MINISTÉRIO DA SAÚDE
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO OSWALDO CRUZ

Doutorado em Programa de Pós-Graduação em Medicina Tropical

Estudos do tropismo de duas linhagens do vírus dengue tipo 2 em modelo experimental murino: análises clínicas, bioquímicas e morfológicas.

Fernanda Cunha Jácome

Rio de Janeiro
Novembro de 2021

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

Orientadoras: Prof. Dr. Debora Ferreira Barreto-Vieira
Prof. Dr. Flávia Barreto do Santos

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Jácome, Fernanda cunha.

Estudos do tropismo de duas linhagens do vírus dengue tipo 2 em modelo experimental murino: análises clínicas, bioquímicas e morfológicas. / Fernanda cunha Jácome. - Rio de Janeiro, 2021.
xiv, 149f f.; il.

Tese (Doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Medicina Tropical, 2021.

Orientadora: Debora Ferreira Barreto-Vieira.
Orientadora: Flavia Barreto dos Santos.

Bibliografia: Inclui Bibliografias.

1. Dengue 2. 2. Linhagens. 3. Modelo murino. 4. Patologia. 5. Tropismo. I. Título.

Elaborado pelo Sistema de Geração Automática de Ficha Catalográfica da Biblioteca de Manguinhos/Icict/Fiocruz com os dados fornecidos pelo(a) autor(a), sob a responsabilidade de Igor Falce Dias de Lima - CRB-7/6930.

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Rio de Janeiro, 04 de novembro de 2021.

Ata da defesa de tese de doutorado acadêmico em Medicina Tropical de **Fernanda Cunha Jácome**, sob orientação da Drª. Debora Ferreira Barreto Vieira e co-orientação da Drª. Flávia Barreto dos Santos. Ao quarto dia do mês de novembro de dois mil vinte e um, realizou-se às nove horas, de forma síncrona remota, o exame da tese de doutorado acadêmico intitulada: **"Estudos do tropismo de duas linhagens do vírus dengue tipo 2 em modelo experimental murino: análises clínicas, bioquímicas e morfológicas"**, no Programa de Pós-graduação em Medicina Tropical do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutora em Ciências - área de concentração: Diagnóstico, Epidemiologia e Controle, na linha de pesquisa: Relação Parasito-Hospedeiro. A banca examinadora foi constituída pelos Professores: Drª. Simone Morais da Costa – IOC/FIOCRUZ (Presidente), Drª. Iranaia Assunção Miranda – UFRJ/RJ, Dr. Eduardo de Mello Volotão – IOC/FIOCRUZ e como suplentes: Drª. Barbara Cristina Euzebio Pereira Dias de Oliveira – IOC/FIOCRUZ e Drª. Kissila Rabelo – UERJ/RJ. Após arguir a candidata e considerando que a mesma demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela Aprovação da defesa da tese de doutorado acadêmico. De acordo com o regulamento do Programa de Pós-Graduação em Medicina Tropical do Instituto Oswaldo Cruz, a outorga do título de Doutora em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, a Presidente da Banca atesta a decisão e a participação da aluna e de todos o membros da banca de forma síncrona remota. A Dra. Jacenir Reis dos Santos Mallet, membro da Comissão de Docentes do Programa, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 04 de novembro de 2021.

Drª. Simone Morais da Costa (Presidente da Banca): Simone Morais da Costa

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Agradecimentos

A toda equipe Programa de Pós-Graduação em Medicina Tropical, do Instituto Oswaldo Cruz, Fiocruz, pelo apoio;

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão de bolsa de estudos durante o período de execução deste trabalho;

À minha querida orientadora Dra Débora Ferreira Barreto-Vieira. Acima de tudo por acreditar em mim. Não fosse pela sua insistência em trazer-me de volta ao mundo acadêmico, eu não estaria escrevendo estas palavras;

À minha querida orientadora Dra Flávia Barreto dos Santos. Agradeço pelo conhecimento partilhado com muito carinho e paciência, pela confiança no meu trabalho e pelas sessões de terapia;

À Dra Ortrud Monika Barth. Obrigada pela amizade, pelas conversas tranquilizadoras e pelo conhecimento transmitido;

A toda equipe do Laboratório de Morfologia e Morfogênese Viral pelo apoio técnico. Em especial, aos mestrandos Ana Luisa e Marcos Alexandre, aos doutorandos Arthur Rasinhas e Gabriela Caldas e ao técnico Raphael Leonardo. Agradeço, ainda, pela amizade e infinita paciência. Essa dissertação, eu dedico a vocês também;

Ao “Team Dengue”, pelas reuniões não só extremamente produtivas, mas também divertidas. Queridos, houve um momento em que, não fosse por vocês, eu teria desistido;

Às Dra Luzia Maria Pinto e Dra Euzinandes Azeredo e a toda equipe do Laboratório de Imunologia Viral, onde parte deste trabalho foi realizada. Obrigada pelo acolhimento e por sempre estarem dispostos a ajudar. Dra Priscila Conrado Guerra Nunes, obrigada pela parceria e apoio;

Ao Dr Marcelo Pelajo, pela ajuda com as análises histopatológicas e a toda equipe do Laboratório de Patologia, em especial ao João Paulo, pela amizade e por toda a ajuda técnica.

Ao Dr. Felipe Aníbal de Carvalho, pela ajuda nas análises estatísticas;

Às equipes da plataforma de Microscopia Eletrônica Rudolph Barth, do Instituto de Ciência e Tecnologia em Biomodelos e do Biotério do Pavilhão Hélio e Peggy Pereira.

Aos meus familiares, em especial minha mãe, meu pai e minha irmã. Obrigada pelo amor e apoio incondicionais;

À Tatá, à Lu e à Cla, porque sim;

Aos amigos e colegas que não foram citados mas, que de alguma forma fizeram parte desta jornada.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001

Para Maria, Sérgio e Flávia.

Mas o que salva a humanidade
é que não há quem cure a curiosidade
(Tom Zé)

Ministério da Saúde

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Resumo

TESE DE DOUTORADO EM MEDICINA TROPICAL

Fernanda Cunha Jácome

A dengue é a arbovirose mais prevalente entre humanos e quatro bilhões de pessoas vivem sob risco de infecção. As manifestações clínicas da dengue são variáveis, e a doença pode se apresentar na forma subclínica ou assintomática. Um quarto dos pacientes desenvolve febre da dengue (FD) ou dengue grave (DG), que pode resultar na falência de diferentes órgãos. Ademias, o envolvimento sistêmico pode estar potencialmente relacionado ao aumento da mortalidade. A fim de compreender melhor o papel da infecção por DENV em diferentes órgãos, o objetivo deste estudo foi investigar os resultados da infecção com duas linhagens distintas do genótipo Asiático/Americano do sorotipo 2 do vírus dengue em fígado, rim, pulmão e coração de um modelo murino. Para tal, camundongos BALB/c foram infectados com as Linhagens I e II de DENV-2 e amostras dos órgãos destes animais foram submetidos à imunohistoquímica, à histomorfometria e a estudos histopatológicos e ultraestruturais. Análises bioquímicas e hematológicas foram realizadas em amostras de sangue. Foi observada uma tendência do aumento do peso dos órgãos dos camundongos infectados com ambas as linhagens. O genoma viral foi detectado em fígado, coração, e músculo esquelético de camundongos infectados com ambas as linhagens e o antígeno do DENV, em rim e coração de camundongos infectados com Linhagem II. As alterações morfológicas foram semelhantes às observadas em casos de dengue humana. Além disso, os parâmetros como peso de órgãos, níveis de ureia, hematócrito e análise morfométrica, mostraram diferenças significativas entre as duas linhagens em camundongos BALB/c infectados, o que demonstrou a adequação deste modelo experimental para estudos de fisiopatologia da dengue nos órgãos avaliados.

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Tropism studies of two lineages of dengue virus type 2 in a murine experimental model: clinical, biochemical and morphological analyses.

Abstract

PHD THESIS IN MEDICINA TROPICAL

Fernanda Cunha Jácome

Dengue is the most prevalent arboviral disease among humans and four billion people live at risk of infection. The clinical manifestations of dengue are variable, and the disease can present itself in a subclinical or asymptomatic form. A quarter of patients develop classic dengue (CD) or severe dengue (SD), which can result in multiple organs failure. Furthermore, systemic involvement can be related to potential increase in mortality. In order to better understand the role of DENV infection in different organs, the aim of this study was to investigate the results of infection with two distinct strains of the Asian/American genotype of dengue virus serotype 2 in liver, kidney, lung and heart of a murine model. For this purpose, BALB/c mice were infected with Lineages I and II and samples of these organs were submitted to immunohistochemistry, histomorphometry, histopathological and ultrastructural studies. Biochemical and hematological analyzes were performed on blood samples. A trend of organ weight increase in mice infected with both strains was observed. The viral genome was detected in liver, heart, and skeletal muscle of mice infected with both lineages and the DENV antigen, in kidney and heart of mice infected with Lineage II. Morphological changes were similar to those observed in human cases of dengue. In addition, parameters such as organ weight, urea levels, hematocrit and morphometric analysis showed significant differences between the two strains in infected BALB/c mice, which demonstrated the suitability of this experimental model for studies of dengue pathophysiology in studied organs.

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Lista de siglas e abreviaturas

^{0}C: graus celsius
µL: microlitros
aa – aminoácido
a.C: antes de cristo
ADE: facilitação dependente de anticorpo (do inglês *antibody dependent enhancement*)
Ae. aegypti: Aedes aegypti
ALT: alanina aminotransferase
AcN: anticorpos neutralizantes
AST: aspartato aminotransferase
BUN: ureia nitrogenada no sangue (do inglês *blood urea nitroge*)
C: capsídeo
CCL2: proteína ligante 2 da quimiocina
CD: grupamento de diferenciação
CE: células edoteliais
CK: creatina quinase
CK-MB: creatina quinase banda miocárdica
cM: corpúsculo de Malpighi
CXCL1: ligante de quimiocina 1
d.C: depois de Cristo
DC-SIGN: Molécula de adesão de células dendríticas
DENV: vírus dengue
DG: dengue grave
dpi: dias pós infecção
E: envelope
FD: febre da dengue
FHD: febre hemorrágica da dengue
g: gramas
GRP78/Bip: proteína de imunoglobulina de ligação
HCT: hematócrito
Hpi: horas pós infecção
Hsp: do inglês "*heat shock protein*"
ICAM-3: Moléculas de adesão intercelular-3
ICTB: Instituto de Ciência e Tecnologia em Biomodelos
IgG: imunoglobulina G
IL: interleucina
IFN: interferon
IP-10: proteína 10 induzida por interferon gama
IRA: insuficiência renal aguda
KDa: kilodaltons
M: membrana
MAVS: proteína de sinalização antiviral mitocondrial
MCP-1: proteína 1 quimioatraente de monócitos
NTPase: nucleosídeo trifosfatase
NS: não estrutural
$NS2B_{CF:}$ proteina não estrutural 2 cofator
$NS3_{PRO:}$ proteina não estrutural 3 protease
NS5-Pol: proteína não estrutural 5 polimerase
OMS: Organização Mundial de Saúde
OPAS: Organização Panamericana de Saúde
ORF: fase aberta de leitura

PNH: primatas não humanos
prM: pré membrana
RE: retículo endoplasmático
RIG-I: do inglês "*retinoic acid-inducible gene I*"
RNA: ácido ribonucleico
SCD: síndrome do choque por dengue
SE: semana epidemiológica
SVS: Secretaria de Vigilância em Saúde
Th1: célula T auxiliar tipo 1
TNFα: fator de necrose tumoral alfa
Th1: célula T auxiliar tipo 1
U/L: unidades por litro
UTR: região não codificante
vRNA: RNA viral

1. Introdução

A dengue, uma das doenças tropicais negligenciadas mais importantes do mundo, é descrita pela Organização Mundial da Saúde (OMS) como a doença viral transmitida por vetores com a dispersão mais rápida e, portanto, tem enorme potencial para causar grandes epidemias em todo o mundo (OMS, 2013; Guzman *et al.*, 2015). A dengue, é atualmente endêmica em mais de 120 países, e as estimativas globais variam. Enquanto um estudo sugere que aproximadamente 50 a 200 milhões de pessoas são infectadas anualmente, com 500.000 episódios de dengue grave (DG) e mais de 20.000 mortes relacionadas à doença (Murray *et al.*, 2013), outros estimam que aproximadamente quatro bilhões de pessoas estão em risco de infecção e 390 milhões são infectadas a cada ano (Bhatt *et al.*, 2013; Stanaway *et al.*, 2016). Como sua incidência aumentou 30 vezes nos últimos 50 anos devido ao crescimento da população humana, urbanização descontrolada e viagens internacionais, a dengue representa uma grande ameaça principalmente para as populações urbanas nas áreas tropicias e subtropicais da América Latina e da Ásia (Guzman *et al.*, 2016; CDC, 2020). Na América Latina, a doença se estabeleceu como um sério problema de saúde pública, com aproximadamente 1,5 milhões de casos anuais (Fischer *et al.*, 2020). O agente etiológico da dengue é o vírus dengue (DENV) e o quadro clínico da infecção pode variar de assintomática a um amplo espectro de manifestações clínicas, desde febre do dengue (FD) à dengue grave (DG), que inclui a dengue hemorrágica (FHD) e a síndrome do choque (SCD).

1.1. Agente etiológico

1.1.1. Classificação, morfologia e genoma

O DENV, arbovírus pertencente ao gênero *Flavivirus*, família *Flaviviridae,* possui quatro sorotipos distintos, porém antigenicamente relacionados, DENV-1, -2, -3 e -4 (ICTV, 2021). Sua manutenção na natureza se dá através de um ciclo de transmissão envolvendo hospedeiros vertebrados e mosquitos hematófagos do gênero *Aedes*, sendo o *Aedes aegypti* o principal vetor e, os humanos os únicos hospedeiros capazes de desenvolver formas clínicas de infecção (Gubler *et al.*, 2002).

As partículas do DENV são esféricas e envoltas por uma membrana bilipídica derivada da célula hospedeira, o envelope, onde estão ancoradas proteínas estruturais do vírus. Apresentam um nucleocapsídeo de simetria icosaédrica e diâmetro de aproximadamente 50 nanômetros. O genoma viral consiste em um RNA positivo de fita simples de aproximadamente 11.000 bases (Wengler *et al.*, 1978). Inclui duas regiões não-codificantes (UTR) 5’ (100

nucleotídeos) e 3’ (400 nucleotídeos) e uma única fase aberta de leitura (ORF), que codifica uma poliproteína precursora, clivada por proteases celulares e viral durante e após a tradução e que dá origem a três proteínas estruturais: proteína de envelope (E), proteína precursora de membrana (prM), clivada mais tardiamente no ciclo replicativo para se tornar a proteína de membrana (M), e proteína do capsídeo (C), e sete proteínas não estruturais (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5, que são expressas em células infectadas (Figura 1), (Brinton *et al.*, 1986; Lindenbach e Rice, 2003; Kuhn *et al.*, 2002; Weaver & Wasilakis, 2009).

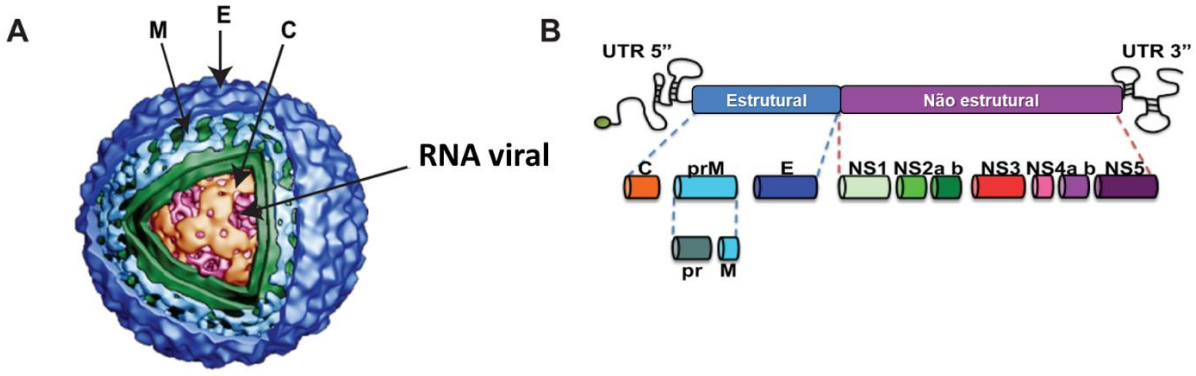

Figura 1: Representação esquemática da partícula (A) e genoma (B) do DENV. Proteína precursora da proteína de membrana (prM); proteína de membrana (M); proteína do envelope (E); proteína do capsídeo (C); proteínas não estruturais 1-5 (NS1-5); regiões não traduzidas (UTR), (Modificado de Del Angel, 2013).

1.1.2. Proteínas virais

A proteína C é o primeiro polipeptídeo viral a ser sintetizado durante a tradução. Tem um peso molecular de aproximadamente 12 kDa, é constituída de 100 aminoácidos (aa) e é rica em resíduos de lisina e arginina. Tem afinidade tanto por ácidos nucleicos quanto por membranas lipídicas da célula hospedeira e encapsula o RNA viral (vRNA) para formar o nucleocapsídeo (Henchal & Putnak, 1990; Byk & Gamarnik, 2016).

A proteína prM (26 kDa) está presente em vírions imaturos e sua clivagem, por uma protease furina da célula hospedeira, dá origem à proteína M (8 kDa/75 aa). A proteína M possui dois domínios transmembrana e um ectodomínio de aproximadamente 40 aa. Está ligada á infectividade e à organização da estrutura da partícula viral (Randolph *et al.* 1990; Velandia & Castellanos, 2011).

A proteína E, com peso molecular de 53 kDa, possui um ectodomínio com três domínios (I, II e III) e um domínio transmembrana, que compreende as regiões de haste e âncora. É responsável pela interação do vírus com os receptores da célula hospedeira, mediando a ligação e fusão da partícula à membrana (Chen *et al.*, 1996; Modis et al., 2004), é alvo da resposta

humoral contra os DENV e contém epítopos importantes para a ligação de anticorpos neutralizantes AnC. Acredita-se que o domínio III, além de mediar a interação vírus-receptores de membrana, contenha resíduos responsáveis pela determinação de tropismo e virulência entre *flavivírus* (Rey *et al.*, 1995; Chen *et al.*, 1996; Weaver & Vasilakis, 2009; Dwivedi *et al*., 2017).

A NS1 é uma proteína altamente conservada e considerada um importante marcador antigênico durante a infecção pelos DENV (Dwivedi *et al*., 2017). Consiste em 352 aa com peso molecular de aproximadamente 40 a 50 kDa, dependendo do seu estado de glicosilação (Muylaert *et al.*, 2010). A proteína, no meio intracelular, existe inicialmente como um monômero; entretanto, no retículo endoplasmático (RE) e na superfície celular, se apresenta como um dímero e sua forma secretada é hexamérica (Crooks *et al.*, 1994; Pryor *et al.*, 1993; Mullet *et al*., 2013). Sua colocalização com o RNA de fita dupla e sua capacidade de se ligar à NS4A sugerem que a NS1 desempenha um papel importante na replicação viral (Mackenzie *et al.*, 1996; Lindenbach & Rice, 1999). Além disso, alguns estudos indicam que a NS1 contribui com o extravasamento vascular e para a trombocitopenia e a hemorragia associados à patogênese da dengue (Carpio & Barrett., 2021).

A NS2A é uma proteína transmembrana hidrofóbica de 22 kDa (218 aa) (Gopala Reddy *et al.*, 2018). Quando localizada nos complexos de replicação viral, no RE rugoso, faz a mediação da síntese do vRNA. Já nos sítios de montagem, no lúmen do RE rugoso e em vesículas do complexo de Golgi, desempenha papel na montagem da partícula viral (Xie *et al.*, 2015). Associada à NS4B, inibe a sinalização de RIG-I e MAVS, garantindo uma replicação eficaz e a disseminação do DENV pelas células do hospedeiro (Dalrymple *et al.*, 2015). A NS2B é uma proteína hidrofóbica de 15 kDa (130 aa) e age como cofator da protease NS3 (Falgout, 1991). Sua associação com a NS3 é essencial para o dobramento, localização e atividade apropriados da serino-protease (Choksupmanee *et al.*, 2012). Ademais, a colocalização de NS2B com o dsRNA sugere que esta proteína pode fazer parte do complexo de replicação viral (García Cordero *et al.*, 2014).

A NS3 é uma proteína hidrofílica, de aproximadamente 70kDa. Sua região N-terminal tem um domínio serino protease e um domínio quimiotripsina, e a região C-terminal apresenta os domínios nucleosídeo trifosfatase (NTPase) e RNA helicase (Dwivedi *et al*., 2017). Por isso, essa proteína é multifuncional e atua como protease, helicase e RNA trifosfatase. (Gorbalenya *et al*..,1989, Li et al.,1999).

A NS4A é uma proteína de membrana de aproximadamente 16 kDa (127 aa), altamente hidrofóbica (Miller *et al.*, 2007). Se localiza em pacotes de vesículas derivadas do RE, onde faz

parte do complexo de replicação viral e induz a remodelação das membranas da célula hospedeira. Já foi demonstrado que a NS4A previne a indução de interferon (Miller *et al.*, 2007; Dalrymple *et al.*, 2015). A NS4B também é uma proteína de membrana, é constituída de 248 aa e tem peso molecular de aproximadamente 27 kDa. Está envolvida na replicação viral e na imunomodulação do hospedeiro (Miller *et al.*, 2006). Ela se localiza nos sistemas de membrana derivadas do RE e é recrutada pela NS4A para fazer parte do complexo de replicação viral. Além disso, a NS4 interage com diversas proteínas virais e do hospedeiro contribuindo para a patogênese da dengue (Zou *et al.*, 2015).

