated to the severe disease occurred in 2002 [77].

In Thailand, a study reported a higher frequency of DENV-2 (35%) and DENV-3 (31%) cases in children during 20 years of investigation [67] and those serotypes were associated to severe cases in children up to 15 years old, in comparison to DENV-1 cases [78]. In our study, children up to 15 years old infected with DENV-2 had

nearly 4-fold increased risk of dying when compared to the same age group in the other serotypes, (Table 3). However, a relationship was also observed on elderly who were infected by any of the serotypes, Table 3. In a DENV-3 epidemic occurred in Havana, Cuba during 2001–2002, 12,889 cases and 81 DHF cases were reported [53]. However, a study in Puerto Rico in 2010 reported that DHF patients were more likely to have been infected by DENV-4 than DENV-1 [59]. In this study, DENV-2 infected individuals had almost 2 times (95%CI: 1.177–3.228) more risk of dying when compared to those infected by DENV-1 (Table 2).

DENV-1 followed by DENV-2 infections were associated with outbreaks of hemorrhagic fever [79]. However, other sequential infections, such as DENV-3 followed by DENV-2, DENV-1 by DENV-3 and DENV-2 by DENV-3 in El Salvador (2000), Cuba (2000–2001) and Brazil (2001–2002), respectively, were associated with severe disease [80]. In DENV-2 infected children previously infected by DENV-3, the occurrence of a more severe disease was also reported [81].

## Conclusions

This study demonstrates that the cause of dengue mortality in Brazil is multifactorial and, although there is much information on the disease epidemiology, information on the causes of dengue mortality is still scarce. Despite all epidemics occurred in the past three decades, the increased severity still leads to a significant number of fatal cases. The analysis performed here, demonstrates how host and viral factors play a role in the disease outcome. Moreover, with the current troublesome clinical differential diagnosis, the performance of distinct laboratorial diagnostic methods is imperative for the disease surveillance in the context of endemic arboviruses scenario.

The study has limitations and those include, in some cases, the quality of the record resulting in the lack of some clinical and/or demographical information, as well the full course of disease during hospitalization. For instance, the analysis of signs and symptoms, infecting serotype, immune response and demographic characteristics were performed only on those cases with data and information available. Dengue cases are still underreported in Brazil and the need of improvement in the proper filling of report forms has been stressed [81]. Despite this, each case record and laboratorial diagnosis results were extensively reviewed and discussed by a physician and laboratory personnel. Another limitation includes the insufficient volume of the samples in some cases for further characterization. Other pathogens were not investigated for, and thus deaths due to bacterial pathogens could not be excluded. Despite the convenience sampling used in this study, the strength of this study lies on the analysis of a considerable number of cases investigated, one of the largest reported so far in the literature and from a comprehensible period (30 years), involving dengue epidemics occurred by the four distinct serotypes in Brazil. Moreover, as a Reference Laboratory, cases were primarily sent for investigation in a time fashion manner.

### Abbreviations

CHIKV: Chikungunya virus; CI: Confidence interval; DENV: Dengue virus; ELISA: Enzyme –linked immunosorbent assay; GLM: Logistic generalized linear models; HI: Haemaglutination inhibition test; ICT: Immunochromatographic test; IFAT: Indirect fluorescent antibody test; IgG: Immunoglobulin G; IgG-ELISA: Immunoglobulin G antibody detection ELISA; IgM: Immunoglobulin M; LABFLA: Laboratory of Flavivirus; MAC-ELISA: Immunoglobulin M antibody capture ELISA; OR: Odd ratio; qRT-PCR: Real-time Reverse Transcriptase PCR (TaqMan) assay; RT-Nested-PCR: Reverse transcriptase-nested polymerase chain reaction; RT-PCR: Reverse transcriptase polymerase chain reaction; WHO: World Health Organization; ZIKV: Zika virus

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### Availability of data and materials

The datasets generated and analyzed during the current study are available on reasonable request.

### Authors' contributions

PCGN, RMR and FBS design the study. PCGN, MRQL, NRCF, FBN, JBSS, MH, TCC, DCL, BSG, SAS, ESMA performed the experiments. PCGN, FBS, AMBF and JCSA analyzed the data. PCGN, TCC, JCSA and FBS wrote the paper. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

The dengue suspected fatal cases analyzed in this study belong to a previously-gathered collection from a Project in the Laboratory of Flavivirus, Oswaldo Cruz Institute, FIOCRUZ, approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil. As a Regional Reference Laboratory for the Brazilian Ministry of Health, suspected cases are received as convenience sampling for diagnosis and informed consent is waived.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note



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## 4.3 ARTIGO 3: ANTIGENEMIA DA NS1 E CARGA VIRAL: POTENCIAIS MARCADORES DE PROGRESSÃO PARA O DESFECHO FATAL DA DENGUE?

**Revista:** Viruses

**Classificação Medicina II:** A2

**Fator de Impacto:** 3.761

**Resumo:** A dengue é um problema mundial caracterizado por uma patogênese multifatorial. Considerando os componentes virais, sabe-se que a alta viremia ou altos níveis secretados da proteína não estrutural 1 (NS1) podem estar associados a gravidade da doença. Nosso objetivo foi caracterizar níveis de viremia e da antigenemia da NS1 em casos fatais e não fatais da dengue, como potenciais marcadores de progressão para o óbito. A antigenemia da NS1 e a viremia foram determinadas em casos fatais (n = 40) e em casos não fatais (n = 40), representativos dos quatro sorotipos do DENV no Brasil. No geral, os casos fatais apresentaram níveis mais altos de NS1 e viremia. Além disso, os casos fatais de infecções secundárias mostraram níveis significativamente mais elevados de NS1 do que os não fatais. Neste estudo, independentemente do desfecho da doença, casos de DENV-1 apresentaram níveis mais elevados de NS1 do que os outros sorotipos. No entanto, os casos fatais de DENV-2 e DENV-4 apresentaram maior secreção de NS1 do que os casos não fatais destes sorotipos. A viremia nos casos fatais foi maior do que os não fatais com DENV-3 e DENV-4 apresentando maiores cargas virais. Componentes virais, como NS1 e RNA viral, podem ser fatores que influenciam no desfecho da doença. No entanto, o estado imunológico do hospedeiro, comorbidades e o suporte médico adequado não podem ser descartados como interferentes no desfecho da doença.

viruses

MDPI

Article

# NS1 Antigenemia and Viraemia Load: Potential Markers of Progression to Dengue Fatal Outcome?

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**Abstract:** Dengue is a worldwide problem characterized by a multifactorial pathogenesis. Considering the viral components, it is known that high viremia or high levels of the secreted nonstructural protein 1 (NS1) may be associated with a more severe disease. We aimed to characterize the NS1 antigenemia and viremia in dengue fatal and non-fatal cases, as potential markers of progression to a fatal outcome. NS1 antigenemia and viremia were determined in Brazilian dengue fatal cases ($n$ = 40) and non-fatal cases ($n$ = 40), representative of the four dengue virus (DENV) serotypes. Overall, the fatal cases presented higher NS1 levels and viremia. Moreover, the fatal cases from secondary infections showed significantly higher NS1 levels than the non-fatal ones. Here, irrespective of the disease outcome, DENV-1 cases presented higher NS1 levels than the other serotypes. However, DENV-2 and DENV-4 fatal cases had higher NS1 antigenemia than the non-fatal cases with the same serotype. The viremia in the fatal cases was higher than in the non-fatal ones, with DENV-3 and DENV-4 presenting higher viral loads. Viral components, such as NS1 and viral RNA, may be factors influencing the disease outcome. However, the host immune status, comorbidities, and access to adequate medical support cannot be ruled out as interfering in the disease outcome.

**Keywords:** dengue; fatal cases; NS1 antigenemia; viraemia load

## 1. Introduction

The incidence of dengue has increased dramatically around the world in recent decades, and an estimated 50–100 million cases occur annually [1]. The disease has a broad clinical spectrum ranging from a self-limiting disease in most individuals to a potentially fatal one [2,3]. Fatal cases may occur in over 10% of the severe cases, with 90% of deaths occurring in children under 15 years of age [4]. However, in recent decades, dengue and severe dengue have become more frequent among adults [5].

In Brazil, dengue has become a major public health problem since the 1980s, and it has accounted for 60–80% of the cases reported in the Americas [6–9]. The co-circulation of four serotypes and the extensive epidemics occurring in Brazilian territory have led to an increased risk of severe and fatal cases [10–13].

Dengue virus (DENV), a single-stranded positive-sense RNA virus, belongs to the *Flavivirus* genus and *Flaviviridae* family and is classified into four antigenically distinct serotypes (DENV-1 to 4). The DENV nonstructural protein 1 (NS1) is a highly conserved specific soluble glycoprotein that plays a role in viral replication [14] and can be detected in patients' serum up to 18 days after the onset of symptoms such as a fever [15], with a peak sensitivity in the first three days of fever [16,17].

Several risk factors for a severe disease have been determined, including exposure to a heterologous DENV serotype, infection by certain viral strains and serotypes, age, gender, and some host genetic variants. High DENV load or secreted NS1 levels have been associated with a more severe disease in endemic populations [18–22], as NS1 is involved in vascular leakage and endothelial hyperpermeability by disrupting the endothelial glycocalyx independently of inflammatory cytokines [23–26]. Here, we sought to characterize the NS1 antigenemia and viremia in dengue fatal cases in comparison to non-fatal ones, representative of the four serotypes, that presented during epidemics that occurred in Brazil.

## 2. Materials and Methods

### 2.1. Ethics Statement

The dengue suspected fatal cases analyzed in this study belong to a collection previously gathered from a Project in the Laboratory of Flavivirus, Oswaldo Cruz Institute, FIOCRUZ, approved (13 May 2014) by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil.

### 2.2. Dengue and Fatal Dengue Cases

Non-fatal dengue and dengue fatal suspected cases were received between January 1990 and December 2013 during an active surveillance program performed by the Laboratory of Flavivirus, IOC/FIOCRUZ, Regional Reference Laboratory for the Brazilian Ministry of Health, sited in Rio de Janeiro. In this study, samples were selected according to their availability, volume, case confirmation as dengue based on the WHO criteria [3], serotype, and signs and symptoms. Non-fatal dengue cases presented mild dengue symptoms and were classified as dengue and dengue without alarm signs [3]. A dengue case was classified as fatal when a dengue suspected fatal case was confirmed as such through a laboratory analysis. No DENV co-infections were identified, and the patients' ages varied from 8 to 80 years old.

DENV infections on non-fatal and fatal cases were analyzed and confirmed by using a laboratorial diagnosis, as follows. Serum samples (up to 8 days after the onset of symptoms) were stored at −70 °C and submitted to virus isolation, molecular methods (reverse transcriptase polymerase chain reaction (RT-PCR), Real-time Reverse Transcriptase PCR (TaqMan) assay (qRT-PCR), NS1 antigen capture ELISA, immunoglobulin M (IgM) antibody-capture MAC-ELISA, and immunoglobulin G (IgG) antibody-capture ELISA (IgG-ELISA) tests. Briefly, for virus isolation and DENV serotyping, the samples were inoculated into C6/36 Aedes albopictus cell line [27] and the serotypes were identified by indirect fluorescent antibody test [28]. An in-house MAC-ELISA was carried out for dengue cases confirmation as described by Nogueira [29]. Alternatively, the Panbio dengue IgM Capture ELISA (Panbio Diagnostics, Queensland, Australia) was used for sera for fatal case confirmations. The IgG-ELISA was previously described by Miagostovich [30] and was performed to characterize the immune response as primary or secondary. The Platelia™ Dengue NS1 Ag-ELISA kit (Biorad Laboratories, Hercules, CA, USA) was used according to the manufacturer's instructions. For the analysis by molecular techniques, the viral RNA was extracted from samples using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RT-PCR for detecting and typing DENV was performed as described previously by Lanciotti [31].

After confirmation by at least one of the laboratorial methods performed, a total of 80 non-fatal and fatal dengue cases, representing the four serotypes, were randomly selected (DENV-1, $n$ = 20

[10 non-fatal and 10 fatal]; DENV-2, $n = 20$ [10 non-fatal and 10 fatal]; DENV-3, $n = 20$ [10 non-fatal and 10 fatal] and DENV-4, $n = 20$ [10 non-fatal and 10 fatal]).

### 2.3. NS1 Antigenemia Quantification

The NS1 antigenemia quantification was performed as previously described by Heringer [32]. Briefly, a standard NS1 antigen curve (ng/mL) based on an equation (y = 1.321x + 0.1271) with $R^2$ = 0.9542 was used and established using synthetic NS1 proteins (Native Antigen Company, Oxforshire, UK) corresponding to the NS1 of DENV-1 (Nauru/Western Pacific/1974), DENV-2 (Thailand/16681/84), DENV-3 (Sri Lanka D3/H/IMTSSA-SRI/2000/1266) and DENV-4 (Dominica/814669/1981) with 10-fold dilution. All samples were tested between days 1 to 8 after the disease onset.

### 2.4. Real Time RT-PCR (qRT-PCR) Assay for Viremia Quantification

DENV viremia quantification was estimated by using the quantitative qPCR system Taqman (PE Applied Biosystems, Foster City, CA, USA) according to the protocol described by Johnson [33].

### 2.5. Statistical Analysis

Statistical analysis was performed using Graphpad PRISM version 6 (GraphPad Software, La Jolla, CA, USA). The *T* and Analysis of variance (ANOVA) tests were used to evaluate the significance of the variable categories. Statistical significance was considered when *p*-value was <0.05.

## 3. Results

### 3.1. Analysis of NS1 Antigenemia

Overall, the NS1 antigenemia was significantly higher in dengue fatal cases when compared to non-dengue fatal ones ($p = 0.01$), Figure 1A. Regardless of the disease outcome, the average of NS1 levels in primary and secondary cases was similar (4.72 ng/mL and 4.92 ng/mL, respectively), Figure 1B. Furthermore, no differences were observed in NS1 levels between non-fatal and fatal primary cases (4.50 ng/mL and 4.65 ng/mL, respectively), Figure 1C. However, fatal cases from secondary infections showed significantly higher NS1 levels than non-fatal ones (4.89 ng/mL versus 3.69 ng/mL, $p = 0.012$), Figure 1D.

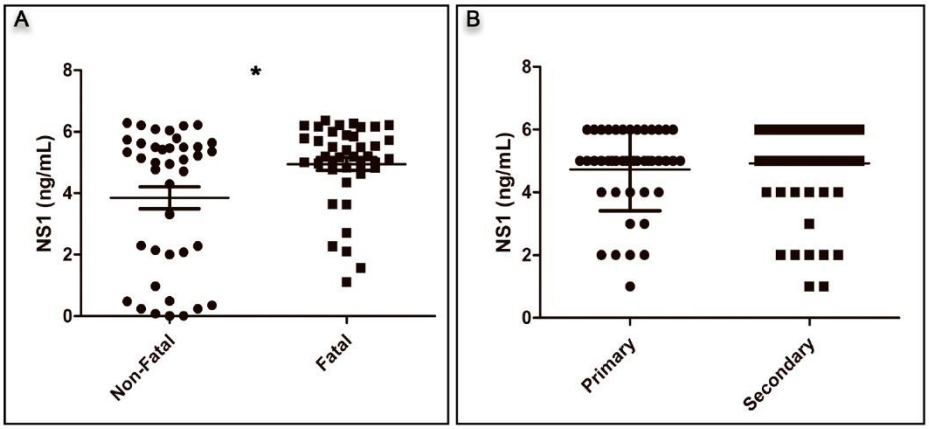


**Figure 1.** *Cont.*

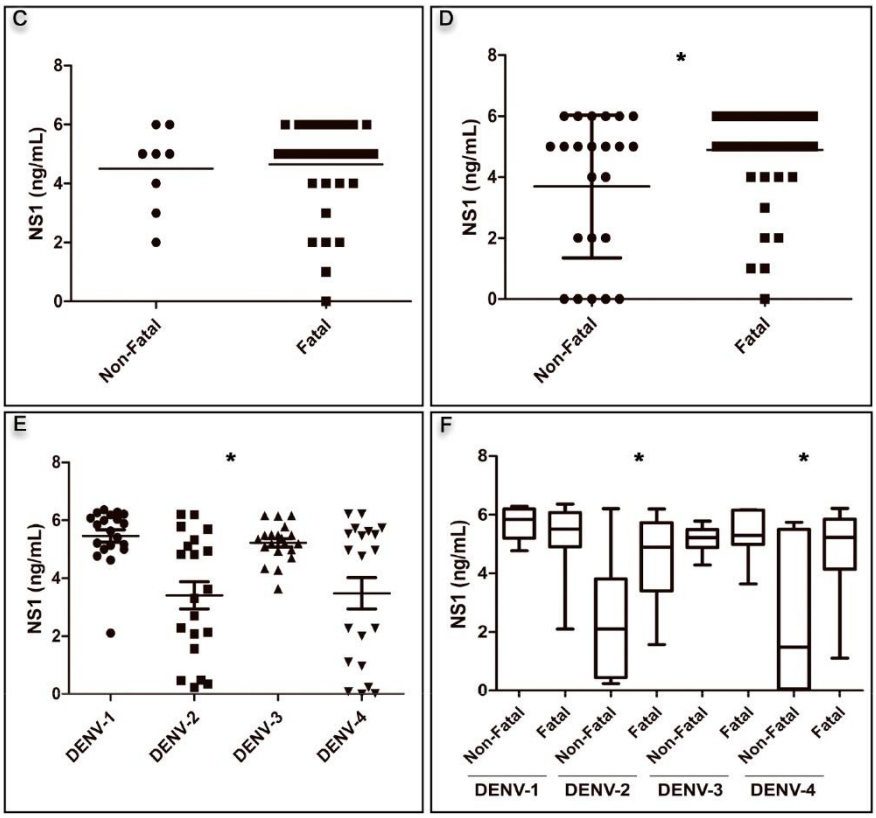


**Figure 1.** Non-structural protein 1 (NS1) antigenemia (**A**) according to the disease outcome (non-fatal: $n = 40$; fatal: $n = 40$), * $p < 0.01$ using $t$ test; (**B**) in the overall analysis of the type of infection (primary: $n = 24$ versus secondary: $n = 45$) using $t$ test; (**C**) in the analysis of primary non-fatal ($n = 8$) versus fatal ($n = 16$) cases by $t$ test; (**D**) in the secondary non-fatal ($n = 23$) and fatal ($n = 22$) cases, * $p < 0.01$ using $t$ test; (**E**) in the analysis of DENV serotype regardless of the disease outcome ($n = 80$) by ANOVA test * $p < 0.01$; (**F**) in the analysis of the distinct DENV serotypes according to the disease outcome (fatal: $n = 10$ and non-fatal/serotype) using $t$ test. For DENV-2, fatal versus non-fatal cases, * $p < 0.01$, and for DENV-4 fatal versus non-fatal cases, * $p < 0.01$.

The overall analysis of the distinct DENV serotypes showed that, regardless of the disease outcome, DENV-1 showed significantly higher NS1 antigenemia, followed by DENV-3, DENV-4, and DENV-2 ($p < 0.001$; Figure 1D, Table 1).

**Table 1.** Overall NS1 antigenemia and RNA viral load according to the distinct DENV serotypes, independent of the disease outcome.

| DENV Serotype | *n* | NS1 Antigenemia (Mean, ng/mL) | *p* | RNA Quantification (Mean, Copies RNA/mL) | *p* |
|---|---|---|---|---|---|
| Dengue 1 | 20 | 5.46 | <0.001 | 1.83 × 106 | =0.001 |
| Dengue 2 | 20 | 3.41 | | 4.73 × 103 | |
| Dengue 3 | 20 | 5.23 | | 1.03 × 109 | |
| Dengue 4 | 20 | 3.48 | | 1.30 × 109 | |

In DENV-2 and DENV-4 fatal cases, the NS1 levels were significantly higher than those observed in non-fatal cases ($p = 0.013$ and $p = 0.018$, respectively, Figure 1F, Table 2). Furthermore, despite the high NS1 levels observed in DENV-1 and DENV-3, no differences were observed between fatal and non-fatal cases with those serotypes (Figure 1F, Table 2).

**Table 2.** NS1 antigenemia and RNA viral load according to the distinct DENV serotype and disease outcome.

| Serotype | Disease Outcome | *n* | NS1 Antigenemia (Mean, ng/mL) | *p* | RNA Quantification (Mean, RNA Copies/mL) | *p* |
|---|---|---|---|---|---|---|
| Dengue 1 | Non-Fatal | 10 | 5.69 | =0.285 | $1.38 \times 10^6$ | =0.266 |
| | Fatal | 10 | 5.27 | | $2.29 \times 10^6$ | |
| Dengue 2 | Non-Fatal | 10 | 2.92 | =0.013 | $3.13 \times 10^1$ | =0.018 |
| | Fatal | 10 | 4.53 | | $9.15 \times 10^3$ | |
| Dengue 3 | Non-Fatal | 10 | 5.16 | =0.663 | $2.15 \times 10^6$ | =0.011 |
| | Fatal | 10 | 5.29 | | $2.06 \times 10^9$ | |
| Dengue 4 | Non-Fatal | 10 | 2.24 | =0.016 | $3.04 \times 10^7$ | =0.731 |
| | Fatal | 10 | 4.73 | | $2.01 \times 10^9$ | |

No differences were observed in the NS1 antigenemia of the different DENV serotypes between the types of infection (primary and secondary). The DENV-1 primary fatal cases had an average NS1 antigenemia of 4.80 ng/mL, and the secondary fatal cases had an average value of 5.66 ng/mL ($p = 0.248$). When DENV-2 fatal cases were analyzed by immune response, also no differences in the average of NS1 levels were observed (3.60 ng/mL and 3.30 ng/mL, respectively, $p = 0.816$). DENV-3 primary fatal cases had an average of 5.04 ng/mL of NS1, while the secondary fatal cases had a value of 5.16 ng/mL ($p = 0.682$). Because of an inadequate sample volume, only two DENV-4 fatal cases could be characterized according to the patient's immune response, and the mean NS1 level was 5.50 ng/mL.

### 3.2. Analysis of Viral RNA Quantification

Overall, dengue fatal cases had a higher viral load than the non-fatal cases, but the difference was not statistically significant ($p = 0.066$), Figure 2A. No difference was also observed when the viremia from primary cases was compared with those from secondary ones ($1.69 \times 10^8$ copies/mL and $5.46 \times 10^8$ copies/mL, respectively), regardless of the disease outcome (Figure 2B). However, in both primary and secondary fatal cases, the mean viremia was significantly higher than that observed in non-fatal primary and secondary cases ($8.19 \times 10^8$ copies/mL versus $2.13 \times 10^4$ copies/mL, $p = 0.0174$, for primary and $3.45 \times 10^8$ copies/mL versus $1.48 \times 10^5$ copies/mL, $p = 0.0048$ for secondary), Figure 2C,D.

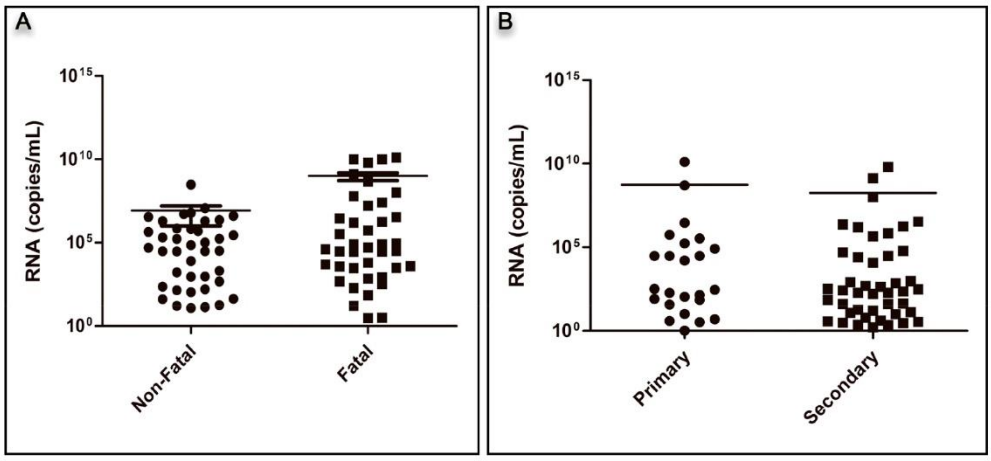


**Figure 2.** *Cont.*

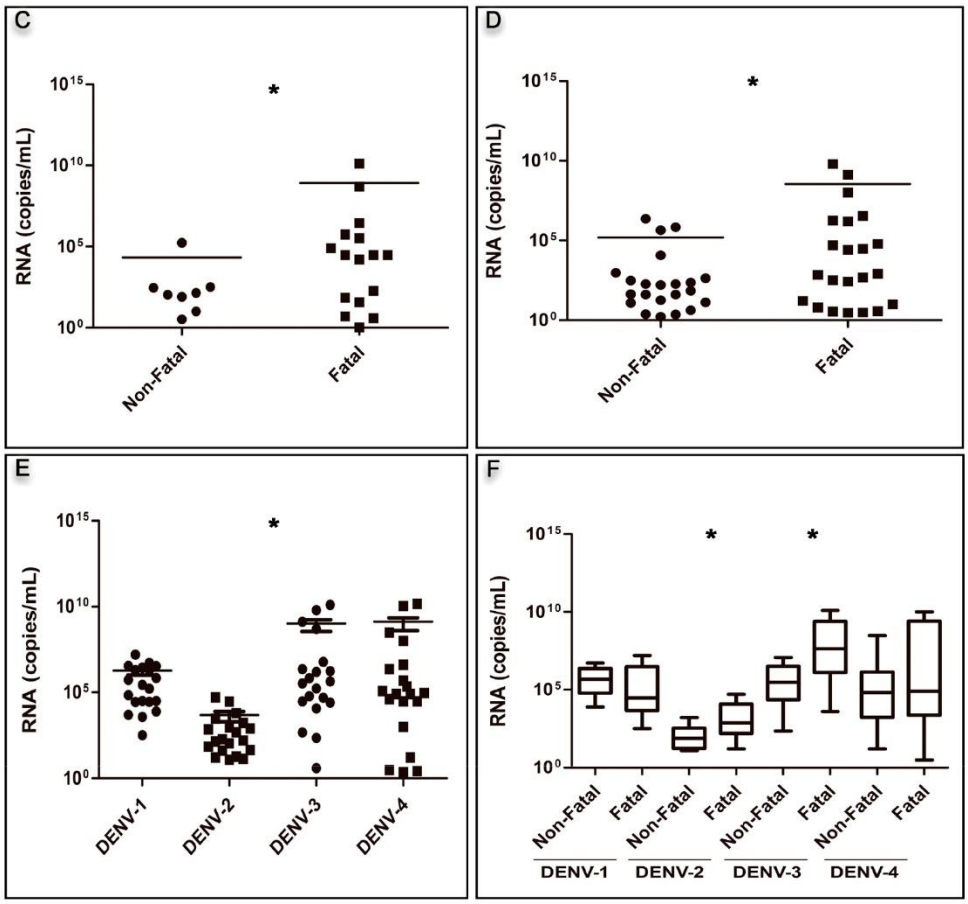


**Figure 2.** RNA viral load quantification by qRT-PCR (**A**) according to the disease outcome (non-fatal: $n = 40$; fatal: $n = 40$) $p = 0.06$ by using $t$ test; (**B**) by type of infection (primary: $n = 24$ versus secondary: $n = 45$) using $t$ test; (**C**) in primary non-fatal ($n = 8$) versus fatal ($n = 16$) cases, * $p < 0.01$ using $t$ test; (**D**) in secondary non-fatal ($n = 23$) versus fatal ($n = 22$) cases, * $p < 0.01$ using $t$ test; (**E**) in the analysis of DENV serotype regardless of the disease outcome ($n = 80$), * $p < 0.01$ by ANOVA test; (**F**) in the analysis of the distinct DENV serotypes according to the disease outcome (fatal: $n = 10$ and non-fatal/serotype) using $t$ test. For DENV-2 fatal versus non-fatal cases, * $p < 0.01$, and for DENV-3 fatal versus non-fatal cases, * $p < 0.01$.