A proteína não estrutural do DENV mais conservada é a NS5. Ela é a maior das proteínas (105 kDa) e desempenha duas funções principais: de RNA polimerase dependente de RNA, necessária para replicação viral, e de RNA metiltransferase, capeando o vRNA durante a tradução da poliproteína (Liu *et al.*, 2010; Klema *et al.*, 2016). Além disso, ela forma um complexo de RNA replicase com a NS3 e interage com proteínas do hospedeiro que estão envolvidas na sinalização de interferon tipo 1 e respostas inatas, inibindo assim as respostas antivirais do hospedeiro (Kapoor *et al.*, 1995).

1.1.3. Ciclo replicativo

A replicação de *flavivírus* ocorre em estreita associação com estruturas membranosas intracelulares derivadas do RE e induzidas pelo vírus, chamadas complexo replicativo, que contém proteínas virais, vRNA e fatores da célula hospedeira (Mackenzie *et al.*, 2005; Miller and Krijnse-Locker, 2008; Salonen *et al.*, 2005; Bäck & Lundkvist, 2011).

A partícula viral infecta uma célula permissiva através da ligação da proteína E a receptores celulares. Vários receptores candidatos já foram identificados, o que sugere que os DENV são capazes de utilizar várias moléculas para entrar na célula (Rodenhuis-Zybert *et al.*, 2010). Dentre estas estão o heparan sulfato (Chen *et al.*, 1997), a "*heat shock protein"* (Hsp) 90 (Reyes-Del Valle *et al.*, 2005), o GRP78/Bip (Jindadamrongwech *et al.*, 2004), um receptor de laminina de alta afinidade de 37-kDa/67-kDa (Thepparit *et al.*, 2004) e a molécula de adesão intercelular específica de célula dendrítica (DC-SIGN) (Lozach *et al.*, 2005). Após a ligação desta proteína a um receptor, a partícula viral entra na célula através de um processo de endocitose mediado por vesículas revestidas por clatrinas. A acidificação do endossoma faz com que a proteína E passe por pequenas alterações conformacionais para que o envelope viral e a membrana do endossoma fusionem-se, resultando na liberação do nucleocapsídeo e do vRNA para o citoplasma (Clyde *et al.*, 2006; Perera *et al.*, 2008; Dwivedi *et al.*, 2017).

O domínio NS5-Pol da proteína NS5 é responsável pela replicação e tradução do genoma viral. Inicialmente, o vRNA atua como RNA mensageiro para a tradução de uma poliproteína. Esta poliproteína é sintetizada no RE, onde também é clivada pela proteína NS3 juntamente com seu cofator NS2B e por proteases da célula, formando três proteínas estruturais (C, prM e E) e cinco proteínas não estruturais (NS1 - NS5). Em seguida, uma fita negativa do vRNA é sintetizada para servir de molde para a replicação do genoma viral, que ocorre em sítios do complexo replicativo denominados pacotes vesiculares (*vesicle packets*). (Clyde *et al.*, 2006; Perera *et al.*, 2008; Welsch *et al.*, 2009; Potisopon *et al.*, 2014). Próximo às membranas do RE, o RNA recém-sintetizado é empacotado pela proteína C para formar o nucleocapsídeo e, inicia-se então a migração das partículas para dentro das cisternas do RE. Durante esse processo, induzido por heterodímeros das proteínas prM/E, as partículas virais adquirem o envelope (Welsch *et al.*, 2009; Rodenhuis-Zybert *et al.*, 2010). Ao migrar pela rede trans-Golgi, a acidificação do pH induz uma modificação conformacional da partícula e uma protease celular, furina, cliva a proteína prM para gerar partículas virais maduras onde a proteína E forma homodímeros. O produto da clivagem da prM pela furina fica associado aos vírions até que estes sejam liberados para o meio extracelular através do processo de exocitose (Figura 2), (Perera *et al.*, 2008; Yu *et al.*, 2008; Fibriansah *et al*, 2021).

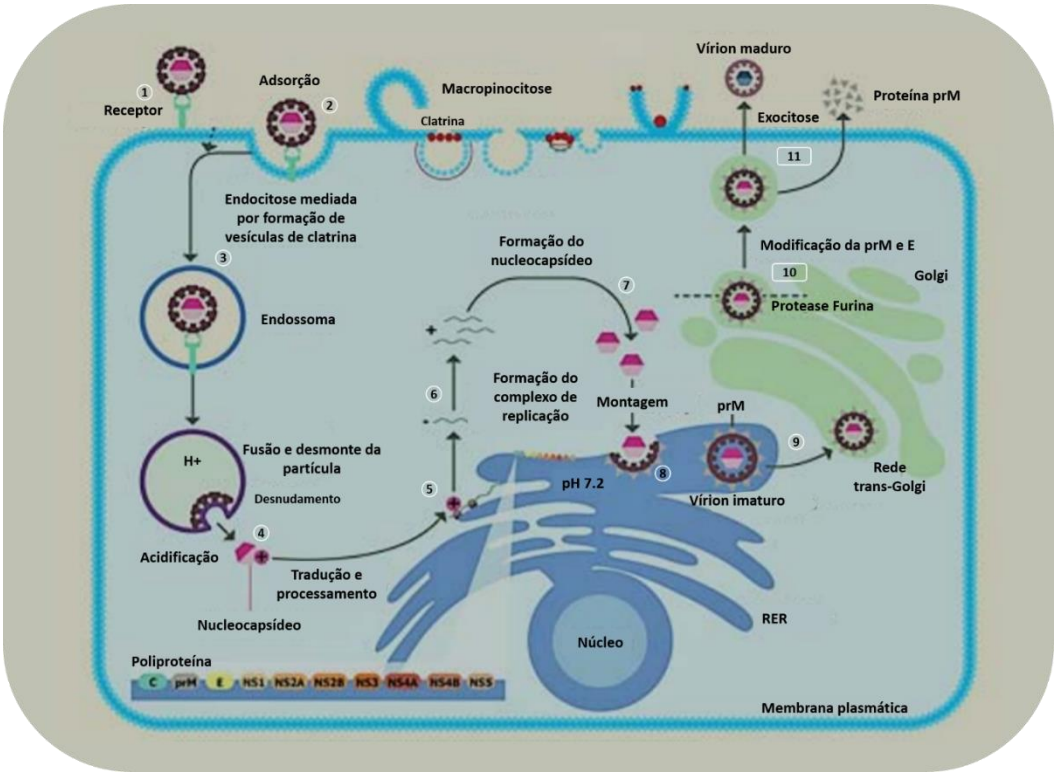

Figura 2: Ciclo replicativo do DENV. Ligação da partícula viral ao receptor da célula hospedeira (1); adsorção da partícula viral (2); endocitose mediada por clatrina e formação do endossoma (3) acidificação do endossoma, fusão das membranas endossomal e viral e liberação do vRNA (4); tradução e processamento da poliproteína (5); formação do complexo de replicação (6); formação do nucleocapsídeo (7); fomação do envoltório viral junto à membrana do retículo endoplasmático (8); migração da partícula viral imatura para o complexo de Golgi (9); maturação da partícula viral no complexo de Golgi (10); liberação da partícula viral madura por exocitose (11). Retículo endoplasmático rugoso (RER), proteína precursora de membrana (prM); proteína do envelope (E) (Modificado de Rodriguez, 2019).

1.2. Epidemiologia

1.2.1. Dengue no mundo

Os primeiros relatos de uma doença semelhante à dengue constam em enciclopédias médicas chinesas datadas da dinastia Chin (265 – 420 a.C) e da dinastia Sung (992 d.C). Além disso, outra doença que não identificada na época, foi responsável por surtos no Caribe, em 1635 e no Panamá, em 1699, pode estar associada ao DENV (Gubler *et al.*, 2006). Entretanto, as primeiras epidemias de dengue ocorreram entre 1779 e 1780 simultaneamente na Ásia, África e América do Norte. Nesta época, a dengue era considerada uma doença benigna e não-fatal contraída por visitantes dos trópicos, e os longos intervalos entre grandes epidemias se justificavam pelo fato de que vírus e mosquitos apenas circulavam entre populações através de viagens marítimas (Gubler *et al.*, 1995).

Uma epidemia global de dengue iniciou-se no sudoeste asiático após a Segunda Guerra Mundial (Halstead *et al*, 1992), e em 1975, casos de FHD tornaram-se a principal causa de hospitalizações e morte entre crianças nesta área (Gubler *et al.*, 1995). A dengue foi reintroduzida no Pacífico no início dos anos 1970 e epidemias causadas pelos 4 sorotipos já

foram reportadas. Na África, o número de epidemias tem crescido significativamente desde a década de 1980, sendo a região leste a área onde há maior ocorrência da doença (Gubler *et al.*, 1995).

Nas Américas, uma campanha com o objetivo de controlar a febre amarela, liderada pela Organização Pan-americana de Saúde, erradicou o mosquito *Ae. Aegypti* durante as décadas de 1950 e 1960. Durante esse período, epidemias de dengue ocorriam esporadicamente em ilhas do Caribe. Com a descontinuação do programa, os DENV voltaram a circular em países do continente americano. Em 1970, o DENV-2 estava presente nas Américas e o DENV-3 apresentava uma distribuição focal na Colômbia e em Porto Rico. O DENV-1 foi introduzido em 1977 causando epidemias na Jamaica, em Cuba, em Porto Rico e na Venezuela. Mais tarde, atingiu outros países caribenhos além de México, América Central e o norte da América do Sul. Em 1981, além da introdução do DENV-4 no leste das ilhas do Caribe, um novo genótipo de DENV-2, proveniente do Sudeste Asiático, causou a primeira grande epidemia de FHD em Cuba. Este genótipo espalhou-se rapidamente e em 1995, 14 países do continente americano já haviam reportado casos confirmados de FHD (Guzman *et al.*, 2003).

Nas últimas décadas, fatores como o crescimento populacional, a urbanização não planejada, viagens globais e mudanças climáticas e ambientais criaram um ambiente extremamente favorável para a transmissão do DENV pelos mosquitos *Ae. aegypti* e consequentemente, a incidência da dengue cresceu dramaticamente no mundo, com mudanças no perfil epidemiológico (Wilson & Chen, 2015, Guzman & Harris, 2015; 2017). Antes da década de 1970 apenas nove países registravam epidemias graves. Hoje, contudo, a doença é endêmica em mais de 120 países da África, Américas, Sudeste Asiático e regiões oeste do Pacífico (Guzman & Harris, 2015, Stanaway *et al.*, 2016).

Em 2013, o número de casos reportados somente na Américas foi de 2,35 milhões. Entretanto, com o crescente número de casos, a dengue vem espalhando-se por novas áreas e atualmente existe na Europa a ameaça de possíveis surtos. Em 2010 foram reportados casos autóctones na França e Croácia. Em 2012, um surto na Ilha da Madeira, em Portugal, resultou em mais de 2000 casos, além de casos importados em outros 10 países europeus. Em 2013 foram notificados casos na Flórida, Estados Unidos e Yunnan, na China. Já em 2014, número crescente de casos de DENV-3 tem afetado países das ilhas do Pacífico (Guzman *et al*, 2014).

O ano de 2016 foi caracterizado por grandes surtos de dengue. Nas Américas, cerca de 2,38 milhões de casos foram relatados, entre estes, 1,5 milhões ocorreram no Brasil. Outras regiões afetadas foram o Pacífico Ocidental, as Ilhas Salomão e a região Africana de Burkina

Faso, que relatou um surto localizado de dengue com 1.061 casos prováveis. Em 2017 houve redução significativa (73%) da incidência da dengue nas Américas e somente Panamá, Peru e Aruba registraram aumento de caso. Em 2019, o maior número de casos já ocorridos em todo o mundo foi reportado e o Afeganistão documentou a transmissão da dengue pela primeira vez (OMS, 2021).

Já em 2020, a dengue afetou vários países, com aumento do número de casos em Bangladesh, Brasil, Ilhas Cook, Equador, Índia, Indonésia, Maldivas, Mauritânia, Mayotte (FR), Nepal, Cingapura, Sri Lanka, Sudão, Tailândia, Timor-Leste e Iêmen. E, em 2021, a dengue continua afetando Brasil, Ilhas Cook, Colômbia, Fiji, Quênia, Paraguai, Peru e Ilha da Reunião (OMS, 2021). A figura 3 mostra a distribuição global do risco de infecção por DENV (OMS, 2021).

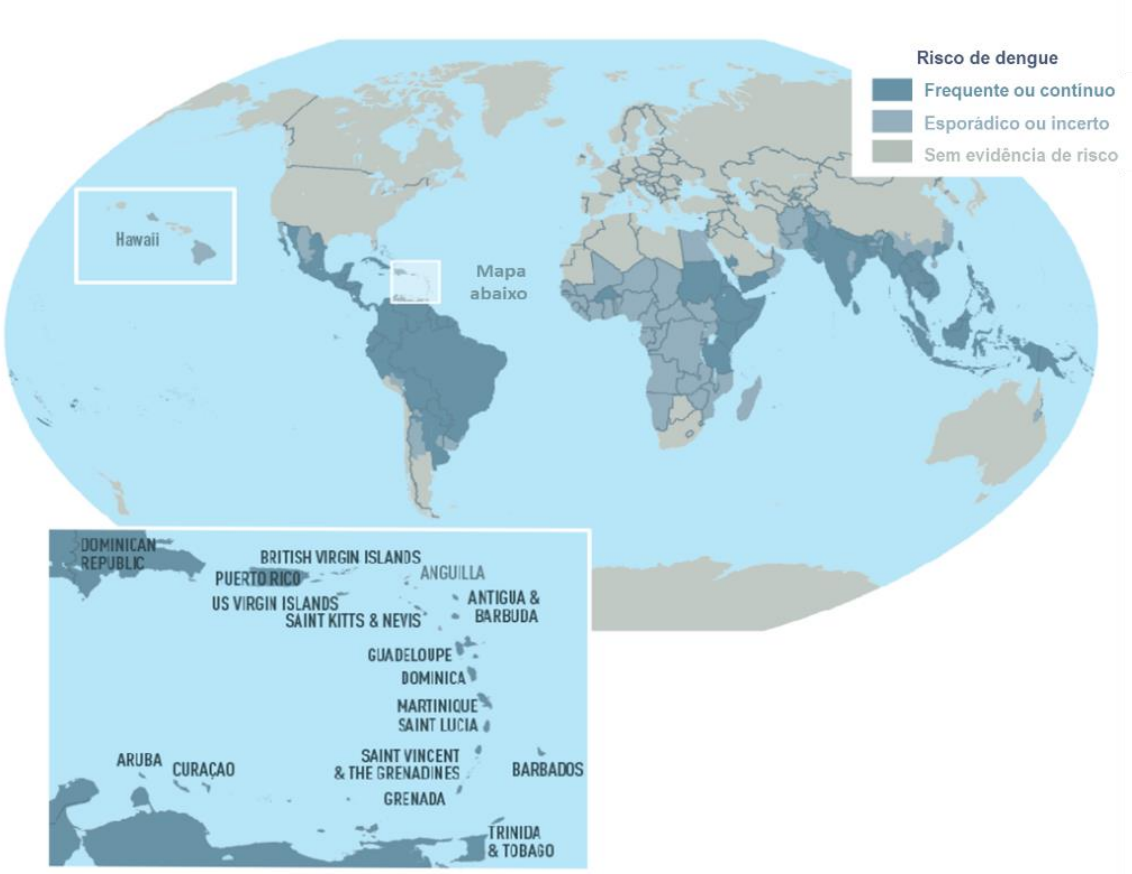

Figura 3: Mapa da distribuição global do risco de infecção por DENV. Área em destaque: Ilhas do Caribe (Modificado de CDC, 2020).

1.2.2. Dengue no Brasil

No Brasil, a primeira epidemia, documentada clínica e laboratorialmente, ocorreu no início da década de 1980, na cidade de Boa Vista, Roraima. Essa epidemia foi causada pelos sorotipos 1 e 4, com sete mil casos notificados, porém a transmissão permaneceu restrita à cidade (Osanai *et al.*, 1983). No entanto, em 1986, o DENV-1 foi isolado em Nova Iguaçu, no

estado do Rio de Janeiro (Schatzmayr *et al.*, 1986). O intenso fluxo de pessoas e a proximidade aos grandes centros urbanos facilitaram a rápida dispersão do vírus, causando uma epidemia explosiva com 92 mil casos reportados neste ano (Nogueira *et al.* ,1999).

Em 1990, um novo surto ocorreu na Região Metropolitana do Rio de Janeiro, na cidade de Niterói, onde o DENV-2 foi isolado pela primeira vez no país. Após a sua introdução, a situação da dengue no país se agravou, sendo notificados os primeiros casos FHD e SCD (Nogueira *et al.*, 1990, 1991, 1993).

A circulação do DENV-3 foi identificada em 2000, no município de Nova Iguaçu, no estado do Rio de Janeiro (Nogueira *et al.*, 2002). Em 2002, esse sorotipo foi responsável por uma grave epidemia no país, que registrou 771.551 casos (Nogueira *et al.*, 2005), correspondendo a 80% dos casos ocorridos nas Américas.

Os anos de 2004 e 2005 foram considerados como interepidêmicos (Araújo *et al.*, 2006). Em 2007, observou-se a reemergência do DENV-2, e a este sorotipo foi atribuída à ocorrência de uma grave epidemia no ano de 2008, com um total de 259.392 casos no estado do Rio de Janeiro (SVS 2009) e um novo perfil epidemiológico, com a ocorrência de casos graves em crianças (Teixeira *et al.*, 2009, Cavalcanti *et al.*, 2011). Em 2009, o DENV-1 substituiu o DENV-2 como sorotipo predominante no país, ocasionando uma grande epidemia, com mais de 1 milhão de casos no ano de 2010 (SVS 2010).

Em julho de 2010, no estado de Roraima, o DENV-4 reemergiu cerca de 30 anos após a sua primeira detecção no país (Temporão *et al.*, 2011). Em 2011 e 2012 o DENV-4 se tornou o sorotipo predominante e, em 2013, foram registrados 1.476.917 casos de dengue e 573 óbitos. Em 2014 foram registrados no país 572.308 e 400 óbitos foram notificados (SVS, 2013, 2014).

No ano de 2015, 1.587.080 casos foram registrados, entre estes, 839 óbitos confirmados, que corresponde a um aumento de 80,4% em relação ao mesmo período no ano anterior (SVS, 2015). Em 2016 foram registrados 1.487.924 casos prováveis de dengue e, 609 casos de óbito foram confirmados. Neste período, os sorotipos 1 e 4 cocircularam, porém o DENV-1 foi o mais prevalente e responsável por aproximadamente 90% dos casos confirmados laboratorialmente (SVS, 2015; 2016). Em 2017, com a cocirculação dos quatro sorotipos, foram registrados 249.056 casos prováveis de dengue e 137 óbitos no país (SVS, 2017). No ano de 2018, houve uma queda no número de casos e foram confirmados 174.724 casos de dengue, dos quais e 155 resultaram em óbito. A região que detectou o maior número de casos foi a Centro-Oeste (SVS, 2018; OPAS, 2021). Em 2019, foram registrados 1.544.984 casos

prováveis de dengue e a Região Centro-Oeste apresentou a maior incidência da doença, 1.349,1 casos/100 mil habitantes. A partir da semana epidemiológica (SE) 44, verificou-se um aumento de incidência de casos na Região Norte, principalmente no Acre, Roraima e Tocantins. Em todo o país, houve 1.419 casos de DG, 18.740 casos de dengue com sinais de alerta e 782 casos de óbito (SVS, 2020).

Em 2020, 1.467.142 casos prováveis de dengue foram reportados, dentre eles, 765.114 foram confirmados laboratorialmente. O número de óbitos, 554, foi menore quando comparado ao ano anterior. Os 4 sorotipos circularam no país, havendo cocirculação de dois, três ou até mesmo quatro sorotipos, em diferentes localidades do país (SVS, 2020; OPAS, 2021).

Neste ano, até a SE 29 (julho de 2021), os sorotipos circulantes eram DENV-1, -2 e -3. O país apresentou 440.012 casos prováveis de dengue, e o 154 óbitos foram confirmados. A região com o maior número de ocorrências foi a Centro-Oeste, onde a incidência registrada foi 46,2 casos/100 mil habitantes. Entretando, acredita-se que haja subnotificação ou atraso nas notificações de casos de arboviroses, já que há no momento uma mobilização das equipes de vigilância para o enfrentamento da pandemia de Covid-19. A figura 4 mostra a identificação dos sorotipos de DENV e distribuição da taxa de incidência de dengue (casos/100mil habitantes), por município, Brasil.

No Brasil,vários fatores têm sido relacionados à disseminação da doença e do vetor no país, como crescimento populacional, migração, viagens aéreas, urbanização inadequada, sistemas de saúde deficientes, densidade populacional e desigualdades socioeconômicas, entre outros Além do número crescente de casos da doença, o aumento de notificações de formas graves nas últimas décadas, pode estar relacionado à circulação concomitante dos diferentes sorotipos do vírus, bem como à virulência das cepas circulantes (de Sousa et al., 2021).

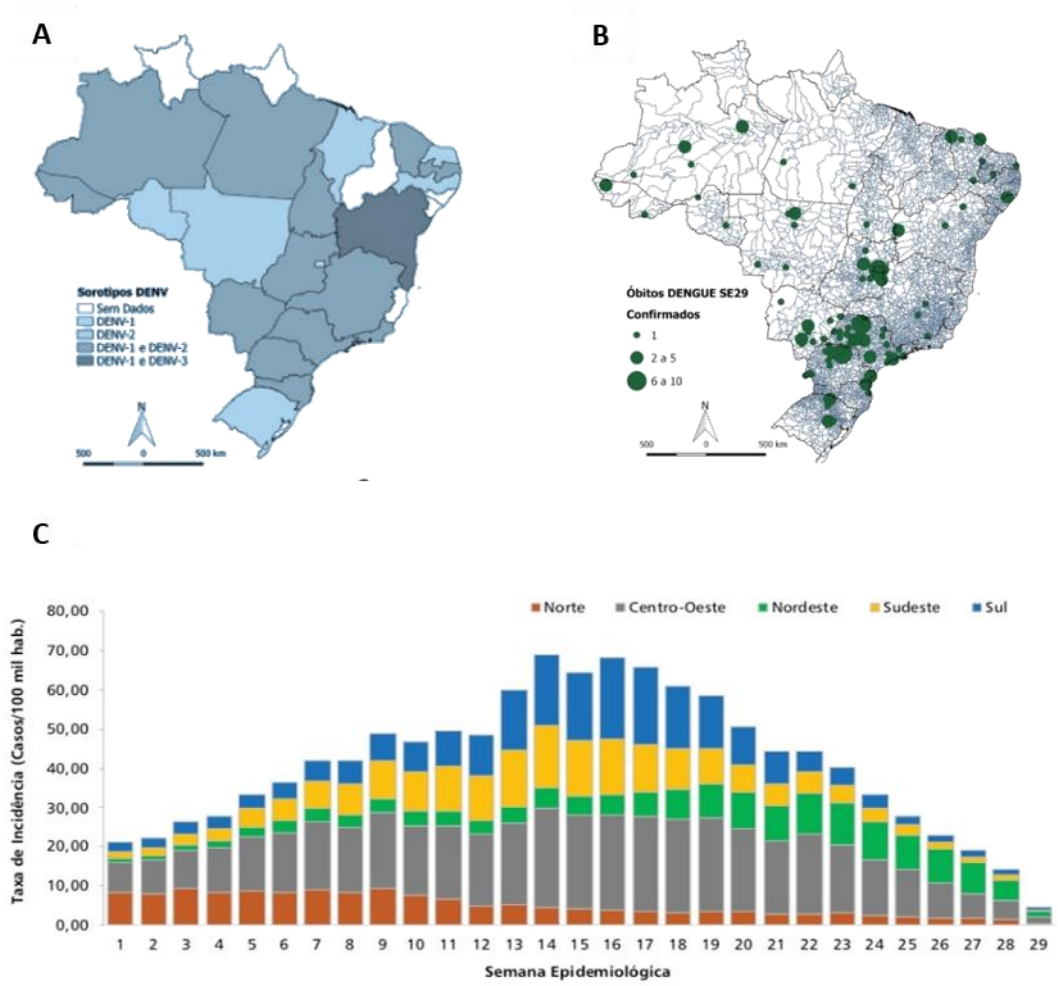

Figura 4: Identificação dose sorotipos DENV (A) e distribuição de óbitos confirmados (B) e da taxa de incidência de dengue (C) (casos/100mil habitantes), por região do Brasil, durante o ano de 2021 até a SE 29 (julho), (modificado de SVS, 2021).

1.3. Origem e diversidade genética do DENV

Investigações prévias sugerem que o DENV se originou em primatas não humanos (PNH) na África e na Ásia - dengue silvestre, com transferência para humanos ocorrendo subsequentemente, de forma independente com todos os quatro sorotipos (Chambers & Monath, 2003; Chen & Vasilakis, 2011). No entanto, a origem dos vírus, sendo asiática ou africana, ainda é controversa. Sugere-se que a diferenciação entre os sorotipos de DENV tenha ocorrido há mais de 2000 anos, com o DENV-2 sendo o primeiro sorotipo a divergir há aproximadamente 400–600 anos, seguido do DENV-4, DENV-1 e DENV-3, e que a dispersão do DENV e adaptação de várias linhagens de *Ae. Aegypti* tenha ocorrido nos útimos 200 anos (Zanotto *et al.*, 1996; Wang *et al.*, 2000; Weaver & Vasilakis 2009). Com base em análises filogenéticas e nos sorotipos existentes, estima-se que são necessários cerca de mil anos para emergência de um novo sorotipo (Sánchez-González *et al.*, 2021).

Variações genéticas entre os sorotipos de DENV são determinantes de *fitness* viral, virulência da cepa infectiva e potencial para causar epidemias, além de influenciar a interação

vírus-resposta antiviral do hospedeiro (Guzman *et al.*, 2015). A variabilidade genética do DENV pode ser atribuída à falta de um mecanismo de correção da RNA polimerase viral. Acredita-se que uma mutação do genoma viral seja produzida a cada ciclo de replicação (Steinhauer *et al.*, 1992; Drake *et al.*, 1993).

Diferenças entre cepas de DENV foram detectadas pela primeira vez através de estudos sorológicos utilizando anticorpos produzidos através da inoculação do vírus em animais de laboratório (Sabin, 1952). Os quatro sorotipos de DENV foram definidos com base em reações cruzadas limitadas, através de testes sorológicos. Já as primeiras evidências genéticas de diferenças entre cepas dentro de um mesmo sorotipo vieram com a utilização do método de *RNA fingerprinting*. As cepas eram agrupadas em topótipos e observou-se que cepas isoladas na mesma região geográfica compartilhavam mais semelhanças do que cepas isoladas em outras áreas (Vezza *et al.*, 1980; Repik *et al.*, 1983 Trent *et al.*, 1990).

Atualmente, o sequenciamento de ácidos nucleicos permite o agrupamento destes vírus em grupos geneticamente distintos, os genótipos, dentro dos quatro sorotipos. Genótipos são definidos como clusters de DENV com diferenças de sequências menores que 6% dentro de uma dada região do genoma e são, geralmente, associados a diferentes áreas geográficas (Rico-Resse *et al.*, 1990; Chen *et al.*, 2011). Além disso, cada genótipo pode ainda ser subdividido em várias linhagens (Holmes & Twiddy, 2003), que têm sido reportadas cada vez mais frequentemente na Ásia e Américas (Oliveira *et al.*, 2010; Faria *et al.*, 2013; Tripathi *et al.*, 2013; Shrivastava *et al.*, 2015; Nunes *et al.*, 2016; Torres *et al.*, 2019).

Baseados no sequenciamento completo do gene E, Weaver e Vasilakis (2009) propuseram a divisão do DENV-1 em cinco genótipos, do DENV-2 em seis genótipos, do DENV-3 em cinco genótipos e do DENV-4 em quatro genótipos (Tabela 1).

No Brasil, circula o genótipo Américas/África ou genótipo V de DENV-1 e quatro linhagens distintas dentro deste genótipo foram descritas (dos Santos *et al.*, 2011; Ribeiro *et al.*, 2021). Para o DENV-2, foi descrita a existência de duas linhagens do genótipo Sudeste Asiático/Americano (Faria *et al.*, 2013; Torres *et al.*, 2019). Já para o DENV-3, quatro linhagens do genótipo Subcontinente Indiano ou genótipo III foram identificadas (Araújo *et al.*, 2009). E, finalmente, para o DENV-4, a existência de dois genótipos (I e II) foi detectada no país (Figueiredo *et al*., 2013)

Tabela 1: Classificação genotípica do DENV baseada na análise filogenética do sequenciamento do gene que codifica a proteína E de acordo com Weaver e Vasilakis (2009).

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

1.3.1. Diversidade genética do DENV-2

Diferentes genótipos dentro de um sorotipo podem induzir respostas imunes variadas (Chambers & Monath et al., 2012) e podem variar em sua capacidade de infectar diferentes células-alvo e, por sua vez, sua capacidade de causar a forma grave da doença (Holmes & Twiddy, 2003).

O genótipo Americano de DENV-2, apesar de maior fitness em *Ae. Aegypti*, em comparação com o genótipo de origem Asiática, é menos transmissível (OhAinle et al., 2011) e está associado a cepas menos virulentas (Holmes & Twiddy, 2003).

A introdução do genótipo Sudeste Asiático/Americano de DENV-2 no Caribe em 1981 foi associada a taxas mais altas da forma grave de dengue (Rico-Hesse *et al.*, 1997) e, no Vietnã, níveis mais altos de viremia em pacientes foram atribuídos à substituição do genótipo Sudeste Asiático/Americano pelo genótipo Asiático 1 do DENV-2 (Vu *et al.*, 2010).

A Linhagem I do genótipo Asiático de DENV-2 foi associada a uma maior taxa de replicação do DENV em células humanas e de mosquito em comparação com o genótipo Asiático/Americano, o que poderia estar associado a uma maior transmissão em humanos e vetores, potencialmente levando a surtos de doenças em regiões onde esta linhagem era dominante (Shrivastava *et al*., 2015). Na Índia, a introdução de uma nova linhagem do genótipo DENV-2 Cosmopolita também foi associada ao aumento das manifestações graves da dengue (Shrivastava *et al*., 2015).

No Brasil, a emergência da Linhagem II do genótipo Sudeste Asiático/Americano de DENV-2 em 2007-2008, resultou na mais grave epidemia de dengue registrada no país até então, com um maior número de hospitalizações e ocorrência de óbitos em crianças e adultos (Teixeira *et al*., 2009; Oliveira *et al.*, 2010, Faria *et al.*, 2013; Nunes *et al.*, 2016).

No Peru, a Linhagem II do genótipo Sudeste Asiático/Americano de DENV-2 também foi associada a um surto grave de dengue em 2010 e 2011, e assim como no Brasil, era geneticamente distinta da Linhagem I deste mesmo genótipo que havia circulado anteriormente (Williams *et al.*, 2014).

1.4. Dengue

1.4.1. Manifestações clínicas e classificação dos casos de dengue

A infecção com um dos quatro sorotipos de DENV fornece imunidade homotípica vitalícia sendo, no entanto, transitória contra os sorotipos heterólogos (Sabin, 1952). Como consequência, a infecção secundária pode resultar em manifestações mais graves, devido à reatividade cruzada de anticorpos não neutralizantes e/ou à proliferação de células T de baixa afinidade (Halstead, 1973; Mathew e Rothman, 2008; Duangchinda *et al.*, 2010; Remmy *et al.*, 2014).

Além disso, a infecção por qualquer um dos sorotipos de DENV pode resultar em um amplo espectro de manifestações clínicas com evolução e desfechos imprevisíveis. A maioria dos casos são assintomáticos ou subclínicos. Geralmente, os pacientes que desenvolvem sintomas de (FD) e apresentam febre branda, auto-limitada, com recuperação espontânea. Entretanto, uma pequena parcela destes evoluem para uma forma grave da doença, caracterizada entre outros sinais clínicos, por coagulopatia, aumento da permeabilidade vascular (Edelman & Hombach *et al.* 2008; Martina *et al.*, 2009).

O período de incubação do DENV pode variar de 3 a 15 dias, mas, em média, é de 4 a 7 dias. A febre é, geralmente, de início súbito e dura em média de 2 a 7 dias. Os sintomas ainda podem incluir cefaléia, dor retro-orbital, sintomas gastrointestinais, mialgia, artralgia e rash. Leucocitose e trombocitopenia também são comuns, além de manifestações hemorrágicas brandas que incluem petéquias, epistaxe e sangramento gengival (Souza *et al.*, 2008; Martina *et al.*, 2009).

A partir da observação de um conjunto de parâmetros clínicos e laboratoriais que possibilita a identificação precoce de casos graves, a OMS propôs uma nova classificalção da dengue que divide a doença em três categorias: dengue sem sinais de aletra, quando o paciente apresenta sintomas clássicos da doença, dengue com sinais de alerta, quando no período de defervescência, os pacientes apresentam sinais como letargia ou inquietação, vômito persistente, sangramento na mucosa, aumento do fígado e dor ou sensibilidade abdominal, e DG, uma complicação potencialmente fatal, envolvendo extravazamento de plasma, acúmulo de fluidos, dificuldade respiratória grave, sangramento e comprometimento de órgãos, ou dengue com ou sem sinais de alerta (Figura 5). Alguns dos sinais de alerta são dor abdominal intensa, vômito persistente, respiração rápida e sangramento nas gengivas (OMS, 2009). Os fatores que levam às diferentes manifestações da dengue ainda não foram compreendidos. No

entanto, as formas mais graves da doença estão frequentemente associadas a infecção secundária, sorotipo e virulência de cepas virais infectantes e fatores genéticos do hospedeiro (Halstead, 2015).

	Critérios	Sinais de alerta*
Dengue com ou sem sinais de alerta	▪ O paciente viajou recentemente ou vive em área endêmica, tem febre e dois dos seguintes sintomas: náusea ou vômito, erupção na pele, dores no corpo, teste do torniquete positivo, leucopenia e qualquer sinal de alerta. ▪ Infecção pelo vírus da dengue confirmada em laboratório	▪ Dor ou sensibilidade abdominal ▪ Vômito persistente ▪ Acúmulo de fluido ▪ Sangramento da mucosa ▪ Letargia ou inquietação ▪ Aumento do fígado (> 2 cm) ▪ Nível de hematócrito aumentado acompanhado por diminuição da contagem de plaquetas
Dengue grave	▪ Vazamento de plasma grave levando à síndrome do choque da dengue e acúmulo de fluido com dificuldade respiratória ▪ Sangramento grave, conforme avaliado por um médico ▪ Envolvimento grave de órgãos, incluindo o fígado (níveis de AST ou ALT ≥1.000), o sistema nervoso central (consciência prejudicada), o coração e outros órgãos	

Figura 5: Quadro com a classificação dos casos de dengue. Confirmação laboratorial de dengue (importante na ausência de sinais de extravasamento de plasma), * Necessitando estrita observação e intervenção médica, AST: aspartato aminotransferase, ALT: alanina aminotransferase (OMS, 2009).

1.4.2. Patogênese

A base fisiopatológica da dengue é multifatorial e a evolução e desfecho da doença dependem de um balanço entre fatores genético e status da imunidade do hospedeiro e características do vírus. O tropismo do DENV por células e tecidos também pode ter um grande impacto no resultado das infecções. Ademais, o sistema imunológico e o endotélio desempenham papéis importantes na patogênese da doença (Martina *et al.*, 2009; Rothman *et al.*, 2011).

Estudos em pele humana mostraram que as primeiras células a serem infectadas pelos DENV são as células de Langerhans e queratinócitos. As células infectadas migram para os linfonodos onde macrófagos e monócitos são recrutados e tornam-se alvos da infecção (Wu *et al.*, 2000, Marovich *et al.*, 2001). Com a disseminação do vírus pelo sistema linfático (viremia primária), diversos tipos celulares, como células dendríticas mielóides e macrófagos do baço e fígado, também são infectados (Jessie *et al.*, 2004; de Macedo *et al.*, 2006; Blackley *et al.*, 2007; Durbin *et al.*, 2008; Kou *et al.*, 2008). Células dendríticas são estimuladas a produzir

mediadores envolvidos nas respostas inflamatória e homeostática do hospedeiro (Bosch *et al.*, 2002; Ho *et al.*, 2004; Chen *et al.*, 2007). Fatores que influenciam a quantidade de células-alvo infectadas, e os níveis de viremia, podem determinar a proporção de diferentes mediadores pró e anti-inflamatórios e a forma com que a resposta inflamatória afeta o sistema hemostático (Suharti *et al.*, 2001; Huerta-Zapeda *et al.*, 2008).

A característica mais marcante da DG é o aumento da permeabilidade vascular que pode resultar em extravasamento de fluidos para as cavidades peritoneal e pleural e choque hipovolêmico. Estudos *in vitro* demonstraram que os DENV podem replicar em células endoteliais (CE), e o antígeno viral já foi detectado na microvasculatura de diversos órgãos (Avirutnan *et al.*, 1998; Huang *et al.*, 2000; Jessie *et al.*, 2004; Póvoa *et al.*, 2014). Sabe-se que os níveis de uma série de mediadores pró-inflamatórios, como TNF-α, IL-8, fator de inibição de migração de macrófagos, proteína quimiotática de monócitos 1, e grupo de proteínas 1 de alta mobilidade estão elevados durante a infecção, o que pode levar ao aumento da permeabilidade vascular (Martina *et al.*, 2009; Chuang *et al.*, 2013).

Várias teorias tentam explicar os mecanismos por trás dos casos mais graves de dengue. Uma delas, sugere que a gravidade da doença esteja relacionada às variações genéticas e antigênicas de diferentes cepas virais. Diferentes graus de virulência em cepas de DENV-2 foram reportados na década de 1980, na África, Vietnã e Malásia e, posteriormente, a origem evolutiva destas cepas foi devidamente traçada e suas distinções genéticas foram confirmadas (Rico-Hesse *et al.*, 1990; 1997; 2003; Mammen *et al.*, 2014).

Durante a fase aguda da dengue, os níveis de NS1 solúvel são particularmente altos e se correlacionam com a gravidade da doença. A proteína é capaz de ligar-se ao heparan-sulfato na superfície de uma ampla variedade de células, inclusive CE, induzindo a produção de citocinas e comprometendo a integridade do glicocálix endotelial. Além disso, anticorpos anti-NS1 podem contribuir para a patogênese da dengue através de reação cruzada com plaquetas, CE e trombina (Falconar *et al.*, 1997; Avirutnan *et al.*, 2006; Chuang *et al.*, 2013; Modhiran *et al.*, 2015).

Apesar de infecções primárias terem potencial para evoluir para a DG (Castellanos *et al.*, 2021; Vallere *et al.*, 2021), infecções secundárias são mais frequentemente associadas a formas mais graves da doença (Gubler *et al.*, 1998; Halstead, 2015). A teoria da amplificação dependente de anticorpo (ADE, do inglês *antibody-dependent enhancement*) postula que os anticorpos heterotípicos, produzidos na infecção primária, se ligam às partículas do sorotipo infectante subsequente sem, no entanto, neutralizá-las. Os complexos imunes formados são

reconhecidos por receptores Fcγ, que facilitam a entrada e replicação dos vírus em células da linhagem mononuclear. Com o aumento da infecção dependente de anticorpos, há consequente aumento da carga viral. Ademais, acredita-se que este mecanismo inicia uma cascata de imunomediadores vasoativos que resulta no aumento da permeabilidade vascular, hipovolemia e choque. (Halstead *et al.*, 1973; 2010; 2015).

Uma outra teoria que justifique a associação de infecções heterólogas com casos graves é a teoria do pecado antigênico original, que diz que durante uma infecção secundária, as células T produzidas durante a infecção primária (o antígeno original) reagem com o sorotipo heterólogo da infecção subsequente. O reconhecimento de peptídeos heterólogos resulta em uma redução no potencial citolítico das células T sem reduzir a produção de citocinas. Respostas patogênicas de células T heterólogas podem contribuir para uma "tempestade de citocinas" que induz a permeabilidade vascular, levando à DG (Mongkolsapaya *et al.*, 2003; Halstead, 2015; Vatti *et al.*, 2017).

Apesar de diversas teorias serem propostas, acredita-se que a ocorrência de casos graves seja multifatorial e inclua comorbidades, fatores nutricionais, genéticos, idade, sexo e estado imunológico do hospedeiro, bem como sorotipo e genótipo viral infectantes (Guzmán & Kourí, 2002; Malavige *et al.*, 2004).

1.4.3. Tropismo nas infecções pelos DENV

Uma série de tipos celulares em diferentes órgãos são susceptíveis aos DENV, e a detecção da fita negativa do vRNA indica que algumas delas suportam a replicação viral (Begum *et al.*, 2019). Estudos realizados em autópsias e biópsias de pacientes demonstraram presença do genoma e do antígeno viral em monócitos e linfócitos circulantes, células linfóides e macrófagos do baço, bem como no fígado, pulmão, baço, cérebro, rim, músculo esquelético, medula óssea e coração, e descreveram alterações histopatológicas nestes órgãos (Jessie *et al.*, 2004; Basílio-de-Oliveira *et al., 2005*; Araújo *et al.*, 2009; Balsitis *et al.*, 2009; Salgado *et al.*, 2010; Lima *et al.*, 2011; Achrya *et al.*, 2010; Misra *et al.*, 2012; Póvoa *et al.*, 2014; Rodrigues *et al.*, 2014; Pagliari *et al.*, 2015; de Souza *et al.*, 2017; Oliveira *et al.*, 2017; Tansir *et al.*, 2017; Nunes *et al.*, 2019A; Cunha *et al.*, 2021).

O envolvimento do fígado é uma característica bastante comum na infecção por DENV (Póvoa *et al.*, 2014, Samanta & Sharma, 2015) e pode ser um efeito direto da replicação do vírus nas células, ou resultado da resposta imunológica do hospedeiro contra o vírus. Hepatócitos e células de Kupffer são suscetíveis ao DENV (de Araújo *et al.*, 2000; Lima *et al.*,

2011; Póvoa *et al.*, 2014) e, após ligarem-se a receptores de DENV, as células de Kupffer liberam citocinas que ativam as células inflamatórias (França *et al.*, 2010; Fernando *et al.*, 2016; Dissanayake & Seneviratne, 2018). Estudos realizados com modelos de camundongo, linhagens celulares de hepatoma HepG2 e Huh7 e culturas primárias de células Kupffer humanas demonstraram que o DENV é capaz de infectar hepatócitos e células Kupffer (Marianneau *et al.*, 1999; Cabrera-Hernandez *et al.*, 2007; El-Bacha *et al.*, 2007; França *et al.*, 2010). Além disso, várias moléculas que atuam como receptores para o vírus ou como fatores de agregação que facilitam a concentração viral na membrana celular antes da ligação aos receptores foram identificadas em células do fígado (Lühn *et al.*, 2007; Cruz-Oliveira *et al.*, 2015). Em humanos, o genoma viral pode ser amplificado a partir de amostras de autópsia de fígado, e o antígeno do DENV foi detectado em hepatócitos, CE e células de Kupffer (de Macedo *et al.*, 2006; de Araújo *et al.*, 2009; Lima *et al.*, 2011).