The overall analysis according to the distinct serotypes showed that DENV-3 and DENV-4 had significantly higher mean viremia compared to DENV-1 and DENV-2 ($p = 0.001$; Table 1; Figure 2E). However, considering the disease outcome, DENV-2 and DENV-3 fatal cases had significantly higher mean viral load compared to the non-fatal cases with the same serotype ($p = 0.018$ and $p = 0.011$, respectively), Table 2, Figure 2F.

Furthermore, when analyzing only the fatal cases, no differences were observed among primary and secondary cases of the distinct DENV serotypes. Primary DENV-1 fatal cases had a mean viral load of $5.80 \times 10^6$ RNA/mL, and the secondary fatal cases of $8.60 \times 10^5$ copies/mL ($p = 0.298$). DENV-2 mean viremia was $6.05 \times 10^3$ copies/mL for the primary fatal cases and $5.22 \times 10^3$ copies/mL for the secondary ones ($p = 0.969$). The mean viral load for DENV-3 fatal cases was $3.22 \times 10^9$ copies/mL for primary and $1.25 \times 10^9$ copies/mL for secondary fatal ones ($p = 0.590$). For DENV-4, no comparison was performed, as only one primary fatal case with $8.00 \times 10^4$ copies/mL and two secondary fatal cases with a mean viral load of $5.00 \times 10^7$ RNA/mL were available.

Cases representative of DENV-1 to 4 ($n$ = 18) were previously sequenced by our group for genotype characterization, and Genotypes V, Southeast Asia, Genotype III, and Genotypes I and II were characterized for DENV-1, DENV-2, DENV-3, and DENV-4, respectively.

## 4. Discussion

Some viral proteins are described to be involved in viral disease pathogenesis, and the NS1 protein has been shown to be a marker of dengue disease severity [18,22,23,34–36]. Moreover, despite NS1 interaction with the complement system [23] and its involvement in the production of inflammatory and immunosuppressive cytokines by inducing immune cells [37,38], it is also suggested that NS1 induces endothelial hyperpermeability in vitro and vascular leak in vivo [24,25,39].

In this study, we observed that NS1 levels in fatal cases were higher than those in non-fatal ones (Figure 1A), and several studies have shared the same observation. An early study by Libraty [18] described the correlation between high circulating levels of NS1 and the development of severe dengue and was corroborated by similar observations in Thailand [23]. While analyzing Brazilian DENV-1 and DENV-4 cases, Allonso [22] observed increased NS1 levels in severe cases when compared to classic dengue. De la Cruz-Hernandez [35] reported that patients with dengue hemorrhagic fever (DHF), infected by DENV-1 showed higher levels of circulating NS1 than those with dengue fever (DF). On the other hand, the study by Duong [16] during an epidemic in Cambodia in 2006 and 2007, showed that NS1 levels significantly correlated with viremia, but a low NS1 ratio was associated with a more severe disease. In Finland, the levels of NS1 antigenemia from travel-acquired dengue cases were not associated with hospitalization [40]. A recent study in Colombia reported the correlation between high circulating levels of NS1 and the development of disease severity in children infected by DENV-1, DENV-2, and DENV-3 [41].

In this study, DENV-1 exhibited higher levels of NS1, followed by DENV-3, DENV-4, and DENV-2 (Table 2), corroborating previous observations [22,42,43]. Furthermore, Chau [42] found that NS1 levels were significantly higher in DENV-1- and DENV-3-infected infants under 18 months of age than in those infected by DENV-2, and also reported increased severity in DENV-3 cases; however, no correlation with viremia was observed. In Vietnam, a study analyzing the kinetics of plasma viremia and soluble NS1 in dengue-infected children reported higher NS1 levels in DENV-1 cases than in DENV-2 ones [43]. Considering the distinct serotypes and the disease outcome, it was shown here that DENV-2, DENV-3, and DENV-4 fatal cases presented higher NS1 antigenemia than non-fatal ones. Despite their non-significance, DENV-1 cases presented an opposite profile (Figure 1F).

Overall, the NS1 magnitude did not vary by type of infection, and no differences were observed in NS1 antigenemia between primary and secondary infections. In contrast, Perdomo-Celis [41] and De la Cruz-Hernandez [35] described that patients presenting primary infections had higher NS1 levels than those with secondary ones.

The NS1 persistence and antigenemia were previously shown to depend on the infecting DENV serotype [16,44], and patients presenting a persistent antigenemia (>5 days of illness) were more likely to develop a more severe disease [36]. However, in our study, the persistence of NS1 antigenemia was not addressed because of the nature of our sampling (convenience samples).

Overall, the fatal cases studied here had a higher viral load than the non-fatal ones, but the difference was not statistically significant (Figure 2A). Several studies of DENV viral quantification published previously showed a correlation between the amount of viral particles and increased disease severity [19,21,45–48]. In Brazil, studies with DENV-2 and DENV-3 [19,21] reported higher viremia in fatal cases and corroborated the observations made in Thailand on DENV-1 and DENV-2 and in Taiwan on DENV-3 [45,47].

Regardless of the disease outcome, no difference was also observed when the viremia from primary cases was compared to that from secondary ones (Figure 2B). On the other hand, the viremia levels were higher in fatal cases from both primary and secondary infections (Figure 2C,D). Tricou [20] observed that the peak in viremia was significantly observed less frequently during secondary than

primary infections for all disease outcomes. In contrast, Perdomo-Celis [41] observed that viremia of primary infections was higher than in patients with secondary infections.

In a pediatric cohort in India, severe dengue was observed in primary and secondary infections; however, no association between viral load and disease severity was reported, despite a correlation with prolonged thrombocytopenia and delayed recovery [49]. This is in agreement with previous observations that showed no differences in viral RNA levels in children with DHF and classic dengue fever in the past [50].

In this study, DENV-4 and DENV-3 had a higher viremia, followed by DENV-1 and DENV-2 (Figure 2E). Tricou [20] similarly demonstrated that DENV-1 infections had higher viremia levels than DENV-2 infections, and, recently, Perdomo-Celis [41] reported that viremia of DENV-1, DENV-2, and DENV-3 were greater in severe cases than in cases without and with warning signs. However, De la Cruz-Hernandez [35] reported that classic DENV-1 and DENV-2 patients presented significantly higher levels of viremia when compared to the DHF ones. Here, all serotypes had higher viremia in fatal cases than in non-fatal ones, but these differences were statistically significant only for DENV-2 and DENV-3 serotypes (Figure 2F).

## 5. Conclusions

Dengue pathogenesis is multifactorial, but viral components, such as secreted NS1 and high viral load, may influence the disease outcome. Therefore, the complex interaction between viral and host immunological determinants should still remain the subject of many studies. In this study, it was observed that, irrespective of the infecting DENV serotype, viremia and NS1 levels were higher in fatal cases than in non-fatal ones, therefore suggesting these parameters as potential biomarkers for increased dengue severity. However, one cannot disregard a patient's ability to resolve the infection or, conversely, to succumb to it and evolve to death, and the factors involved in the latter, such as the availability of adequate and prompt assistance.

**Author Contributions:** Priscila Conrado Guerra Nunes, Monique da Rocha Queiroz Lima and Flávia Barreto dos Santos designed the study. Priscila Conrado Guerra Nunes, Monique da Rocha Queiroz Lima, Manoela Heringer, Thaís Chouin-Carneiro, Cintia Damasceno dos Santos Rodrigues performed the experiments. Priscila Conrado Guerra Nunes, Monique da Rocha Queiroz Lima, and Flávia Barreto dos Santos analyzed the data. Ana Maria Bispo de Filippis and Rita Maria Ribeiro Nogueira supported the study. Priscila Conrado Guerra Nunes, Monique da Rocha Queiroz Lima, Thaís Chouin-Carneiro, and Flávia Barreto dos Santos wrote the paper.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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## 4.4 ARTIGO 4: DETECÇÃO DAS PROTEÍNAS NS1 E NS3 DO DENGUE EM PLACENTA E CORDÃO UMBILICAL DE MORTE MATERNA E FETAL

**Revista:** Journal of Medical Virology

**Classificação Medicina II:** B1

**Fator de Impacto:** 1.988

**Resumo:** No Brasil, a dengue é um problema de saúde pública com a ocorrência de epidemias explosivas. Este estudo relata um caso fatal materno e fetal e as alterações de tecidos de placenta e cordão umbilical ocasionadas pelo dengue, analisados por métodos moleculares e imuno-histoquímicos. A detecção da NS3 e NS1viral demonstrou a presença do DENV em diferentes células da placenta e do cordão umbilical. Neste último, o DENV-2 foi detectado com $1,02x10^4$ cópias de RNA/mL. Foi demonstrado que os marcadores de DENV analisados aqui, podem ser uma abordagem alternativa para a investigação de casos fatais de dengue, especialmente envolvendo morte materna e fetal.

# Detection of Dengue NS1 and NS3 Proteins in Placenta and Umbilical Cord in Fetal and Maternal Death

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In Brazil, dengue is a public health problem with the occurrence of explosive epidemics. This study reports maternal and fetal deaths due to dengue and which tissues of placenta and umbilical cord were analyzed by molecular methods and immunohistochemistry. The dengue NS3 and NS1 detection revealed the viral presence in different cells from placenta and umbilical cord. In the latter, DENV-2 was detected at a viral titer of $1{,}02 \times 10^4$ amounts of viral RNA. It was shown that the DENV markers analyzed here may be an alternative approach for dengue fatal cases investigation, especially involving maternal and fetal death. ***J. Med. Virol.*** © 2016 Wiley Periodicals, Inc.

**KEY WORDS:** dengue; NS1; NS3; maternal and fetal death; Brazil

## INTRODUCTION

Dengue virus (DENV) is an arthropod-borne virus belonging to the *Flaviviridae* family, genus *Flavivirus* and has four antigenically distinct serotypes (DENV 1–4) [Lindenbach and Rice, 2003]. The viral RNA encodes three structural proteins (capsid [C], membrane [M] and envelope [E] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [Miller., 2010]. The NS1 is secreted into the bloodstream during viral replication [Xu et al., 2006] and NS3 is a protease which cleaves the precursor polyprotein into functional proteins [Falgout et al., 1991; Keelapang et al., 2004]. The detection of both proteins confirms viral replication.

Dengue is currently one of the most important arboviral diseases in the world in terms of morbidity and mortality. In Brazil more than eight million dengue cases were reported over the past 29 years and, the state of Rio de Janeiro (RJ) in particular, has been important for the disease epidemiology, since DENV-1 introduction and spread in 1986. In 2010, a total of 1,011,548 dengue cases were reported in the country and Rio de Janeiro, alone, notified 29,824 cases and 86 deaths [SVS, 2014].

Dengue epidemiology has been characterized by an increased incidence and spread, followed by increased number of severe cases [Teixeira et al., 2013]. Despite endemic in several regions of the country, few reports of dengue infection in pregnant women and the



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consequences for the fetus are available. The occurrences of dengue fever in pregnant women have been reported since 1948 in Asia. In Brazil, the vertical dengue transmission has also been described previously, and the consequences during pregnancy for the mother and the fetus depend on the severity of the disease and pregnancy period the infection occurs [Figueiredo et al., 1994; Maroun et al., 2008; Mota et al., 2012; Machado et al., 2013]. A report performed in southeast Brazil described correlations of dengue incidence and maternal mortality [Mota et al., 2012]. Here, we report the detection of NS1 and NS3 dengue proteins in the placenta and umbilical cord of a dengue infected pregnant woman suggesting vertical transmission and resulting in maternal and fetal death.

## MATERIALS AND METHODS

### Case Investigation

In November of 2010, a 23-year-old pregnant woman was admitted to a maternity ward in Rio de Janeiro, Brazil, with complaints of abdominal pain in the lower abdomen, vomiting, diarrhea, 32–33-week-old pregnancy and audible fetal heartbeat. After lipothymy and hypotension, the obstetric evaluation revealed an inaudible fetal heartbeat and the ultra-sound showed an apparently dead fetus. The patient evolved to shock and during the intervention, a large blood extravasation into the abdominal cavity occurred and subsequent death was reported. DENV-2 was the infecting serotype identified by molecular techniques on umbilical cord (manuscript submitted). Placenta and umbilical cord were paraffin-embedded for histopathological analysis and, during the fatal case investigation process DENV infection was suggested (Soares ACG, personal communication).

### Histopathological Analysis, Molecular Diagnosis and Immunohistochemistry

The samples analyzed in this study were received as part of an ongoing Project in the Flavivirus Laboratory, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil–Regional Reference Laboratory of Dengue and Yellow Fever Diagnosis for the Brazilian Ministry of Health, approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health-Brazil.

Tissues samples from the patient necropsy were paraffin-embedded, fixed in 10% formalin, cut (4 μm), deparaffinized in xylene and rehydrated with alcohol, as described elsewhere [Póvoa et al., 2014]. Tissue sections were stained with hematoxylin and eosin for histological examination and visualized in a Nikon ECLIPSE E600 microscope.

Immunohistochemistry assays for the detection of NS3 and NS1 antigens were performed according to the protocol described by Póvoa et al. [2014]. For NS3 detection, anti-DENV-3 polyclonal antibodies (raised in Swiss mouse inoculated with DENV-3) [Póvoa et al., 2014] were used, while detection of the NS1 was performed using a polyclonal serum obtained

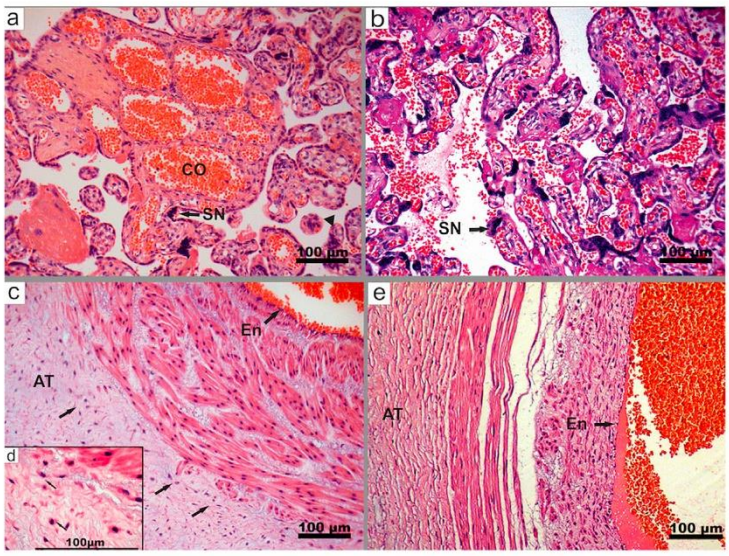


Fig. 1. Histopathological analysis of the placenta and umbilical cord from a pregnant woman infected with DENV, stained with H.E. (**a**) Placenta of the dengue patient showing corioangiosis (CO), villous hypotrophy (arrow head) and areas with syncytial knots (SN). (**b**) Placenta of a control non-dengue patient. (**c** and **d**) Umbilical cord of the dengue patient reveling an increase number of macrophages (black arrow) inside the adventitia tunica (AT) of the artery. (**e**) Umbilical cord of a non-dengue patient exhibiting tunics with normal structures. Endothelium (En).

from rabbit immunized with a DNA vaccine encoding the DENV-2 NS1 gene [Costa et al., 2006].

For molecular diagnosis, the viral RNA was extracted from paraffin-embedded tissues using the PureLink FFPE RNA Isolation Kit (Invitrogen, Carlsbad, CA). The viral RNA quantification was determined using the protocol described by Poersch [de Oliveira Poersch et al., 2005], using a Taqman quantitative Real Time RT-PCR system (PE Applied Biosystems, Foster City, CA).

## RESULTS

The histopathological analysis of the dengue patient placenta (Fig. 1a) showed areas of corioangiosis and villous hypotrophy, while the control placenta (non-dengue patient) exhibit normal villous structures (Fig. 1b). In the umbilical cord, an increased number of macrophages in the adventitia tunica of the two arteries were observed (Fig. 1c and d) when compared to the control (Fig. 1e). The immuhistochemistry assay revealed the presence of the DENV NS3 antigen in macrophages inside the placental villus of the dengue case (Fig. 2a). In the umbilical cord, the NS3 was detected in endothelium (Fig. 2c) and macrophages in the adventitia tunica (Fig. 2e). Interestingly, in the umbilical cord, the dengue NS1 protein was detected both in macrophages in the adventitia tunica (Fig. 2g) and in the endothelium of arteries (Fig. 2h). As expected, no NS3 or NS1 antigens were detected in control tissues obtained from a non-dengue patient (Fig. 2b, d, i and j).

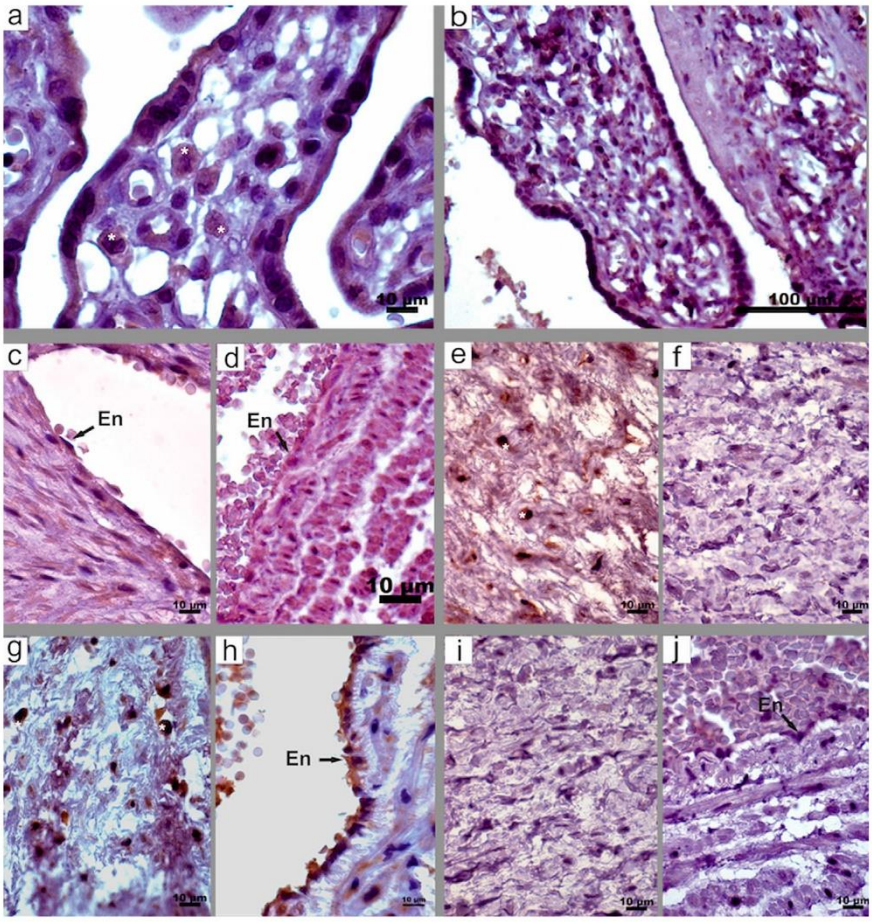


Fig. 2. Immunohistochemistry for detection of dengue antigens. (**a**) Detection of the DENV NS3 protein in macrophage of the placental villous of dengue patient (*). (**b**) Negative control of the placenta of a non-dengue patient incubated with anti-NS3 antibody. (**c**) Detection of the NS3 protein in endothelium (En) of umbilical cord. (**d**) Negative control endothelium of the umbilical cord of a non-dengue patient incubated with anti-NS3 antibody. (**e**) Detection of the NS3 protein in macrophage inside the adventitia tunica of umbilical cord of the dengue patient (*). (**f**) Negative control macrophage of the umbilical cord of a non-dengue patient incubated with anti-NS3 antibody. (**g**) Detection of the DENV NS1 protein in macrophage inside the adventitia tunica and (**h**) in the endothelium of umbilical cord of the dengue patient (En). (**i** and **j**) Negative control of the umbilical cord of a non-dengue patient incubated with anti-NS1 antibody in macrophage and endothelium.

DENV-2 was the infecting serotype identified only in the umbilical cord, with viral titer of $1{,}02 \times 10^4$ copies/ml at a Ct of 28.

## DISCUSSION AND CONCLUSIONS

Over the past 50 years, dengue has progressively achieved the status of a pandemic, and approximately 4 billion people are at risk of infection with an estimated 390 million cases and 20,000 deaths occurring annually [Bhatt et al., 2013; Messina et al., 2015].

During pregnancy, the fetus may be susceptible to DENV infection, especially during the critical period of organogenesis or in late pregnancy, even though the primary infection may not manifest symptoms in the mother [Kliks et al., 1988; Figueiredo et al., 1994; Maroun et al., 2008; Pouliot et al., 2010 Mota et al., 2012].

The study by Ribeiro et al. [2012] investigated 28 placentas of pregnant women infected by DENV and the main pathological changes observed were hypoxia (causing edema of the villous stroma, excessive formation of nodes syncytial and corioangiosis), deciduitis, coriodeciduitis, intervillositis and villositis. Here, we also report such observations specially the corioangiosis and increased number of macrophages either in the placenta or in umbilical cord.

The immunohistochemical assay revealed the presence of dengue NS1 and NS3 antigens, in macrophages present in placental villus and inside the adventitia tunica of umbilical cord, as well as in the endothelium of the two arteries of the umbilical cord. The endothelium is the first barrier vasculature in dengue viral infection. The endothelial cells may also contribute directly to the pathogenesis by altering capillary permeability and allowing its replication, inducing cytokine secretion by modulating the complement cascade or transforming the endothelium in a target immune responses [Dalrymple and Mackow, 2012].

Since the NS3 protein is a non-structural protein which is present only inside dengue infected cells, these results may suggest viral replication in macrophages of both the placenta and umbilical cords and in the endothelium of the arteries. Immunohistochemistry test by using anti-NS1 antibodies confirmed the results obtained with anti-NS3. The NS1 is also a non-structural DENV protein, and therefore indicates virus replication. However, since the NS1 is present inside infected cells as well as secreted to extracellular medium, its presence may not be an indicative of virus presence in the detected cells. Nevertheless, the combination of both tests, with NS3 and NS1, reinforced results suggesting virus replication in those cells. In addition, those observations are reinforced by the high titer of DENV-2 RNA detected in the umbilical cord.

As far as we know, this is the first report showing the DENV NS1 and NS3 protein in macrophage and endothelium in the placenta and umbilical cord, suggesting a possible DENV vertical transmission.

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## 4.5 ARTIGO 5: INVESTIGAÇÃO DE MÚLTIPLOS TECIDOS DE NATIMORTO DE INFECÇÃO MATERNA POR DENV-4: CARACTERIZAÇÃO HISTOPATOLÓGICA E DE MEDIADORES INFLAMATÓRIOS

**Revista:** Viruses

**Classificação Medicina II:** A2

**Fator de Impacto:** 3.761

**Resumo:** A associação entre o DENV e a transmissão vertical com distúrbios na placenta consiste em uma preocupação mundial. Neste estudo, investigamos um caso de infeção materno-fatal por DENV-4 ocorrido em epidemia no 2013, que resultou no óbito fetal. Tecidos de múltiplos órgãos disponíveis *post-mortem*, foram investigados quanto às alterações histopatológicas e aos mediadores inflamatórios. Durante a 29ª semana gestacional, a gestante apresentou complicações graves da infecção por dengue e óbito fetal intrauterino. A análise *post-mortem* dos órgãos fetais demonstrou a presença de DENV por RT-PCR no cérebro e detecção da NS3 específica para o DENV. O vírus foi identificado na placenta (principalmente em células Hofbauer) e em vários tecidos fetais periféricos, como cérebro, fígado, pulmões e baço. Análises histológicas da placenta e dos órgãos fetais revelaram diferentes tipos de anormalidades teciduais, que incluíram inflamação, hemorragia, edema, necrose na placenta, bem como desorganização tecidual no feto, como parênquima esponjoso, inflamação microglial, esteatose, hialinose arteriolar, células inflamatórias nos septos alveolares e desorganização do folículo linfoide esplênico. O aumento da celularidade (macrófagos, células de Hofbauer e linfócitos TCD8+, bem como a regulação de mediadores inflamatórios como IFN-γ, TNF-α, RANTES/CCL5, MCP1/CCL2 e VEGF/R2 foram detectados no fígado, pulmão, baço, cérebro e placenta que suportam a inflamação dos tecidos periféricos da placenta e do feto. O vínculo materno-fetal sob uma influência viral leva à ativação imune materna, um processo complexo que muda de forma dinâmica à medida que a gravidez evolui.

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# [Viruses] Manuscript ID: viruses-437089 - Accepted for Publication

2 mensagens

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Dear Dr. Paes,

We are pleased to inform you that the following paper has been officially accepted for publication:

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Kind regards,
Dr. Jason Kindrachuk
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Article

# A stillborn multiple organs' investigation from a maternal DENV-4 infection: histopathological and inflammatory mediators characterization

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**Abstract:** Dengue virus (DENV) is an emerging virus involved in outbreaks in Brazil. The association between the virus and vertical transmission, with disorders in the placenta, has raised a worldwide concern. On the 29th gestational week, a pregnant woman presented severe complications due to DENV infection leading to maternal and fetus deaths. Postmortem analysis of fetal organs demonstrated the presence of DENV by RT-PCR in the fetal brain and DENV NS3 staining in placenta and several peripheral fetal tissues, such as brain, liver, lungs and spleen. Histological analysis of the placenta and fetal organs revealed different types of tissue abnormalities, which included inflammation, hemorrhage, edema, necrosis in placenta and tissue disorganization in the fetus, such as spongiform parenchyma, microglial inflammation, steatosis, hyalinose arteriolar, inflammatory cells in the alveolar septa and disorganization of the lymphoid follicle. Increased cellularity (macrophage, Hofbauer cells and TCD8+ lymphocytes) and, up-regulation of inflammatory mediators such as IFN-γ, TNF-α, RANTES/CCL5, MCP1/CCL2 and VEGF/R2 were detected in liver, lung, spleen, brain and placenta, supporting placental and fetus peripheral tissues inflammation. Maternal infection leading to the production of those vascular mediators may alter the vascular permeability, facilitating the virus entry and tissue and barrier dysfunction.

**Keywords:** Dengue 4, Pregnancy, Fetal death, Cytokines, Inflammatory mediators.

## 1. Introduction

Dengue is a mosquito-borne viral disease endemic in many countries in tropical and subtropical regions worldwide [1], and approximately half of world's population is at risk of infection by one of the four Dengue virus (DENV) serotypes [2]. In Brazil, dengue emerged as a public health problem, after DENV-1 introduction in 1986 [3] and, after 32 years, the four DENV serotypes are currently co-circulating [4].