As alterações hepáticas causadas pela infecção viral variam desde o aumento assintomático das transaminases até a insuficiência hepática aguda, com desfecho fatal (Trung *et al.*, 2010; Kularatne *et al.*, 2018; Devarbhavi *et al.*, 2020). A alteração clínica mais comumente relatada é a elevação dos níveis das enzimas hepáticas alanina aminotransferase (ALT) e aspartato aminotransferase (AST), que é observada nos primeiros dias após o início da febre, apresentando um pico no período de convalescença, e já foi associada à tendência ao sangramento espontâneo (Wichmann *et al.*, 2007; Fernando *et al.*, 2016; Kularatne *et al.*, 2018). A hepatomegalia também é comum; entretanto, é mais frequentemente observada em pacientes com DG (Kuo *et al.*, 1992; Samanta & Sharma, 2015; Wang *et al.*, 2016). Com relação à histopatologia, alterações lipídicas (esteatose macro e microvesicular), necrose hepatocelular, inchaço dos hepatócitos seguido de balonização, hiperplasia, destruição das células de Kupffer, corpúsculos de Councilman, infiltrados celulares no trato portal e focos de hemorragia e edema foram observados na necropsia de amostras de casos fatais de dengue (Burke *et al.*, 1968; Kularatne *et al.*, 2005; Basílio-de-Oliveira *et al.*, 2005; Leong *et al.*, 2007; Limonta *et al.*, 2012; Póvoa *et al.*, 2014).

A infecção por DENV pode levar a danos renais. Esses danos podem resultar de efeito citopático resultantes da ação viral, mediadores inflamatórios liberados em resposta à infecção, de instabilidade hemodinâmica, rabdomiólise, hemólise ou lesão glomerular aguda (Lima *et al.*, 2008; Póvoa *et al.*, 2014; Lim *et al.*, 2019). Níveis elevados de ureia e creatinina, proteinúria, hematúria, glomerulonefrite e lesão renal aguda foram relacionados à dengue (Gulati *et al.*, 2007; Lizarraga *et al.*, 2014; Oliveira *et al.*, 2015; Vakrani *et al.*, 2017). A lesão renal aguda e a insuficiência renal aguda são complicações significativas da dengue, e os pacientes com DG

têm maior probabilidade de desenvolvê-las (Kuo *et al.*, 2008; Mallhi *et al.*, 2015; Oliveira *et al.*, 2015; Eswarappa *et al.*, 2019; Diptyanusa *et al.*, 2021). As taxas de mortalidade em que há lesão renal aguda são de 1% para FD, 12% - 40% para FHD e 60% para SCD (Prassad *et al.*, 2019).

A presença de DENV no rim já foi demonstrada através da detecção do antígeno viral em célular tubulares e endoteliais e em macrófagos e monócitos nos vasos sanguíneos deste órgão (Jessie *et al.*, 2004; Lima *et al.*, 2011; Póvoa *et al.*, 2014; Nunes *et al.*, 2019B; Cunha *et al.*, 2021). Além disso, estudos ultraestruturais por microscopia eletrônica de transmissão revelaram estruturas reticulares de microtúbulos, dilatação do RE em células necróticas e partículas elétron-densas semelhantes a vírus em glomérulos, sugerindo infecção viral (Boonpucknavig *et al.*, 1976; Wiersinga *et al.*, 2006; Póvoa *et al.*, 2014). Análises de amostras de rins de casos humanos de dengue revelou dano no parênquima e circulatório (Póvoa *et al.*, 2014; Nunes *et al.*, 2019B). Ademais, necrose tubular, caracterizada pela presença de núcleos picnóticos em células epiteliais, espessamento da membrana basal glomerular, proliferação mesangial, congestão glomerular, hialinose, fibrose intersticial focal, infiltrado mononuclear difuso e focos de hemorragia, nas regiões cortical e medular, e aumento das populações de células $CD68^+$ e $CD4^+$ também foram relatados (Repizo *et al.*, 2014; Pagliari *et al.*, 2018; Póvoa *et al.*, 2018; Nunes *et al.*, 2019B).

Manifestações pulmonares durante a dengue são, geralmente, brandas e a infecção afeta principalmente as vias aéreas superiores (Halsey *et al.*, 2012; Rodrigues *et al.*, 2014; de Almeida *et al.*, 2017). Achados incluem efusão pleural, edema pulmonar não-cardiogênico, hemorragia acompanhada ou não de hemoptise, pneumonite e insuficiência respiratória aguda grave (Marchiori *et al.*, 2009, 2012, 2020; Rodrigues *et al.*, 2014; de Almeida *et al.*, 2017). Em amostras provenientes de casos de óbito, pode-se observar infiltrado inflamatório mononuclear, espessamento de septo interalveolar, hiperplasia de macrófagos alveolares, formação de membrana hialina com hipetrofia e hiperplasia de pneumócitos do tipo II, edema intersticial, focos de hemorragia e congestão alveolar difusa. Além disso, megacariócitos e fragmentos celulares semelhantes a plaquetas foram vistos no espaço alveolar (Basílio-de-Oliveira *et al.*, 2005; Póvoa *et al.*, 2014; Rodrigues *et al.*, 2014; de Oliveira *et al.*, 2017). O DENV também já foi detectado em amostras de pulmão (Jessie *et al.*, 2004; Lima *et al.*, 2011, Póvoa *et al.*, 2014) assim como uma série de citocinas pró-inflamatórias foram identificadas em tecido pulmonar indicando que as alterações podem ser resultado da resposta inflamatória do hospedeiro (de Oliveira *et al.*, 2017; Póvoa *et al.*, 2018).

A dengue apresenta uma ampla gama de manifestações cardíacas; entretanto, o envolvimento do coração não é comum e está quase sempre relacionado a casos graves da doença (Gulati *et al.*, 2007, Estofolete *et al.*, 2019). Em um estudo realizado com crianças, o envolvimento cardíaco foi mais proeminente (72,7%) em crianças com DG (Abhinayaa *et al.*, 2021). A fisiopatologia do envolvimento cardíaco na dengue não é inteiramente compreendida, e as disfunções cardíacas podem ser causadas por efeito indireto de citocinas produzidas e liberada pela resposta inflamatória do hospedeiro ou do resultado direto da infecção das fibras cardíacas pelos DENV (Warke *et al.*, 2003; Salgado *et al.*, 2010; Oliveira *et al.*, 2017). A alteração mais comumente reportada é a miocardite (Lee *et al.*, 2009; Salgado *et al.*, 2010; Weerakoon *et al.*, 2011; Pareda *et al.*, 2015; Bhatt *et al.*, 2020; Cunha *et al.*, 2021). Os níveis de enzimas marcadoras de lesão cardíaca, creatina quinase (CK), creatina quinase banda miocárdica (CK-MB) e Troponina I, podem apresentar-se elevados (Wichmann *et al.*, 2009; Miranda *et al.*, 2013a; Pareda *et al.*, 2015; Tahir *et al.*, 2015). A infecção pode resultar em quadros de arritmia ventricular e atrial, bradicardia e taquicardia sinusal, hipocinesia, choque cardiogênico, efusão pericardiaca, e cardiomiopatia dilatada, cardiomegalia, falência cardíaca e até infarto do miocárdio (Obeyesekere *et al.*, 1973; Pelupessy *et al.*, 1989; Lee *et al.*, 2008; Salgado *et al.*, 2010; Shivanthan *et al.*, 2015; Pareda *et al.*, 2015; Tahir *et al.*, 2015).

O genoma e antígeno viral já foram detectados em fibras cardíacas, macrófagos, monócitos e CE do coração (Basílio-de-Oliveira *et al.*, 2005; Araújo *et al.*, 2009; Salgado *et al.*, 2010, Lima *et al.*, 2011; Póvoa *et al.*, 2014) e a presença da fita negativa do vRNA em CE, mioblastos, células intersticiais do miocárdio e macrófagos indicam a infecção do coração pelos DENV (Póvoa *et al.*, 2014). Além disso, estudos realizados com amostras de necrópsia revelaram edema intersticial e focos de hemorragia, degeneração de fibras cardíacas e extensas áreas de necrose, infiltrado inflamatório formado principalmente por células mononucleares e fibroblastos, fibras cardíacas apresentando núcleos apoptóticos e alteração da heterocromatina, degeneração de miofilamentos, e alteração mitocondrial (Weerakoon *et al.*, 2011; Miranda *et al.*, 2013a, 2013b; Póvoa *et al.*, 2014; Oliveira *et al.*, 2017).

Mialgia e fraqueza muscular são sintomas comumente apresentadas por pacientes de dengue; entretanto, o envolvimento muscular durante a dengue é benigno e autolimitado (Misra *et al.*, 2012). A elevação níveis de CK durante a infecção já foi documentada (Misra *et al.*, 2012; Tansir *et al.*, 2017; Madhusankha *et al.*, 2021). Paralisia flácida aguda já foi relatada e a insuficiência renal aguda na dengue pode ser causada por rabdomiólise ou miosite (Malheiros *et al.*, 1993; Davis *et al.*, 2004; Kalyta *et al.*, 2005; Acharya *et al.*, 2010; Misra *et al.*, 2015; Tansir *et al.*, 2017; Gulati *et al.*, 2020). A infecção de miotubos e células satélites do músculo

esquelético já foi confirmada em estudos *in vitro,* através da deteção das proteínas E, C e NS1 do DENV e os níveis da quimiocina IP-10 se elevaram frente a infecção por estes vírus (Warke *et al.*, 2008; Salgado *et al.*, 2010). Análises de biópsias de músculo esquelético revelaram infiltrado inflamatório perivascular, proliferação mitocondrial, focos de necrose com ou sem miofagocitose e hemorragia intersticial (Malheiros *et al.*, 1993; Davis *et al.*, 2004; Acharya *et al.*, 2010; Misra *et al.*, 2012). Além disso, presença de edemas, focos de hemorragia e alterações metabólicas podem ser responsáveis pela fraqueza muscular transiente (Misra *et al.*, 2012).

1.5. Vacinas

Em consequência das dificuldades encontradas na implementação de programas de controle do mosquito vetor, juntamente com o número crescente de casos de dengue no mundo, o desenvolvimento de uma vacina tornou-se uma das grandes prioridades da OMS. Entretanto, o desenvolvimento de uma vacina contra a doença tem se mostrado uma tarefa desafiadora, já que casos mais graves da doença são frequentemente associados a infecções secundárias por DENV com um sorotipo heterólogo, e uma vacina ideal precisa conferir resposta protetora contra cada um dos quatro sorotipos de forma robusta e balanceada. Além disso, a falta de um modelo experimental adequado também se apresenta como uma dificuldade (Del Angel & Reyes-del Valle, 2013; Wilder-Smith *et al.*, 2020). Até o momento, há uma vacina licenciada e uma série de outras em diferentes fases de testes clínicos.

A Dengvaxia (CYD-TDV), desenvolvida pela Sanofi-Pasteur, foi aprovada e licenciada pela OMS para uso em indivíduos entre 9 e 45 anos (Guy *et al.*, 2010), em diversos países onde a doença é endêmica, dentre eles México, Filipinas, Brasil e El Salvador. É uma vacina quimérica baseada no vírus atenuado da vacina contra a febre amarela (cepa vacinal 17D) e os genes prM e E do envelope dos quatro sorotipos de DENV são substituídos no genoma do vetor atenuado (Vannice, Durbin & Hombach, 2016). Estudos clínicos de fase III demonstraram que a eficácia de vacina varia de acordo com a idade, status sorológico e sorotipo. Embora a CYD-TDV tenha reduzido a incidência da dengue, foi detectado o aumento do risco de pacientes sem contato prévio com o vírus antes da vacinação desenvolverem DG durante os 30 meses após a administração da primeira dose (Hadinegoro *et al.*, 2015; Sridhar *et al.*, 2018). Dado o cenário, a OMS recomenda que os países que consideram a implementação da vacina CYD-TDV como parte do programa de controle da dengue realizem uma triagem sorológica pré-vacinação para que somente pessoas soropositivas para dengue sejam vacinadas (Arien & Wilder-Smith, 2018).

Entre as vacinas que estão em ensaio clínico, duas estão em fases mais avançadas de estudo. A vacina candidata da Takeda, TAK-003, tetravalente de vírus atenuado por passagem

em células, foi desenvolvida a partir de uma cepa DENV-2 atenuada e vírus quiméricos contendo os genes prM e E do DENV-1, -2, -3 e -4 clonados no arcabouço do DENV-2 atenuado (Osorio *et al.*, 2016). A TAK-003 encontra-se na fase III dos testes clínicos e ao contrário da Dengvaxia, apresenta proteínas NS da dengue no vetor DENV-2. Estudos clínicos de fase II demonstraram que a vacina candidata induziu respostas de AcN e soroconversão para todos os quatro DENV, além de resposta mediada por células T de reatividade cruzada (Osorio *et al.*, 2016; Saez-Lorens *et al.*, 2018). Um estudo de Fase III, envolvendo mais de 20.000 crianças saudáveis e adolescentes entre 4 e 16 anos de idade para receber duas doses de vacina, está sendo conduzido em 8 países na Ásia e na América Latina (Wilder-Smith *et al.*, 2020). Na primeira parte da fase III (até12 meses pós imunização), a eficácia da TAK-003 para a promoção de proteção para a população foi de 80,9% e a eficácia para a proteção contra hospitalizações foi de 95,4%. Já na segunda parte da fase III (até 18 meses após a imunização), a eficácia geral da vacina foi de de 80,2%. Além disso, eficácia variou por sorotipos individuais: DENV-1, 69,8%, DENV-2, 95,1%; DENV-3, 48,9%; DENV-4, 51,0% (Biswal *et al.*, 2019; 2020).

A vacina candidata do "*National Institute of Health"*, TV003/TV005, uma vacina tetravalente, atenuada pela deleção de 30 nucleotídeos da região 3 'UTR dos DENV-1, -3 e -4 e uma quimera de DENV-2/DENV-4, está na terceira fase de testes clínicos no Brasil (Instituto Butantan). A TV003/TV005 foi capaz de induzir respostas de células CD4+ similares às observadas na imunidade natural. Avaliação de segurança, viremia da vacina, e a resposta de anticorpos indicou que uma única dose é suficiente para induzir produção de anticorpo contra os quatro sorotipos e induzir resposta robusta de células T (Kirkpatrick *et al.*, 2015; Weiskopf *et al.*, 2015; Durbin *et al.*, 2016; Angelo *et al.*, 2017).

A tabela 2 apresenta uma visão geral das principais vacinas tetravalentes candidatas, com suas respectivas abordagens tecnológicas e estágios de desenvolvimento (Prompetchara *et al.*, 2020).

Tabela 2: Panorama geral do desenvolvimento dos principais candidatos vacinais tetravalentes contra a dengue (Modificada de Prompetchara, 2020).

	Vacina / estratégia	Laboratório	Testes clínicos
Quimera atenuada	**CYD, Denvaxia®:** Quimera (cepa 17 D do vírus da febre amarela + genes E e M dos DENV)	Sanofi-Pasteur	Licenciada, avaliação pós-licenciamento em andamento
	TAK-003, DENVax: DENV-2 PDK-53 atenuado como vetor. Substitui a prM e E de outros sorotipos (quimeras DENV-2 / -1, -2 / -3 e -2 / -4)	US CDC/Inviragen/ Takeda	Fase III
	TV003/TV005: Atenuada pela deleção de 30 nucleotídeos da 3' UTR deDENV-1, -3 e -4, e uma quimera DENV-2/DENV-4	US NIH	Fase III
Vírus inativado	**TDENV PIV** DENV purificado e inativado com formalina + adjuvantes	WRAIR/GSK	Fase I
Vacina de DNA	**TVDV:** Proteínas prM/E expressas em vetor Plasmidial	US NMRC	Fase I
Vacina de subunidade	**V180:** Subnidade da proteína E	Hawaii Biotech Inc. /Merck	Fase I
Heterologous prime/boost	**TLAV-prime/PIV-boost:** Combinação vírus atenuado purificado + virus inativado purificado	US Army Medical Research/Materiel Command	Fase I

United Stares (US),*Centers for Disease Control and Prevention* (CDC), *National Institute of Health* (NIH), *Walter Reed Army Institute of Research* (WRAIR), *GlaxoSmithKline* (GSK), *Naval Medical Research Center* (NMRC).

1.6. Modelos experimentais para o estudo de infecção pelos vírus dengue

Devido à falta de uma vacina tetravalente completamente eficaz, que não apresente riscos de desenvolvimento do quadro grave da doença, e de um tratamento específico para dengue, além da necessidade de se compreender os mecanismos fisiopatológicos que resultam em diferentes manifestações da doença, o estabelecimento de modelos animais é de grande relevância (Torresi *et al.*, 2017; Wilder-Smith *et al.*, 2019; OMS, 2021).

Um modelo experimental ideal para estudar a dengue deve mimetizar a progressão da doença conforme esta ocorre em humanos (Chan *et al.*, 2015). Até o momento, não existem modelos experimentais que atendam a esse requisito (Zompi & Harris, 2012), o que dificulta a compreensão dos mecanismos da patogênese do DENV e o desenvolvimento de drogas e vacinas (Oliveira *et al.*, 2016). Um dos maiores desafios no que se refere ao desenvolvimento de um modelo animal adequado é a baixa replicação viral, quando camundongos imunocompetentes são inoculados com isolados clínicos, e a ausência de sinais clínicos da doença em PNH (Zompi *et al.*, 2012). Entretanto, vários modelos já foram propostos e têm sido de grande importância para a elucidação de vários aspectos da patogênese da dengue (Zompi & Harris, 2012, Chan et al., 2015; Na *et al.*, 2017).

1.6.1. Primatas não-humanos

Os PNH são naturalmente infectados pelos DENV e mantém o ciclo de transmissão selvagem (Gubler *et al.*, 1998). Os quatro sorotipos do DENV são capazes de infectar e replicar em PNH, produzindo viremia e induzindo AcN. Os PNH apresentam íntima relação genética com seres humanos e resposta imunológica similar e, apesar de tal modelo não apresentar sinais clínicos, ele tem sido utilizado para a investigação da resposta imunológica e para testes de imunogenicidade e eficácia de vacinas candidatas antes que estas entrem em fase de testes clínicos (Zompi *et al.*, 2012; Clark *et al.*, 2013; Zellweger *et al.*, 2014; Azami *et al.*, 2020; Sundaram *et al.*, 2020; Uno & Ross, 2021).

1.6.2. Modelos murinos

O estabelecimento de um modelo murino tem sido desafiador, já que isolados clínicos, além de não causarem patologia grave, apresentam baixa ou nenhuma replicação em camundongos imunocompetentes (Zompi *et al.*, 2012; Zellwegwer *et al.*, 2014). A maioria dos modelos propostos utilizam camundongos humanizados ou imunodeficientes, utilizam vias invasivas de inoculação (Faugout *et al.*, 1990; Reut *et al.*, 1996) ou amostras de vírus adaptados (Johnson *et al.*, 1999; Gonçalves *et al.*, 2018; Chen & Diamond, 2020).

1.6.2.1. Camundongos imunodeficientes humanizados

Camundongos humanizados são animais imunodeficientes transplantados com tecidos ou que expressam genes humanos. A capacidade destes modelos de desenvolver fenótipos celulares idênticos aos observados em humanos os tornam ferramentas importantes para a investigação de patógenos humanos ou processos de doenças *in vivo* e são bastante utilizados para estudos da patogênese da dengue, principalmente da resposta de células T frente a infecção (Legrand *et al.,* 2009; Skelton *et al.*, 2018; Coronel-Ruiz *et al.,* 2020). Este modelo, quando infectado por DENV, produz viremia e desenvolve sinais clínicos compatíveis com a dengue como febre, exantema e eritema, trombocitopenia, aumento dos níveis de citocinas e até mesmo hemorragia branda no fígado (An, 1999 *et al.*; Bente *et al.*, 2005; Kuruvilla *et al.*, 2007; Mota *et a.l*, 2011; Cox *et al.*, 2012; Frias-Staheli *et al.*, 2014). Além disso, os anticorpos detectados no soro destes camundongos humanizados infectados foram capazes de neutralizar o DENV (Kuruvilla *et al.*, 2007; Jaiswal *et al.*, 2009; 2012).

1.6.2.2. Camundongos imunodeficientes interferon-deficientes

Camundongos deficientes em interferon (INF) deficientes são amplamente utilizados em estudos sobre patogênese, ADE, da ação protetora de vacinas candidatas e da resposta antiviral do hospedeiro já que suportam replicação robusta de DENV, demonstram sinais de patologia grave e são capazes de desenvolver resposta imune protetora (Wollner *et al.*, 2021). Os sinais apresentados por estes modelos são: permeabilidade vascular, elevados níveis de citocinas no soro, trombocitopenia, leucocitose, hematócrito elevado e hemorragia gastrointestinal. O antígeno ou genoma viral pode ser detectado em diferentes órgãos, como fígado, rim e cérebro e no trato gastrointestinal em diferentes linhagens de camundongos imunocomprometidos (Balsitis *et al.*, 2010; Zellweger *et al.*, 2010; Sarathy *et al.*, 2018).

1.6.2.3. Camundongos imunocompetentes

Embora a suscetibilidade de camundongos imunodeficientes ao DENV seja questionada pela comunidade científica, estes modelos suportam a replicação viral e a disseminação de vírus pelo organismo desses animais os tornam aliados para uma melhor compreensão do envolvimento de múltiplos órgãos na patogênese da dengue. Suas respostas imunológicas eficientes são importantes para estudos de imunopatogênese, eficácia de vacinas e desenvolvimento de terapias anti-DENV (Tabela 3). Além disso, os sintomas de DG podem ser reproduzidos em modelos imunocompetentes quando infectados com altas doses do vírus ou com cepas adaptadas (Huang *et al.*, 2000; Shresta *et al.*, 2004; Gonçalves *et al.*, 2012;

Christofferson *et al.*, 2013; Quinnan *et al.*, 2014; Frei *et al.*, 2018; Pinto *et al.*, 2015; Uno & Ross, 2021).

Tabela 3: Modelos murinos imunocompetentes para estudos da patogênese da dengue e testes de eficácia de terapias antivirais e vacinas candidatas contra a dengue (modificada de Chen & Diamond, 2020).

Camundongo	Cepa viral	Via de inoculação	Alterações
BALB/c	DENV-1 Hawaii, DENV-2 Tr1751, DENV-3 H87, DENV-4 H241	Intracraniana	Produção de citocinas, perda de peso, letalidade
BALB/c	DENV-2 NGC	Intracraniana	Morbidade, letalidade, neuropatologia Produção de citocinas
C3H/HeN e C57BL/6J	DENV-2 454009 A	Intracraniana	Hemorragia, trombocitopenia
C57BL/6J	DENV-2 Thailand/16681/84 NS1	Intradérmica/ Intravenosa	Extravasamento vascular
C57BL/6	DENV-2 Eden2	Intraperitoneal	Trombocitopenia
ICR e BALB/c	DENV-1 Mochizuki	Intraperitoneal (células K562 infectadas)	Viremia,, letalidade
BALB/c	DENV1-4	Intraperitoneal (células K562 infectadas)	Viremia, produção de citocinas
BALB/c	DENV-2	Subcutânea	Extravasamento vascular
C3H/HeN	DENV-2 454009A	Intravenosa	Viremia, bleeding time

1.6.2.3.1. Camundongos BALB/c

Camundongos BALB/c infectados experimentalmente com DENV pela via intravenosa são capazes de produzir viremia entre o 2º e o 11º dia pós-infecção (dpi), atingindo o pico no sétimo dia (Paes *et al.*, 2005; Barth *et al.*, 2006). O vRNA já foi detectado em amostras de baço, fígado, cérebro, coração, pulmão, rim e saliva (Oliveira *et al.*, 2016; Rasinhas *et al.*, 2017; Salomão *et al.*, 2018; Caldas *et al.*, 2019, Kangussu *et al.*, 2020). Dentre os sinais clínicos observados neste modelo podemos citar aumento da temperatura corporal, trombocitopenia, alteração da função cardíaca, e elevação das enzimas hepáticas, fosfatase alcalina, ureia e creatinina. Sinais neurológicos, como a paralisia, e morte são observados quando os camundongos são infectados com cepas adaptadas pelas vias intraperitoneal ou intracraniana. Estes camundongos são capazes de expressar uma resposta imunológica contra DENV produzindo uma série de mediadores da resposta inflamatória e AcN (Atrasheuskaya et al.,

2003; Oliveira *et al.*, 2016; Rasinhas *et al.*, 2017; Amorim, 2019; Caldas, 2019 *et al.*, Kangussu *et al.*, 2020; Sakinah *et al.*, 2021).

Semelhantes aos casos humanos de dengue, em camundongos BALB/c vários órgãos são afetados durante a infecção. Neste modelo, as alterações são geralmente focais e limitadas a pequenas áreas. Amostras de fígado apresentam vacuolização hepatocelular, edema intersticial, infiltrado inflamatório mononuclear, capilares sinusóides parcialmente colapsados e vasos sanguíneos dilatados, esteatose, eritrócitos internalizados por hepatócitos, aumento da população de hepatócitos binucleados, rompimento de vasos sanguíneos, hemorragia e hepatócitos apoptóticos e necróticos (Atrasheuskaya *et al.*, 2003; Paes *et al.*, 2005; 2009; França *et al.*, 2010; Sakinah *et al.*, 2017; 2021).

No rim de camundongos BALB/c infectados com DENV, são observados tanto atrofia quanto aumento do volume glomerular. Além disso, já foram reportados aumento da celularidade mesangial, inclusões citoplasmáticas em células epiteliais dos túbulos contorcidos proximais, ausência da cápsula de Bowman, sinais de congestão com presença de transudato e células infiltradas no interstício (Barreto *et al.*, 2004, Caldas *et al.*, 2019). Em amostras de pulmão, pode-se observar espessamento de septo interalveolar com infiltrado celular majoritariamente mononuclear, congestão vascular, macrófagos alveolares, células inflamatórias, debris nucleares e eritrócitos no espaço alveolar, pequenos focos de hemorragia, edema e hiperventilação, hiperplasia do epitélio bronquiolar e rompimento de fibras elásticas de septos interalveolares (Atrasheuskaya *et al.*, 2003; Barth *et al.*, 2006; Barreto 2007 *et al.*, 2009; Caldas *et al.*, 2019).

Amostras de coração apresentam pericardite, rarefação do citoplasma de fibras cardíacas, focos de hemorragia e áreas de congestão vascular. Ademais, Estudos ultraestruturais revelam alterações na estrutura dos discos intercalares, tráfego intenso de vesículas de membrana entre CE e fibras cardíacas, bem como plaquetas aderidas às paredes de capilares e mitocôndrias apresentando degeneração de suas cristas (Rasinhas *et al.*, 2017; Jácome *et al.*, 2018, Kangussu *et al.*, 2020). No cérebro, nota-se edema e infiltrado perivascular e infiltrado de células da microglia na região *Cornu ammonis* 1 do hipocampo e, o cerebelo apresenta degeneração da camada de Purkinje, desmielinização com infiltrado de células da microglia e focos de hemorragia (Atrasheuskaya *et al.*, 2003, Salomão *et al.*, 2018).

Como camundongos BALB/c são imunocompetentes, eles são utilizados para estudos sobre o envolvimento da resposta imune do hospedeiro na imunopatogênese da dengue (Zompi e Harris, 2014, Chen & Diamond, 2020). Em camundongos BALB/c infectados com uma cepa

adaptada de DENV-2, pela via intraperitoneal, IgG anti-DENV-2 foi detectado até o 28° dpi e níveis séricos de IL-6, IL-1β, TNFα e IFN-γ se elevaram durante a infecção (Atrasheuskaya *et al.*, 2003). Em outro estudo, os níveis séricos de IL-12p70, TNF-α e MCP-1 aumentam em camundongos infectados pela via intracraniana com vírus adaptado, e que também apresentam anticorpos anti-NS1 a partir quinto dia de infecção (Oliveira *et al.*, 2016). Durante a infecção com cepas não adaptadas, este modelo mostra um aumento na população de células $CD86^{+}$, $CD11c^{+}$, consideradas apresentadoras de antígenos no baço, e $CD8^{+}$, $CD45RB^{low}$ no sangue. Ademais, em tecido cardíaco, os níveis de IL-1, TNF-α, IL-6, CXCL1 e CCL2 mostraram-se elevados quando comparados ao grupo controle negativo (Salomão *et al.*, 2018; Kangussu *et al.*, 2020).

Com relação a estudos de eficácia de vacinas, terapias e antivirais, o tratamento com soro anti-TNF retardou o aparecimento dos sinais clínicos e reduziu a taxa de mortalidade de 100% para 40% em modelo letal de camundongos BALB/c e o tratamento de camundongos neonatos com soro de camundongos imunizados com uma vacina de DNA expressando prM-E reduziu a mortalidade em 100% (Atrasheuskaya *et al.*, 2003; Feng *et al.*, 2020). A imunização de camundongos BALB/c com domínio III da proteína E do DENV-2, com plasmídeo de DNA que codifica o gene NS1 ou com vacinas tetravalentes ou de vírus inativado tetravalente induziu a produção de anticorpo (Konish *et al.*, 2005; Costa *et al.*, 2006; Zhang *et al.*,2006; Feng *et al.*, 2020; Sundaram *et al.*, 2020). Além disso, a vacina de DNA expressando o antígeno prM de DENV-3 induziu a resposta celular, ativando células T de memória efetoras (Feng *et al.*, 2020). De modo semelhante, vacinas de DENA expressando NS1 ou a proteína E de DENV-2 induziram resposta celular, com produção de IFN (Pinto *et al.*, 2019).

Desta forma, camundongos BALB/c suportam a replicação viral e a disseminação de vírus pelo organismo, o que os tornam aliados na investigação do envolvimento de múltiplos órgãos na patogênese do DENV. Suas respostas imunológicas eficientes são importantes para estudos de imunopatogênese e eficácia de vacinas e tratamentos anti-DENV. Além disso, os sintomas de DG podem ser reproduzidos em modelos imunocompetentes quando infectados com altas doses do vírus ou com cepas adaptadas (Atrasheuskaya *et al.*, 2003; Chen & Diamond, 2020; Sakinah *et al.*, 2021).

1.7. Justificativa

O DENV-2 foi detectado pela primeira vez no Brasil em 1990 e sua introdução no país, resultou em um aumento no número de casos e na ocorrência dos primeiros casos de FHD e SCD. Desde então, o sorotipo já foi isolado em todos os estados do país (Nogueira *et al.*, 1991;

1993; 2007; Bezerra et al., 2021) e foi responsável por mais duas grandes epidemias, em 1998 e 2008 com diferentes perfis, tendo sido esta última responsável por um maior número de hospitalizações, casos graves e óbitos (Siqueira *et al.*, 2011). Os vírus que circularam em ambas as epidemias pertencem ao genótipo Sudeste Asiático/Americano, contudo, estudos filogenéticos identificaram uma nova linhagem (Linhagem II) em 2008 (Oliveira *et al.*, 2010; Faria *et al.*, 2013).

O DENV-2 tem sido, tradicionalmente, o sorotipo mais estudado devido à sua associação com grandes epidemias, manifestações clínicas mais graves e, frequentemente, com casos de FHD/SCD. No entanto, os fatores que determinam a evolução da doença para a DG em certos indivíduos ainda não estão completamente esclarecidos.

Estudos de quantificação de DENV realizados por Vaughn *et al.* (2000) e Araújo *et al.* (2009) associaram altas cargas virais com a gravidade da doença e um estudo realizado por Nunes *et al.* (2016) demonstrou que a viremia detectada em pacientes infectados pelos DENV-2 pertencentes à Linhagem II foi mais elevada quando comparada à viremia induzida pela Linhagem I, sendo esta maior viremia associada aos casos graves da Linhagem II, sugerindo que a linhagem é um fator importante para a gravidade da doença. Além disso, nas últimas décadas, observou-se o aumento do número de relatos de acometimento de diferentes órgãos durante a infecção pelos DENV no Brasil, o que pode estar relacionado a introdução da Linhagem II no ano de 2008 (Nunes *et al.*, 2019A).

Na patogêse das infeções por DENV, o fígado é considerado um órgão alvo, sendo o mais estudado. No entanto, durante a infecção, pode haver envolvimento cardíaco, renal, pulmonar, neurológico, esplênico, gastrointestinal e até mesmo ocular (Gulati *et al.*, 2007, Martina *et al.*, 2009, Estofolete *et al.*, 2019). Como a maioria dos relatos de alterações histopatológicas são baseados em amostras obtidas de casos fatais (Basílio-De-Oliveira *et al.*, 2005; Limonta *et al.*, 2012; Póvoa *et al.*, 2014), existe dificuldade em avaliar o grau de alterações presentes em diferentes órgãos em pacientes com a forma mais branda da doença.

O estabelecimento de modelos animais experimentais para o estudo da interação vírus/hospedeiro é de grande relevância para a pesquisa sobre patogênese, imunidade, desenvolvimento de fármacos e desenho e teste de vacinas. Contudo, o estabelecimento destes modelos tem sido um desafio, uma vez que os vírus epidêmicos circulantes não infectam naturalmente espécies não-humanas.

Estudos prévios demonstram que camundongos BALB/c imunocompetentes, quando infectados experimentalmente com DENV não neuroadaptado, apresentam infecção e, alguns

sinais clínicos, bem como alterações teciduais semelhantes aos observados em casos humanos infectados pelo DENV, foram observados. (Barreto *et al.*, 2004; 2007; Barth *et al.*, 2006; Paes *et al.*, 2005, 2009; Rasinhas *et al.*, 2017; 2018; Caldas *et al.*, 2018; Salomão *et al.*, 2018)

Neste estudo foram realizadas infecções experimentais de camundongos BALB/c com cepas representantes das duas Linhagens de DENV-2 isoladas de casos humanos, não neuroadaptadas, para a avaliação do impacto destas infecções, bem como o envolvimento de diferentes órgãos. Com isso, pretendemos contribuir para o entendimento dos fatores e mecanismos envolvidos na patogênese das infecções pelo DENV.

2. Objetivos

2.1. Objetivo geral

- Investigar o tropismo de duas linhagens brasileiras do genótipo Asiático/Americano de DENV-2 em modelo murino imunocompetente e comparar as alterações induzidas pelas infecções virais em diferentes órgãos.

2.2. Objetivos específicos

- Comparar sinais clínicos e o envolvimento hepático, através da avaliação das alterações histopatológicas e de transaminases, caracterização da população hepatocitária e detecção viral, frente à infecção independente por duas Linhagens do genótipo Asiático/Americano de DENV-2 utilizando como modelo experimental camundongos imunocompetentes BALB/c.
- Avaliar e comparar as alterações morfológicas e bioquímicas do rim de camundongos BALB/c infctados por duas Linhagens do genótipo Asiático/Americano de DENV-2, bem como detectar vírus neste órgão.
- Investigar o impacto da infecção de Linhagens do genótipo Asiático/Americano de DENV-2 em pulmão e músculo estriado cardíaco e esquelético de camundongos BALB/c, caracterizando as alterações morfológicas e bioquímicas e detectando o vírus nestes tecidos.

3. Metodologia e Resultados

As metodologias utilizadas nesta Tese e resultados obtidos serão apresentados sob forma de artigos científicos publicados e/ou aceitos para publicação, e serão listados na ordem em que serão apresentados. As tabelas 4 e 5 mostram, de maneira resumida, os resultados expostos nos artigos.

Artigo 1: Jácome FC, Caldas GC, Rasinhas ADC, de Almeida ALT, de Souza DDC, Paulino AC, Leonardo R, Barth OM, Dos Santos FB, Barreto-Vieira DF. Comparative analysis of liver involvement caused by two DENV-2 lineages using an immunocompetent murine model. Sci Rep. 2021 May 6;11(1):9723. doi: 10.1038/s41598-021-88502-2. PMID: 33958631; PMCID: PMC8102549.

Artigo 2: Jácome FC, Caldas GC, Rasinhas ADC, de Almeida ALT, de Souza DDC, Paulino AC, da Silva MAN, Barth OM, Dos Santos FB, Barreto-Vieira DF. Brazilian Dengue Virus Type 2-Associated Renal Involvement in a Murine Model: Outcomes after Infection by Two Lineages of the Asian/American Genotype. Pathogens. 2021 Aug 26;10(9):1084. doi: 10.3390/pathogens10091084. PMID: 34578117; PMCID: PMC8467194.

Artigo 3: Fernanda Cunha Jácome 1, Gabriela Cardoso Caldas 1, Arthur da Costa Rasinhas, Ana Luisa Teixeira de Almeida, Daniel Dias Coutinho de Souza, Amanda Carlos Paulino, Marcos Alexandre Nunes da Silva, Derick Mendes Bandeira, Ortrud Monika Barth, Flavia Barreto dos Santos, Debora Ferreira Barreto-Vieira. Immunocompetent Mice Infected by Two Lineages of Dengue Virus Type 2: Observations on the Pathology of the Lung, Heart and Skeletal Muscle. Aceito para publicação na revista Microorganisms.

3.1. Artigo 1: **Análise comparativa do envolvimento do fígado causado por duas linhagens DENV-2 usando um modelo murino imunocompetente**

Revista: Scientific Reports

Resumo: Dengue (DEN) é a arbovirose mais prevalente entre humanos e quatro bilhões de pessoas vivem sob risco de infecção pelo vírus (DENV). As manifestações clínicas da DEN são variáveis e a doença pode se apresentar de forma subclínica ou assintomática. Um quarto dos pacientes desenvolve febre do dengue (FD) ou dengue grave (DG), que é potencialmente letal e envolve alterações da permeabilidade vascular, hemorragia grave e lesão de órgãos. O envolvimento do fígado é uma característica bastante comum na DEN, e as alterações variam desde a elevação assintomática das transaminases até a insuficiência hepática aguda. Desde a sua introdução no Brasil em 1990, foram detectadas duas cepas do genótipo Asiático/Americano do DENV-2: Linhagem I, que foi responsável por um surto em 1991, e Linhagem II, que causou uma grave epidemia em 2007-2008 e com perfil epidemiológico diferente. Até o momento, estudos sobre diferentes cepas do mesmo sorotipo/genótipo e sua associação com a gravidade da doença são escassos. Além disso, um dos maiores desafios no estudo da patogênese da DEN e no desenvolvimento de terapias medicamentosas e vacinais é a ausência de um modelo animal que reproduza a doença como ela ocorre em humanos. Os principais objetivos deste estudo foram avaliar a suscetibilidade de camundongos BALB/c infectados experimentalmente por duas cepas distintas de DENV-2 e caracterizar possíveis diferenças nos sinais clínicos e alterações induzidas no fígado resultantes dessas infecções. Para tal, os camundongos foram separados em dois grupos, cada grupo sendo infectados com uma das linhagens e. De acordo com os tempos de infecção pré-estabelecidos, os animais e seus fígados foram pesados, amostras de sangue foram coletadas para análises hematológica e dosagem das transaminases hepáticas e, amostras de fígado foram submetidas a estudos histopatológicos, histomorfométricos e de detecção viral. Os camundongos infectados pelas duas linhagens DENV-2, independentemente, ganharam menos peso do que os não infectados; no entanto, seus fígados eram ligeiramente mais pesados. Níveis aumentados de AST e ALT foram observados em camundongos infectados, e o número de plaquetas aumentou nas primeiras 72 horas de infecção e subsequentemente diminuiu. Independente da Linhagem infectante, os camundongos infectados apresentaram leucocitose, mas em momentos de infecção diferentes. As alterações histopatológicas induzidas por ambas as linhagens foram semelhantes e comparáveis às alterações observadas em casos fatais de DEN. O genoma viral foi detectado em duas amostras de fígado. Os resultados demonstram a suscetibilidade dos camundongos BALB/c a ambas as linhagens de DENV-2 e sugerem que as alterações induzidas por essas cepas são semelhantes, embora para alguns parâmetros se manifestem em momentos diferentes da infecção.

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Comparative analysis of liver involvement caused by two DENV-2 lineages using an immunocompetent murine model

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Dengue (DEN) is the most prevalent arbovirus among humans, and four billion people live at risk of infection. The clinical manifestations of DEN are variable, and the disease may present subclinically or asymptomatically. A quarter of patients develop classical dengue (CD) or severe dengue (SD), which is potentially lethal and involves vascular permeability changes, severe hemorrhage and organ damage. The involvement of the liver is a fairly common feature in DEN, and alterations range from asymptomatic elevation of transaminases to acute liver failure. Since its introduction in Brazil in 1990, two strains of Dengue virus (DENV) serotype 2 (DENV-2) have been detected: Lineage I, which is responsible for an outbreak in 1991, and Lineage II, which caused an epidemic greater than the previous one and had a different epidemiological profile. To date, studies on different strains of the same serotype/genotype and their association with disease severity are scarce. In addition, one of the greatest challenges regarding the study of DEN pathogenesis and the development of drug and vaccine therapies is the absence of an animal model that reproduces the disease as it occurs in humans. The main goals of this study were to assess BALB/c mouse susceptibility experimentally infected by two distinct DENV-2 strains and characterize possible differences in the clinical signs and alterations induced in the liver resulting from those infections. Mice infected by the two DENV-2 lineages gained less weight than uninfected mice; however, their livers were slightly heavier. Increased AST and AST levels were observed in infected mice, and the number of platelets increased in the first 72 h of infection and subsequently decreased. Mice infected with both lineages presented leukocytosis but at different times of infection. The histopathological changes induced by both lineages were similar and comparable to the changes observed in DEN fatal cases. The viral genome was detected in two liver samples. The results demonstrate the susceptibility of BALB/c mice to both DENV-2 lineages and suggest that the changes induced by those strains are similar, although for some parameters, they are manifested at different times of infection.

DEN is considered the most important arboviral disease in the world and classified by the World Health Organization (WHO) as the vector-borne viral disease with the fastest dispersal; thus, it has enormous potential to cause major epidemics worldwide[1,2]. The disease is currently endemic in over 125 countries, and global estimates vary. While a study suggests that approximately 50 to 200 million people are infected annually, with 500,000 episodes of SD and more than 20,000 deaths related to the disease[3], others estimate that approximately four billion people are at risk of infection and 390 million are infected each year[4,5].

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DEN's etiological agent is the dengue virus (DENV), an arbovirus belonging to the genus *Flavivirus*, family Flaviviridae. DENV presents four distinct but antigenically related serotypes, DENV-1, -2, -3 and -4, and is maintained in nature by a transmission cycle involving vertebrate hosts and blood-sucking mosquitoes of the genus *Aedes*, with humans being the only host capable of developing clinical forms of infection[6]. The viral particles are enveloped and spherical, present an icosahedral nucleocapsid and measure approximately 50 nm in diameter, and the viral genome consists of a single-stranded positive RNA of approximately 11,000 bases[7], which encodes three structural proteins (E [envelope protein], M [membrane protein], C [core protein]), that constitute the viral particle and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) that are expressed in infected cells[8,9].