Despite its first detection in Roraima, north of Brazil in 1981 [5], it was only in 2010, that DENV-4 was re-introduced [6] and spread to other states in the country, including Rio de Janeiro (RJ), in the Southeast region [7]. Due to the populations' susceptibility to this newly introduced serotype, explosive epidemics in the country were a real threat, and the impact of the DENV-4 emergence in an endemic region, where other three serotypes were circulating, was unknown [8]. DENV-4 spread countrywide, and caused explosive epidemics in the following years. In 2013, Brazil experienced an intense epidemic, with the co-circulation of the four serotypes and a total of 1,452,489 cases were reported. RJ reported alone, a total of 213,058 dengue cases, about 15% of the whole country [9]. In fact, DENV-4 was responsible for the highest number of cases in RJ, and the metropolitan region was responsible for most cases occurred during 2013.

The disease has a broad clinical spectrum, from asymptomatic and oligosymptomatic forms to severe conditions [10] and currently, the World Health Organization (WHO) 2009 guidelines classify the illness as dengue without warning signs, dengue with warning signs and severe

dengue [11]. Severe dengue can be characterized by severe bleeding, severe organ involvement and severe plasma leakage, and most deaths are associated to this condition. In the absence of supportive care, severe dengue fatality can occur in approximately 4% of the cases [12]. Despite DENV-4 is known as a mild serotype, it has been associated to severe and fatal cases in Brazil [8]. Histopathological analysis in dengue fatal cases has demonstrated alterations and/or inflammatory reactions in the liver, spleen, kidney, lung, heart and central nervous system [13–21].

Pregnant women and neonates are considered a group of increased risk to a more severe disease [22,23]. Although there is no consensus regarding the effects of the disease on this vulnerable group, some studies indicate that vertical transmission may occur and cause serious consequences, such as preterm delivery and fetal death [24–28]. There is some evidence that the risk of severe dengue and hospitalization is higher among pregnant women compared to non-pregnant ones [29] and the maternal natural immunosuppression during pregnancy may favor the occurrence of a more severe infection [30]. Immunohistochemical analysis revealed a systemic involvement of infection with mononuclear cells targeted to all of the tissues analyzed. Assessment of local cytokine response showed increased levels of IFN-γ- and TNF-α-expressing cells in all tissues that evidenced a consistent pro-inflammatory induction and inflammatory mediators.

Maternal–fetal interface studies are still in need, as several agents, including arboviruses can be transmitted from mother to her offspring, leading to a wide spectrum of outcomes [31]. Despite the efforts and studies worldwide [23,27–30,32–41], the burden of dengue during pregnancy on the mother/child pair, is not fully understood, but shall be undoubtedly be taken seriously, especially where multiple arboviruses co-circulate, such as in Brazil [27]. Here, we sought to investigate multiple organs of a fetus from an abortion occurred in a DENV-4 infected pregnant woman during the outbreak in RJ in 2013.

## 2. Materials and Methods

*Case:* A 29-week-old pregnant woman, 36 years old, resident of Itaboraí, metropolitan region of RJ, started a febrile illness with vomiting, arthralgia, headache and epigastria on 03/16/2013. She was admitted to a hospital in Rio de Janeiro on 03/17/2013 presenting a leukocyte count of 13,000/mm$^3$, hematocrit of 33.7%, platelet count of 276.000/mm$^3$ and a positive result for DENV NS1 antigen. She was dismissed and requested to return within 48 hours for new evaluation. On 03/22/13, the patient returned to the health unit with pain in the lower limbs, vaginal bleeding, leukocyte count of 14,000/mm$^3$, hematocrit of 34.3% and platelet count of 112,000 mm$^3$ and, was hospitalized in the ICU. On the following day, she presented intense bleeding, blood pressure of 13x10, vomiting with blood, leukocyte count of 445,000/mm$^3$, hematocrit of 26.5% and platelet count of 56,000/mm$^3$. The ultrasound revealed a stillborn and

vaginal delivery was performed. The fetus was detached from the placenta and an autopsy was performed. Fragments of liver, spleen, brain, lung and placenta were collected and sent to the Flavivirus Laboratory, FIOCRUZ on 03/27/2013 for case investigation. After vaginal delivery, she remained in the ICU, but died 11 days later, on 04/02/13 and no autopsy was performed at that time. The case was classified as Dengue with Complications (DCC), according the criteria established by the Brazilian Ministry of Health in 2000. DCC was established to define severe dengue cases that did not meet the 1997 WHO criteria for dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).

*Ethical Considerations:* The samples used in this study were received as convenience samples at the Flavivirus Laboratory, Oswaldo Cruz Institute, FIOCRUZ, Regional Reference Center for Dengue, Yellow Fever, Chikungunya and Zika for the Brazilian Ministry of Health. This study was approved by the Research Ethics Committee (CEP 274/05 and CAAE: 57221416.0.1001.5248) of the Oswaldo Cruz Foundation, Ministry of Health, Brazil.

*Molecular diagnosis, histopathological analysis and immunohistochemistry:* Tissues samples from necropsy were paraffin-embedded, fixed in 10% formalin, cut (4μm), deparaffinized in xylene and rehydrated with alcohol, as described elsewhere [18]. For the paraffin-embedded viral RNA extraction, three 5-μm slices of each fragment were used and submitted separately to the PureLink ™ FFPE RNA Isolation Kit (Invitrogen, CA, USA). The reverse transcriptase polymerase chain reaction (RT-PCR) for DENV identification and serotyping was performed as described by Lanciotti et al. [42]. Tissues sections were stained with hematoxylin and eosin for histological examination and visualized by light microscopy (Olympus, Tokyo, Japan) and digital images were obtained using Image Pro Plus software version 4.5. For immunohistochemical studies, antigen retrieval was performed by heating the tissue in the presence of EnVision Flex target retrieval solution high pH (Dako, CA, USA), or citrate buffer. Tissues were blocked for endogenous peroxidase with 3% hydrogen peroxidase in methanol and rinsed in Tris-HCl (pH 7.4). To reduce non-specific binding, sections were incubated for 30 min at room temperature. Samples were then incubated overnight at 4 ° C with anti-DENV NS3 recombinant antibody [18], rabbit anti-human CD4 monoclonal antibody clone SP35 (Spring Bioscience, CA, USA), mouse anti-human CD8 Clone C8/144B (Dako, CA, USA), macrophage antibody CD68 clone EBM11 (Dako, CA, USA), anti-MCP 1 monoclonal antibody (Novus Biologicals, CO, USA), anti-TNF alpha antibody, Clone ab6671 (Abcam, MA, USA), goat IFN-γ (D-17) polyclonal antibody (Santa Cruz Biotechnology, TX, USA), anti-RANTES antibody, Clone ab189841 (Abcam, MA, USA); anti-VEGF/R2 (Spring Bioscience, CA, USA).The next day, the sections were incubated with REVEAL COMPLEMENT secondary antibody (Spring Bioscience, CA, USA) for 10 min, and a REVEAL-HRP secondary antibody conjugate (Spring Bioscience, CA, USA) for 15 min at room temperature. Reaction was revealed with diaminobenzidine (Spring Bioscience, CA, USA) as chromogen and sections were counterstained on Harris hematoxylin (Dako, CA, USA).

## 3. Results

Fragments of liver, lung, spleen, brain, and placenta were available and, after viral RNA extraction, the RT-PCR detected DENV-4 infection only in the fetal brain. In the brain, an increase in microglial cells and glial cells was observed in the white matter region (Figure 1B and 1C). As expected, the brain tissue of a non-infected fetus (negative control) showed pyramidal neurons layer and white matter with regular structures (Figure 1A). Meningeal thickening presented mononuclear inflammatory infiltrate and DENV NS3 protein was detected in endothelium, macrophages and microglial cells in the white matter (Figure 1D and 1E). The inflammatory infiltrates were more diffuse in the white matter with the predominance of microglial cells positive for CD68+ (Figure 1G), while the control only showed CD68 staining in monocytes in the cerebral capillary (Figure 1H). CD8+ T cells were observed in the meningeal, pia mater and cerebral parenchyma (Figure 1I and 1J). Microglial cells expressing RANTES, IFN-γ, MCP-1 and VEGF/R2 were identified in the spongiform parenchyma and TNF-α was detected in endothelial cells in the meninger's vessels (Figure 1L, 1M, 1N, 1O and 1P).

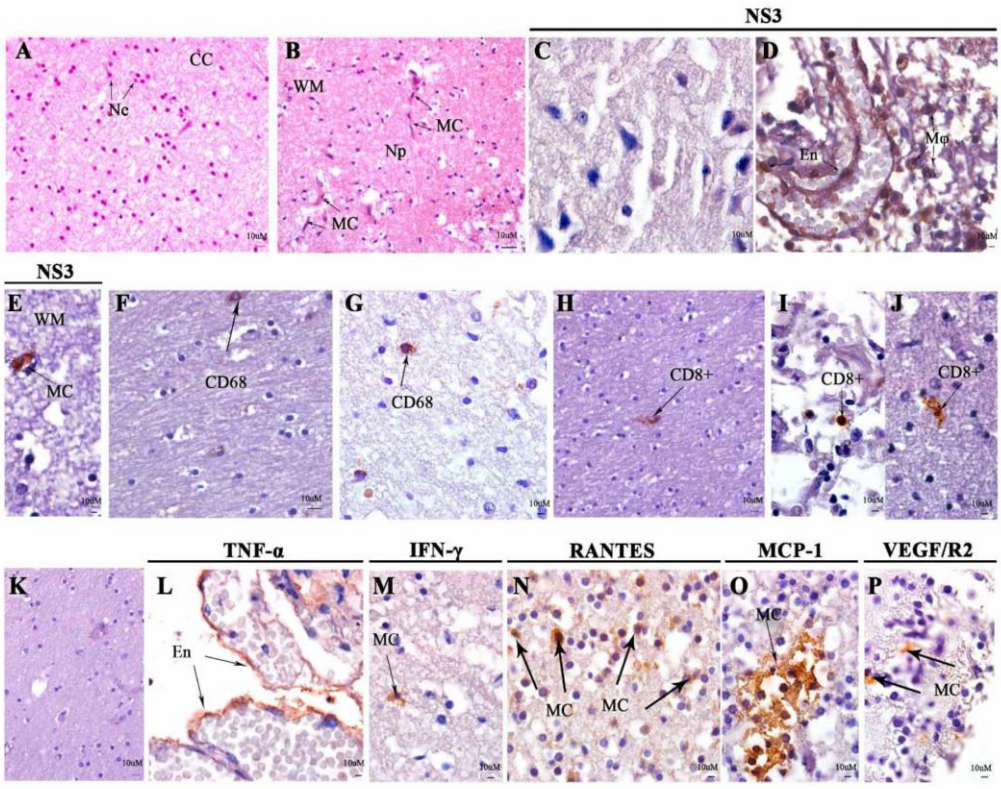


**Figure 1:** Histopathological and immunohistochemistry analysis of the fetal brain. **(A)** Brain of a non-DENV case presenting normal aspect: cerebral cortex (CC) and neurons (Ne). **(B)** White matter (WM) region of the stillborn brain presenting neuropile (Np) and microglial cells (MC). **(C)** DENV NS3 staining by immunohistochemistry in negative control. **(D)** DENV NS3 protein present in circulating macrophages (Mø) and endothelial cells (En) in blood vessels in the meningeal region and **(E)** microglial cells (MC) in white matter (WM). CD68 detection in negative control **(F)** and DENV case **(G)**. CD8+ T cell detection in vessels in negative control **(H)**, meningel region

**(I)** and parenchyma **(J)** both in DENV cases. **(K)** Representative negative control non-DENV of cytokine and inflammatory mediators. **(L)** Endothelial cells (En) TNF-α expressing in DENV-case. IFN-γ **(M)**, RANTES **(N)**, MCP-1 **(O)** and VEGF/R2 **(P)** expressing in microglial cells (MC) from DENV-case parenchyma.

In the placenta, vacuolization around the maternal decidual cells associated to vascular areas containing mononuclear infiltrate and vascular congestion, was observed. Hemorrhage and mononuclear infiltrate were observed in the chorionic villi (Figure 2B and 2C). In the maternal region, DENV NS3 protein was detected in the endothelial cells from the vessels, in the cytotrophoblasts cells, Hofbauer cells in chorionic villi, macrophages and decidual cells (Figure 2E and 2G). Moreover, circulating macrophages and monocytes expressing CD68+ cells in the intervillous space and Hofbauer cells were detected (Figure 2I and 2J). Also in the maternal region, activated CD8+ T cells were detected in the chorionic villus (Figure 2L and 2O). Expression of TNF-α, IFN-γ, RANTES and MCP-1 was observed in macrophages, Hofbauer cells, cytotrophoblasts and endothelial cells. In addition, VEGF/R2 was detected in endothelium and lymphocytes (Figure 2Q and 2U). Controls exhibited normal chorionic villi syncytiotrophoblasts, cytotrophoblasts and endothelial cells (Figure 2A).

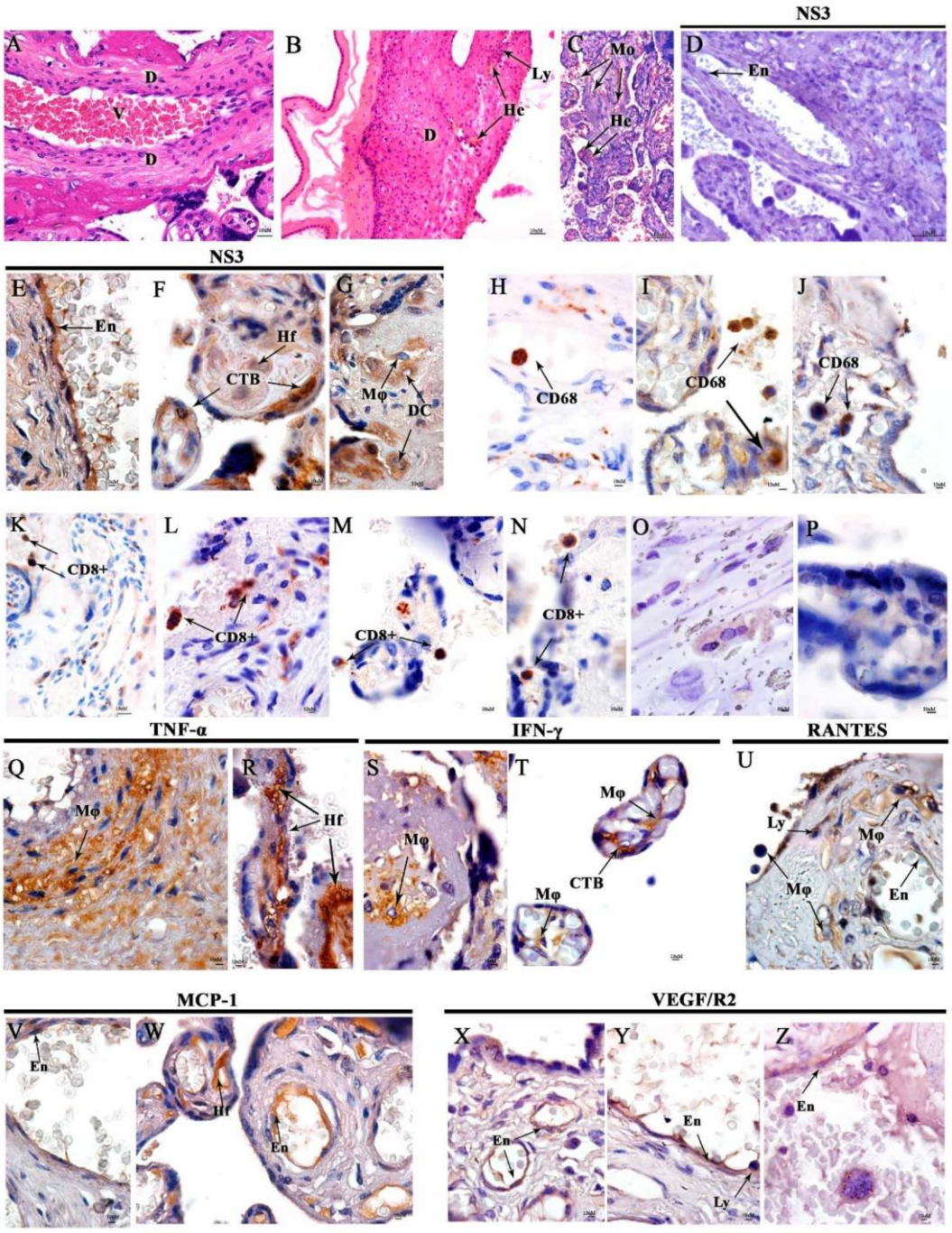


**Figure 2:** Histopathological and immunohistochemistry analysis of the placenta. **(A)** Non-DENV patient stained with H.E. and presenting normal maternal decidua **(D)** and blood vessels (V). **(B)** Maternal decidua (D) from the DENV case presenting focal hemorrhage and lymphocytic infiltrates. **(C)** Chorionic villus with mononuclear infiltrate (Mo) and haemorrhage (He) and also intervillus space in the DENV case. **(D)** Placenta control with no DENV NS3 protein detection. **(E)** DENV NS3 protein detection in endothelial cells (En) from the maternal vessel, **(F)** in the cytotrophoblasts (CTB) and Hofbauer cells (Hf), **(G)** in decidual cells (DC), and macrophages (Mø) in

the infected placenta. **(H)** CD68 detection in negative control and in the DENV case **(I-J). (K)** $CD8^+$ T cells detection in negative control and in the DENV case **(L-N). (O-P)** Representative negative control of cytokine and inflammatory mediators from a non-DENV case (Q-R) Expressing TNF-α in macrophages (Mø) and Hofbauer cells (Hf) in the DENV-case. **(S)** Expressing IFN-γ in macrophages (Mø) in maternal region, **(T)** in Macrophages (Mø) and cytotrophoblasts (CTB) in chorionic villi**. (U)** Expressing RANTES in Macrophages (Mø), endothelial cells (En) and lymphocytes (Ly) in the maternal region. **(V)** Endothelial cells (En) expressing MCP-1 in the maternal region and, **(W)** endothelial cells (En) and Hofbauer cells (Hf), both in chorionic villi. **(X-Z)** Macrophages (Mø), endothelial cells (En) and lymphocytes (Ly) expressing VEGF/R2.

The liver's negative control showed normal parenchyma and regular central vein and portal space (Figure 3A). The stillborn liver showed a diffuse area of necrotic hepatocytes with mononuclear infiltrate, microesteatosis and macrosteatosis, hyperplasia of Kupffer cells, polyploidy, discrete area of lymphocyte infiltrate in the sinusoidal capillary, thickening of the endothelium in the central vein and presence of edema (Figure 3B and 3D). DENV NS3 protein was detected in the Kupffer cells and hepatocytes (Figure 3F) and CD68+ was expressed the hyperplasic Kupffer cells and circulating macrophages (Figure 3G and 3H). $CD8^+$ and $CD4^+$ T cells were identified inside the hepatocyte's cytoplasm (Figure 3J and 3K). Kupffer cells and endothelial cells were expressing TNF-α in macrophages and lymphocytes (Figure 3O) and Kupffer cells expressing IFN-γ and RANTES (Figure 3P and 3Q). Circulating Kupffer cells and macrophages were expressing MCP-1 and VEGF/R2 in the sinusoidal capillaries (Figure 3R and 3S). The control liver tissue showed low density of positive cells (Figure 3N).

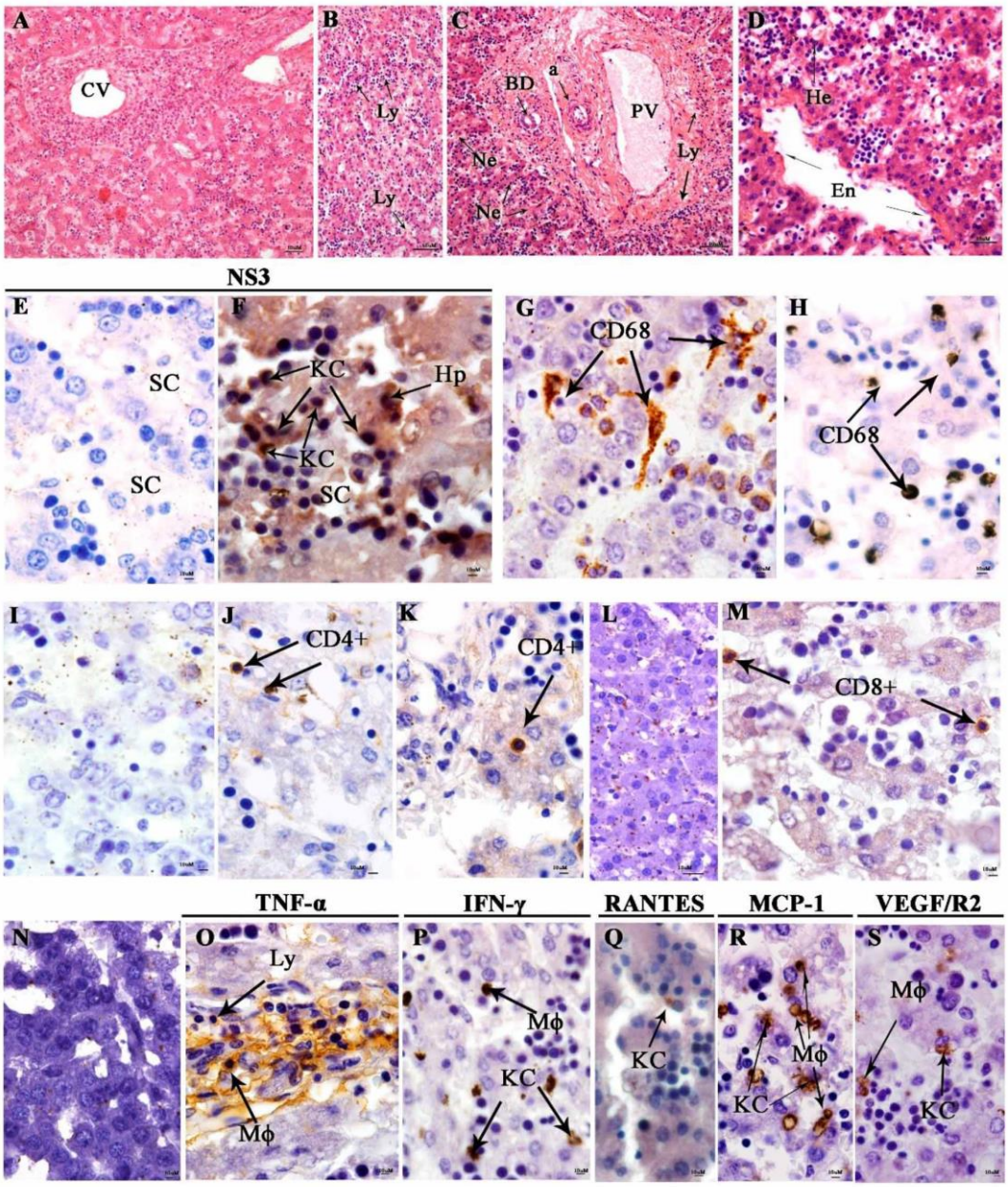


**Figure 3:** Histopathological and immunohistochemistry analysis of the liver. **(A)** Liver of a non-dengue case stained with H.E, presenting a normal central vein (CV) aspect. Stillborn liver with lymphocytes infiltrate (Ly) in the lobular center **(B)**, **(C)** area of necrotic hepatocytes (Ne), with normal bile duct (BD) and lymphocytes infiltrate (Ly) in the peri-portal vein (PV), **(D)** thickening of the endothelium (En) in the lobular center and presence of hemorrhage (He). **(E)** Sinusoids capillaries of the negative control without DENV NS3 staining. **(F)** DENV NS3 protein in Kupffer cells (HPC) and hepatocytes (Hp) near the sinusoids capillaries. CD68 cells staining in the control **(G)** and in the dengue case **(H)**. No evidence of $CD4^+$ **(I)** and $CD8^+$ T cells **(L)** in the negative control. $CD4^+$ **(J-K)** and $CD8^+$ **(M)** T cells detection in the dengue case. **(N)** Representative negative control of cytokine and inflammatory mediators from a non-DENV case. **(O)** TNF-$\alpha$ expression in macrophages (Mø) and

lymphocytes (Ly). **(P, R-S)** IFN-γ, MCP-1 and VEGF/R2 in macrophages (Mø) and Kupffer cells (HPC). **(Q)** RANTES expression in Kupffer cells (HPC).

In the lung, we observed an increase of mononuclear infiltrate around the bronchus, hyalinosis and mononuclear infiltrate in the muscular tunic, in the pulmonary artery layers and focal area of alveolar thickening (Ht) (Figure 4B and 4D). DENV NS3 protein was detected in the endothelial cells, monocytes and macrophages in the alveolar septum (Figure 4F and 4G). Cells expressing $CD68^{+}$and $CD8^{+}$ in the alveolar septum were observed (Figure 4I and 4K). TNF-α, IFN-γ, RANTES, MCP-1 and VEGF/R2 and MCP-1 were detected in macrophages and endothelial cells in the pulmonary vein (Figure 4M and 4R). The control lung tissue showed a low density of positive cells (Figure 4L).