DEN presents a wide spectrum of clinical manifestations with unpredictable evolution, and different organs are involved[10]. In addition to manifesting classic dengue symptoms, the disease can present different clinical forms, including asymptomatic cases, undifferentiated febrile illness, or severe hemorrhagic disease, such as DHF and DSS[11]. Observations of a set of clinical and laboratory parameters have led to the early identification of severe cases, and the WHO[12] classifies DEN as dengue with or without warning signs and indicates that SD is a potentially fatal outcome involving plasma leakage, fluid accumulation, respiratory distress, severe bleeding, and organ impairment. The factors leading to the different DEN manifestations are yet to be understood. However, severe forms of the disease are often associated with secondary infections, the DENV serotype, viral strain virulence and host genetic factors[13].

Liver involvement is a fairly common feature in DENV infection[14,15] and may be a direct effect of viruses infecting cells because hepatocytes and Kupffer cells are susceptible to DENV[14,16,17] or it may represent an immune response against the virus because after recognizing DENV particles, Kupffer cells and macrophages release cytokines that activate inflammatory cells[18–20]. The tissue alterations caused by the infection range from an asymptomatic increase in transaminases to acute liver failure, which has fatal outcomes[21–23]. The most commonly reported clinical alteration is the elevation of ALT and AST levels, which are observed in the initial days after onset of fever and peak in the period of convalescence[18,22]. Hepatomegaly is common as well; however, it is more frequently observed in patients with DHF[15,24,25].

Studies carried out with mouse models, HepG2 and Huh7 hepatoma cell lines and primary cultures of human Kupffer cells have demonstrated that DENV is able to infect hepatocytes and Kupffer cells[19,26–28]. Several molecules that act either as receptors for the virus or as attachment factors that facilitate viral concentration on the membrane before binding to receptors have been identified in liver cells[29,30]. In humans, the viral genome could be amplified from autopsy samples, and DENV antigen has been detected in hepatocytes, endothelial cells and Kupffer cells[16,17,31].

Histological changes, such as fatty changes (macro- and microvesicular steatosis), hepatocellular necrosis, hepatocyte swelling followed by ballooning degeneration, Kupffer cell hyperplasia and destruction, Councilman bodies and cellular infiltrates at the portal tract, hemorrhage foci and edema, were noted in necropsy samples of DEN fatal cases[14,32–39]. Most reports of histopathologic changes are based on samples obtained from fatal cases. Thus, it is difficult to assess the degree of changes present in patients with milder disease, which leads researchers to seek animal models to study DEN pathogenesis in different organs.

Genetic variability of DENV can be attributed to the lack of a mechanism underlying transcriptional fidelity. At each round of genome replication, a mutation is produced[40,41]. Phylogenetic and molecular epidemiological data characterize DENV into different genotypes, which are generally associated with different geographic areas[42]. Based on the complete sequencing of the E gene, Weaver and Vasilakis[43] proposed the characterization of DENV-1 into 5 genotypes, DENV-2 into 6 genotypes, DENV-3 into 5 genotypes and DENV-4 into 4 genotypes.

In Brazil, the DENV-2 S*outheast Asian*/American genotype is currently circulating. After its introduction in the country in the 1990s, two DENV-2 outbreaks occurred in Rio de Janeiro (1998 and 2008)[44]. Phylogenetic studies have shown that the strains from both epidemics belonged to the Asian/American genotype; however, isolates from the 2008 outbreak grouped together and gave rise to a new distinct lineage (Lineage II) from the lineage that was initially introduced in the country (Lineage I)[45,46].

During the 1998 epidemic, more than 700 thousand cases were reported, mostly affecting the portion of the population between 20 and 40 years old[44,47]. In addition to introducing a new epidemiological profile of the disease that presents increased severity in children ≤ 15 years old, the 2008 epidemic led to 806,036 cases countrywide. In Rio de Janeiro, approximately 322,000 DEN cases and 252 deaths were reported[47–51]. DENV-2 Lineage II was associated with higher viremia in patients with SD than in patients with CD[52], however, the role played by different lineages of a genotype is not completely understood.

The establishment of animal models is of great importance due to the lack of an effective tetravalent vaccine and a specific treatment for DEN and the need to understand the pathophysiological mechanisms leading to the different manifestations of the disease[53–55].

Although BALB/c mice may be less susceptible to DENV infection[56], viral replication and dissemination have already been observed in this murine model. When infected by an epidemic DENV-2 strain, BALB/c produces viremia between the 2nd and 11th days after infection[57], and when adapted strains are used, viremia peaks on the 6th day postinfection[58]. Moreover, DENV-infected BALB/c present clinical signs similar to those observed in humans, such as increased transaminase levels and thrombocytopenia. Infection by neuroadapted strains cause severe disease with anorexia, weight loss, anemia, limb paralysis, and shock and lead to death[19,57–59].

Similar to DEN human cases, multiple organs in BALB/c mice are affected by DENV infection, and the viral genome and antigen have been detected in the spleen, liver, brain, heart, lung, kidney and saliva[60–64]. Furthermore, histopathological studies on the livers of infected BALB/c mice have shown inflammatory cell infiltrates, intracellular edema, sinusoid capillary collapse, hemorrhage, hepatocellular vacuolization, increased binucleate hepatocyte population and hepatocyte death[19,57–59,65].

Different serotypes and DENV strains may present differences in tissue tropism[20,60]. As previously mentioned, one of the factors that may interfere with the DENV infection outcome is the virulence of viral strains[13]. Here, we aimed to assess the susceptibility of BALB/c mice to two distinct DENV-2 lineages and characterize the impact of these lineages in the murine model liver.

Results

No mice died over the course of this study. All animals were euthanized at 24 hpi, 48 hpi, 72 hpi, 7 dpi or 14 dpi. Clinical signs, such as petechiae, tremors or diarrhea, as well as neurological signs, such as paralysis, were not observed.

Body temperature. At 72 hpi, there was a decrease in the mean temperature of both infected and noninfected mice (means: negative control = − 0.223 °C, Lineage I = − 0.67 °C and Lineage II = − 0.355 °C). After 7 and 14 dpi, a small temperature increase was observed in noninfected mice (means: 0.715 °C and 0.307 °C, respectively). The same did not occur with infected mice, whose mean temperature showed small decreases at 7 and 14 dpi (means: Lineage I = − 0.626 °C and − 0.006 °C, respectively; Lineage II = − 0.024 °C and − 0.24 °C, respectively). The difference between body temperature variation of the noninfected group and the group infected with Lineage I was significant at 7 dpi ($p < 0.05$).

Body weight variation. On average, mice from all experimental groups gained weight; however, aside from Lineage I at 72 hpi (mean = 0.709 g), the weight gain of infected mice was lower compared to that of the control group. At 72 hpi, a small increase in body weight was observed in both noninfected and infected mice. The average weight gain of Lineage II-infected mice was 0.311 g, and the variation among noninfected mice was 0.429 g. On the seventh day after infection, noninfected mice and mice infected with Lineage II presented an increase in body weight (means: 1.069 g and 0.703 g, respectively), while the mean body weight of mice infected with Lineage I was slightly lower than that at 72 hpi (mean = 0.673 g). After 14 dpi, the mean for noninfected mice was 1.623 g, and for infected mice, it was 1.02 g (Lineage I) and 1.339 g (Lineage II). The difference between the body weight gain of the groups infected with Lineages I and II of DENV-2 was significant at 72 hpi ($p < 0.01$), and the difference between the negative control group and mice infected with Lineage I was significant at 7 dpi ($p < 0.05$) and 14 dpi ($p < 0.001$) (Fig. 1).

Liver weight variation. Comparing the mean liver weight of noninfected mice (1.529 g) and mice infected with Lineage I, it is possible to observe that the infected group presented heavier livers (mean = 1.547 g) at 72 hpi and the means were lower than that in control group after 7 dpi (1.493 g at 7 dpi and 1.442 g at 14 dpi). In mice infected with Lineage II, the opposite was observed. At 72 hpi, the mean liver weight (1.494 g) was lower than that of the control group and subsequently increased, presenting higher means of 1.560 g and 1.607 g at 7 and 14 dpi, respectively. The difference between the means of mice infected with Lineages I and II was significant at 14 dpi ($p \leq 0.05$) (Fig. 2).

Liver weight/ body weight ratio (%). The average percentage of liver weight in relation to body weight of mice infected with Lineage I was slightly higher at 72 hpi (5.55%) than that observed in uninfected mice (5.42%). However, it decreased at subsequent kinetic points (7 dpi = 5.331%; 14 dpi = 5.323%). When comparing the averages of mice infected with Lineage II and uninfected mice, a slight increase was observed on the third and seventh days of infection (mean = 5.47% and 5.569%, respectively). After 14 days of infection, the liver weight/body weight ratio showed a slight decrease (mean = 5.75%); however, it remained greater than that of the control group (Fig. 3).

Hematological parameters. *Platelets.* The mean number of platelets present in blood samples from mice in the control group was 1103.89 thousand/mm^3. In blood samples from mice infected with both DENV-2 strains, a slight increase was observed in the number of platelets at 72 hpi, with an average of 1304.43 thousand/mm^3 for Lineage I and 1308.57 thousand/mm^3 for Lineage II. On the seventh and fourteenth days after infection, the number of platelets in the infected samples decreased; however, except for the group infected with Lineage I at at 7 dpi and 14 dpi (means = 1038.7 thousand/mm^3 and 1103.3 thousand/mm^3, respectively) and the group infected with Lineage II at 14 dpi (mean = 1002.4 thousand/mm^3), the infected group averages were not lower than that of the control group (Fig. 4).

Hematocrit. The HCT of blood samples from uninfected mice was 50.022% on average. In mice infected with DENV-2 Lineage I, the averages observed three and seven days after infection were higher at 51.157% and 51.325%, respectively. In mice infected with Lineage II, the HCT decreased at 72 hpi and 7 dpi (48. 643% and 48.822%, respectively) and increased on the fourteenth day (50.44%), slightly surpassing the mean of the negative control group (Fig. 4). The difference between the group infected with Lineage I and Lineage II was statistically significant ($p < 0.01$) at 72 hpi. The difference between mice infected with Lineage II at 72 hpi and 14 dpi was also significant ($p < 0.05$).

Leukocytes. Seventy-two hours after infection, the leukocyte count of blood samples from mice infected with DENV-2 Lineage I (mean = 3.143 thousand/mm^3) and Lineage II (mean = 4.2 thousand/mm^3) was higher than that observed in the control group (mean = 2.356 thousand/mm^3). The difference between the control group and the group infected with Lineage II was statistically significant ($p < 0.05$). On the seventh day of infection, the

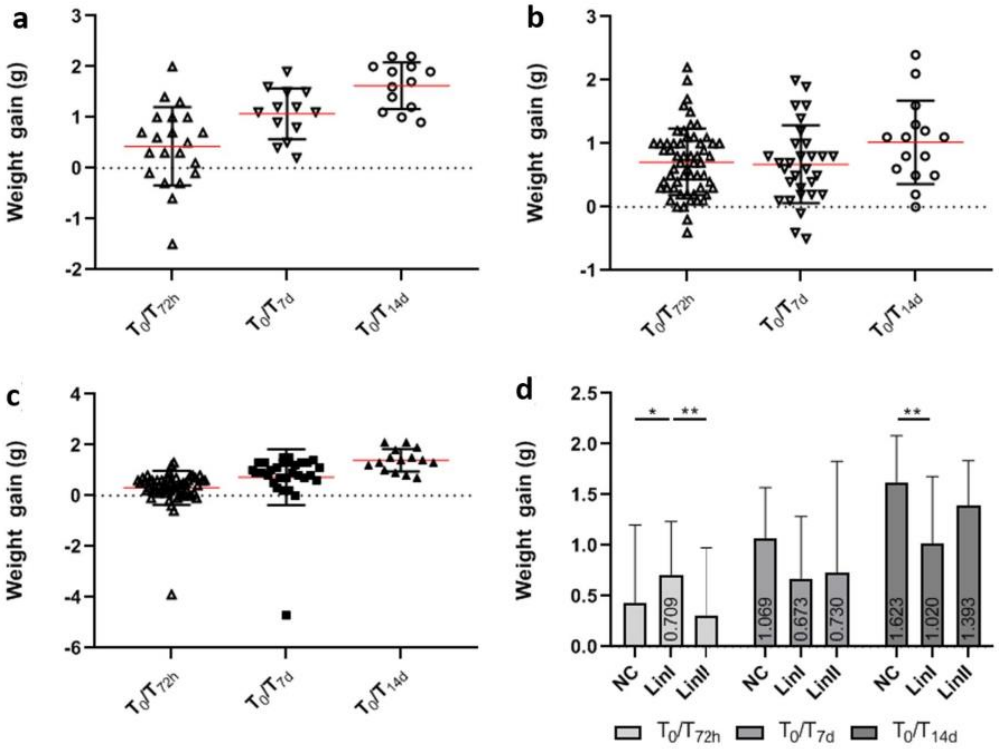

Figure 1. Body weight gain of BALB/c mice uninfected and infected with DENV-2 Lineages before infection (T_0), 72 hpi (T_{72h}), 7 dpi (T_{7d}) and 14 dpi (T_{14d}). (**a**) negative control; (**b**) DENV-2 Lineage I; (**c**) DENV-2 Lineage II; (d) comparison of body weight gain means. NC: (n: T_0/ T_{72h} = 21; T_0/ T_{7d} = 13; T_0/ T_{14d} = 13); Lin I: (N: T_0/ T_{72h} = 58; T_0/ T_{7d} = 30; T_0/ T_{14d} = 15); Lin II: (N: T_0/ T_{72h} = 58; T_0/ T_{7d} = 30; T_0/ T_{14d} = 15). n: number of mice, NC: negative control, Lin: Lineage, hpi: hours post-infection, dpi: days post-infection, $^*p < 0.05$, $^{**}p < 0.01$.

white blood cell count of mice infected with Lineage I was lower than that of uninfected mice (2.34 thousand/mm^3), and the average number of individuals infected with Lineage II was equal to 3.664 thousand/mm^3. At 14 dpi, the group infected with Lineage I showed an average of 2.75 thousand/mm^3, and the group infected with Lineage II showed an average of 3.08 thousand/mm^3 (Fig. 4).

Biochemical parameters. *Aspartate aminotransferase (AST).* The average level of AST in serum samples from uninfected mice was 158.6 U/L. Except for the group infected with Lineage II euthanized on the third day of infection (mean = 143.2 U/L), the infected mice generally showed increased levels of the enzyme when compared to the control group. In mice infected with Lineage I, the level of AST was higher on 24 hpi (mean = 221 U/L), decreased on the second day of infection (mean = 174 U/L) and then slightly increased at 72 hpi (mean = 176.2 U/L). In mice infected with Lineage II, the AST levels were also higher compared with the control group on the first day of infection (mean = 185.6 U/L); however, the concentration of the enzyme peaked at 48 hpi (mean = 324.8 U/L). On the third day after infection, AST levels were lower than those in the control group (Fig. 5).

Alanine aminotransferase (ALT). The mean level of ALT present in the sera of uninfected mice was 85.6 U/L. In mice infected with Lineage I, the concentration of aminotransferase was higher in the serum of mice 24 hpi (mean = 144.6 U/L). On the second day of infection, the average was 86.4 U/L, and on the third day of infection, it was 118.2 U/L. In samples from mice infected with Lineage II at 24 hpi, the ALT levels were lower than those in the control group (mean = 61 U/L). The enzyme concentration reached its peak on the second day of infection (mean = 204 U/L) and decreased on the third day (mean = 107 U/L) (Fig. 5).

Viral genome detection. Viral RNA was detected in two liver samples. One mouse was infected with Lineage I (1.2×10^{-1} copies of RNA/µl), and one mouse was infected with Lineage II (3.93×10^8 copies of RNA/µl).

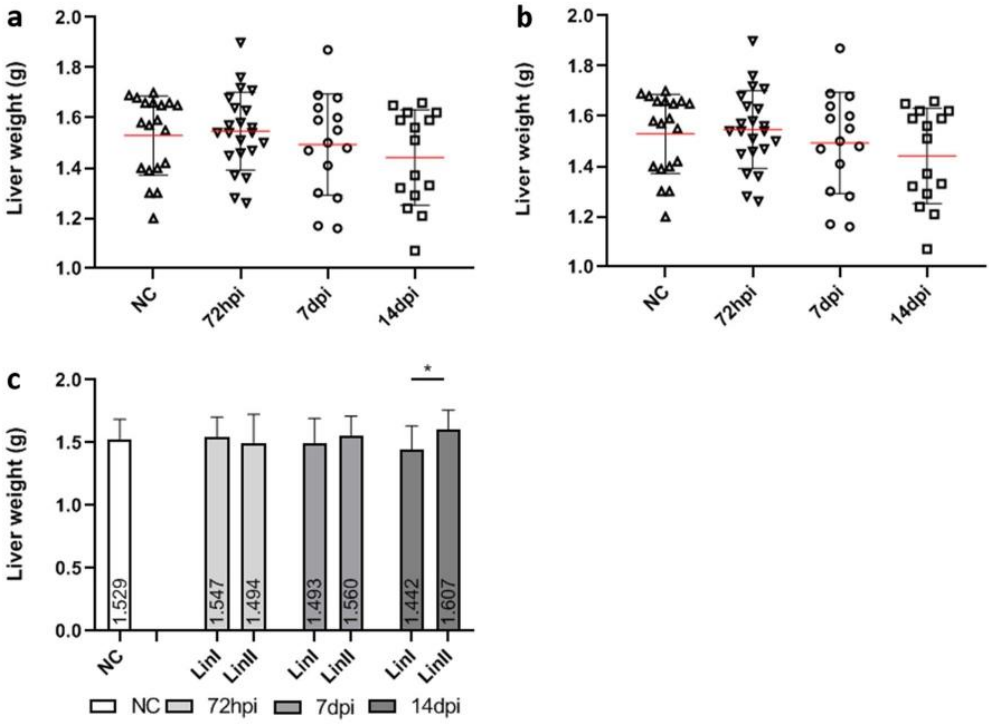

Figure 2. Liver weight of BALB/c mice uninfected and infected with DENV-2 Lineages 72 hpi, 7 and 14 dpi. (**a**) DENV-2 Lineage I; (**b**) DENV-2 Lineage II; (**c**) comparison of liver weight means. NC: (n = 19); Lin I: (n: 72 hpi = 22; 7dpi = 15; 14 dpi = 15); Lin II: (n: 72 hpi = 22; 7dpi = 15; 14 dpi = 15). n: number of mice, NC: negative control, Lin: Lineage, hpi: hours post-infection, dpi: days post-infection, $^{*}p < 0.05$.

Histopathology. The images of histological sections (Figs. 6 and 7)are representative of changes observed in the liver samples of BALB/c mice infected with DENV-2 Lineage I or II and euthanized at 72 hpi. Table 1 shows the number of infected mice whose livers presented each alteration.

Liver samples from noninfected mice showed intact parenchyma, with no signs of edema, congestion, steatosis or hemorrhage. The hepatocytes did not show nuclear or cytoplasmic alterations. Sinusoid capillaries were not dilated and did not present cellular infiltration or hemorrhaging, and Kupffer cells did not present histopathological alterations (Fig. 6a). Morphological alterations induced by both DENV-2 lineages in BALB/c mouse livers were focal and similar except for two cases. The most frequently observed alterations were inflammatory infiltrates, which is commonly observed next to the portal area (Fig. 6b), hepatocyte swelling and cytoplasmic loss, suggesting hepatic cell ballooning (Fig. 6c), hepatocyte nuclear area enlargement and chromatin pattern alterations (Fig. 6d), and vascular congestion (Fig. 6e). Sinusoid capillary dilation (Fig. 6f), which is more pronounced around centrolobular veins, and nuclear atypia, which is characterized by nuclear inclusions (Fig. 7b) or thin and regularly scattered chromatin, giving a relatively homogeneous appearance to the nuclei (Fig. 7a), were also seen. Only one mouse infected with DENV-2 Lineage II presented lipid droplets within the liver parenchyma (Fig. 7d), suggesting macrovesicular steatosis and focal hemorrhage (Fig. 7e). Signs of necrosis, or citoplasmic rarefaction, were only seen in samples infected with Lineage II (Fig. 7c).

Morphometry. Hepatocyte counting showed a significant increase in the number of binucleate cells in mice infected with both DENV-2 lineages at 72 hpi compared with the control group (Fig. 8a). Morphometrical analysis showed that the percentage of binucleate hepatocytes was 25.41% in mice infected with Lineage I ($p < 0.001$), 28.1% in mice infected with Lineage II ($p < 0.001$) and 20.05% in noninfected mice. However, when all hepatocytes were accounted for, a significant decrease in the cell population of infected mice was noted. Samples of mice infected with Lineage I presented 32.4% fewer cells than control samples ($p < 0.001$), and samples of mice infected with Lineage II presented 28.7% fewer cells ($p < 0.001$) (Fig. 8b).

Discussion

The DENV-2 introduction in Rio de Janeiro in 1990 led to the first cases of SD in the state and country[44,66]. Its re-emergence in 2007 caused a great DEN epidemic in 2008[49]. Although the DENV-2 that reemerged in 2007–2008 still belongs to the Southeast Asia genotype, two distinct strains (Lineage I and Lineage II) within this genotype

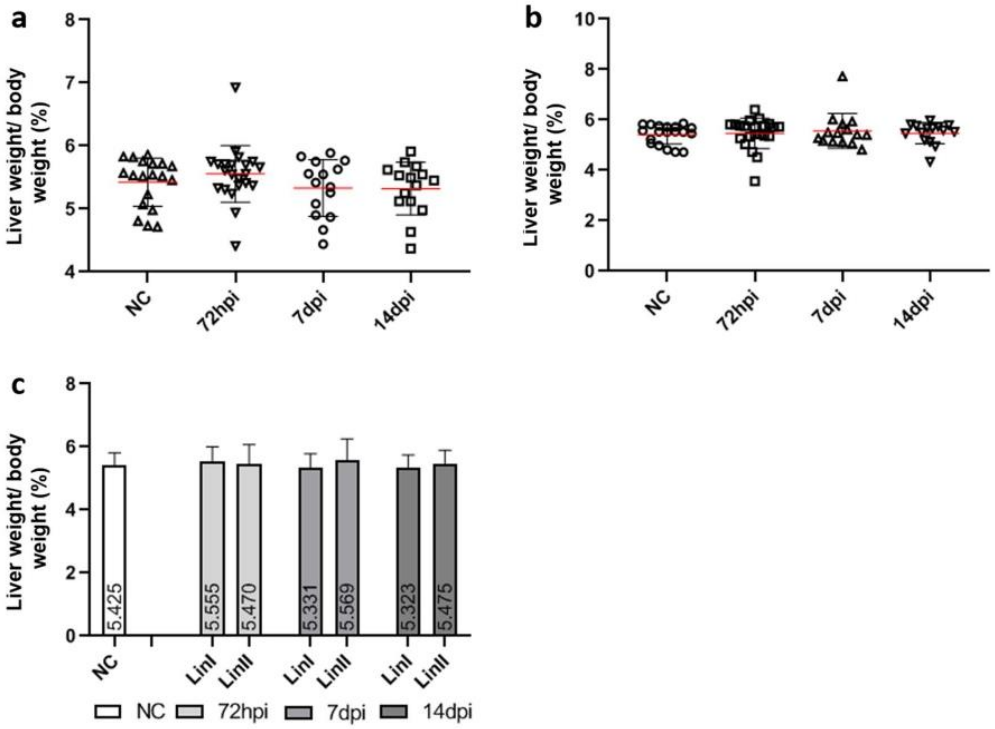

Figure 3. Liver weight / body weight ratio (%) of BALB/c mice uninfected and infected with DENV-2 Lineages 72 hpi, 7 and 14 dpi. (**a**) DENV-2 Lineage I; (**b**) DENV-2 Lineage II; (**c**) comparison of ratio means. NC: (n = 19); Lin I: (n: 72 hpi = 22; 7 dpi = 15; 14 dpi = 15); Lin II: (n: 72 hpi = 22; 7dpi = 15; 14 dpi = 15). n: number of mice, NC: negative control, Lin: Lineage, hpi: hours post-infection, dpi: days post-infection.

have been described[45,46]. Genetic variations between both strains were observed; however, no consistent differences in the genome that could be correlated with the severity of the disease have been identified to date[46,67]. Therefore, the factors that determine why certain DEN patients present mild symptoms and others develop severe disease are still not well defined. This study assessed the susceptibility of BALB/c mice to two distinct strains of DENV-2 and characterized alterations induced by each strain.

DEN patients may manifest acute fever that persists for three to seven days after the virus incubation period[68]. In this study, the average temperature variation was very small. Interestingly, only the control group showed a slight increase in temperature at 7 and 14 dpi. In studies carried out in BALB/c mice infected with DENV-3 and DENV-4 by our group, an increase in temperature at 72 hpi was observed[64,69] (unpublished data). However, the absence of a temperature rise and other clinical signs in this same animal model infected with DENV-2 have been previously reported[19,65] and may be related to asymptomatic DEN because only 25% of the cases of the disease show symptoms[12].

Although no neurological signs were observed in this study, paralysis was reported in BALB/c mice infected with DENV-2[58,65]. In addition, DENV-1-infected BALB/c mice experienced mild hemorrhage in the brain but no signs of neurological disease[60].

The weight gain among infected mice was lower than that of the control group, which may be associated with a loss of appetite, as observed in human DEN cases and immunocompetent models infected with DENV-2[11,58,70–72]. A cohort study conducted with DEN patients in a geriatric clinic reported that anorexia was the main complaint and that weight loss was greater during infection[72]. Among children, anorexia was reported in 78% of DF cases and in 91.2% of patients presenting DHF[70]. In DENV-3-infected mice, Caldas[64] observed a slight increase in mouse weight in comparison to the control group; nevertheless, the actual weight variation of each individual was not assessed (unpublished data).

During DENV infection, changes in blood component counts are commonly observed and indicate disease prognosis. Among the changes, thrombocytopenia (platelet count below 100,000/mm^3) is a hallmark for both mild and severe forms of DEN and may result from infection of bone marrow hematopoietic cell populations, which reduces their proliferative capacity[73,74], platelet deposition in the microvascular bed, aggregation with leukocytes or destruction from peripheral blood[75]. At 72 hpi, the platelet count of infected mice was higher than that of the control group. At subsequent times of infection, the platelet count decreased and reached values slightly lower than those of the control group. A decrease in platelet count is to be expected and has been observed in immunocompetent mice infected with DENV-2[58]; however, the slight thrombocytosis at 72 hpi, which was

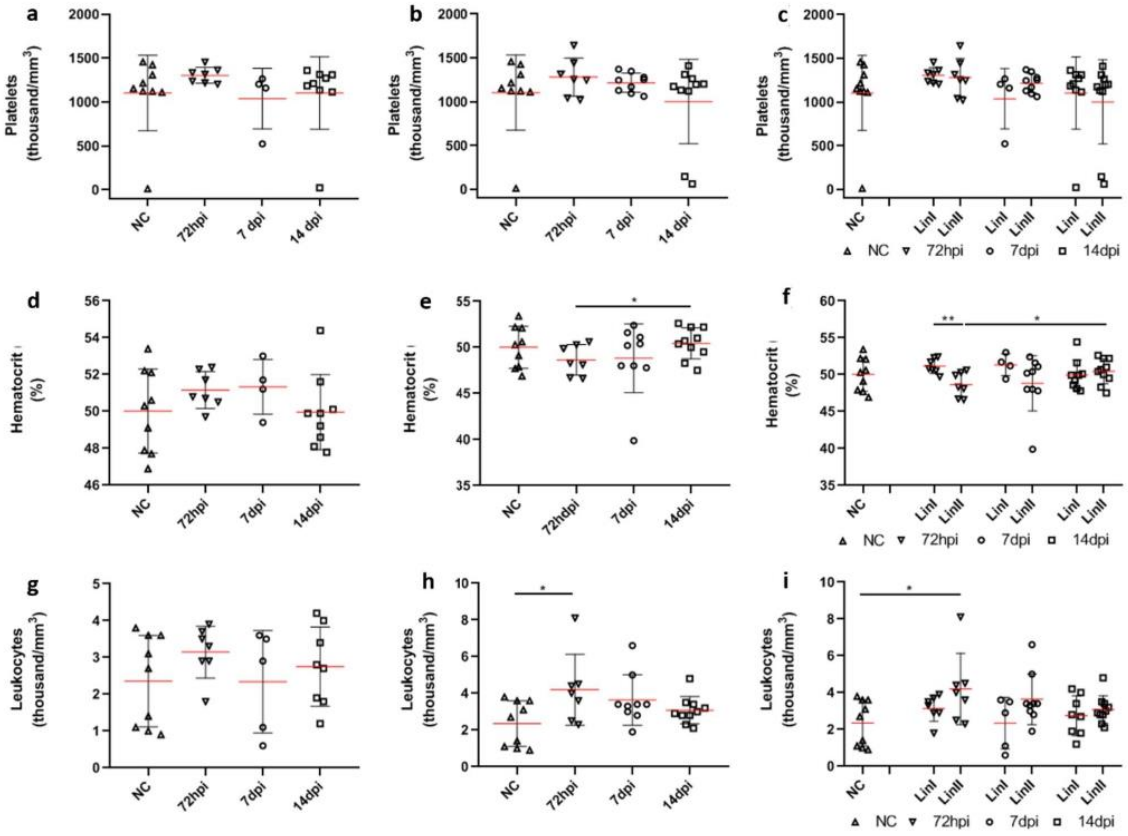

Figure 4. Hematological parameters of BALB/c mice uninfected and infected with DENV-2 Lineages 72 hpi, 7 and 14 dpi. Platelet count (thousands/mm^3): (**a**) DENV-2 Lineage I; (**b**) DENV-2 Lineage II; (**c**) comparison of platelet count means. Hematocrit (%): (**d**) DENV-2 Lineage I; (**e**) DENV-2 Lineage II; (**f**) comparison of hematocrit means. Leukocyte count (thousand/mm^3): (**g**) DENV-2 Lineage I; (**h**) DENV-2 Lineage II; (**i**) comparison of leukocyte count means. NC: (n = 10); Lin I: (n: 72 hpi = 10; 7dpi = 10; 14 dpi = 10); Lin II: (n: 72 hpi = 10; 7dpi = 10; 14 dpi = 10). NC: negative control, n: number of mice, hpi: hours post-infection, dpi: days post-infection, $^*p < 0.05$, $^{**}p < 0.01$.

also observed in mice infected with DENV-3[64] (unpublished data), could be a response to factors triggered by infection that increase thrombopoietin production[76].

Hemoconcentration can be observed as a result of plasma leakage[11]. An increase of the HCT of 20% over the baseline is a sign of DHF, and its maximum elevation coincides with shock[77]. HCT significantly increased in BALB/c mice infected with DENV-3[64] (unpublished data). Mice infected with DENV-2 Lineage I presented a very slight increase (less than 2%) in HCT at 72 hpi and 7 dpi, and this observation was corroborated by histopathological analyses since plasma leakage was not observed.

The leukocyte count is variable, and although leukopenia is more frequently reported, there are cases of leukocytosis associated with SD[78]. Our study showed an increase in the leukocyte count of mice infected with both lineages of DENV-2; moreover, this change was more preeminent in mice infected with Lineage II. After peaking at 72 hpi, the leukocyte count started decreasing. Another mouse model infected with DENV-2 also showed leukocytosis followed by leukopenia[79], and DENV-3-infected BALB/c mice presented a decrease in the number of leukocytes at 72 hpi, 7 dpi and 14 dpi[64].

Liver involvement is commonly seen in DEN, and although more frequently associated with SD, it is also present in nonsevere cases of DEN[18,80]. Liver function abnormalities induced by DENV infection range from a mild rise in transaminase and bilirubin levels to acute liver failure, which may lead to death[22,81,82]. ALT and AST are considered indicators of liver abnormalities, as they are released into the bloodstream following liver cell injury[83]. Elevated levels of these enzymes are an early marker of DEN. Transaminase levels are higher in patients presenting DHF or DSS, and the increase is usually mild or moderate; however, an increase in enzyme levels of more than tenfold has been reported[20,22,84]. An increase in transaminase levels has also been seen in immunocompetent mice infected with DENV-2[19,57,59]. Our studies also showed an increase in AST and ALT levels at all times of infection in mice infected with Lineage I, while samples infected with Lineage II showed higher values at 24 and 48 hpi (AST) and 48 and 72 hpi (ALT).

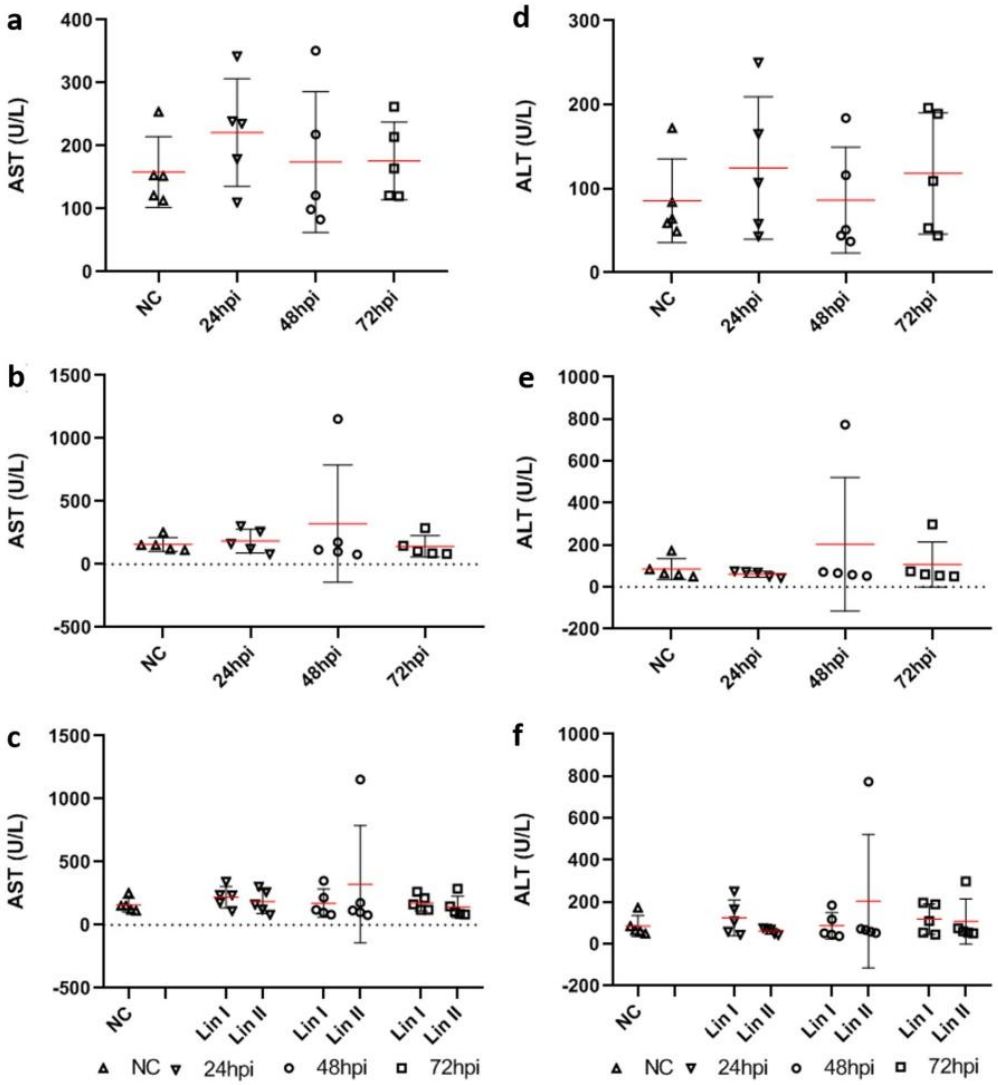

Figure 5. Transaminases levels (U/L) of BALB/c mice uninfected and infected with DENV-2 Lineages 24, 48 and 72 hpi. AST: (**a**) DENV-2 Lineage I; (**b**) DENV-2 Lineage II; (**c**) comparison of AST levels means. ALT: (**d**) DENV-2 Lineage I; (**e**) DENV-2 Lineage II; (**f**) comparison of ALT levels means. NC: (n = 5); Lin I: (n: 24 hpi = 5; 48 hpi = 5; 72 hpi = 5); Lin II: (n: 24 hpi = 5; 48 hpi = 5; 72 hpi = 5). NC: negative control, n: number of mice, hpi: hours post-infection.

The average liver weight of mice infected with both DENV-2 strains increased slightly and exceeded that of the control group at 72 hpi in mice infected with Lineage I and at 7 and 14 dpi in mice infected with Lineage II. Liver enlargement is commonly observed in DEN, mainly in SD cases[11,85], and may result from edema due to vascular permeability or accumulation of fat within hepatocytes[2,86]. A study carried out with SD patients showed a high prevalence (72.7%) of hepatomegaly and associated painful hepatomegaly with increased levels of ALT[85,87]. When analyzing the liver weight/body weight ratio, we observed that it varied similarly to the liver weight, with this ratio higher at 72 hpi on average in mice infected with Lineage I and at all points of infection in the Lineage II group. These results suggest that the heavier liver in infected mice is not solely related to body weight gain and may be a consequence of infection. The reduction in body weight gain observed in infected mice and detection of the viral genome in macerates of the liver reinforce this hypothesis.

DENV infection-induced histopathological changes can be observed in the livers of experimentally infected immunocompetent mice as well as in autopsy samples[14,19,34,57,58,65,88,89]. Although only two liver samples tested positive for the viral genome (Lineage I: 1/10 and Lineage II: 1/10), a number of alterations were observed in our samples. The micrographs presented are representative of alterations observed in our samples. Alterations were

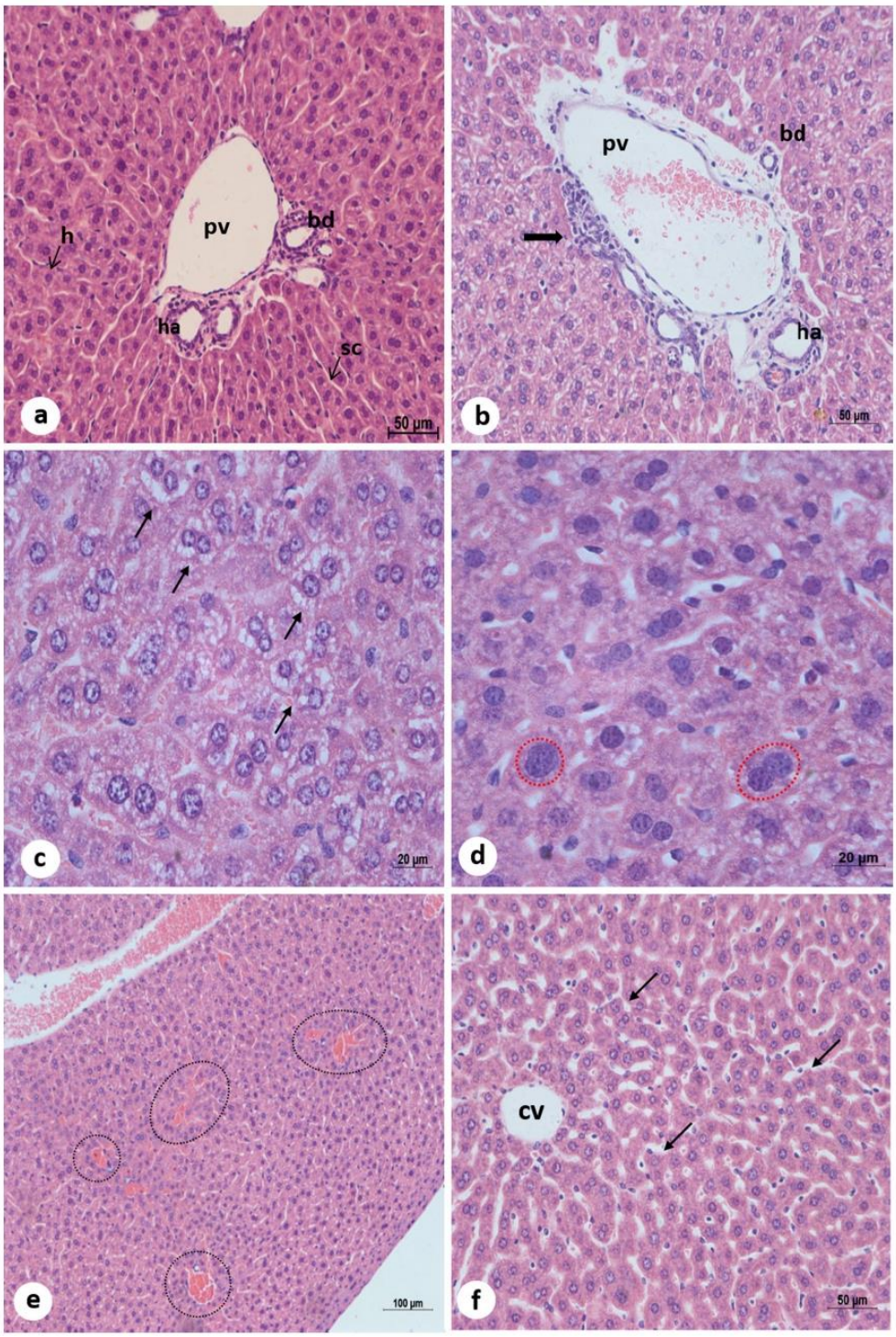

Figure 6. Liver micrographs of BALB/c mice. H&E staining. (**a**) negative control. (**b**–**d**) mice infected with DENV-2 Lineage I (Lin I) or II (Lin II). (**b**) (LinII) inflammatory infiltrate (arrow). (**c**) (Lin I) hepatic cell ballooning (arrows) (**d**) (Lin I) enlarged cell nucleus (red-dotted outline). (**e**) (Lin II) vascular congestion (dashed outline). (**f**) (Lin II) dilation of sinusoid capillaries (arrows). pv: portal vein. bd: bile duct. ha: hepatic artery. sc: sinusoid capillary. h: hepatocytes.