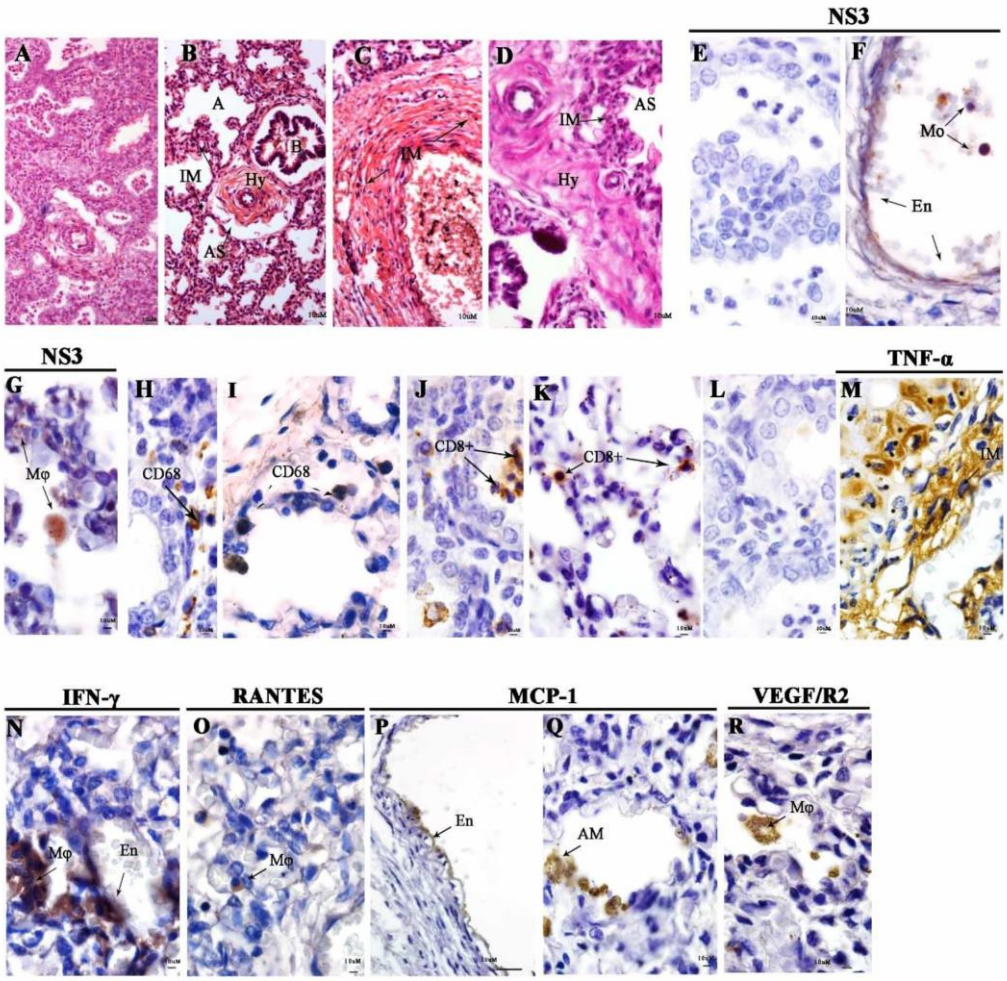


**Figure 4:** Histopathological and immunohistochemistry analysis of the lung. **(A)** Lung of a non-dengue case. In the stillborn lung, we observed the **(B)** presence of mononuclear infiltrates (IM) around the bronchus and hyalinosis (Hy). **(C)** Mononuclear infiltrates (IM) in the muscular tunica and **(D)** focal area of alveolar hyalinosis (Hy) with mononuclear infiltrate (IM). **(E)** Negative control without DENV NS3 staining. **(F)** Detection of DENV NS3 in monocytes (Mo) and endothelial cells (En) in vessels and **(G)** macrophage (Mø), in the alveolar space. **(H-I)** Presence of CD68 and **(J-K)** $CD8^+$ T cells in the alveolar space. **(L)** Representative negative control of cytokine and inflammatory mediators from a non-DENV case. **(M)** TNF-$\alpha$ in mononuclear infiltrate (IM) **(N)** IFN-$\gamma$ in macrophages and endothelial cells (En), **(O)** RANTES in macrophages (Mø), **(P-Q)** MCP-1 in endothelial cells (En) and alveolar macrophages (AM) and **(R)** VEGF/R2 in macrophages (Mø).

In the spleen, vascular congestion and follicle disorganization in the white pulp were observed (Figure 5B). DENV NS3 was detected in splenic macrophage in the follicle in germinative center of the white pulp (Figure 5D and 5E). $CD68^+$ expression was observed in activated macrophage cells that presented degenerated cytoplasm (Figure 5F) and $CD8^+$ T cells in the red pulp area

(Figure 5H). TNF-α and IFN-γ were expressed in macrophages in germinal center areas in the white pulp, but around the central arteriole and splenic macrophage in the red pulp (Figure 5J and 5L). RANTES was also expressed in endothelial cells and macrophages with expansive cytoplasm (Figure 5M and 5N), and vascular mediators MCP-1 and VEGF/R2 were present in the white pulp (Figure 5O and 5Q). The control spleen tissue showed low density of positive cells (Figure 5I).

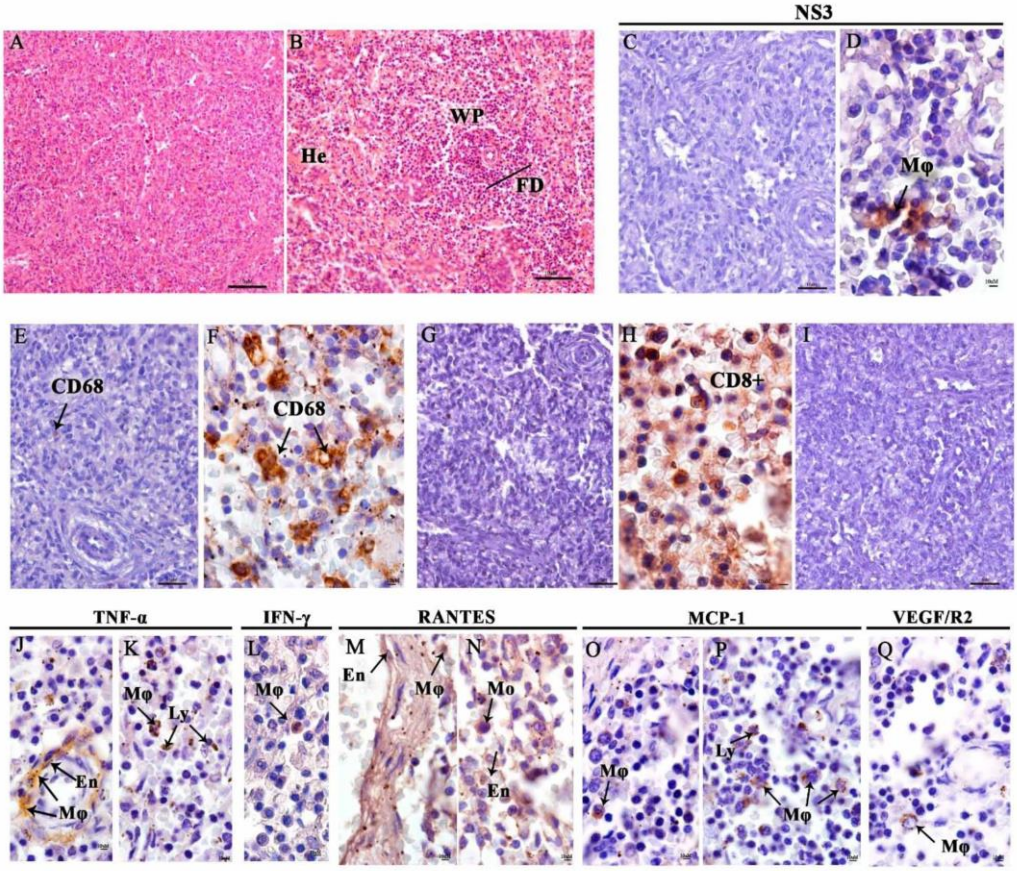


**Figure 5:** Histopathological and immunohistochemistry analysis of the spleen. **(A)** Spleen of a non-dengue case. **(B)** In the stillborn spleen, hemorrhage (He) in the red and white pulps, and follicle disorganization (FD) in the white pulp, were observed. **(C)** Negative control without NS3 staining. **(D)** Detection of DENV NS3 protein in macrophage (Mø). **(E-F)** Presence of CD68. Negative control without TCD8⁺ cells **(G). (H)** Presence of T CD8⁺ cells in red pulp. **(I)** Representative negative control of cytokine and inflammatory mediators from a non-DENV case. **(J-K)** TNF-α in macrophages (Mø), endothelial cells (En) and lymphocytes (Ly), **(L)** IFN-y in macrophages, **(M-N)** RANTES in macrophages (Mø), endothelial cells (En) and monocytes (Mo), **(O-P)** MCP-1 in macrophages (Mø) and lymphocytes (Ly) and **(Q)** VEGF/R2 only in macrophages (Mø).

## 4. Discussion

The risk of dengue infection in pregnancy is still inconclusive and controversial [43]. However, some authors consider that pregnant women are more likely to progress to more severe forms of the disease [23,27,29,30,36,41]. A recent study reported that DENV vertical transmission rates might vary between 18.5% and 22.7%. Moreover, mother-to-child DENV transmission occurs both at the beginning and at the end of the pregnancy, being more frequent when maternal dengue occurs late during gestation, near delivery [24]. Evidences suggest that symptomatic dengue fever during pregnancy may be associated with adverse fetal outcomes, such as miscarriage, stillbirth, prematurity, and low birth weight [23,40,44–46]. Here, alterations such as inflammation, hemorrhage, edema and necrosis were found in the placenta and brain, spleen, liver and fetal lung. In addition, the presence of DENV-specific NS3 protein indicates viral replication in these tissues. Similar changes caused by DENV were reported in adult fatal cases [15,18,47].

Nunes et al. [48] analyzing the placenta from a DENV-2 case, reported areas of chorioangiosis and placental villous, hypotrophy and infiltration of macrophages in the adventitia tunica of the artery in the umbilical cord. Similarly, Ribeiro et al. [49] analyzing placentas during dengue epidemics occurred in Rio de Janeiro, identified signs of hypoxia, choriodeciduitis, deciduitis and intervilositis, corroborating the observations here. However, Rabelo et al. [50] reported the presence of large diffuse areas of fibrinoid necrosis in the maternal decidua from a zika vertical transmission, with evidenced diffuse edema, fibrosis, vascular endothelial thickening, degeneration, vascular congestion and focal areas of mononuclear cells or perivascular inflammatory infiltrates.

The viral transmission to the fetal-placental tissues can occur through the maternal vascular endothelium to the endovascular extra viral trophoblasts; by infected maternal blood macrophages, which transmit the infection to placental trophoblasts and by paracellular routes from maternal blood to the fetal capillaries [51,52]. Moreover, it has been reported previously that the potential mechanisms by which a maternal infection may result in fetal death outcome, includes direct fetal infection and organ damage, placental infection resulting in decreased transmission of nutrients and oxygen, and maternal illness with increased production of cytokines and chemokines [53].

The role of cytokines in the pathogenesis of dengue severity has been demonstrated and is a consensus that inflammatory response associated with deregulated cytokine production is critical for development of severe cases, besides the virus-mediated pathogenesis. The significant increase in several soluble inflammatory mediators, “cytokine storm”, is a well-known event and is present in the most severe forms of the disease [54]. Furthermore, activated $CD4^+$ and $CD8^+$ T cells generated in response to DENV infection, may produce cytokines and inflammatory mediators, lyse infected target cells for viral control and tissue injury [55–57].

TNF-$\alpha$ is a pleiotropic cytokine that regulates many physiological and pathological functions such as cell survival, apoptosis, migration and inflammation [58]. In fact, TNF$\alpha$ is associated with dengue severity in patients [59,60] inducing vascular permeability, as well as, metalloproteinases [61] that would act on endothelial cells. Importantly, pregnancy is associated with a Th2 response with a decrease of Th1 induction and, the balance of pro and anti-inflammatory cytokines is critical for implantation, placental development and pregnancy outcome [62]. However, a cytokine imbalance

could be responsible for alterations in the placental environment, and be involved in unexplained recurrent miscarriages. Therefore, TNF-$\alpha$ plays essential role during pregnancy, for instance, in the trophoblast turnover and renewal. At the same time, TNF might be detrimental to pregnancy, causing complications such as miscarriage and preeclampsia [63]. RANTES is important in the recruitment of leukocytes to inflamed sites [64], and previous studies found low circulating levels of RANTES in the blood of dengue patients, while high expression was found in the hepatic tissue of fatal cases [65,66]. In severe dengue, the increased expression of RANTES, MCP-1 and VEGF/R2, associated with increased permeability of endothelial cells, may be indicative of a dysfunction of the blood-brain and placental barriers, increasing viral dissemination and inflammation [67–71].

## 5. Conclusions

The production of vascular mediators during the maternal dengue infection may alter the vascular permeability, facilitating virus entry and barrier dysfunction, mainly inflaming the placenta and fetal brain. The infection exacerbation occurs, concomitantly, with the viral spread to the peripheral regions, causing infection in DENV target organs such as the liver, lung and spleen and which play a role on the uptake circulation of infected cells, leading to a widespread infection in the fetus. Despite the reports suggesting that stillbirth might result from direct viral transmission to the fetus, the role of the maternal infection in this outcome, remains to be fully elucidated [45]. This study has some limitations and those include the quality of the record resulting in the lack of some clinical information and the fact that, after the mother's death, no autopsy was performed, therefore, no additional tissues were available, besides the placenta.

## 6. Patents

**Author Contributions:** PCGN, MVP and FBS conceptualized the work, PCGN, FCCR, NGS, CCJ, KR, ELA performed the experiments and formal analysis; PCGN, JMCOC, RMRN, FBS participated directly in the investigation, RMRN, JMCOC, FBS, MVP, CABO, provided resources, PCGN, MVP wrote the original draft of the paper and FBS, ELA, MVP, JJC, CBO reviewed and edited the paper.

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# 5 DISCUSSÃO

As infecções por arbovírus têm se tornado um importante problema de saúde pública em todo o mundo nas últimas décadas. Os arbovírus são considerados patógenos emergentes ou re-emergentes com base em sua dispersão geográfica e impacto crescente em populações suscetíveis. A infecção pelo DENV, uma vez de ocorrência rara, é agora estimada como a arbovirose mais comum em todo o mundo, com transmissão ocorrendo em pelo menos 128 países e com aproximadamente 4 bilhões de pessoas em risco (BHATT et al., 2013; STANAWAY et al., 2016), na África, Américas, Mediterrâneo Oriental, Sudeste Asiático e Pacífico Ocidental. Porém, as regiões das Américas, Sudeste da Ásia e Pacífico Ocidental têm sido as mais afetadas (WHO, 2018b).

Dado o crescente número de casos, a distribuição geográfica e o impacto sanitário, social e econômico dos surtos por arbovírus, estimar seu real custo representa um fator crucial, no entanto, ainda desafiador. Na fase aguda das infecções, o amplo espectro de manifestações, desde o quadro brando aos mais graves e fatais, apresentado pelos indivíduos infectados, pode resultar na classificação equivocada, principalmente quando vários arbovírus co-circulam (BEATTY et al., 2011), como no Brasil.

A vigilância epidemiológica é essencial para a identificação e investigação de surtos e epidemias, no entanto, pode subestimar a real incidência da doença. De fato, esse cenário pode ser devido à natureza das infecções por DENV com um alto percentual de casos assintomáticos (BHATT et al., 2013) e porque a procura à assistência pode variar com base no acesso aos cuidados médicos (WHO, 2018b).

Estudos em dengue têm sido realizados principalmente nas Américas (39%) e na Ásia (33%) e, neste contexto, o Brasil tem sido um dos países mais envolvidos na implementação das pesquisas de vigilância em dengue nas últimas duas décadas, seguido de Cingapura, Tailândia e Índia (FRITZELL et al., 2018). Entre 1995 e 2018, mais de 21 milhões de casos de dengue foram notificados nas Américas, cerca de 77% destes (16.576.541 milhões), somente na América do Sul. O Brasil foi responsável 56% dos casos de todo continente americano e 73% dos casos da América do Sul (PAHO, 2018). No mesmo período (1995-2018), um total de 10.124 casos fatais causados por dengue ocorreu nas Américas, sendo 7.557 na América do Sul e, desse total, 5.052 só no Brasil, o que representa cerca de 49% de todos os óbitos do continente

americano e 67% dos óbitos da América do Sul (PAHO, 2018). No entanto, diante dos problemas associados às subnotificações da doença no país (SILVA et al., 2016), estes números podem ser ainda maiores.

A progressão clínica na dengue grave pode ser dinâmica e por vezes imprevista, levando o indivíduo ao óbito rapidamente em um curto período de tempo.Vários fatores de risco para uma doença grave e fatal foram determinados, e incluem a exposição a um sorotipo heterólogo de DENV, infecção por certos sorotipos e/ou genótipos, idade, sexo e algumas variantes genéticas do hospedeiro (KATZELNICK; HARRIS, 2018).

Nesta tese, visamos analisar os casos fatais de dengue ocorridos no Brasil em 30 anos (1986-2015), realizando análises que englobaram três aspectos, importantes para o entendimento deste desfecho: epidemiológico, virológico e patológico.

## 5.1 ANÁLISE DESCRITIVA DOS ASPECTOS EPIDEMIOLÓGICOS, CLÍNICOS E LABORATORIAIS DOS CASOS FATAIS SUSPEITOS DE DENGUE RECEBIDOS NO LABFLA, IOC/FIOCRUZ E DOS DADOS DISPONÍVEIS NA BASE DE DADOS DO MINISTÉRIO DA SAÚDE, 1986 A 2015

Neste estudo, uma revisão dos casos fatais suspeitos de dengue recebidos no LABFLA, IOC/FIOCRUZ em 30 anos, foi apresentada e discutida em consonância com as observações do estudo ecológico que utilizou dados secundários de casos fatais de dengue obtidos à partir do Sistema Nacional de Informações sobre Agravos de Notificação (SINAN) e do Sistema de Informação sobre Mortalidade (SIM), ambos mantidos pelo Ministério da Infância. Saúde.

O LABFLA do Instituto Oswaldo Cruz no Rio de Janeiro, como Laboratório Regional de Referência de para o Ministério da Saúde, vem apoiando o programa de vigilância de dengue no Brasil, desde a primeira confirmação do caso de dengue em abril de 1986, quando DENV-1 foi isolado durante um surto de doença exantemática no município de Nova Iguaçu, no Rio de Janeiro (SCHATZMAYR et al., 1986). Nos anos seguintes, a doença se tornou um problema de saúde pública em quase todo território nacional, com epidemias ocorrendo em diversos estados do país (NOGUEIRA; DE ARAÚJO; SCHATZMAYR, 2007). Deste então, a

vigilância da doença tem sido considerada uma importante ferramenta para a previsão de surtos e epidemias (DE SIMONE et al., 2004).

O aumento de casos de dengue no Brasil levou ao estabelecimento e consolidação de uma rede nacional de diagnóstico de dengue em 1989 (SCHATZMAYR; NOGUEIRA; MIAGOSTOVICH, 1996; SIQUEIRA et al., 2005) para monitorar a transmissão e dispersão deste vírus recém-introduzido. Cada estado brasileiro conta com um Laboratório Central (LACEN), onde amostras de casos suspeitos de dengue recebidas de centros de saúde e os hospitais públicos, são testadas. A rede nacional é apoiada por Laboratórios de Referência Regionais, responsáveis pelas cinco regiões brasileiras: Instituto Evandro Chagas (IEC), também Laboratório Referência Nacional, o Instituto Adolfo Lutz (IAL), o LACEN Distrito Federal; LACEN Recife e o LABFLA/IOC, no Rio de Janeiro.

Em 1990, foi registrada a introdução do DENV-2 também no estado do Rio de Janeiro e, um agravamento dos quadros clínicos e notificação dos primeiros casos de FHD/SCD no país, foram reportados (NOGUEIRA et al., 1990; NOGUEIRA; DE ARAÚJO; SCHATZMAYR, 2007). A introdução do novo sorotipo causou novas epidemias em diversos estados e naquele ano foram notificados 40.279 casos e oito óbitos (SES/SINAN, 2017a, 2017b).

A introdução do DENV-3 no estado do Rio de Janeiro no final de 2000 (NOGUEIRA; MIAGOSTOVICH; SCHATZMAYR, 2000), resultou no ano seguinte, na cocirculação dos três sorotipos (DENV-1, DENV-2 e DENV-3) e, na maior e, até então, mais grave epidemia do país, no ano de 2002 (DE SIMONE et al., 2004; NOGUEIRA et al., 2002). Em 2002, 696.472 casos foram notificados e 150 óbitos confirmados (SES/SINAN, 2017a, 2017b).

Entre 2002 e 2007, o DENV-3 foi o sorotipo predominante, porém o DENV-2 reemergiu em 2007, resultando em uma extensa epidemia no ano de 2008, com um aumento significativo no número de FHD em menores de 15 anos, representando cerca de 50% dos casos de dengue e 86% das mortes ocorrendo em indivíduos nesta faixa etária (TEIXEIRA et al., 2008).

A análise das cepas isoladas após a emergência deste sorotipo apontou a existência de linhagens distintas daquelas que circularam na década de 1990 (OLIVEIRA et al., 2010), no entanto, não foram observadas diferentes genômicas virais relacionadas aos quadros mais graves (FARIA et al., 2013). Por outro lado, NUNES et al., (2016) descreveram que a viremia dos pacientes infectados pela linhagem emergente (Linhagem II) era mais elevada do que àquela dos pacientes infectados na década de 1990. Além disso, os casos graves infectados pela linhagem II apresentavam uma viremia 1.000 vezes maior. Naquele ano, o país notificou 632.680 casos e 561 óbitos por dengue (SES/SINAN, 2017a, 2017b). Os fatores que levaram à

gravidade dessa epidemia ainda não foram determinados e ficou demonstrado que, as autoridades de saúde experimentaram dificuldades óbvias no controle da epidemia, situação que causou pânico e insegurança em toda a sociedade brasileira (TEIXEIRA; BARRETO, 2009).

Em 2009, o DENV-1 reemergiu resultando em uma epidemia em 2010, com um total de 1.011.548 casos suspeitos e 656 óbitos notificados (SES/SINAN, 2017a, 2017b), porém os casos graves e óbitos foram relacionados a evidência de comorbidades (SIQUEIRA JR et al., 2011). No ano seguinte, houve uma diminuição no número de notificações, com 764.032 casos de dengue no país e 482 óbitos (SES/SINAN, 2017a, 2017b).

Até o ano de 2010 era relatada, principalmente, a cocirculação de DENV-1 e DENV-2, quando o DENV-4 foi identificado em Roraima e no Amazonas. Menos de 20 casos de DENV-4 foram confirmados naquele estado ao longo do segundo semestre de 2010. O risco da introdução do DENV-4 no país era iminente, uma vez que este sorotipo circulava em países vizinhos, como a Venezuela e Colômbia (GUZMÁN; KOURÍ, 2002a). No Rio de Janeiro, este novo sorotipo foi identificado, pela primeira vez, em 2011 (NOGUEIRA; EPPINGHAUS, 2011; TEMPORÃO et al., 2011). Em 2012, 589.591 casos de dengue e 327 óbitos foram registrados e, em 2013, o maior número de casos havia sido notificado no país (1.452.489 casos e 674 óbitos), (SES/SINAN, 2017a, 2017b).

A presença do DENV-4 propiciou o risco, não apenas do desenvolvimento de manifestações mais graves em pessoas previamente infectadas por outros sorotipos, mas pela possibilidade de aumento de casos. Com exceção da região Sul, um rápido aumento na incidência do DENV-4 foi reportado no país nos anos seguintes (SALLES et al., 2018).

O ano de 2014 foi marcado por uma queda nas notificações, no entanto, em 2015, 1.688.688 casos prováveis de dengue e 846 óbitos foram reportados no Brasil (SES/SINAN, 2017a, 2017b). As taxas de letalidade foram as mais altas vividas no país, com óbitos confirmados em todo os estados.

Casos autóctones de CHIKV e ZIKV foram registrados pela primeira vez no Brasil em 2014 e 2015, respectivamente (SVS/MS, 2015), quando epidemias de dengue ainda ocorriam no país. Neste cenário, 1.500.535 casos prováveis de dengue foram registrados no ano de 2016 (SES/SINAN, 2017b), no entanto, acredita-se que a cocirculação desses outros arbovírus, tenha resultado na redução dos casos de dengue nos anos 2017 e 2018.

Com esta nova situação epidemiológica, um novo desafio e problemática em potencial para a investigação da patogênese da dengue é a ocorrência de coinfecções por estes arbovírus, uma

vez que os desfechos virológicos e imunológicos para o paciente, ainda não são conhecidos. O diagnóstico laboratorial é um desafio em que esses arbovírus são endêmicos e, o diagnóstico etiológico mostra-se imperativo para a caracterização dos casos. Apesar dos poucos estudos relatando coinfecções por DENV e ZIKV e até CHIKV, é sugerido que pacientes coinfectados não apresentem um desfecho clínico mais grave (AZEREDO et al., 2018; CHAHAR et al., 2009; WAGGONER et al., 2016).

De acordo com os dados obtidos à partir do SINAN e SIM do Ministério da Saúde, Brasil, em 30 anos (1986-2015), foram notificados 11.084.755 casos suspeitos de dengue, com a confirmação de 5.399 óbitos em todo o país, e a doença tornou-se um grave problema de saúde pública em vários estados brasileiros (SES/SINAN, 2017a, 2017b). Historicamente, as regiões com maior incidência de casos fatais de dengue e dengue no Brasil têm sido o Sudeste, seguida pela região Nordeste. Em 30 anos, a região Sudeste registrou 43% (n = 2.225) de todas as mortes por dengue no país. São Paulo confirmou 945 casos fatais, o Rio de Janeiro, 738, Minas Gerais e Espírito Santo reportaram 430 e 196 óbitos, respectivamente. No Nordeste, os estados com maior número de casos fatais foram Ceará, Pernambuco, Bahia e Maranhão. A região Centro-Oeste foi responsável por 18% dos casos fatais e o estado de Goiás reportou 600 óbitos. No norte do país, apenas 7% das mortes foram confirmadas e o Pará foi o estado que reportou o maior número de mortes por dengue (n = 141) no período. A região Sul, historicamente menos afetada por casos de dengue, relatou, consequentemente, o menor número de casos fatais de dengue (2%) e, apenas o Paraná (n = 108) e Rio Grande do Sul (n = 4), relataram casos fatais por dengue.

Neste período, o LABFLA, recebeu casos suspeitos de dengue, atendendo as exigências do Ministério da Saúde para monitorar a doença no país. Um total de 1.047 casos fatais suspeitos de dengue, representativos das regiões Norte, Nordeste, Centro Oeste e Sudeste do país, foram recebidos e analisados em 1986 a 2015, e 34,3% (359/1.047) foram confirmados como dengue por qualquer uma das metodologias laboratoriais utilizadas no laboratório. De fato, apesar das limitações e distintas sensibilidades que algumas técnicas laboratoriais podem apresentar, sua contribuição para a vigilância e confirmação da doença, é clara. Em uma revisão sobre o diagnóstico laboratorial das infecções por dengue realizada pelo LABFLA/IOC, de 1986 a 2011, foi demonstrado que a implementação de novas técnicas pode melhorar o diagnóstico, aumentando a detecção e confirmação de casos durante períodos epidêmicos e inter-epidêmicos (DOS SANTOS et al., 2013).

O isolamento viral em células foi possível em 15,2% (46/302) dos casos confirmados, enquanto que a RT-PCR contribuiu com 46,5% (153/329) para a confirmação dos casos. A RT-PCR em tempo real contribuiu confirmando a infecção em 60,5% (78/129) dos casos fatais. Embora o isolamento viral seja considerado o padrão-ouro para o diagnóstico de dengue, a sensibilidade da detecção molecular do genoma viral oferece uma grande vantagem nos períodos epidêmicos.

A confirmação dos óbitos por ELISA de antígeno NS1 foi de 67,2%, (207/308) nos casos com até 7 dias de doença. O ELISA de captura de antígeno NS1 foi estabelecido no final de 2007 como uma abordagem alternativa para o diagnóstico precoce de infecções por DENV e, em 2008, o Ministério da Saúde do Brasil estabeleceu esta metodologia em unidades sentinelas em todo o país (DOS SANTOS et al., 2013). O teste de captura de antígeno NS1 é um método sorológico que permite o diagnóstico precoce de infecções por DENV, mesmo em laboratórios com recursos limitados (ANDRIES et al., 2012). A taxa de detecção do anticorpo IgM anti-DENV foi de 65,1% (218/335) nos casos fatais confirmados de dengue. Os métodos sorológicos ainda são a ferramenta mais útil para o diagnóstico da doença durante as epidemias, pois são mais acessíveis e de fácil execução (TANG; OOI, 2012), e a confirmação de casos por MAC-ELISA é diretamente relacionado ao período de coleta da amostra (PEELING; OLLIARO, 2016; SINGLA et al., 2016b). A contribuição da imunohistoquímica foi confirmada em 59,9% (18/34) dos casos fatais analisadas a partir tecidos embebidos em parafina disponíveis.

Entre as teorias propostas para explicar o elevado grau de variação de manifestações clínicas causadas por DENV, estão a relacionada com a virulência das cepas virais (COLOGNA; ARMSTRONG; RICO-HESSE, 2005; RICO-HESSE, 1990, p., 2003; ROSEN, 1977), a teoria de infecções sequenciais (HALSTEAD, 1988; HALSTEAD; NIMMANNITYA; COHEN, 1970; SIMMONS et al., 2006) e teoria integral de múltipla causalidade (KOURI; GUZMÁN; BRAVO, 1987), que integra os fatores individuais (idade, sexo, raça, estado nutricional, comorbidades), fatores epidemiológicos (imunidade, competência e densidade vetorial, a intensidade de circulação viral e o intervalo de tempo entre infecções por diferentes sorotipos) e fatores de virulência, principalmente relacionados aos sorotipos e cepas de origem asiática (GUZMAN et al., 2016; KATZELNICK; HARRIS, 2018).

Apesar dos quatro sorotipos de DENV produzirem as mesmas manifestações clínicas e padrões semelhantes de disseminação sistêmica, diferenças biológicas são observadas entre eles

(BARA; CLARK; REMOLD, 2013; MESSER et al., 2012). Associações entre determinados sorotipos ou genótipos e a gravidade da doença, potencial epidêmico e eficiência de transmissão já foram descritos, mas estas associações podem ser influenciadas por outros fatores, além das características virais intrínsecas, como imunidade ao hospedeiro, capacidade do mosquito vetor de se infectar e de transmitir o vírus para os seres humanos e as condições, pouco conhecidas, que influenciam o deslocamento de um genótipo por outro (HALSTEAD, 2012; RICO-HESSE, 2003).

Os DENV-2 e DENV-3 são os sorotipos mais associados aos casos fatais. Em nosso estudo, o DENV-2 foi identificado em 43,9% (83/189) dos casos fatais e foi associado a um risco 5 vezes maior de morte quando comparado ao DENV-1. Da mesma forma, o DENV-3 causou 32,8% (62/189) de óbitos e apresentou um aumento de 3 vezes para o óbito. Além disso, estudos anteriores relataram que as infecções secundárias pelo DENV-2, principalmente pelo genótipo asiático que circula no Brasil, levaram a um aumento de casos graves, como a FHD/ SCD (GUZMÁN; KOURÍ, 2002b; PAWITAN, 2011; THEIN et al., 1997). De fato, a taxa de mortalidade foi duas vezes maior após a introdução da nova linhagem do genótipo asiático (Linhagem II) em 2007 (FARIA et al., 2013; NUNES et al., 2016; TEIXEIRA et al., 2008).

O DENV-3 circulante no Brasil pertence ao genótipo III, também de origem asiática, e tem sido associado à doença grave ocorrida em 2002 (ARAÚJO et al., 2009). Na Tailândia, um estudo relatou uma maior frequência de casos de DENV-2 e DENV-3 em crianças durante 20 anos de investigação (NISALAK et al., 2003) e esses sorotipos foram associados a casos graves em crianças com até 15 anos, em comparação com casos de DENV-1 (FRIED et al., 2010).

Neste estudo, febre, mialgia, náusea, cefaléia, mal-estar, choque hipovolêmico, trombocitopenia, dor abdominal e hipotensão foram descritos nos casos fatais recebidos pelo LABFLA/IOC e analisados. Menos comumente observados foram hepatomegalia e derrame pleural. Apenas quatro casos entraram em coma e um caso apresentou esplenomegalia. As manifestações hemorrágicas mais freqüentemente observadas foram petéquias, epistaxe, sangramento gengival, hematêmese e hematúria, corroborando as observações descritas em Cuba (GONZÁLEZ et al., 2005; GUZMÁN et al., 1999), Cingapura (ONG et al., 2007), Malásia (SAM et al., 2013), Índia (GAUTAM et al., 2016) e Taiwan (WANG et al., 2009).

A gravidade da doença que acompanha uma primo-infecção por DENV está diretamente relacionada com a idade. Em crianças suscetíveis, a primo-infecção por DENV é geralmente assintomática ou leve, enquanto em adultos, quadros clássico são observados.

Quadros clínicos mais graves são observados em idosos ou em aqueles com doenças crônicas, como diabetes mellitus, doença pulmonar obstrutiva crônica ou doença cardiovascular (WHO, 2009).

Em nosso estudo, crianças com até 15 anos de idade infectadas pelo DENV-2 apresentaram risco quase 4 vezes maior de evoluir ao óbito quando comparadas à mesma faixa etária infectada pelos outros sorotipos. Entretanto, uma relação também foi observada em idosos que foram infectados por qualquer um dos sorotipos. No entanto, um estudo em Porto Rico, em 2010, relatou que os pacientes com DHF eram mais propensos a terem sido infectados pelo DENV-4 do que pelo DENV-1 (SHARP et al., 2013).

Neste estudo, os indivíduos infectados pelo DENV-2 tiveram quase 2 vezes mais risco de evoluir a óbito quando comparados aos infectados pelo DENV-1. DENV-1 seguido por infecções por DENV-2 foram associados com surtos de FHD no passado (HALSTEAD, 1988). No entanto, outras infecções sequenciais, como DENV-3 seguido por DENV-2, DENV-1 por DENV-3 e DENV-2 por DENV-3 em El Salvador (2000), Cuba (2000-2001) e Brasil (2001-2002), respectivamente, foram associados com doença grave (GUZMAN; KOURI, 2003). A ocorrência de uma doença mais grave também foi relatada em crianças infectadas pelo DENV-2 que haviam sido previamente infectadas pelo DENV-3 (OHAINLE et al., 2011).

Estudos recentes no Brasil também relacionaram diferentes sorotipos de DENV a quadros mais graves da doença. Um estudo realizado em Goiânia em 2013-2014 reportou uma relação maior dos casos graves com o DENV-1 do que DENV-4 (ROCHA et al., 2017). A investigação de dois momentos epidêmicos distintos em Mato Grosso do Sul (2010 e 2013) reportou que, casos ocorridos em 2010, quando DENV-2 circulava, apresentaram uma sintomatologia mais grave, além de internações, quando comparados àqueles ocorridos durante a epidemia de DENV-4, em 2013 (FARIA et al., 2016) corroborando com nossas observações.

O sexo tem sido considerado por alguns autores como fator de risco para a gravidade da doença. Estudos na Ásia e nas Américas mostram que as mulheres são mais propensas a terem dengue e correm maior risco de desenvolver formas mais graves do que os homens (ANDERS et al., 2011; DETTOGNI et al., 2015; GARCÍA et al., 2011; HALSTEAD; NIMMANNITYA; COHEN, 1970). A análise dos dados disponibilizado pelo Ministério da Saúde (SINAN/SIM) demonstrou que, no período de 30 anos, um total de 2.682 óbitos por dengue ocorreram em indivíduos do sexo masculino e 2.455 do sexo feminino no Brasil, portanto, uma distribuição homogênea de óbitos entre os sexos foi observada.

Na casuística do LABFLA/IOC não foram observadas diferenças quando analisamos o desfecho fatal em relação ao sexo do paciente, corroborando os achados de WANG et al., (2009), THOMAS et al., (2008) , e os dados do país. De forma semelhante, o estudo realizado por PINTO et al., (2016), analisando os preditores de mortalidade em pacientes do Amazonas, entre 2001 e 2013, mostrou que os pacientes com dengue grave não apresentavam risco aumentado de morte, relacionada ao sexo do paciente. No entanto, um estudo realizado no Vietnã associou um risco maior para a gravidade da doença em mulheres, com risco 1,57 vezes maior de evoluir para a óbito do que os homens (ANDERS et al., 2011). Da mesma forma, SAM et al., (2013) relatou casos fatais em 9 de 10 mulheres analisadas. Por outro lado, MORAES et al., (2013) relataram que as mulheres tinham menor probabilidade de evoluir à óbito por dengue do que os homens. O estudo de ARAÚJO et al., (2012), em 84 casos fatais, relatou morte em 54% dos homens. Além disso, LEO et al., (2011) relatou que 67,9% dos homens infectados com dengue evoluíram para óbito em comparação às mulheres.

Em uma análise multivariada, realizada na Argentina e recém-publicada por BYRNE et al., (2018), ficou demonstrado que as mulheres tinham um risco aumentado de desenvolvimento de DCSA quando comparados com homens, porém esse risco não era mais significativo quando ajustado para a idade e imunidade pré-existente ao DENV.

WHITEHORN; SIMMONS, (2011) relataram que a idade é um fator importante para dengue grave e óbito, e crianças vietnamitas com até 5 anos de idade apresentavam 4 vezes mais chances de desenvolver uma doença mais grave do que o grupo de 11-15 anos de idade. Por outro lado, GARCÍA-RIVERA e RIGAU-PÉREZ, (2003) demonstraram que os idosos tinham 6 vezes mais risco de morte, que os adultos jovens e quase 2 vezes mais que os lactentes.

Em nossa análise, durante todo o período do estudo, observamos que as faixas etárias 0-15 e 51-96 anos apresentaram 1,744 e 2,945 vezes mais risco de evolução ao óbito, respectivamente. Quando analisamos os períodos epidêmico e inter-epidêmico, foi demonstrado que, até 2006, as maiores taxas de dengue e dengue grave no Brasil ocorreram em pacientes com mais de 15 anos de idade. Esse mesmo padrão foi observado em uma epidemia em 2010 em Porto Rico, onde os adultos foram responsáveis por 49,7% dos casos graves e casos fatais de dengue (SHARP et al., 2013). No Paquistão, um maior número de casos graves foi observado em indivíduos com mais de 30 anos em 2011 (AHMED et al., 2013).

O padrão inicial de casos graves em adultos jovens apresentou mudanças significativas nos últimos anos no Brasil. Em 2007, durante a re-emergência do DENV-2, o

aumento das taxas de hospitalização e dengue grave em crianças com 15 anos ou menos foram relatados (CAVALCANTI et al., 2011; TEIXEIRA et al., 2008), semelhante às observações no sudeste da Ásia. Esses dados corroboram os encontrados em nosso estudo, pois foi observado que, durante a epidemia de 2008, causada pelo DENV-2, os casos fatais em menores de 15 anos foram mais frequentes e não observados em outras epidemias, e crianças de 0 a 15 anos infectadas com DENV -2 apresentaram maiores chances de evoluírem ao óbito.

Com a cocirculação de vários sorotipos de DENV no Brasil, os adultos são menos propensos a permanecer suscetíveis à infecção (RODRÍGUEZ-BARRAQUER et al., 2014). Em nossa análise, observamos que as faixas etárias 51-96 anos apresentaram 2,945 vezes maior chance de morte quando comparados com a faixa etária de 16-30 anos, 1,68 vezes mais do que 0-15 anos e 2,53 vezes mais do que 31-50 anos. Foi demonstrada também que a chance de evoluir ao óbito é alta nesse grupo, independente do sorotipo infectante quando comparados com os outros grupos etários.

LEE et al., (2018) observaram uma maior soroprevalência de dengue em indivíduos acima de 65 anos, nas epidemias de 2013 à 2015 em Hong Kong e na Malásia, uma maior incidência de óbitos em pacientes acima de 67 anos foi reportada (MD-SANI et al., 2018). Essa maior incidência de óbitos neste grupo etário pode estar associada à dificuldade de manejo da doença em uma população com alta frequência de comorbidades (AMÂNCIO et al., 2014).

Vários estudos mostraram que as infecções secundárias estão relacionadas ao aumento do risco de dengue grave e óbito (CHANGAL et al., 2016; DHANOA et al., 2017; GUZMÁN et al., 1999; SANGKAWIBHA et al., 1984; THEIN et al., 1997). Em nosso estudo, os casos fatais por infecções primárias foram mais frequentes do que os secundários ($p = 0,001$). A análise dos casos fatais ocorridos em 2002 pelo DENV-3 também relatou uma maior frequência de infecções primárias (ARAÚJO et al., 2009). Apesar destas observações, uma revisão sistemática sobre a mortalidade da dengue, aponta casos fatais como mais comuns em indivíduos que apresentavam infecções secundárias e nenhum dos relatos associou casos de óbitos à infecção primária (CARABALI et al., 2015).

O maior número de casos fatais devido a infecções secundárias foi uma característica da epidemia do DENV-2 em 2007-2008. Além disso, foi demonstrado que crianças menores de 15 anos, apresentando infecção secundária, apresentaram quatro vezes mais chances para um desfecho fatal. De fato, estudos anteriores relataram que as infecções secundárias pelo DENV-2, principalmente pelo genótipo asiático que circula no Brasil, levaram a um aumento de casos graves (GUZMÁN et al., 1999; PAWITAN, 2011; THEIN et al., 1997).

O estudo de NISALAK et al., (2003) demostrou que pacientes com infecções secundárias apresentavam cinco vezes mais risco de desenvolver FHD do que àqueles apresentando infecções primárias. Da mesma forma, foi descrito que crianças infectadas pelo DENV-2, que foram previamente expostas ao DENV-3, apresentaram um desfecho mais grave (OHAINLE et al., 2011).

Em uma coorte de 97 pacientes pediátricos na Índia, a evolução da gravidade da doença foi maior em infecções secundárias e em aproximadamente um terço das infecções primárias (SINGLA et al., 2016a).

## 5.2 ANÁLISE DA ANTIGENEMIA DE NS1 E VIREMIA COMO POTENCIAIS MARCADORES DE EVOLUÇÃO AO ÓBITO

Algumas proteínas virais são envolvidas na patogênese e a proteína NS1 demonstrou ser um marcador da gravidade da dengue (ALLONSO et al., 2014; AVIRUTNAN et al., 2006; DE LA CRUZ HERNÁNDEZ et al., 2013; LIBRATY et al., 2002a; THOMAS, 2015), Isso deve-se à produção anticorpos de reação cruzada, levando à depleção plaquetária, apoptose de células endoteliais e ativação do complemento, com danos aos tecidos do hospedeiro (AKEY et al., 2014; AMORIM et al., 2014; AVIRUTNAN et al., 2011; BEATTY et al., 2015; FALCONAR; MARTINEZ, 2011; KUROSU et al., 2007; LIN et al., 2002; MALAVIGE; OGG, 2017; MARTINA; KORAKA; OSTERHAUS, 2009; MULLER; YOUNG, 2013; PUERTA-GUARDO; GLASNER; HARRIS, 2016; RASTOGI; SHARMA; SINGH, 2016).

Neste estudo, observamos que os níveis de NS1 nos casos fatais foram maiores que nos casos não fatais e vários estudos corroboram com a nossa observação. LIBRATY et al., (2002) descreveu a correlação entre altos níveis circulantes de NS1 e o desenvolvimento de dengue grave, resultados semelhantes foram descritos na Ásia e nas Américas (ALLONSO et al., 2014; AVIRUTNAN et al., 2006; DE LA CRUZ HERNÁNDEZ et al., 2013; PERDOMO-CELIS; SALGADO; NARVÁEZ, 2017). Um estudo realizado na Colômbia descreveu a correlação entre altos níveis circulantes de NS1 e o desenvolvimento da gravidade da doença em crianças infectadas DENV-1, DENV-2 e DENV-3 (PERDOMO-CELIS; SALGADO; NARVÁEZ, 2017).

Por outro lado, o estudo de DUONG et al., (2011) durante uma epidemia no Camboja em 2006 e 2007 mostrou que os níveis de NS1 se correlacionaram com a viremia, mas uma

baixa relação NS1 foi associada a doença grave. Porém, estudos realizados na Finlândia não encontraram associação entre níveis de NS1 com a hospitalização (ERRA et al., 2013).

Foi observado que o DENV-1 exibiu níveis mais elevados de NS1, seguido por DENV-3, DENV-4 e DENV-2, corroborando observações prévias (ALLONSO et al., 2014; BICH CHAU et al., 2010; DUYEN et al., 2011). Além disso, BICH CHAU et al., (2010) reportou que os níveis de NS1 foram significativamente mais elevados em crianças infectadas com DENV-1 e DENV-3 do que com o DENV-2 e relataram maior gravidade em casos de DENV-3. No Vietnã, um estudo em crianças infectadas com dengue relatou níveis mais elevados de NS1 em casos de DENV-1 do que em DENV-2 (DUYEN et al., 2011).

Considerando os distintos sorotipos e o desfecho da doença, foi demonstrado que os casos fatais de DENV-2, DENV-3 e DENV-4 apresentaram maior antigenemia do NS1 do que os não fatais. Apesar da não significância, os casos de DENV-1 apresentaram perfil oposto.

No geral, a magnitude de NS1 não variou por tipo de infecção, e não foram observadas diferenças na antigenemia de NS1 entre infecções primárias e secundárias. No entanto, PERDOMO-CELIS; et al., (2017) e DE LA CRUZ HERNÁNDEZ et al., (2013) descreveram que pacientes com infecções primárias apresentavam níveis mais elevados de NS1 do que aqueles com secundárias.

A antigenemia da NS1 é dependente do sorotipo de DENV (DUONG et al., 2011; FOX et al., 2011) e, pacientes que apresentavam uma antigenemia persistente foram mais propensos a desenvolver um quadro mais grave da doença (PARANAVITANE et al., 2014). Entretanto, em nosso estudo, a persistência da antigenemia NS1 não foi abordada devido à natureza de nossa amostragem (amostras de conveniência).

Vários estudos, mostraram uma correlação entre a quantidade de partículas virais e o aumento da gravidade da doença (CHEN et al., 2005; DE ARAÚJO et al., 2009b; MURGUE et al., 2000; NUNES et al., 2016; VAUGHN et al., 2000; WANG et al., 2003).

Em nosso estudo, observamos que os casos fatais estudados tiveram uma maior viremia do que os não fatais. Todos os sorotipos apresentaram uma maior viremia nos fatais do que nos não fatais, porém, apenas os sorotipos DENV-2 e DENV-3 apresentaram diferenças estatísticas significativas. No Brasil, estudos com DENV-2 e DENV-3 observaram uma maior viremia em casos fatais (DE ARAÚJO et al., 2009b; NUNES et al., 2016), corroborando os dados de estudos realizados na Tailândia e Taiwan (VAUGHN et al., 2000; WANG et al., 2003).

Neste estudo, o DENV-4 e o DENV-3 apresentaram maior viremia, seguido pelo DENV-1 e DENV-2. TRICOU et al., (2011) demonstraram de forma semelhante que, as

infecções por DENV-1 apresentaram uma maior viremia do que as infecções por DENV-2 e, recentemente, PERDOMO-CELIS et al., (2017) relataram que a viremia de DENV-1, DENV-2 e DENV-3 eram maiores nos casos graves do que nos não graves. No entanto, DE LA CRUZ HERNÁNDEZ et al., (2013) relataram que os pacientes não graves, infectados pelo DENV-1 ou DENV-2, apresentavam viremia significativamente mais elevada, quando comparada com os graves.

Por outro lado, foram observados aqui, níveis de viremia maiores nos casos fatais de infecções primárias do que em secundárias. TRICOU et al., (2011) observoaram que o pico da viremia foi significativamente menor durante as infecções secundárias do que primárias, para todos os desfechos da doença. Em contraste, PERDOMO-CELIS et al., (2017) observaram que a viremia nas infecções primárias foi maior do que nas secundárias.

## 5.3 CARACTERIZAÇÃO DAS ALTERAÇÕES HISTOPATOLÓGICAS MARCADORES VIRAIS E MEDIADORES INFLAMATÓRIOS DE ÓBITOS MATERNO E FETAL DE DENGUE

Gestantes e neonatos também são considerados grupos de risco ao desenvolvimento de formas graves da doença (BRASIL; LUPI, 2017; PAIXAO et al., 2018; WAKIMOTO et al., 2015). Estudos prévios realizados no mundo (ADAM et al., 2010; CHAU et al., 2009; CHITRA; PANICKER, 2011; KARIYAWASAM; SENANAYAKE, 2010; LIBRATY et al., 2009; MACHAIN-WILLIAMS et al., 2018; PENGSAA et al., 2006; TIEN DAT et al., 2018) e no Brasil (ARGOLO et al., 2013; BRAGA et al., 2016; FEITOZA et al., 2017; LEITE et al., 2014; MACHADO et al., 2013; NASCIMENTO et al., 2017; PAIXAO et al., 2018), já avaliaram os impactos das infecções pelos DENV nestes grupos.

A ocorrência de arboviroses durante a gravidez é uma preocupação adicional, devido à possibilidade de transmissão vertical e envolvimento fetal (BEAUFRÈRE et al., 2018; BRASIL et al., 2016; BRASIL; LUPI, 2017; BRASIL; NIELSEN-SAINES, 2016; FRANÇA et al., 2016; FRITEL et al., 2010; GÉRARDIN et al., 2008; HALAI et al., 2017; MARINHO et al., 2017; MARTINES et al., 2016; MLAKAR et al., 2016). Devido a cocirculação dos arbovírus, um estudo recente já reportou morte fetal associado à coinfecção com ZIKV e CHIKV (PRATA-BARBOSA et al., 2018). Um caso de coinfecção de CHIKV e DENV durante a gravidez resultou em cesariana de emergência (RAHIM et al., 2018).

Apesar da alta incidência da doença, estudos relacionados às consequências materno-fetais da infecção pelo DENV durante gestação ainda são limitados. Além disso, ainda não há um consenso a respeito dos efeitos da infecção em gestantes e/ou neonatos, contudo alguns estudos apontam que a transmissão vertical pode ocorrer e apresentar desfechos graves, como partos prematuros e óbito materno-fetal (BASURKO et al., 2009; NASCIMENTO et al., 2017; PAIXÃO et al., 2016).

Embora a gestação seja considerada um fator de risco para o curso clínico da doença, estudos prévios não encontraram associação entre a gravidade da infecção materna e a doença neonatal (KARIYAWASAM; SENANAYAKE, 2010; RIBEIRO et al., 2013). No entanto, sugere-se que a imunossupressão natural materna durante a gestação possa favorecer a ocorrência de infecções de maior gravidade, causando danos à saúde da mãe e feto (FEITOZA et al., 2017). Recentemente, um estudo no Brasil reportou um risco de óbito materno 3 vezes maior em casos de dengue e de 450 vezes, quando a gestante apresentava FHD (PAIXAO et al., 2018).

No México, das gestantes infectadas por DENV em 2013, 65,9% foram diagnosticados com DSSA, 18,3% com DCSA e 15,9% com DG. Gestantes com DG (38,5%) apresentaram sofrimento fetal, foram submetidos a cesáreas de emergência e este quadro foi associado à hemorragia obstétrica (30,8%), pré-eclâmpsia (15,4%) e eclâmpsia (7,7%). Gestantes que não apresentaram DG tiveram gestações a termo, deram à luz por via vaginal e tiveram bebês aparentemente saudáveis com peso normal ao nascer (MACHAIN-WILLIAMS et al., 2018).

No Vietnã, uma investigação de gestantes infectadas por DENV em 2015 demonstrou que 90% apresentavam positividade para antígeno NS1 e infecção primária, 20% tiveram partos prematuros e 5%, com natimorto. Todos os neonatos nascidos vivos receberam alta hospitalar sem intercorrências e não foi reportado óbito materno (TIEN DAT et al., 2018).

Durante a gestação, o feto pode ser suscetível à infecção por DENV, especialmente durante o período crítico de organogênese ou no final da gravidez (FIGUEIREDO; CARLUCCI; DUARTE, 1994; KLIKS et al., 1988; MAROUN et al., 2008; MOTA et al., 2012; POULIOT et al., 2010).

Um estudo recente avaliou gestantes durante uma epidemia na Guiana Francesa entre 2012-2013 e reportou uma taxa de transmissão vertical de 18,5%, com transmissão viral, tanto no início quanto no final da gravidez foi possível verificar que é mais frequente quando infecção

materna ocorre tardiamente durante a gestação próxima ao parto e que recém-nascidos podem apresentar dengue neonatal com sinais de alerta que necessitaram de transfusão de plaquetas. Além disso, ressalta que, se houver febre durante os 15 dias anteriores ao parto, o sangue do cordão e a placenta devem ser amostrados e testados para o vírus, e o recém-nascido deve ser monitorado de perto durante o período pós-parto (BASURKO et al., 2018).

Analisamos fragmentos da placenta e cordão umbilical de um caso de óbito materno-fetal por dengue além de fragmentos da placenta e de tecidos fetais. O DENV-2 foi o sorotipo infectante identificado no cordão umbilical (no primeiro caso) e o DENV-4 foi detectado no cérebro fetal pela técnica de RT-PCR.

Nas placentas de ambos os casos, observamos alterações na região materna com a detecção da proteína NS1 em macrófagos na túnica adventícia e no endotélio de artérias. A NS3 foi identificada em macrófagos no interior da vilosidades da placenta e no cordão umbilical, no endotélio, macrófagos e na túnica adventícia, no primeiro caso. Já no segundo caso, a proteína NS3 do DENV foi detectada em células endoteliais dos vasos da região materna, em células citotrofoblásticas e células Hofbauer na vilosidades coriônicas e em macrófagos e células deciduais da região materna.

A proteína NS1 é requerida no ciclo replicativo viral, porém é secretada (sNS1) para meio extracelular e por esse motivo, sua detecção pode não ser um indicativo de presença do vírus nas células detectadas (FLAMAND et al., 1999; MASON, 1989). A NS3, por outro lado, que está envolvida na replicação (GORBALENYA et al., 1989; LI et al., 1999), quando encontrada sugere a replicação viral no interior das células. Neste contexto, os resultados obtidos neste estudo podem sugerir replicação viral em macrófagos e em células endoteliais no tecido materno. Além disso, essas observações são reforçadas pelos altos títulos do RNA detectado no cordão umbilical (DENV-2) e no cérebro do feto (DENV-4). Embora a quantificação viral no tecido do cérebro fetal não tenha sido possível, foi o único tecido que apresentou resultado positivo na técnica de RT-PCR convencional, indicando uma maior quantidade de RNA neste tecido do que nos outros.

Os dois casos foram provenientes de demanda espontânea e diante disso, não foi possível se obter amostras de tecido fetal (DENV-2) e materna, de ambos os casos, o que prejudica de certa forma, a conclusão final. A investigação também se torna um desafio pela ausência de dados mais detalhados a acerca dos casos na ficha epidemiológica disponível e que acompanha o caso.

A análise de placentas de gestantes infectadas por DENV durante epidemias ocorridas no Rio de Janeiro entre 2002 e 2010 também identificou o antígeno viral no tecido e sinais de hipóxia, coriodeciduite, deciduite e intervilosite foram encontrados no citoplasma do trofoblasto, estroma viloso e decídua. Além disso, foi sugerido que a IHQ pode ser usada como um método de confirmação laboratorial em gestantes, especialmente em áreas endêmicas, quando o material fixado é o único disponível (RIBEIRO et al., 2017).

As observações clínicas de pacientes em estudos *post-mortem* por dengue têm fornecido informações importantes sobre a fisiopatologia da doença, embora ainda existem lacunas para esta compreensão. As principais alterações morfológicas em todos os órgãos foram congestão e edema. Estas observações eram esperadas, uma vez que dengue grave é geralmente associada a um aumento da permeabilidade vascular, levando ao extravasamento de plasma (CARLOS et al., 2005; KITTIGUL et al., 2007; PÓVOA et al., 2014; SRIKIATKHACHORN, 2009).

A transmissão viral para o feto via placenta pode ocorrer através do endotélio vascular materno para os trofoblastos por monócitos maternos infectados, que transmitem a infecção para os trofoblastos da placenta, também por vias paracelulares do sangue materno aos capilares fetais (COYNE, 2016; DELORME-AXFORD; SADOVSKY; COYNE, 2014). Além disso, já foi relatado anteriormente que os mecanismos potenciais pelos quais uma infecção materna pode resultar em morte fetal incluem infecção fetal direta e dano a órgãos, infecção placentária resultando em diminuição da transmissão de nutrientes e oxigênio, produção aumentada de citocinas e quimiocinas (MCCLURE; GOLDENBERG, 2009).

Com a disponibilidade dos tecidos de cérebro, fígado, pulmão e baço fetal, do caso de DENV-4, foi possível ampliar a investigação e caracterizar os tipos celulares dos infiltrados e citocinas e quimiocinas, que já foram amplamente descritas como participantes no desfecho grave da doença (ALDINUCCI; COLOMBATTI, 2014; BOZZA et al., 2008; BRAGA et al., 2001; DE-OLIVEIRA-PINTO et al., 2012; GAGNON; ENNIS; ROTHMAN, 1999; GILLE et al., 2001; KUBELKA et al., 2010; KURANE et al., 1989, 2011; LEE; LEONG; WILDER-SMITH, 2016; LOCKSLEY; KILLEEN; LENARDO, 2001; PÓVOA et al., 2016; SRIKIATKHACHORN et al., 2007; STAMATOVIC et al., 2003; TSENG et al., 2005; YAMADA et al., 2003).

No cérebro, um aumento nas células da microglia e células da glia foi observado na região da substância branca. O fígado apresentou área difusa de hepatócitos necróticos com infiltrado mononuclear, microesteatose e macrosteatose, hiperplasia de células de Kupffer, poliploidia, discreta área de infiltrado linfocitário em capilar sinusoidal, espessamento do

endotélio na veia central e presença de edema. No pulmão, um aumento do infiltrado mononuclear em torno do brônquio, hialinose e infiltrado mononuclear na túnica muscular das artérias e área focal do espessamento alveolar, foram observados. No baço, a congestão vascular e a desorganização folicular na polpa branca foram observadas. Todas as alterações descritas corroboraram observações prévias de análises de tecidos de casos fatais por dengue em adultos (AHSAN; AHMAD; RAFI, 2018a; BASÍLIO-DE-OLIVEIRA et al., 2005; BHAMARAPRAVATI; TUCHINDA; BOONYAPAKNAVIK, 1967; CAROD-ARTAL et al., 2013; DE MOURA MENDONÇA et al., 2011; DE SOUZA et al., 2017; KULARATNE et al., 2014; LEONG et al., 2007; LIMONTA et al., 2012; MIAGOSTOVICH et al., 1997; PÓVOA et al., 2014; PUNGJITPRAPAI; TANTAWICHIEN, 2008; RODRIGUES et al., 2014; SENEVIRATNE; MALAVIGE; DE SILVA, 2006; WANG et al., 2007).

O DENV pode infectar diversos tipos celulares em diferentes tecidos e órgãos e análises histopatológicas demonstraram presença viral em monócitos e macrófagos no fígado, pulmão, baço, cérebro, rim, medula óssea e coração (AHSAN; AHMAD; RAFI, 2018; BALSITIS et al., 2009; DE ARAÚJO et al., 2009; HUERRE et al., 2001; JESSIE et al., 2004; LIMA et al., 2011; MIRANDA et al., 2013; PAGLIARI et al., 2016; PÓVOA et al., 2014, 2016; RATHI et al., 2013). A replicação viral nesses tecidos foi confirmada pela detecção da proteína NS3 específica para DENV no interior de macrófagos no cérebro, fígado, pulmão e baço do feto.

Acredita-se que o ponto crítico da fisiopatologia da dengue seja o extravasamento plasmático de curta duração mediado pela reposta imunológica do hospedeiro, principalmente no que se trata de mediadores solúveis, resultando em derrames, choques, hemorragias e até no envolvimento do sistema nervoso central pela alteração da barreira hemato-encefálica. São descritas elevações nas concentrações de citocinas inflamatórias (*cytokine storm*) em pacientes com manifestações mais graves, sugerindo um papel fundamental destes mediadores no choque hipovolêmico (ASSUNÇÃO-MIRANDA et al., 2010; BETHELL et al., 1998; BOZZA et al., 2008; CHEN et al., 2006; DE-OLIVEIRA-PINTO et al., 2012; GREEN et al., 1999; MUSTAFA et al., 2001; VAN DE WEG et al., 2013).

Neste estudo, níveis aumentados de células CD68, $TCD8^{+}$ além de IFN-γ, TNF-α, RANTES, MCP-1 e VEGF/R2 foram observados em todos os tecidos. As células T, NK, monócitos, macrófagos, hepatócitos e células endoteliais proliferam e produzem citocinas e mediadores inflamatórios para controle viral e, consequentemente, ocasionamdo a lesão tecidual (GAGNON; ENNIS; ROTHMAN, 1999; KURANE et al., 1989, 2011).

O papel das citocinas na patogênese da gravidade da dengue tem sido demonstrado e é consenso que a resposta inflamatória associada à produção desregulada de citocinas é fundamental para o desenvolvimento de casos graves, além da patogênese mediada pelo vírus (LEE; LEONG; WILDER-SMITH, 2016).

O TNF-α é uma citocina pleiotrópica que regula muitas funções fisiológicas e patológicas, como sobrevivência celular, apoptose, migração e inflamação (LOCKSLEY; KILLEEN; LENARDO, 2001). De fato, o TNFα estava associado à gravidade dos pacientes com dengue (BOZZA et al., 2008; BRAGA et al., 2001), induzindo permeabilidade vascular, bem como metaloproteinases (KUBELKA et al., 2010) que atuariam nas células endoteliais. No entanto, o desequilíbrio de citocinas pode ser responsável por alterações do ambiente placentário e pode estar envolvido em abortos recorrentes inexplicáveis. Deste modo, o TNF-α desempenha um papel essencial durante a gravidez, por exemplo, na renovação do trofoblasto. Por outro lado, o TNF pode ser prejudicial, causando complicações como aborto espontâneo e pré-eclâmpsia (HAIDER; KNÖFLER, 2009). Estudos in vitro com HBV e HIV-1 utilizando células trofoblásticas mostraram que o TNF-α favorece um ambiente adequado para a infecção vertical desses vírus. (LEE et al., 1997; LI et al., 2007).

Altas concentrações de IFN-γ no soro do paciente com dengue podem indicar um alto risco de progressão da doença para dengue grave (ZHANG et al., 2017). Além disso, foi descrito que o IFN-γ poderia induzir o insucesso da gravidez pela moderação de células natural killer (NK) (LI et al., 2015), aumentando a apoptose decidual (BORBELY et al., 2012). In vitro, o IFN-γ demonstrou ser citotóxico para trofoblasto humano (YUI et al., 1994) e pode inibir a sua proliferação (BERKOWITZ et al., 1988). Foi demonstrado que os níveis de IFN-γ no sangue e no tecido endometrial eram maiores em pacientes com perda recorrente de gravidez do que em mulheres com fertilidade normal (COMBA et al., 2015). Além disso, a expressão preferencial de TNF-a e IFN-y pode resultar em anormalidades placentárias e embrionárias de crescimento e subsequente morte fetal (CARP, 2004).

A RANTES é importante no recrutamento de leucócitos para locais inflamados (ALDINUCCI; COLOMBATTI, 2014) e estudos prévios encontraram baixos níveis circulantes da RANTES no sangue de pacientes com dengue, enquanto alta expressão foi encontrada no tecido hepático de casos fatais (DE-OLIVEIRA-PINTO et al., 2012).

A proteína quimiotática de monócitos (CCL2 / MCP-1) é uma quimiocina produzida por macrófagos, linfócitos T, fibroblastos, queratinócitos e células endoteliais e causa a abertura de junções oclusivas de células endoteliais infectadas in vitro por DENV (STAMATOVIC et al., 2003). A expressão induzida pelo fator de crescimento endotelial vascular (VEGF) em células endoteliais vasculares eleva as alterações de permeabilidade endotelial in vivo (YAMADA et al., 2003).

A produção desses mediadores vasculares irá alterar a permeabilidade vascular, causando disfunção de barreira, facilitando a entrada do vírus e inflamação, principalmente na placenta e no cérebro fetal. Essa exacerbação da infecção pode ocorrer concomitantemente com a dispersão do vírus para a parte periférica, causando infecção nos órgãos-alvo da infecção por dengue como o fígado, pulmão e baço

# 6 CONCLUSÕES

- Foi observada uma maior ocorrência de óbitos por dengue a partir da última década e uma mudança nos padrões de mortalidade associada à doença no Brasil.
- Tanto em períodos epidêmicos quanto inter-epidêmicos, a região Sudeste foi a que mais registrou óbitos por dengue no país, seguida da região Nordeste.
- Nos últimos 30 anos, DENV-2 foi o sorotipo responsável pelo maior número de óbitos, seguido do DENV-3 e foi demonstrado que pacientes infectados por DENV-2 e DENV-3 apresentaram maior probabilidade de evoluir para óbito, independente do sexo.
- De uma forma geral, a faixa etária de 51 a 96 anos apresentou maior probabilidade de desfechos fatais, no entanto, uma maior frequência de óbitos em menores de 15 anos foi observada nos anos de 2007 e 2008, durante a epidemia causada por DENV-2.
- A maioria dos óbitos foi associada à infecção primária
- Na epidemia de 2008, crianças com até 15 anos e com infecção secundária apresentaram uma probabilidade 4 vezes maior de evoluir ao óbito.
- Casos fatais apresentaram significativamente maior antigenemia de NS1 e viremia quando comparados aos casos não fatais, sendo casos de DENV-1 com antigenemia e de DENV-4 com maior viremia.
- A análise da placenta de casos fatais de infecção materna e fetal demonstrou alterações histopatológicas e celulares relacionadas à infecção viral, com a detecção de NS1 e NS3 em macrófagos e endotélio, sugerindo replicação viral.
- O aumento na quantidade de células de defesa, bem como a presença de mediadores inflamatórios detectados no fígado, pulmão, baço, cérebro e placenta, indicam a inflamação dos tecidos.
- A dispersão viral para os tecidos periféricos do feto, combinada à produção dos mediadores inflamatórios nos tecidos do feto e placenta podem resultar em um aumento da permeabilidade vascular, consequente disfunção de barreira e facilitação da entrada do vírus, impactando no desfecho da doença.

# 7 PERSPECTIVAS

Prentendemos analisar as alterações histopatológicas dos casos fatais por dengue de adultos infectados pelos quatro sorotipos causadores da doença. O objetivo é avaliar se existem diferenças no tropismo e nas lesões causadados pelos diferentes sorotipos.

Em amostras frescas avaliaremos também os níveis de citocinas e quimiocinas produzidas na infecção pelos DENV, afim de estabelecer marcadores imunológicos de progressão ao óbito.

Pretendemos também analisar os casos fatais de dengue ocorridos após a introdução do ZIKV e CHIKV, com a perspectiva de estudar as coinfeções por esses arbovírus

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# 9 ANEXOS:

## 9.1 ARTIGOS SUBMETIDO: LESÃO RENAL EM CASOS FATAIS POR DENV-4: VIREMIA, RESPOSTA IMUNE E PERFIL DE CITOCINAS

**Submetido a Revista:** The Journal of Infectious Diseases

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**Resumo:** As manifestações clínicas da dengue variam de assintomática a doença grave, incluindo falência múltipla de órgãos. No entanto, o comprometimento renal e seu desfecho, como a lesão renal aguda, são complicações pouco estudadas. O envolvimento renal na dengue pode estar potencialmente relacionado ao aumento da mortalidade, possivelmente devido aos efeitos indiretos da resposta imune do hospedeiro. Devido à falta de estudos baseados em autópsia renal, foram realizadas investigações *post mortem* do vírus da dengue tipo 4 (DENV-4) em quatro casos fatais ocorridos durante a epidemia de dengue no Brasil. Provavelmente devido à alta carga viral, várias lesões foram observadas no tecido renal, como infiltração mononuclear difusa ao redor do glomérulo na região cortical e nos vasos medulares, hialinose arteriolar, infiltrado linfocitário, aumento da fibrose capsular, túbulo contorcido proximal (PCT) danos, edema e espessamento da membrana basal dos vasos. Essas alterações foram associadas à infecção por DENV-4, confirmada pela proteína NS3 específica para DENV, indicativa de replicação viral. A presença exacerbada de células mononucleares em vários locais de tecido renal culmina na secreção de citocinas e quimiocinas pró-inflamatórias. Além disso, pode-se sugerir que a lesão do tecido renal aqui observada pode ser devida à combinação de alta carga viral e resposta imune exacerbada do hospedeiro.

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# Renal injury in DENV-4 fatal cases: viremia, immune response and cytokines profile

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| Abstract: | Clinical manifestations of dengue range from asymptomatic to severe disease, including multiple organs failure. However, the kidney involvement and its outcome, such as acute kidney injury, are poorly studied complications. Renal involvement in dengue can be potentially related to an increased mortality, possibly owing to indirect effects of the host immunity. Due to the lack of renal autopsy-based studies, post-mortem dengue virus type 4 (DENV-4) investigations were performed in four fatal cases occurred during dengue epidemics in Brazil. Probably due the high viral load, several lesions were observed in the renal tissue, such as diffuse mononuclear infiltration around the glomerulus in the cortical region and in the medullary vessels, hyalinosis arteriolar, lymphocytic infiltrate, increased capsular fibrosis, proximal convoluted tubule (PCT) damage, edema, PCT debris formation, thickening of the |

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| | basal vessel membrane. These changes were associated with DENV-4 infection, confirmed by the DENV specific NS3 protein, indicative of viral replication. The exacerbated presence of mononuclear cells at several renal tissue sites culminates in the secretion of proinflammatory cytokines and chemokines. Moreover, it can be suggested that the renal tissue injury observed here, may be due to the combination of both high viral load and exacerbated host immune response. |
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# Renal injury in DENV-4 fatal cases: viremia, immune response and cytokines profile

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**Abstract (197 words):**

Clinical manifestations of dengue range from asymptomatic to severe disease, including multiple organs failure. However, the kidney involvement and its outcome, such as acute kidney injury, are poorly studied complications. Renal involvement in dengue can be potentially related to an increased mortality, possibly owing to indirect effects of the host immunity. Due to the lack of renal autopsy-based studies, post-mortem dengue virus type 4 (DENV-4) investigations were performed in four fatal cases occurred during dengue epidemics in Brazil. Probably due the high viral load, several lesions were observed in the renal tissue, such as diffuse mononuclear infiltration around the glomerulus in the cortical region and in the medullary vessels, hyalinosis arteriolar, lymphocytic infiltrate, increased capsular fibrosis, proximal convoluted tubule (PCT) damage, edema, PCT debris formation, thickening of the basal vessel membrane. These changes were associated with DENV-4 infection, confirmed by the DENV specific NS3 protein, indicative of viral replication. The exacerbated presence of mononuclear cells at several renal tissue sites culminates in the secretion of proinflammatory cytokines and chemokines. Moreover, it can be suggested that the renal tissue injury observed here, may be due to the combination of both high viral load and exacerbated host immune response.

**Keywords:** Dengue 4, Fatal case, Viremia, Histopathology, Cytokines, Inflammatory mediators

## Introduction

Dengue virus (DENV) have four-related, but antigenically distinct serotypes (DENV-1 to 4). The viral genome consists of a single RNA strand of positive polarity [1,2], which encodes three structural proteins (C, prM and E) , seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5), and the presence of the non-structural proteins is indicative of viral replication [3–5].

In Brazil, the first DENV-4 cases leading to this serotype spread nationwide, occurred in Roraima and Amazonas in 2010, about 30 years after the first detection in the country [6]. In 2012, DENV-4 was prevalent and detected in 63% of the cases reported in Brazil [7]. Despite the highest notification in 2013 (1,452,289 cases), DENV-4 circulation was associated to mild cases [8,9], but severe and fatal cases due to DENV-4 were reported [10]. Although there has been no definitive association of the different DENV serotypes with the clinical course of the disease, it has been suggested that DENV-2 and DENV-3 are more frequently associated to a severe disease [11].

Infection by one DENV serotype provides a lifelong immunity only against the specific serotype, but no cross protective immunity is provided against the others. Most patients experience asymptomatic and a mild disease, but a small proportion may evolve to a severe disease, mostly characterized by plasma leakage and hemorrhagic manifestations, which includes multiple organs failure [12].

Several reports suggest an increase in the incidence of dengue-complicated infections affecting different organ systems, such as gastrointestinal, hepatic, respiratory, cardiac, neurological and renal [13–18]. However, studies evaluating renal tissues from dengue fatal cases are scarce. Renal involvement in dengue can potentially be related to an increased mortality, possibly due to indirect effects of the host immunity [19].

Therefore, post-mortem studies on the kidney of dengue infected individuals may provide important information on the disease immunopathology. In the present study, post-mortem investigations were performed in kidney tissues from DENV-4 fatal cases occurred during dengue epidemics in Brazil, as the pathogenic mechanisms involved in the severe forms of the diesase are still a challenge.

## Material and Methods

### Ethical Considerations

The samples used in this study were received as convenience samples at the Pathological Anatomy Laboratory, National Infectology Institute (Instituto Nacional de Infectologia Evandro Chagas/INI), FIOCRUZ and were investigated in a collaboration with the Flavivirus Laboratory, Oswaldo Cruz Institute, IOC, FIOCRUZ, Regional Reference Laboratory for the Brazilian Ministry of Health, in a study approved by the Research Ethics Committee (CEP 274/05 and CAAE: 57221416.0.1001.5248) of the Oswaldo Cruz Foundation, Ministry of Health, Brazil.

### Dengue fatal cases

Young adults (20-33 years old) fatal cases (n=4), were investigated as dengue suspected case during epidemics occurred in 2012 and 2013 in Brazil. All cases died within 3 to 4 days after the fever onset and no comorbidity was reported. All cases were confirmed as dengue, by a positive result on the tissue by immunohistochemistry and RT-PCR that confirmed DENV-4 as the infecting serotype.

Case 1: A 27 years old female patient, living in Manaus, North region of Brazil, presented fever, myalgia, bleeding, and headache. Leptospirosis was investigated and presented a negative result. Death occurred in January of 2012.

Case 2: A 22 years old male patient resident of Pernambuco, Northeast Brazil, presented fever with back and lower limb pain, vomiting with blood, respiratory changes, and was quite agitated. He evolved into cardiorespiratory arrest, doctors initiated resuscitation maneuver and performed ortho-tracheal intubation. Intubation showed large amounts of blood from the lower airways. According to family members the patient had a history of fever and lower back pain for 3 days. Leptospirosis was investigated and presented a negative result. Death occurred in 2013.

Case 3: A 33 years old female resident of Mato Grosso do Sul, Midwest region of the country, presented petechia, metrorrhagia, signs of hemorrhagic shock, lowering of consciousness level and hemorrhagic shock. She evolved to death 4 days after the onset of the symptoms in 2012. Leptospirosis was investigated and presented a negative result

Case 4: A 20 years old male patient resident of Pernambuco, presented a persistent cough with hemoptysis for 4 days, which evolved into massive hemoptysis. There was diffuse pulmonary hemorrhage and unspecified hemorrhagic syndrome, dyspneic wheezing and snoring. Thorax X ray with eradication of 2/3 of the lung and acute exacerbation (AE) of 1/3. The patient evolved to death 4 days after the onset of the symptoms, in 2013. Leptospirosis was investigated and presented a negative result.

Dengue was investigated as a differential diagnosis.

Molecular diagnosis, histopathological analysis and immunohistochemistry

Kidney tissues samples from necropsy were paraffin-embedded, fixed in 10% formalin, cut (4µm), deparaffinized in xylene and rehydrated with alcohol, as described elsewhere

[20]. For the paraffin-embedded viral RNA extraction, three 5-µm slices of each fragment were used and submitted separately to the PureLink ™ FFPE RNA Isolation Kit (Invitrogen, CA, USA). The conventional reverse transcriptase polymerase chain reaction (RT-PCR) for DENV identification and serotyping was performed as described by Lanciotti et al. [21]. DENV-4 quantification was performed by real-time RT-PCR technique described by Johnson et al. [22] using a Taqman quantitative Real Time RT-PCR system.

Kideny were stained with sections (5 mm thick) were then treated with different stains (hematoxylin–eosin, Masson's trichrome or PAS). examination and visualized by light microscopy (Olympus, Tokyo, Japan) and digital images were obtained using Image Pro Plus software version 4.5. For immunoperoxidase assay, antigen retrieval was performed by heating the tissue in the presence of EnVision Flex target retrieval solution high pH (Dako, CA, USA), or citrate buffer. Tissues were blocked for endogenous peroxidase with 3% hydrogen peroxidase in methanol and rinsed in Tris-HCl (pH 7.4). To reduce non-specific binding, sections were incubated for 30 min at room temperature. Samples were then incubated overnight at 4 ° C with anti-DENV NS3 recombinant antibody (Póvoa et al., 2014), mouse anti-human CD8 Clone C8/144B (Dako, CA, USA), macrophage antibody CD68 clone EBM11 (Dako, CA, USA), anti-MCP 1 monoclonal antibody (Novus Biologicals, CO, USA), anti-TNF alpha antibody, Clone ab6671 (Abcam, MA, USA), Rb anti-FLK-1 and VEGF/R2 (Spring Bioscience, CA, USA). The next day, the sections were incubated with REVEAL COMPLEMENT secondary antibody (Spring Bioscience, CA, USA) for 10 min, and a REVEAL-HRP secondary antibody conjugate (Spring Bioscience, CA, USA) for 15 min at room temperature. Reaction was revealed with diaminobenzidine

144 (Spring Bioscience, CA, USA) as chromogen and sections were counterstained on
145 Harris hematoxylin (Dako, CA, USA).
146
147 For immunofluorescence and CD68/NS3 co-staining, the paraffin embedded tissues
148 were cut to 4 μm and the slides were stained overnight at 4° C with anti-DENV NS3
149 recombinant antibody [20] and anti-human monoclonal CD68 (Dako, CA, USA).
150 Sections were incubated with Alexa 488 conjugated rabbit anti-mouse IgG, goat anti-
151 rabbit IgG conjugated to Alexa 555 or goat anti-mouse IgG conjugated to Alexa 555
152 (ThermoFisher, USA) and analyzed using a Zeiss LSM 510 Meta confocal microscope
153 (Carl Zeiss, Oberkochen, Germany).
154

155 **Results**

156 DENV-4 was the infecting serotype identified and quantified in all kidney tissues by
157 molecular techniques and, the mean viral quantification was $3.34x10^9$ copies of
158 RNA/mL (cases 1 to 4 with $5.85x10^9$, $4.56x10^9$, $1.89x10^9$ and $1.06x10^9$ copies of
159 RNA/mL, respectively).

160 The histopathological analysis showed vascular congestion of glomerular capillary and
161 medullar region, in all kidney tissues (Figs 1B, 1E and 1H). As expected, in the kidney
162 of non-dengue patient, a regular structure of the glomerulus and normal structure with
163 preserved distal and proximal convoluted tubules were observed (Fig 1A). Isolated
164 glomerular obsolescence (Fig 1B and Fig 1F) and focal inflammatory infiltrate in
165 cortical (Fig 1B and Fig 1F) and medullary region (Figs 1G and 1H), was present in
166 case 1. In the proximal convoluted tubules (PCT), loss of the apical cell portion and
167 cytoplasmic remains (star) in light were observed in cases 2 and 4 (Figs 1D and 1F), and
168 congestion of peritubular capillaries were showed in cases 1, 2 and 4, (Figs 1B, 1E and

1H). Discrete focus of isolated hyalinosis was observed in case 1 and case 3 showed a prominent arteriolar hyalinosis (Figs 1D).

The glomeruli presented visceral epithelial cells and tubular cells at confluence (Figs 1B), as well as, glomeruli presenting hyperplastic epithelial cells in the cortical region (Figs 1F) present in case 1. In addition to the changes described in the renal glomerulus, a focus of interstitial fibroedema and tubular atrophy with discrete mononuclear inflammatory infiltrate, focal cell necrosis, granular material in the fibrous endarteritis lumen into the intertubular arteries, and focal hemorrhage were observed only in case 3 (Fig 1C). Immunohistochemistry assay demonstrated the presence of DENV NS3 protein in macrophages and mesangial cells in the renal glomerulus (Fig 2B), parietal leaflet of the Bowman's capsule (Fig 2B) and in isolated macrophages of the cortical region (Fig 2B).Compared to the case control, there was an increase in the CD68 cellular infiltration (Figs 2E and 2F) and $CD8^+$ T cells (Figs 2H and 2I) in the cortical and medullary regions. There was also an increase of TNF-α in the peritubular mononuclear cells infiltrate (Fig 2K), increase of macrophages expressing MCP-1 in the capillary peritubular region (Fig 2L) and increase in VEGF/ R2 expression in peritubular macrophages in the medullary region (Fig 2 M). DENV NS3 and $CD68^+$ double stainined cells were also observed (Fig 2O).

## Discussion

DENV can infect several cell types in different tissues and organs. Studies in autopsies and biopsies of patients have demonstrated viral presence in monocytes and macrophages in the liver, lung, spleen, brain, kidney, bone marrow and heart [20,23–26].

192 Dengue renal involvement varies from elevated serum creatinine, acute tubular necrosis,
193 hemolytic uremic syndrome, proteinuria, glomerulopathy, nephrotic syndrome and
194 acute renal injury [27]. Despite the remarkable evidence that the transient increase in
195 serum creatinine is linked to increased mortality [28], acute renal injury is a poorly
196 studied complication in dengue. The available data indicates the presence of acute renal
197 injury occurs in 0.83% to 14.2% of dengue patients, depending on the methodology and
198 the population evaluated [18,29–34].

199 Kidney damage can be induced by viral infection, in which a direct viral cytopathic
200 effect may occur on glomerular and tubular cells. An immune-mediated *in situ*
201 mechanism triggered by viral antigens in the glomeruli, can cause tissue damage and
202 deposition of immunocomplexes and antiviral antibodies and expression of
203 inflammatory mediators are released in response to exacerbated inflammation in the
204 intertubular vessels [35]. Analysis of autopsies or biopsies of human cells infected by
205 DENV using immunohistochemistry and *in situ* hybridization techniques detected viral
206 antigens on tubular epithelial cells [23,25,36].

207 Jessie et al. [23] analyzed human tissues from DENV-1 infected patients and found
208 viral antigens as discrete granular deposits within the tubule-lining cells, but found no
209 viral RNA in the samples. Here, on the other hand, high viral titers in all DENV-4 cases
210 were observed. Basilio-de-Oliveira et al [25] demonstrated in a fatal elderly DENV-3
211 case, hemorrhage in the glomerular capillaries and proximal convoluted tubules. Renal
212 medullary tissue also presented a mononuclear infiltrate around the collection ducts,
213 with pockets of hemorrhage, interstitial edema and vascular congestion. Similarly, our
214 cases presented more diffuse and focal infiltrates. Moreover, congestion, peritubular

hemorrhage in case 3, and congestion in all cases, both in the medullary and in the cortical area, were identified.

Histological analysis revealed circulatory and parenchymal damage, presenting acute tubular necrosis, characterized by desquamation of necrotic cells and loss of the basal membrane mainly in contorted proximal tubules, thrombotic microangiopathy and glomerulopathy [20,37–40]. Studies have reported immune complex-mediated lesion in the glomerulus in patients with DENV infections, and have suggested this as a possible mechanism for the described urinary abnormalities [41,42]. The histopathological changes exhibited a characteristic lesion of tubular necrosis, seen in cases with comorbidities, although our cases were of young adults, these alterations have already been related to cases with diabetes [43].

In three DENV-3 fatal cases, Póvoa et al. [20] described the presence of acute tubular necrosis, characterized by desquamation of necrotic cells and loss of the basal membrane, mainly in contorted proximal tubules but also, to a lesser extent, in the distal tubules, with mold formation of cellular debris, similarly, in the DENV-4 cases analyzed here, suggesting that renal damage caused by DENV is not serotype-specific. In this study, we observed the presence of vascular congestion around and within the glomerulus in the cortical and medullary region, arteriolar hyalinosis, damaged mesangial cells, lymphocytic infiltrate in the cortical and medullary region, fibrous increase in the bowman's capsule, thickened basement membrane in the cortical region associated with DENV-4 infection and replication, demonstrated by the presence of the DENV NS3 protein [20,44].

238 Arteriolar hyalinosis occurs by glomerular hyperfiltration [45] and this lesion is not
239 exclusive to a specific disease, being observed in arterioles of normal individuals with
240 advanced age [46], but it may occur earlier and intensely in cases of hypertension and
241 diabetes mellitus. However, this is the first study to describe this renal alteration in
242 young adults fatal cases and caused by serotype 4.
243
244 The analysis in this study revealed an inflammatory environment with mononuclear
245 cells in several sites in the renal tissue. The evaluation of the response in the renal and
246 medullary cortex showed increased detection of macrophages ($CD68^+$) and $CD8^+$ T cells
247 in the peritubular space and around vessels in the medullary. $CD68^+$ cells showed their
248 co-expression with the DENV NS3 protein, confirming the participation of those cells
249 during DENV-4 infection in renal tissue. In addition, migration of $CD68^+$ and $CD8^+$ T
250 cells could indicate cells playing a role in the cytotoxic response in such lesions, as a
251 way to contain viral infection, leading to secretion of proinflammatory cytokines and
252 chemokines [47] in the cortical and medullary regions of the kidney. Furthermore, it
253 may be suggested that the renal tissue injury observed here, may be due to the
254 combination of both high viral load identified in those tissues and the exacerbated host
255 immune response.
256
257 Dengue may cause vascular overload and reduction of intravascular volume. This may
258 result in reduction of renal perfusion leading to acute tubular necrosis, and involvement
259 of multiple organ failure in severe dengue [36,48,49]. Moreover, intense capillary
260 leakage may lead to multiple organ dysfunction [50] and those observations
261 corroborate the changes observed in the other organs of thoses cases, systemic lesions in
262 several tissues were observed (data not shown).

263

264 Retrospective case series demonstrate that acute renal injury induced by dengue is a
265 highly morbid and fatal complication and is associated with prolonged hospitalization
266 [34,36]. However, it is known that the present changes observed here can not be
267 analyzed isolated, since the changes in dengue are dynamic and the virus can be
268 disseminated to all vital organs of the host. However, studies such as the one presented
269 here, may be useful as an alert so that the identification of renal injury parameters can
270 be observed in dengue cases and that proper management is performed.

271

272 **Data Availability**

273 All data generated or analyzed during this study are included in this article.

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277 **Conflict of Interest**

278 The authors declare no conflict of interest exists.

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## Author Contributions

PCGN, MVP and FBS conceptualized the work, PCGN, FCCR, NGS, CCJ, KR and LSR performed the experiments and formal analysis; PCGN, JMCOC, RMRN, FBS participated directly in the investigation, RMRN, JMCOC, FBS, MVP, ELA, CABO, RBO provided resources, PCGN, MVP wrote the original draft of the paper and FBS, ELA, MVP, NGS, KR reviewed and edited the paper.

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## Figure and Legends

**Figure 1:** Renal histological analysis

**(A)** Non-dengue control evidencing cortical region with presence of renal glomerulus (GR) and proximal contiguous tubules (PCT) and distal tubules (DCT). **(B)** Dengue case showing the presence of inflammatory infiltrate (Ly), hyalinoses (Hy) arteriole (a), vascular congestion (VC) in the renal glomerulus (RG), thickened Bowman's capsule (BC) and damage (star) PCT. **(C)** Dengue case showing tubular debris (D), edema (E) and pockets of hemorrhage (He). **(D)** Presence of hyalinoses (Hy) arteriole (a) and

438 damage (star) PCT, in dengue case. **(E)** Glomerular and peri-glomerular vascular (VC)
439 congestion, presence of Bowman's capsule (BC) and damage (star) PCT, in the case of
440 dengue stained with Masson. **(F)** Peritubular lymphocyte infiltrate (Ly), Bowman's
441 capsule (BC), damage (star) PCT and preserved DCT, in dengue case stained with PAS.
442 **(G)** Vascular congestion (VC) and lymphocytic infiltration (Ly) in the spinal cord of the
443 fatal case due to dengue.

444

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450

451 **Figure 2:** Immunohistochemistry of viral staining, subpopulations and cytokines /
452 chemokines

453 **(A)** non-dengue case control without DENV NS3 staining. **(B-C)** Detection of DENV
454 NS3 in the dengue case, within macrophages (Mø), Mesenchymal cells (MC),
455 endothelial cells (En) in the cortical region and macrophages (Mø) in the medullar
456 region. **(D)** control with CD68 staining inside the glomerulus. **(E-F)** Expression of
457 CD68-labeled cells in the cortical and medullary regions of the dengue case. **(G)** control
458 with $CD8^+$ peritubular staining **(H-I)** Expression of $CD8^+$ T cells in the medullary and
459 cortical region of the DENV-4 case. **(J)** Representative negative control of cytokine and
460 inflammatory mediators. **(K)** DENV-4 case with mononuclear infiltrate (Inf) expressing

461 TNF-α, macrophages (Mø) expressing MCP-1 **(L)** and macrophages (Mø) expressing
462 VEGF/R2 **(M)**. Cells showing double staining (green and red) were observed in control
463 **(N)** and fatal dengue cases **(O)**.

464

Figure 1

Click here to access/download;Figure;FIGURE 1 (1).tif

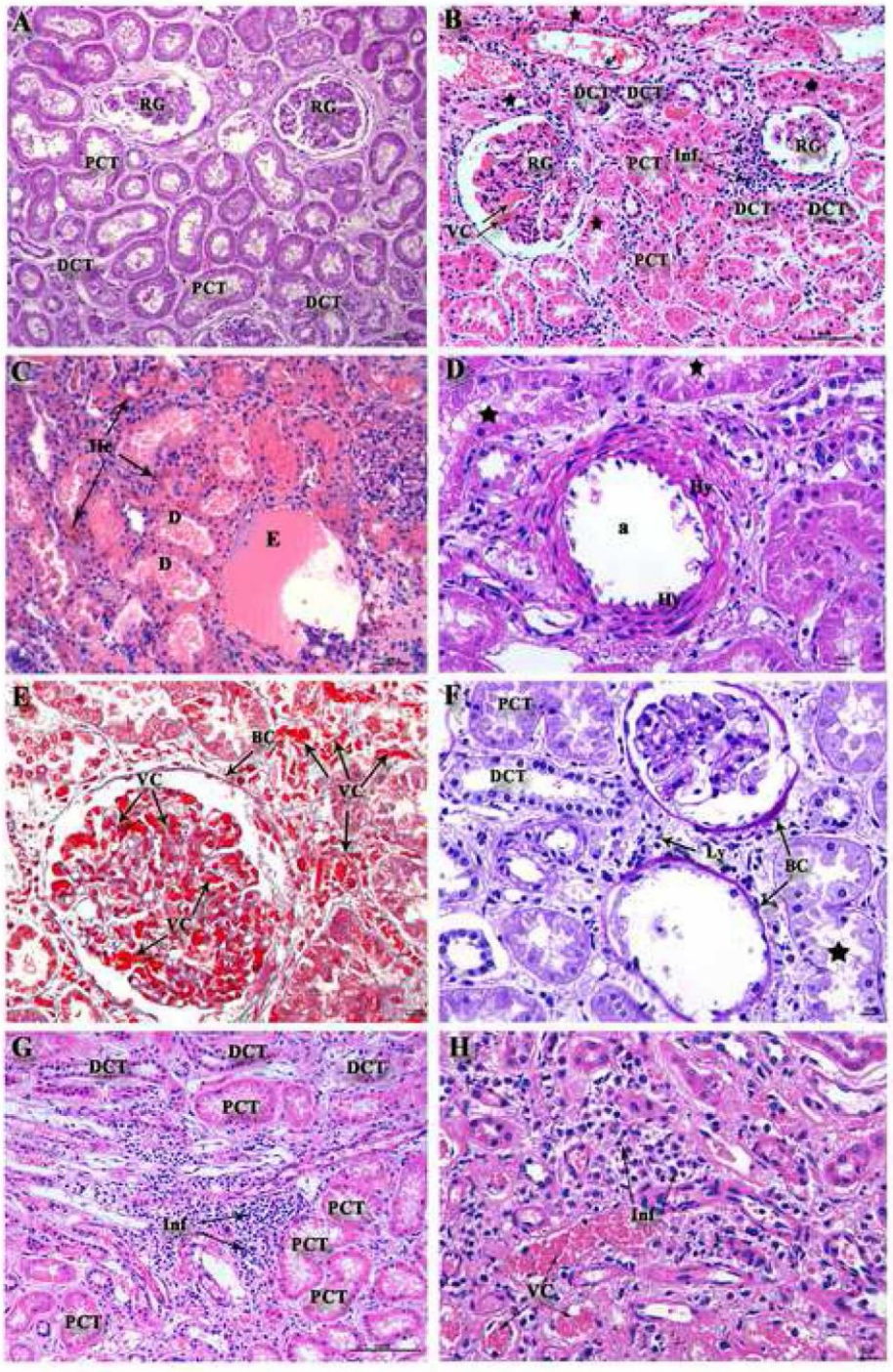
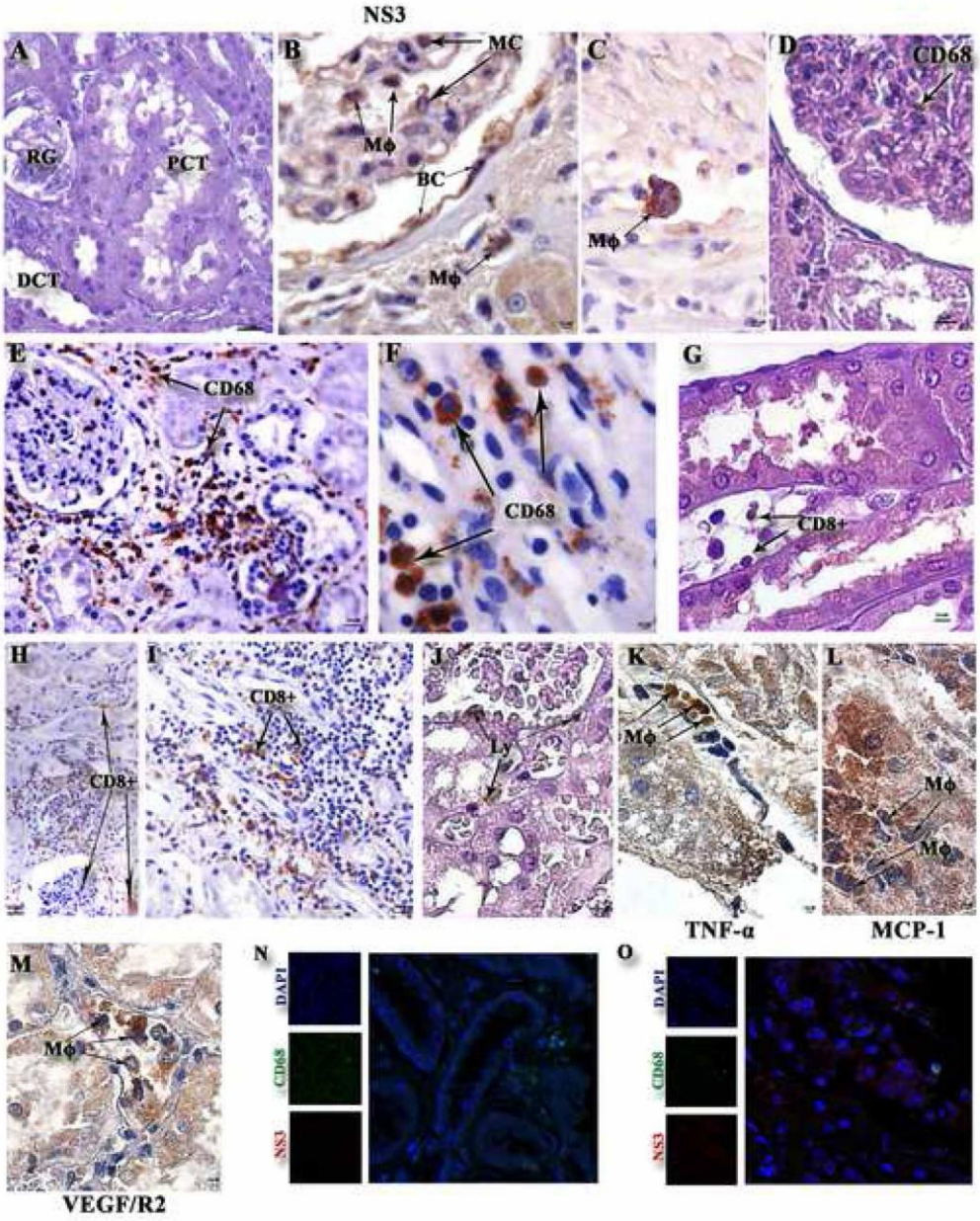


Figure 2

Click here to access/download;Figure;FIGURE 2 (1).tif



## 9.2 ARTIGOS PUBLICADOS DURANTE A TESE:

MULTIPLE SCLEROSIS JOURNAL | MSJ

*Case Report*

# Zika virus found in brain tissue of a multiple sclerosis patient undergoing an acute disseminated encephalomyelitis-like episode

**Soniza Vieira Alves-Leon, Monique da Rocha Lima, Priscila Conrado Guerra Nunes, Leila Maria Cardão Chimelli, Kissila Rabelo, Rita Maria Ribeiro Nogueira, Fernanda de Bruycker-Nogueira, Elzinandes Leal de Azeredo, Paulo Roberto Bahia, Fernanda Cristina Rueda Lopes, Jorge P Barreto Marcondes de Souza, Fabricia Lima Fontes-Dantas, Marciano Viana Paes, Elba RS Lemos and Flavia Barreto Santos**

*Abstract*

**Background:** A range of different neurological manifestations has been reported in fetuses and adults after Zika virus (ZIKV) infection.

**Objective:** We describe a detection of the ZIKV in the brain tissue from a multiple sclerosis (MS) patient with acute disseminated encephalomyelitis (ADEM)-like event in Rio de Janeiro, Brazil.

**Methods:** Biological samples collected during the hospitalization were tested by serology and molecular diagnostic for various infectious agents. Histopathological analysis was performed using the anti-flavivirus group 4G2 monoclonal antibody, anti-ZIKV non-structural 1 (NS1) monoclonal antibody, and anti-CD4, CD8, and CD11b antibodies.

**Results:** Anti-ZIKV IgM and IgG antibodies were positive in the serum and urine. A brain biopsy showed ZIKV protein in brain cells and T CD8 infiltration in brain tissue.

**Conclusion:** Our data describe the coexistence of a recent central nervous system (CNS) ZIKV infection accompanied by a severe ADEM-like syndrome outcome in a patient with clinical history of MS. A de novo immune response concomitant with ZIKV infection might be involved in the mechanism of the ADEM-like syndrome and response to immunotherapy. The present report reinforces the importance of providing the differential diagnosis of acute episodes of MS exacerbation in an environment prone to ZIKV expression.

*Keywords:* Multiple sclerosis, Zika virus, acute disseminated encephalomyelitis, differential diagnosis, immunotherapy

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### Introduction

Multiple sclerosis (MS) is an inflammatory and autoimmune disease of the central nervous system (CNS).[1] Acute disseminated encephalomyelitis (ADEM) is an acute inflammatory demyelinating disease that, as MS, predominantly affects the white matter and occurs with a temporal relationship to post-infection or post-vaccination.[2]

Zika virus (ZIKV) targets human brain cells, provokes an immune activation, and in contrast with asymptomatic patients, reports of neurological disorders are usually severe.[3,4] Here, we provide evidence of brain exposure to ZIKV in the case of a Brazilian MS patient confirmed infected with ADEM-like syndrome and the diagnostic challenge during the MS course and treatment.

### Case report

A 35-year-old female was diagnosed with MS in 2012. MS symptoms were a cervical myelitis, and a magnetic resonance imaging (MRI) examination showed a T2 weighted-image (WI) hyperintense

SCIENTIFIC REPORTS

OPEN

# BALB/c mice infected with DENV-2 strain 66985 by the intravenous route display injury in the central nervous system

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**Dengue is a mild flu-like arboviral illness caused by dengue virus (DENV) that occurs in tropical and subtropical countries. An increasing number of reports have been indicating that dengue is also associated to neurological manifestations, however, little is known regarding the neuropathogenesis of the disease. Here, using BALB/c mice intravenously infected with DENV-2 strain 66985, we demonstrated that the virus is capable of invading and damaging the host's central nervous system (CNS). Brain and cerebellum of infected animals revealed histological alterations such as the presence of inflammatory infiltrates, thickening of pia matter and disorganization of white matter. Additionally, it was also seen that infection lead to altered morphology of neuroglial cells and apoptotic cell death. Such observations highlighted possible alterations that DENV may promote in the host's CNS during a natural infection, hence, helping us to better understand the neuropathological component of the disease.**

Dengue is a mosquito-borne disease that represents a major health problem especially in tropical and subtropical regions worldwide. The disease is caused by dengue virus (DENV), which comprises four antigenically different serotypes (DENV-1 to DENV-4) belonging to the *Flaviviridae* family. Dengue burden has been expanding since 1960s as it grew side by side with the world's population. Nowadays, around 390 million people are infected every year, of which about 25% are of clinical relevance[1]. Symptoms of dengue are usually similar to the regular flu, however, a small fraction of cases may evolve to a severe hemorrhagic form that is eventually responsible for about 20,000 deaths in an annual basis[2,3].

An intriguing fact that has drawn attention in dengue is the involvement of the host's central nervous system (CNS) in the course of infection. CNS-related symptoms of dengue were first reported as an acute encephalopathy in 1976[4] and, classically, these manifestations have been treated as rare phenomena in humans[5,6]. Back in 1998,

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# First detection of dengue virus in the saliva of immunocompetent murine model

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The lack of an experimental animal model for the study of dengue pathogenesis is a limiting factor for the development of vaccines and drugs. In previous studies, our group demonstrated the susceptibility of BALB/c mice to infection by dengue virus (DENV) 1 and 2, and the virus was successfully isolated in several organs. In this study, BALB/c mice were experimentally infected intravenously with DENV-4, and samples of their saliva were collected. Viral RNA extracted from the saliva samples was subjected to qRT-PCR, with a detection limit of 0.002 PFU/mL. The presence of DENV-4 viral RNA was detected in the saliva of two mice, presenting viral titers of $10^6$ RNA/mL. The detection of DENV RNA via saliva sampling is not a common practice in dengue diagnosis, due to the lower detection rates in human patients. However, the results observed in this study seem to indicate that, as in humans, detection rates of DENV RNA in mouse saliva are also low, correlating the infection in both cases. This study reports the first DENV detection in the saliva of BALB/c immunocompetent mice experimentally infected with non-neuroadapted DENV-4.

Key words: dengue 4 - saliva - BALB/c mice - immunocompetent murine model

Dengue (DEN) is an emerging disease, prevailing in urban and suburban areas of tropical and subtropical countries. World Health Organization (WHO) data show that annually at least 100 million infections occur in over 100 countries in which the disease is endemic. Other sources suggest that worldwide this number could be almost four-fold higher, closer to 390 million infections per year (Bhatt et al. 2013).

Classified as an arbovirus (arthropod-borne virus), the DEN virus (DENV) is a member of the *Flaviviridae* family, genus *Flavivirus*, and can be discriminated into four antigenically distinct serotypes: DENV serotype 1 (DENV-1), DENV-2, DENV-3, and DENV-4 (Reiner et al. 2016, WHO 2016). The virus is transmitted by the bite of *Aedes aegypti* or *Ae. albopictus* mosquitoes. Successful infection results in DEN (San Martín et al. 2010).

DENV is a spherical particle measuring approximately 40-60 nm in diameter, with a lipid envelope and icosahedral nucleocapsid that measures about 30 nm (Barth 2010). The viral genome comprises a single stranded, positive polarity RNA molecule, which is approximately 11 kilobases (kb) in length. The genome codifies three structural proteins, those of the capsid (C), the membrane (M) and the envelope (E), and seven non-structural proteins: NS-1, NS-2A, NS-2B, NS-3, NS-4A, NS-4B, and NS-5 (Guzman et al. 2010, Yamashita et al. 2016).

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Despite being the only natural vertebrate hosts for DENV, non-human primates are not preferred as an animal model for experimental DEN infection, failing to show signs of the disease as observed in humans (Clark et al. 2013). The absence of a suitable animal model that successfully replicates the disease as it occurs naturally not only hampers the development of efficient vaccines and therapeutics, but also hinders a better understanding of the viral mechanisms of immunopathogenesis (Oliveira et al. 2016). Although some DENV strains induce limited viremia in some mouse strains, the overwhelming majority of immunocompetent mouse models do not present with clinical signs of DENV infection (Sarathy et al. 2015). Our group verified the susceptibility of immunocompetent BALB/c mice when infected by the intraperitoneal and intravenous routes with DENV non-neuroadapted viral strains. Focal alterations in the lung, heart, kidney, and hepatic tissue have been demonstrated (Paes et al. 2005, Barreto et al. 2007, Barreto-Vieira et al. 2015, Jácome et al. 2015). The virus particles were isolated in the *Ae. albopictus* C6/36 cell line inoculated with the supernatant of a macerate of the lung, cerebellum, kidney, and liver of infected animals. Viral antigen was detected in liver endothelial cells and in hepatocytes (Paes et al. 2005). A peak in viremia was detected on the 7th day post-infection (Paes et al. 2005).

Thus far, there have been no reports regarding the detection of DENV in the saliva of a DENV animal model. However, the virus has been detected in the saliva of infected human patients (Cuzzubbo et al. 1998, Balmaseda et al. 2003, Poloni et al. 2010, Yap et al. 2011, Anders et al. 2012, Andries et al. 2015).

The aim of the present study was to detect DENV in the saliva of an immunocompetent animal model,

PLOS | CURRENTS OUTBREAKS

# Clinical and Laboratory Profile of Zika and Dengue Infected Patients: Lessons Learned From the Co-circulation of Dengue, Zika and Chikungunya in Brazil

*February 15, 2018* · *Research Article*

## *Citation*

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PLOS | CURRENTS OUTBREAKS

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

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BMC Infectious Diseases

RESEARCH ARTICLE Open Access

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

Manoela Heringer[1], Thiara Manuele A Souza[1], Monique da Rocha Q Lima[1], Priscila Conrado G Nunes[1], Nieli Rodrigues da C Faria[1], Fernanda de Bruycker-Nogueira[1], Thaís Chouin-Carneiro[1], Rita Maria R Nogueira[2] and Flavia Barreto dos Santos[1*]

**Abstract**

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

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

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

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

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

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BRIEF REPORT

# Evidence of dengue virus replication in a non-traumatic spleen rupture case

Luiz José de Souza[1,2] · João de Azevedo[3] · Liza Ingride Acha Kohler[3] · Lorena de Freitas Barros[3] · Mariana Arêdes Lima[3] · Emiliana Mandarano Silva[4] · Ronaldo Mohana-Borges[4] · Priscila Conrado Guerra Nunes[5] · Marciano Viana Paes[6]

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**Abstract** The present report describes a case of splenic rupture due to dengue, a rare complication of dengue that should be considered in any patient with suspected dengue disease who started with left upper quadrant abdominal pain and hypotension. The pathophysiology of this entity is not yet well elucidated, but one of the theories present in the literature is that it is due to a depletion of coagulation factors and platelets leading to intra-splenic hemorrhage and rupture. The RT-PCR technique detected serotype 1 and histopathological studies of the spleen revealed significant atrophy of lymphoid follicles and extensive hemorrhage areas. Besides histopathological observations, virus replication was investigated by detection of dengue antigens, especially the non-structural 3 protein (NS3) in endothelial cells and splenic macrophages. This important complication has serious clinical repercussions and high mortality, due to the diagnostic difficulty and many factors that usually confuse or delay its diagnosis. Therefore, it is of the utmost importance to recognize their manifestations and their management to try to best minimize their consequences and mortality.

**Keywords** Splenic rupture · Dengue · NS3 detection · Acute abdomen

Priscila Conrado Guerra Nunes and Marciano Viana Paes contributed equally.

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## Introduction

Dengue virus (DENV) is an arbovirus, classifiable in the *Flaviviridae* family, which has four distinct serotypes (DENV 1–4) [1]. The viral RNA genome encodes three structural proteins: capsid, membrane and envelope, and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [2]. The disease caused by DENV is classified as classical dengue and dengue hemorrhagic fever; however, a new classification was recently recommended: dengue fever without signs of alarm, dengue fever with signs of severe dengue fever [3].

Dengue is an infection that can lead to several complications, such as neurological (encephalitis, stroke, Guillain-Barre syndrome), cardiac (myocarditis), gastrointestinal (liver failure, acute acalculous cholecystitis) or even an acute abdomen [4]. Spontaneous rupture is a very rare condition independent of etiology, even rarer when resulting from dengue infection. It is most common in infections such as malaria, typhoid fever and infectious mononucleosis. The mechanism underpinning acute abdomen formation is still unclear, but it is believed to be due to depletion of coagulation factors and platelets leading to intra-splenic hemorrhage and its consequent rupture [5].

frontiers in Immunology

CASE REPORT
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# Placental Histopathology and Clinical Presentation of Severe Congenital Zika Syndrome in a Human Immunodeficiency Virus-Exposed Uninfected Infant

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In the large Zika virus (ZIKV) epidemic that occurred in Brazil in 2015, the intrauterine fetal exposure to ZIKV was associated with a significant risk of developing microcephaly and neurological disorders in the infected infants. ZIKV-associated disease has since been reported in 24 countries in the Americas. At present, definitive evidence is lacking regarding the intrauterine co-exposure to ZIKV and other viral infections and whether the coinfection impacts the risk of acquiring either infection or disease severity. Here, we provide evidence of intrauterine exposure to both ZIKV and human immunodeficiency virus (HIV) infections, causing congenital Zika syndrome in an HIV-exposed uninfected infant. Clinical, imaging and laboratory examinations of the pregnant woman and the newborn were performed. Histopathology, ZIKV/HIV-specific immunoassays, and ultrastructural evaluation of the placenta were performed. The Zika-asymptomatic, HIV-positive pregnant woman underwent ultrasounds revealing fetal cerebral ventriculomegaly, microcephaly, and brain atrophy. Her baby girl was born small for gestational age and with the neurological sequelae of congenital Zika syndrome. The evaluation of the abnormally large term placenta revealed severe damage to the maternal decidua and chorionic villi, cells positive for ZIKV-specific antigens but not for HIV antigens, and intracellular membranous clusters of virus-like particles approximately 25 nm in diameter. The rapid progression and severity of the congenital Zika syndrome may be related to the uncontrolled HIV disease in the mother. The poor inflammatory response observed in the placenta may have reduced the inherent risk of mother-to-child transmission of HIV.

**Keywords: Zika virus, placenta, congenital Zika syndrome, histopathology, microcephaly, human immunodeficiency virus**

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Immunity, Inflammation and Disease

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ORIGINAL RESEARCH

# Human T cell responses to Dengue and Zika virus infection compared to Dengue/Zika coinfection

Jessica Badolato-Corrêa[1], Juan Camilo Sánchez-Arcila[1], Thiara Manuele Alves de Souza[1], Luciana Santos Barbosa[1,2], Priscila Conrado Guerra Nunes[1], Monique da Rocha Queiroz Lima[1], Mariana Gandini[3], Ana Maria Bispo de Filippis[4], Rivaldo Venâncio da Cunha[5,6], Elzinandes Leal de Azeredo[1], & Luzia Maria de-Oliveira-Pinto[1]

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**Keywords**
Dengue, Zika: T lymphocytes

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Jessica Badolato-Corrêa and Juan Camilo Sánchez-Arcila contributed equally to this work.

## Abstract

**Introduction:** Zika virus (ZIKV) and dengue virus (DENV) co-circulated during latest outbreaks in Brazil, hence, it is important to evaluate the host cross-reactive immune responses to these viruses. So far, little is known about human T cell responses to ZIKV and no reports detail adaptive immune responses during DENV/ZIKV coinfection.

**Methods:** Here, we studied T cells responses in well-characterized groups of DENV, ZIKV, or DENV/ZIKV infected patients and DENV-exposed healthy donors. We evaluated chemokine receptors expression and single/multifunctional frequencies of IFNγ, TNF, and IL2-producing T cells during these infections. Even without antigenic stimulation, it was possible to detect chemokine receptors and IFNγ, TNF, and IL2-producing T cells from all individuals by flow cytometry. Additionally, PBMCs' IFNγ response to DENV NS1 protein and to polyclonal stimuli was evaluated by ELISPOT.

**Results:** DENV and ZIKV infections and DENV/ZIKV coinfections similarly induced expression of CCR5, CX3CR1, and CXCR3 on CD4 and CD8 T cells. DENV/ZIKV coinfection decreased the ability of $CD4^+$ T cells to produce $IFN\gamma^+$, $TNF^+$, $TNF^+IFN\gamma^+$, and $TNF^+IL2^+$, compared to DENV and ZIKV infections. A higher magnitude of IFNγ response to DENV NS1 was found in donors with a history of dengue infection, however, a hyporesponsiveness was found in acute DENV, ZIKV, or DENV/ZIKV infected patients, even previously infected with DENV.

**Conclusion:** Therefore, we emphasize the potential impact of coinfection on the immune response from human hosts, mainly in areas where DENV and ZIKV cocirculate.

## Introduction

Dengue virus (DENV) and Zika virus (ZIKV) belong to Flaviviridae family and both diseases affect significantly human health. These viruses are mainly transmitted by *Aedes aegypti* or *albopictus* infected mosquitoes. Other routes of infection, including sexual, maternal, and blood transfusions, have been recently reported for ZIKV [1]. DENV

Journal of Medical Virology 88:1130–1136 (2016)

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

**Priscila Conrado Guerra Nunes, Simone Alves Faria Sampaio, Nieli Rodrigues da Costa, Marcos Cesar Lima de Mendonça, Monique da Rocha Queiroz Lima, Saraiva Eliane M Araujo, Flávia Barreto dos Santos, Jaqueline Basto Santos Simões, Bianca de Santis Gonçalves, Rita Maria Ribeiro Nogueira, and Ana Maria Bispo de Filippis***

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Dengue virus-type 2 (DENV-2) caused three outbreaks, in the years 1990, 1998, and 2008, in Rio de Janeiro, Brazil. The 2008 outbreak was the most severe in reported cases, hospitalizations, and deaths. To investigate virological and epidemiological factors that may have contributed to the pathogenic profile of 2008 epidemic, 102 patients sera obtained during the epidemic and inter-epidemic periods of three outbreaks were analysed by qRT-PCR to estimate viremia levels and their correlation with the clinical, immunological, and demographic patient characteristics. DENV-2 isolates from the outbreaks were sequenced. Two DENV-2 lineages (I and II) of the American/Asian genotype were confirmed, each exclusive for 1990–2002 and 2007–2011, respectively. The mean viremia level in the 2008 samples was two orders of magnitude higher than that of the 1990–2002 samples. Severe dengue cases increased from 31% in 1990–2002 to 69% in 2007–2011; in patients aged ≤15 years, from 3% in 1990–2002 to 37% in 2007–2011. The DENV-2 lineage II and younger age significantly contributed to the pathogenic profile of 2008 epidemic in Rio de Janeiro. ***J. Med. Virol. 88:1130–1136, 2016.*** © 2016 Wiley Periodicals, Inc.

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

## INTRODUCTION

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



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

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

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ORIGINAL ARTICLE

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

**Nieli Rodrigues da Costa Faria[a,b], Victor Edgar Fiestas Solorzano[b], Rita Maria Ribeiro Nogueira[a], Thaís Chouin-Carneiro[a], Priscila Conrado Guerra Nunes[a], Jaqueline Bastos Santos Simões[a], Fernanda de Bruycker Nogueira[a], Monique da Rocha Queiroz Lima[a], Luzia Maria de Oliveira Pinto[b], Claire Fernandes Kubelka[b], Rivaldo Venâncio da Cunha[c], Elzinandes Leal de Azeredo[b] and Flavia Barreto dos Santos[a,*]**

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

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

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

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

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

## Introduction

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

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

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

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Case Report

# Fatal case of co-infection with dengue virus and *Neisseria meningitidis* during a dengue epidemic in the state of Rio de Janeiro, Brazil

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**Introduction:** Dengue and meningococcal disease are caused by two different agents: a flavivirus and a Gram-negative bacterium, respectively. The first symptoms of both diseases can be indistinct and a rapid and accurate diagnosis is crucial, considering that both diseases are associated with high morbidity and mortality, representing a major public-health problem in Brazil.

**Case presentation:** We report a fatal case of co-infection of dengue virus (DENV) and *Neisseria meningitidis* in a 54-year-old patient. The serum tested positive for DENV NS1 antigen, and *N. meningitidis* serogroup C was detected by *nspA*-PCR. Following the initial positive result for DENV infection, rRT-PCRwas performed and DENV-4 was confirmed.

**Conclusion:** Our report highlights the importance of accurate differential diagnosis during periods of high circulation of DENV, in order to provide adequate management and an improved outcome.

**Keywords:** Dengue; meningococcal disease; diagnostic; PCR; RT-PCR.

## Introduction

Despite being aetiologically distinct diseases, both dengue, caused by one of four serotypes of dengue virus (DENV), and meningococcal meningitis, caused by the Gram-negative bacterium *Neisseria meningitidis*, can initially present with similar clinical symptoms, such as high fever, dizziness (potentially evolving to central nervous system injuries), petechial rash or an occasionally fatal shock syndrome (Coureuil *et al.*, 2013; Stephens *et al.*, 2007).

Dengue is hyperendemic in Brazil, with outbreaks occurring almost yearly. The co-circulation of all four DENV serotypes in all Brazilian states has increased the incidence of severe cases, hospitalizations and death secondary to dengue infection (Siqueira *et al.*, 2005). Since the first reports of dengue in the 1980s, more than 10 million cases have been diagnosed (Brasil Ministério da Saúde SVS/MS, 2016).

Abbreviation: DENV, dengue virus.

During 2012, 565510 cases of dengue infection were reported in Brazil, including 4055 cases of severe dengue infection and 284 related deaths (Brasil Ministério da Saúde SVS/MS, 2016; van Panhuis *et al.*, 2014; Zambrano & San Martin, 2014; Pan American Health Organization (PAHO) 2016, PAHO/World Health Organization programme – dengue, http://www.paho.org/hq/index.php?option=com_topics&view=article&id=1&Itemid=40734, accessed on 25 May 2016. In the state of Rio de Janeiro, a total of 181169 cases with 43 deaths were reported in 2012. In the same year, a total of 2083 cases of meningococcal disease were notified, with 440 deaths in Brazil. At the same time in Rio de Janeiro, a total of 390 cases of meningococcal disease with 96 deaths were reported (SVS/SES-RJ, 2016).

Unlike dengue, almost all pathogenic *N. meningitidis* serogroups are preventable by immunization; however, meningococcal disease is still endemic in our country, being the leading cause of bacterial meningitis in Brazil. The incidence of meningococcal disease in Brazil ranges from 1–1.5

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ORIGINAL ARTICLE

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

**Manoela Heringer, Rita Maria R. Nogueira, Ana Maria B. de Filippis, Monique R. Q. Lima, Nieli R. C. Faria, Priscila C. G. Nunes, Fernanda B. Nogueira and Flávia B. dos Santos***

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

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

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

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

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

## Introduction

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

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

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

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# Heart Compromise and Detection of Dengue Virus-Like Particles in Cardiac Tissue of Experimentally Infected Murine Model

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**Abstract:** *The involvement of the myocardium in human cases of dengue has been reported, but the mechanism leading to myocarditis, one of the most commonly observed pathologies, remains unclear. In this study, BALB/c mice were infected with different strains of non-neuroadapted dengue virus serotype 2 and morphological analysis of heart were performed by transmission electron microscopy. For detection and quantification of the Viral RNA, Real-Time Reverse Transcriptase PCR assay was performed. Our analyses showed involvement of heart in DENV infection. DENV-like particles were observed inside endothelial cells and cardiomyocytes. DENV RNA was detected in 15 heart and four serum samples. In three samples, we observed titers higher than that of the inocula.*

**Keywords:** *Endothelial cells, dengue virus, heart, BALB/c mice.*

## 1. Introduction

With more than one-third of the world's population living in areas at risk for infection, dengue is a leading cause of illness and death in the tropics and subtropics. Around 400 million people are infected yearly [1]. It is caused by the Dengue virus (DENV), a group four serologically distinct RNA viruses (DENV-1, DENV-2, DENV-3 and DENV-4) which are transmitted to humans by the bite of the vector mosquitoes *Aedes aegypti* (*Ae. aegypti*) and *Aedes albopictus (Ae. albopictus)*. Asymptomatic and undifferentiated febrile illness is common. Subsequent infections with different serotypes of DENV are common, as the neutralizing antibodies against the primary infectious virus does not effectively protect from other serotypes [2]. Secondary infections and primary infections in elder children and adults are associated with more severe manifestations. According to the World Health Organization [3], DENV infections are divided into two categories: dengue, subdivided into dengue without warning signs and with warning signs and severe dengue (SD). The main characteristics of SD are increasing of vascular permeability without morphological damage to the capillary endothelium, thrombocytopenia, altered number and function of leucocytes, altered haemostasis and liver injury [4]. It is not yet clear which cell type or tissues are involved in the replication of DENV. DENV antigen was observed in different tissue and cell samples from SD patients, including myocardial endothelium and cardiomyocytes, Kupffer and sinusoidal endothelial cells of the liver, macrophages and vascular endothelium in the lung, spleen, lymph node, thymus and kidney tubules [5-9]. Cardiac involvement in dengue has been reported in few studies, usually resulting in a benign and self-limited disease [8, 10, 11]. Immunohistochemistry of heart from fatal cases of dengue showed distinct perinuclear staining in endothelial cells and cardiomyocytes as small granular deposits within the cytoplasm[9]. Although cases of a more severe disease with progression to cardiogenic shock and death have been increasingly described, [9, 11-14], the pathogenesis of myocardial lesions has not been elucidated. Herein, we aim to present evidence of heart involvement during DENV infection by using immunocompetent BALB/c mice inoculated with a non-neuroadapted DENV-2.

 

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

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ABSTRACT

Dengue virus type 1 (DENV-1) was first isolated in Brazil in 1986 in the state of Rio de Janeiro (RJ) and during 25 years, this serotype emerged and re-emerged causing explosive epidemics in the country. Here, we aimed to present the phylogeny and molecular characterization based on the envelope gene (E) of DENV-1 (n = 48) isolated during epidemics occurred from 1986 to 2011. Six full coding region genomes of DENV-1 were fully sequenced and possible genomic recombination events were analyzed. The results showed that the Brazilian DENV-1 isolates analyzed belong to genotype V (Americas/Africa), but grouping into distinct clades. Three groups were identified, one dating from 1986 to 2002 (lineage 1a), a second group isolated from 2009 to 2011 and a representative strain isolated in 2002 (lineage 2), and a group of strains isolated from 2010 to 2011 (lineage 1b). The lineages 1a and 1b were more closely related to the American strains, while lineage 2 to the Asian strains. Amino acids (aa) substitutions were observed in the domains I and III of the E protein and were associated to the lineages segregation. A substitution on $E_{297}$ differentiated the lineage 1a from the lineages 1b and 2. Substitutions on $E_{328}$, $E_{394}$ (domain III), $E_{428}$ and $E_{436}$ (stem region) differentiated lineages 1a, 1b and 2. With the exception of the C gene, all the others genes analyzed allowed the DENV-1 classification into the distinct genotypes. Interestingly, the E gene's domain III and stem regions alone were able to characterize the distinct lineages, as observed by the analysis of the entire E gene and the complete coding region. No recombinant events were detected, but a strain belonging to lineage 1a was closely related to a known recombinant strain (AF513110/BR/2001).



## 1. Introduction

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

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

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Short communication

# A simple heat dissociation method increases significantly the ELISA detection sensitivity of the nonstructural-1 glycoprotein in patients infected with DENV type-4

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A B S T R A C T

The secreted form of the dengue virus (DENV) nonstructural-1 (NS1) glycoprotein has been shown to be useful for the diagnosis of DENV infections in patients' serum samples. In a number of studies, the sensitivity of the commercially available DENV NS1 glycoprotein detection assays was higher against some DENV serotypes (DENV-1 > DENV-3 > DENV-2 = DENV-4) than others and were also lower using patients' serum samples with secondary versus primary DENV infections. In this study, 471 DENV-4 positive acute phase patients' serum samples were selected from a large panel collected in Brazil from March 2011 to October 2012 by RT-PCR and/or virus isolation followed by serotype determination. The sera from primary (n = 228) and secondary (n = 238) DENV-4 infections were identified using IgM and IgG capture ELISAs. The sensitivity of a commercial DENV NS1 glycoprotein detection ELISA was then assessed when these serum samples were not pre-treated or pre-treated by acid or heat dissociation prior to being tested. Acid and heat dissociation of patients' serum samples with primary and secondary DENV-4 infections increased significantly the sensitivity of the DENV NS1 glycoprotein detection ELISA from 54.4% to 77.2% ($p < 0.05$) and 82% ($p < 0.05$) and from 39.1% to 63.9% ($p < 0.05$) and 73.1% ($p < 0.05$), respectively. Treatment of DENV infected patients' serum samples using simple and rapid heat dissociation step (100 °C for 5 min) was, therefore, shown to be very useful for increasing the sensitivity of the DENV NS1 glycoprotein detection ELISA using serum samples from either primary or secondary DENV infected patients.



Dengue fever (DF) is a mosquito-borne viral disease of public health significance, caused by one of four dengue virus serotypes (DENV-1 to DENV-4) and mainly transmitted by *Aedes aegypti* mosquitoes. Hyper-endemic DENV transmission of one or more serotypes occurs in most countries of the Americas (WHO, 2013). In Brazil, dengue became a public health problem after the introduction of DENV-1 in 1986. In July of 2010, DENV-4 was isolated in Roraima, 28 years after its first detection in that same State and soon this serotype spread other States of the country (Nogueira and Eppinghaus, 2011).

The DENV nonstructural-1 (NS1) glycoprotein, exists as membrane-associated protein and secreted form and was demonstrated to circulate in the acute phase of the disease by antigen capture ELISAs, up to the ninth day after the onset of the symptoms of primary and secondary infections (Alcon et al., 2002). The diagnostic sensitivity of the DENV NS1 glycoprotein detection ELISA could exceed 90% for serum samples from patients with primary DENV infections, and its circulation persisted for several days after the fever subsided (Chaterji et al., 2011; Dussart et al., 2008; Tricou et al., 2011). However, using serum samples from patients with secondary DENV infections, the DENV NS1 glycoprotein antigenaemia was shown to be shorter and the detection sensitivity using this ELISA was lower (60–80%) (Guzman et al., 2010). The anamnestic IgG responses generated against this glycoprotein during the primary DENV infection were hypothesized to account for these findings, since the formation of circulating immune complexes (CICs) which contained IgG–DENV NS1 glycoprotein would reduce

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## 9.3 CAPÍTULOS DE LIVRO PUBLICADOS DURANTE A TESE:

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*Chapter 1*

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

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***Nieli Rodrigues da Costa Faria,***
***Priscila Conrado Guerra Nunes, Manoela Heringer,***
***Thaís Chouin-Carneiro, Patricia Carvalho de Sequeira,***
***Monique da Rocha Queiroz Lima,***
***Rita Maria Ribeiro Nogueira,***
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## ABSTRACT

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

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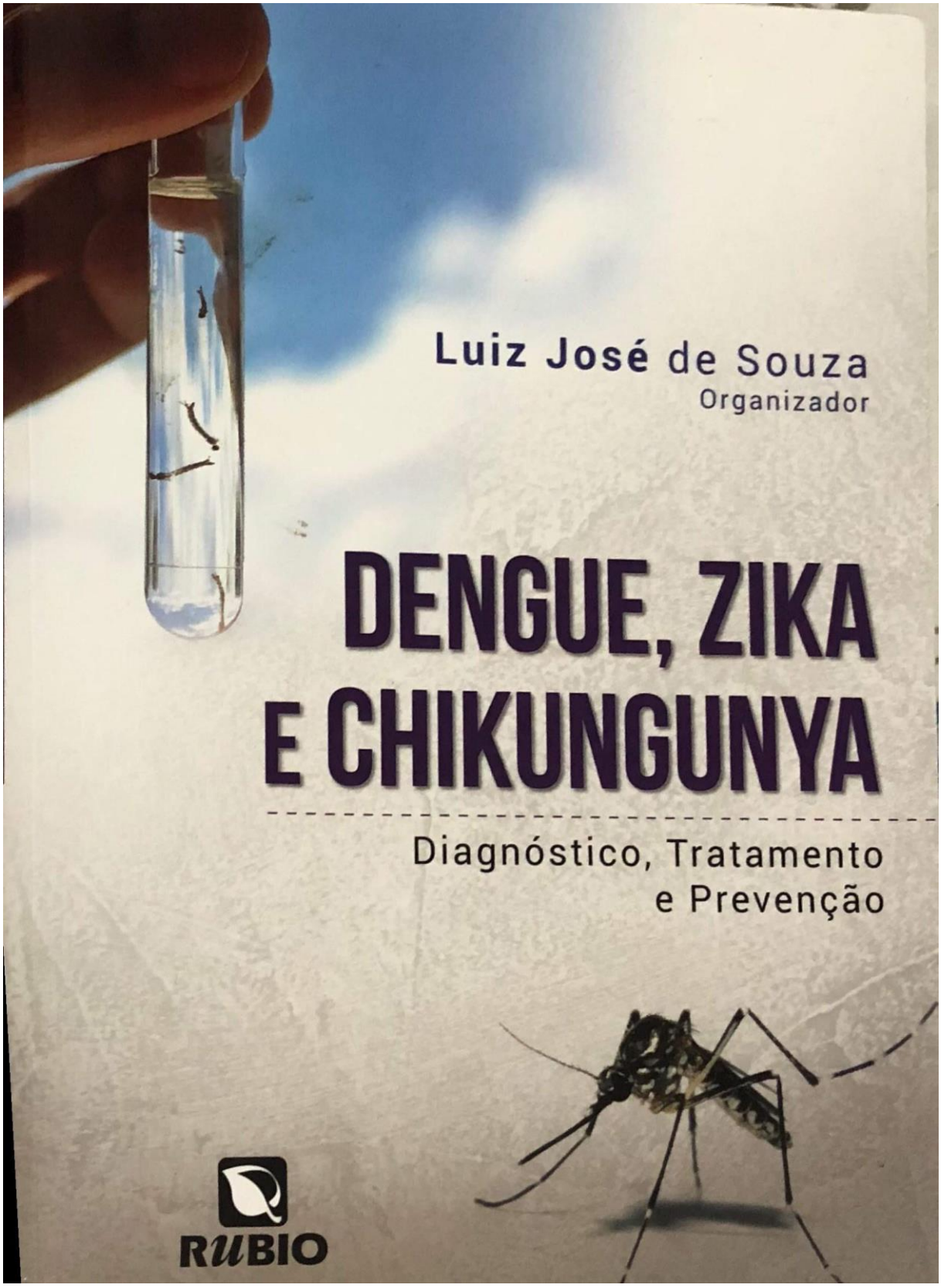
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e Prevenção
RUBIO

CAPÍTULO

12

# Zika na Gravidez

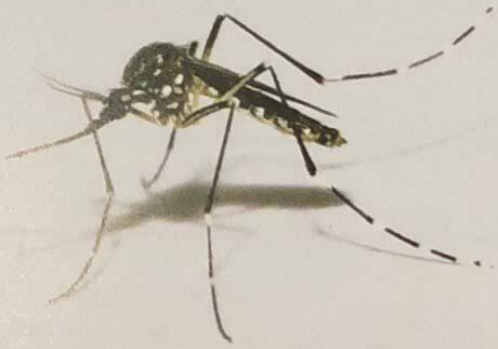
Flavia Barreto dos Santos
Priscila Conrado Guerra Nunes
Patrícia Carvalho de Sequeira
Roberta Peixoto Gava

CAPÍTULO

11

# Zika

Flavia Barreto dos Santos
Priscila Conrado Guerra Nunes
Patrícia Carvalho de Sequeira
Luiza Nascentes Machado
Mariana Arêdes Lima
Roberta Peixoto Gava



## 9.1 PARECER DO COMITÊ DE ÉTICA EM PESQUISA

Ministério da Saúde
FIOCRUZ
Fundação Oswaldo Cruz
Instituto Oswaldo Cruz
COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS-CEP FIOCRUZ-IOC

Rio de Janeiro, 30 de março de 2012.

**PARECER**

Título do Projeto: "**Dengue no Brasil: vigilância virológica, epidemiologia molecular e padronização de método sorológico utilizando antígenos recombinantes**"

Registro do Projeto no CEP Fiocruz-IOC: **274/05**

Pesquisador (a) Responsável: Rita Maria Ribeiro Nogueira

Instituição Proponente: **Fiocruz/IOC**

Deliberação: **APROVADO**

Em resposta à carta datada de 10 de novembro de 2011 enviada pelo pesquisador responsável ao CEP Fiocruz/IOC solicitando a extensão do prazo de execução do projeto, após a análise do relatório parcial, tendo por referência as diretrizes e normas da resolução CNS 196/96, foi decidido pela **APROVAÇÃO** de extensão do prazo por 02 (dois anos) a partir deste parecer.

Informamos que deverão ser apresentados relatórios parciais/anuais e relatório final do projeto de pesquisa.

Além disso, qualquer modificação ou emenda ao protocolo original deverá ser submetida para apreciação do CEP Fiocruz/IOC.

**José Henrique da Silva Pilotto**
Coordenador
Comitê de Ética em Pesquisa com Seres Humanos
(**CEP Fiocruz-IOC**)

| COMPOSIÇÃO DO CEP FIOCRUZ-IOC | |
|---|---|
| Adalberto Rezende Santos – **Membro** | Kycia Maria Rodrigues Do Ó - **Membro** |
| Adriana Lima Vallochi – **Membro** | Márcia de Cássia Cassimiro – **Coordenadora Adjunta** |
| Carlos Augusto Ferreira Andrade - **Membro** | Marcos Adriano Lessa - **Membro** |
| Carlos Norberto Veraldo – **Rep. Usuários** | Maria Regina Reis Amendoeira – **Coordenadora Adjunta** |
| Catarina Macedo Lopes – **Membro** | Paulo Roberto Vasconcellos-Silva - **Membro** |
| Dumith Chequer Bou-Habib - **Membro** | Vera Bongertz - **Membro** |
| Gerson Rosenberg – **Membro** | Yara Hahr Marques Hökerberg – **Membro** |
| José Henrique da Silva Pilotto – **Coordenador** | |

FUNDAÇÃO OSWALDO CRUZ - FIOCRUZ/IOC

## PARECER CONSUBSTANCIADO DO CEP

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** DENGUE, ZIKA E CHIKUNGUNYA: UMA ABORDAGEM MULTIDISCIPLINAR EM APOIO À INVESTIGAÇÃO DESTAS ARBOVIROSES NO BRASIL

**Pesquisador:** FLAVIA BARRETO DOS SANTOS

**Área Temática:**

**Versão:** 4

**CAAE:** 57221416.0.1001.5248

**Instituição Proponente:** FUNDACAO OSWALDO CRUZ

**Patrocinador Principal:** FUN CARLOS CHAGAS F. DE AMPARO A PESQUISA DO ESTADO DO RIO DE JANEIRO - FAPERJ
FUNDACAO OSWALDO CRUZ

**DADOS DO PARECER**

**Número do Parecer:** 1.920.256

**Apresentação do Projeto:**

"As epidemias causadas por dengue, chikungunya e zika constituem um sério problema de Saúde Pública brasileira, com grande impacto para sociedade. Dadas às limitadas opções de prevenção e controle, é demonstrado que o diagnóstico laboratorial possui um papel fundamental para o tratamento oportuno dos pacientes. Os quatro sorotipos de DENV variam em termos de patogenicidade e virulência, e a imunopatologia da doença pode estar diretamente associada ao vírus, às diferenças nos sorotipos e genótipos. O CHIKV introduzido no país é conhecido por causar uma doença considerada benigna até então, porém, apresentações atípicas e complicações neurológicas, cardíacas, renais, oculares e de pele, já foram reportadas. A infecção pelo ZIKV não era associada a complicações grave, no entanto, relatos da síndrome de Guillain-Barré foram descritos. O aumento significativo de casos de microcefalia em fetos, possivelmente associada à infecção pelo ZIKV foi reportado. O presente Projeto é resultante da formação de uma Rede proposta que visa atender às demandas emergenciais de enfrentamento do dengue, zika e chikungunya no âmbito da Saúde Pública nacional e propõe uma abordagem multidisciplinar que contribua nas áreas da vigilância epidemiológica, aspectos clínicos, diagnóstico laboratorial, fisiopatologia das infecções e desenvolvimento tecnológico. A integração entre a rede ocorrerá

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