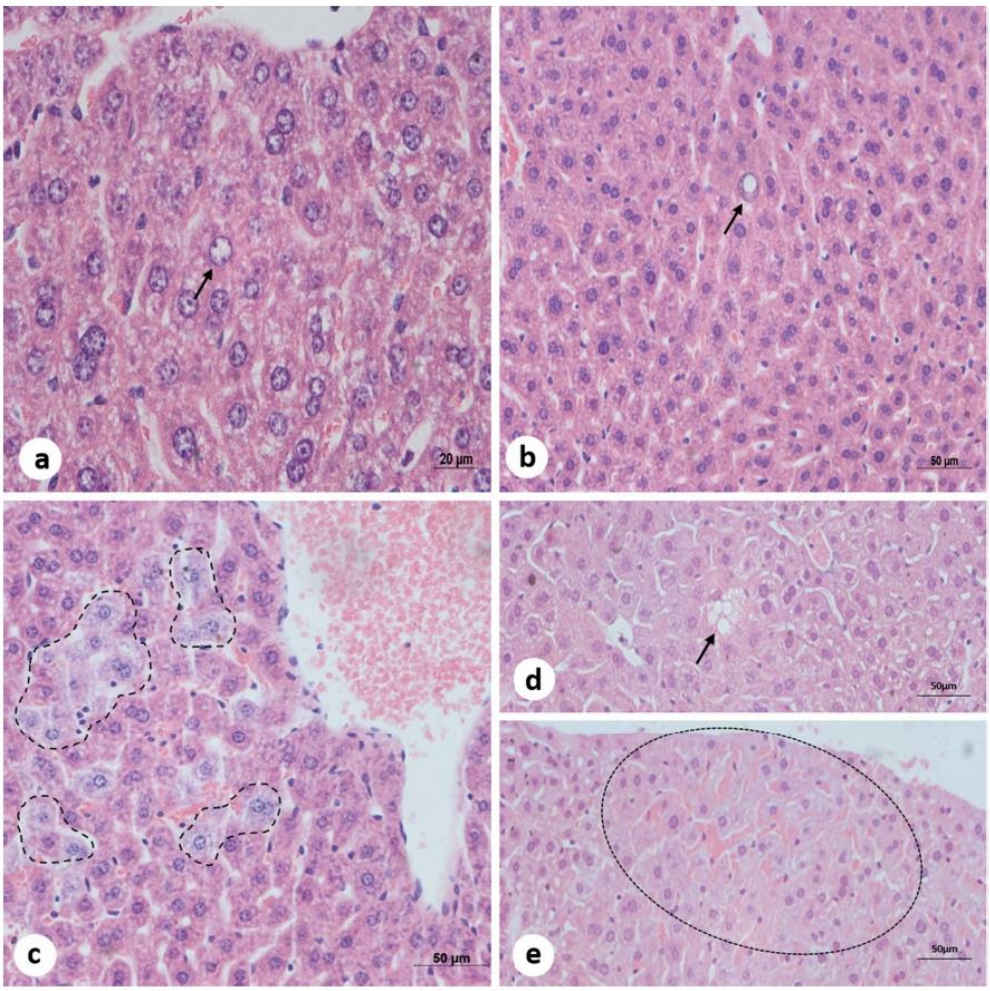

Figure 7. Liver micrographs of BALB/c mice infected with DENV-2 Lineage I (Lin I) or II (Lin II). H&E staining. (**a**) (Lin I), (**b**) (LinII) nuclear atypias (arrows). (**c**) (Lin II) signs of necrosis (dashed outline). (**d**) (Lin II) macrovesicular steatosis (arrow). (**e**) (Lin II) focal haemorrhage (dashed outline).

	DENV-2		
Alterations	Lineage I (%)	Lineage II (%)	Total (%)
Inflammatory cell infiltration	7/10 (70)	8/10 (80)	15/20 (75)
Hepatic cell ballooning	7/10 (70)	8/10 (80)	15/20 (75)
Enlarged cell nucleus	7/10 (70)	8/10 (80)	15/20 (75)
Vascular congestion	6/10 (60)	8/10 (80)	14/20 (70)
Sinusoid capillary dilation	9/10 (90)	1/10 (10)	10/20 (50)
Nuclear atypia	7/10 (70)	3/10 (30)	10/20 (50)
Necrosis	0/10 (0)	2/10 (20)	2/20 (10)
Macrovesicular steatosis	0/10 (0)	1/10 (10)	1/20 (5)
Haemorrhage	0/10 (0)	1/10 (10)	1/20 (5)
Signs of necrosis	0/10 (0)	1/10 (10)	1/20 (5)

Table 1. Histopathological alterations observed in liver samples of BALB/c infected with DENV-2 Lineages I or II. Number of mice whose livers presented the alteration/total number infected mice.

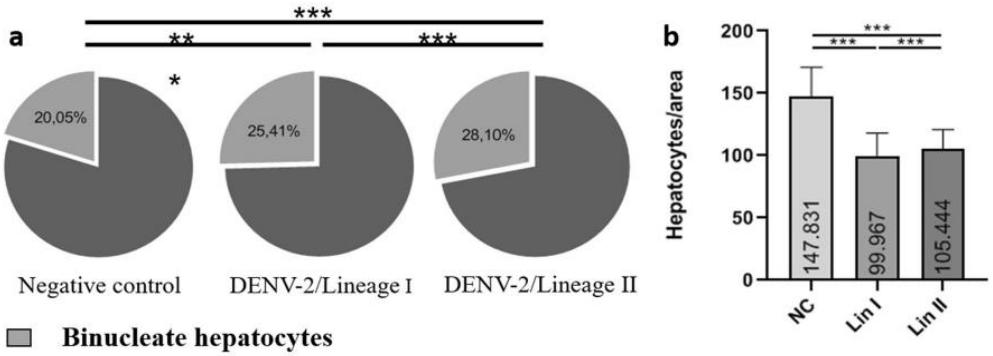

Figure 8. Hepatocyte count from samples of BALB/c mice uninfected and infected with DENV-2 Lineages I or II euthanized 72 hpi. (**a**) population of binucleated hepatocytes in negative control samples; (**b**) population of binucleate hepatocytes in samples infected with DENV-2 Lineage I; (**c**) population of binucleate hepatocytes in samples infected with DENV-2 Lineage II; (**d**) hepatocyte count per captured area. NC: negative control; Lin: Lineage, hpi: hous post-infection. ***$p < 0.001$.

focal, as reported elsewhere[88,90]. The most frequently observed change was inflammatory cell infiltration, which corroborates previous findings in BALB/c mice infected with DENV-2, -3 and -4[57,58,64,69,88]. Upon DENV infection, Kupffer cells and macrophages release cytokines and chemokines that activate inflammatory cells. Vasodilation is a result of proinflammatory cytokine release by Th1 cells[20]. Sinusoid capillary dilation was more frequently observed in samples infected with Lineage I (90%), whereas the frequency among samples infected with the other lineage was 10%. This alteration has been observed in other studies using the same mouse model[64,88,90].

Hepatic cell ballooning is presumably caused by the influx of fluids into the cell due to damage to the cytoplasmic membrane[91]. This alteration was present in 70% and 80% of samples infected with Lineage I and Lineage II, respectively, and has been reported as a result of viral infection[92,93]. DENV infection is known to alter lipid metabolism[94]. Macrovesicular steatosis was observed in one sample infected with Lineage II. These changes were observed in studies performed by our group with BALB/c mice infected with DENV-3 and -4[64,69] (unpublished data). Intracellular accumulation of fat occurs in different DENV-infected human cells as well as in the *A. albopictus* C6/36 cell line and may play a role in viral production[95,96].

In this study, enlargement of the nucleus was observed. Kariocytomegaly can be caused by hepatocyte polyploidy. There is no mention of hepatocyte kariocytomegaly in DEN; however, the increased number of hepatic cells with enlarged nuclei has been associated with liver damage[97,98]. Other atypical-looking nuclei presented inclusions and altered chromatin patterns, which were also seen in the livers of BALB/c mice infected with DENV-3[64] (unpublished data). Nuclear inclusions accumulate, in the nuclear matrix, substances that are not found in the nucleus under normal circumstances and can be caused by viral infection. Hepatocytes may be a result of glycogen accumulation[99]. Paes[57] has also observed nuclear inclusions in liver samples of BALB/c mice and described them as lipid-like. Further investigation is necessary to identify the nature of the inclusion.

Hepatocyte polyploidization and binucleation are features of liver growth and physiology, can be associated with disease and could be favorable for pathogens or induced by them to improve survival[100]. Moreover, binucleate cells may be more capable of responding to a major demand for protein synthesis[101–103]. Our infected samples presented a significant increase in binucleation. The same change was observed in BALB/c mice by Sakinah[65] and by our group in an ongoing study on reinfection[104]. Binucleate cells may be formed by nuclear division or cell fusion[105,106], and several viruses have fusogenic activity[107,108]. Grizzi[100] suggested that binucleation is a cell's response to hepatic illness because it increases with progression of the necroinflammatory state. It can also be a sign of tissue regeneration because it was reported to increase after partial hepatectomy[109]. Binucleation may have increased as a response to infection. A decrease in the total number of hepatocytes was observed in infected mice, which may indicate cell death. Indeed, some of our samples presented signs of necrosis, as seen in other studies with DENV-2[57,58,88] and DENV-4[69] (unpublished data).

SD disease is associated with extensive involvement of the endothelium[74]. Vascular permeability plays an important role in SD pathogenesis[11,110]. Studies have shown that an increase in the number of infected hepatic endothelial cells coincides with the onset of SD[111] and that vascular permeability is caused by inflammatory mediators rather than by infection of endothelial cells or cell death[110,112,113]. Although ours is not a SD model, focal hemorrhage was observed in one liver sample, suggesting altered vascular permeability. This finding is in accordance with human case reports[14,34] and studies of this same experimental model infected with DENV-3 and DENV-4[64,69] (unpublished data).

The outcome of infection and tissue tropism can be influenced by infective strain virulence[13,20,60]. Indeed, a study carried out with patient sera reported that the viremia level of Lineage II samples was two orders of magnitude higher than that of Lineage I samples and an increase in the number of SD cases occurred after DENV-2 strain emergence in 2007[52]. Although the viral genome was detected in only two samples (one of each lineage), the titer found in the liver of mice infected with Lineage II was higher.

In this study, two groups of BALB/c mice were experimentally infected with either Lineage I or Lineage II. No rise in temperature was observed among infected mice. A decrease in body weight gain was observed in infected mice. Although the 72 hpi mean weight gain of mice infected with Lineage II was significantly lower than that of mice infected with the other lineages ($p < 0.01$), at the end point of the experiment (14 dpi), it was inferior among mice infected with Lineage I ($p < 0.01$). It is noteworthy that while infected mice gained less weight, the livers of infected individuals were heavier than control group 72 hpi in Lineage I infected mice and 7 and 14 dpi in mice infected with Lineage II. The difference between the infected groups was statistically significant at 14 dpi ($p < 0.05$). At 72 hpi, the viral load of a liver sample infected with Lineage II was 3.93×10^8 copies of RNA/µl (higher than Lineage I) and liver weight of this group was inferior compared to the other lineage, and these characteristics may be related to differences between the strains, the time of infection at which each lineage manifests symptoms, or host genetic factors. Nevertheless, more studies are necessary to generate conclusive findings.

The platelet count increased on the first three days of infection in both groups and declined subsequently. The mean number of platelets was lower than that in the control group at 7 dpi in mice infected with Lineage I, whereas the mean was inferior to that in the control group at 14 dpi in mice infected with Lineage II. Nonetheless, there was no statistically significant difference among the groups.

Similarly, the number of leukocytes increased at 72 hpi in both groups and decreased afterwards. The leukocyte count of Lineage II was higher than that of the control group at all times of infection, and at 72 hpi, the difference between means was statistically significant ($p < 0.005$). At seven dpi, the leukocyte count was slightly lower in mice infected with Lineage I than in the control group, but there was no significant difference between the infected and noninfected groups.

Regarding the HCT, the curves of the infected group presented opposite profiles. Although the HCT increased at 72 hpi and 7 dpi in mice infected with Lineage I, the values were lower than that of the control group 14 dpi; however, the HCT values in mice infected with Lineage II were greater than that of the control group at 14 dpi. Significant differences were observed in the HCT between the infected groups at 72 hpi ($p < 0{,}001$). Regarding mice infected with Lineage II, the difference was significant between the groups euthanized at 72 hpi and 14 dpi ($p < 0.005$).

The AST and ALT levels of the Lineage I group were higher than that in the control group at all times of infection, while the AST and ALT levels of the Lineage II group were lower than that in the noninfected mice at 24 hpi and 48 hpi, respectively. The levels of AST and ALT peaked at 24 hpi in mice infected with DENV-2 Lineage I (rise of 28.2% and 40.8% in relation to the control group, respectively) and at 48 hpi in mice infected with Lineage II (increase of 51.1% and 58% compared to the control group, respectively). The highest difference between the control group and Lineage II group concerning transaminase peak levels could be associated with the higher viral load detected in the sample infected with this lineage. Paes[57,59] reported that AST and ALT peaked at the same time as the viral load in liver of BALB/c mice infected with DENV-2, although a cohort study conducted with both SD and nonsevere dengue patients did not find a correlation between viral titer and level of liver transaminases[18]. No significant difference was observed.

Histopathological alterations induced by the two lineages were focal and mostly similar, and the frequency at which alterations were observed varied. Some changes were seen at a much higher frequency in mice infected with Lineage I (sinusoid capillary dilation and nuclear atypia), while others were only seen in samples infected with the other lineage (macrovesicular steatosis and hemorrhage). There was a significant difference between the infected groups concerning the number of binucleate hepatocytes, which were higher in Lineage II-infected samples, and hepatocyte count, which was higher in Lineage I-infected samples. Morphological changes were mild compared to those reported in fatal cases[14,22,34], and the samples were from mice euthanized at 72 hpi. However, in mice infected with Lineage II, the livers were heavier later in the infection; thus, it would be interesting to investigate tissue from mice euthanized at 7 and 14 dpi as well.

In addition to the circulation of viral strains of Asian origin, the current hyperendemicity of DEN highlights the need to investigate the role of these viruses in the occurrence of severe and fatal cases. Furthermore, after the reemergence of serotype 2 in 2017 and the consequent change in the predominance of the circulating serotype from DENV-1 to DENV-2[114], there was an explosion of DEN cases in Brazil in 2019, with 905,912 probable cases reported until August[115]. Thus, the establishment of experimental models for the study of this serotype is relevant. Here, we have demonstrated that although the changes induced by the infection of the two DENV-2 strains are similar in terms of certain alterations, differences are observed in the infection timeline and intensity.

Material and methods

Ethical statements. All procedures performed in this study were approved by the Animal Ethics Committee (protocol L-041/2015) and the Human Research Ethics Committee (protocol 247/05) of Instituto Oswaldo Cruz, Fundação Oswaldo Cruz. All methods were carried out in accordance with the relevant guidelines and regulations and animal experimentation was in compliance with the ARRIVE guidelines for *in-vivo* studies.

Virus. DENV-2 strains BR/RJ66985/2000 (GenBank #HQ012518) and BR/RJ0337/2008 (GenBank #HQ01253), representative of Lineage I and Lineage II[45,46] were isolated from patient sera at Flavivirus Laboratory, IOC, FIOCRUZ, during the epidemics of 2000 and 2008, respectively. The serotype was confirmed by indirect immunofluorescence using a DENV-type-specific monoclonal antibody (3H5) and reverse transcription polymerase chain reaction (RT-PCR)[116,117].

Viral stock. Viral stocks were prepared by inoculating 100 µl of each strain into 175 cm^2 cell culture bottles containing mosquito Ae. albopictus C6/36 cell line at a concentration of 5×10^5 cells/ml. Titers of both strains

(BR/RJ66985/2000:$10^{6.66}$ $TCID_{50}$/1 ml and BR/RJ0337/2008:10^{9} $TCID_{50}$/1 ml) were calculated by the Reed & Muench method[118]. The viruses did not undergo any passages through the mouse brain for neuroadaptation.

Mice. For the experimental infection, two-month-old male BALB/c mice, provided by Instituto de Ciência e Tecnologia em Biomodelos (ICTB) of FIOCRUZ, were used. During the animal experimentation period, the animals were kept under controlled temperature, photoperiod, nutrition and hydration conditions.

Study design. For infection with both lineages of DENV-2 (BR/RJ66985/2000 and BR/RJ0337/2008), BALB/c mice were inoculated by the intravenous route (IV) through the caudal vein. The inoculum volume was 100 µl, and the viral concentration was 10,000 $TCID_{50}$/0.1 ml. The mice were anesthetized and euthanized (0.2 ml of ketamine, xylazina and tramadol solution) at 24, 48, 72 h postinfection (hpi) and 7 or 14 days postinfection (dpi) according to their experimental group. Blood was sampled by cardiac puncture before euthanasia and centrifuged in a refrigerated centrifuge (4 °C) for 10 min at 5000 rotations per minute to separate the serum from the cellular components. Liver samples destined for morphological analysis were fixed in Millonig buffered formalin. The samples destined for molecular studies were stored in a -80 °C freezer. Biochemical and hematological tests were carried out immediately after sample collection. Body temperature and weight were verified preinfection and before euthanasia and at 72 hpi, 7 dpi and 14 dpi. All liver samples were weighed immediately after harvesting, and to evaluate whether the liver weight increase was due to DENV infection and not just a consequence of body weight gain, a ratio (100×liver weight/body weight) between both measurements was calculated. Noninfected mice were used as negative controls.

Body temperature. The body temperature of noninfected (n = 21) and infected (n = 58/lineage) mice was verified immediately before infection (T_0) and at 72 hpi, 7 dpi and 14 dpi, and variation means (72 hpi-T_0, 7 dpi-T_0 and 14 dpi-T_0) were calculated (Fig. 9, Table 2). Temperature was determined by dipping the measuring extremity of a digital thermometer in mineral oil and then gently inserting it into the mouse rectum for one minute.

Body weight variation. The body weights of noninfected (n = 21) and infected (n = 58/lineage) mice were measured immediately before infection (T_0) and at 72 hpi, 7 dpi and 14 dpi, and the variation (72 hpi-T_0, 7 dpi-T_0 and 14 dpi-T_0) in the mean weight was calculated (Fig. 9, Table 2).

Liver weight. Livers of noninfected (n = 19) and infected (n = 52/lineage) mice were weighed immediately and at 72 hpi, 7 dpi and 14 dpi, and the mean weight was calculated (Table 2).

Hematological analysis. For both DENV-2 lineages, 30 mice were infected. The mice were divided into three groups of 10 animals, and each group was euthanized at different times after infection (72 hpi, 7 dpi and 14 dpi). Before euthanasia, the mice were anesthetized and blood was collected by cardiac puncture. Blood from noninfected mice (n = 10) was collected on the same day as the 14 dpi group. To avoid coagulation, samples were stored in EDTA-coated tubes. Platelets and leucocytes were counted, and hematocrit (HCT) was measured in collaboration with ICTB utilizing a Poch 100-iV DIFF platform (Sysmex, Kobe) (Table 3).

Biochemical analysis. For both DENV-2 lineages, 15 mice were infected. The mice were divided into three groups of five animals, and each group was euthanized at different times after infection (24, 48 and 72 hpi). Prior to euthanasia, the mice were anesthetized and blood was collected by cardiac puncture. Blood samples were then centrifuged for 10 min at 5000 rotations per minute to separate the serum from the cellular components. Blood from noninfected mice (n = 5) was collected on the same day as the 72 hpi group. ALT and AST blood levels were measured by dry chemistry testing using a Vitros 250 system (Ortho clinical—Jonhson & Jonhson) and in collaboration with ICTB (Table 3).

Nucleic acid extraction and quantitative RT-PCR. For each DENV-2 lineage, 5 BALB/c mice were infected and euthanized at 72 hpi. Five noninfected mice were used as a negative control. Viral RNA was extracted from liver samples of infected and noninfected mice. Organ samples were macerated with 500 µl of Leibovitz medium (Sigma). RNA was extracted from 140 µl of supernatant of macerated liver samples by using a QIAmp Viral RNA mini kit (Qiagen) as described by the manufacturer's protocol. Viral RNA quantitation was carried out using the primers and probes DEN2-R (5-ACCATAGGAACGACACATTTCC-3) and DEN2-F (5-CAA CGCATTGTCATTGAAGGA-3) and (FAM-5-AGGGCCTTGATTTTCATCTTACTGACAGC-3-TAMRA). TaqMan Fast Virus One-step Master Mix (Applied Biosystems) was used for the amplification reaction. Five microliters of extracted RNA and a mix containing 12.5 µl of reaction mix, 1 µl of DEN2-F and DEN2-R primers (Sigma), 0.75 µl of DEN2-P probe, 3.65 µl of nuclease-free water (Gibco), 1 µl of $MgSO_4$ and 0.5 µl of SuperScript III Platinum One-Step Quantitative RT-PCR (Invitrogen) were applied to a 96-well microplate. The assay was performed using a 7500 FAST platform (Applied Biosystems). Thermal cycling parameters were as follows: reverse transcription at 50 °C for 15 min (min), 1 cycle of enzyme activation at 95 °C for 2 min, 1 cycle of denaturation at 95 °C for 15 s, 40 cycles of annealing/elongation at 60 °C for 1 min (Table 3).

Bright field microscopy. For each DENV-2 lineage, 10 mice were infected. Five noninfected mice were used as a negative control. At 72 hpi, the mice were euthanized and liver samples were collected and fixed in Millonig buffered formalin. The samples were then dehydrated in decreasing concentrations of ethanol, clarified in

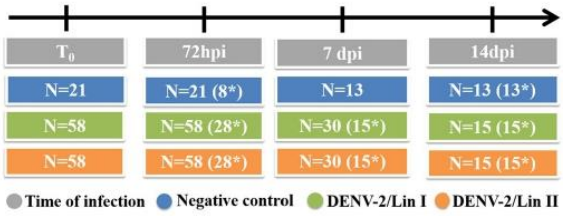

Figure 9. Number of mice used in clinical analysis at each time of infection. T_0: before infection, hpi: hours post infection, dpi: days post infection, (*) mice euthanized at that time of infection, Lin: Lineage.

	Body temperature/weight				Liver weight		
	T_0	72hpi	7dpi	14dpi	72hpi	7dpi	14dpi
DENV-2/Lin I	58	58	30	15	22	15	15
DENV-2/Lin II	58	58	30	15	22	15	15
Negative Control	21	21	13	13			19
Total				137			123

Table 2. Number of mice used for clinical analysis and liver weight analysis. T_0: before infection, hpi: hours post infection, dpi: days post infection, Lin: Lineage.

	Histopathology/qRT-PCR	Hematological analysis			Biochemical analysis		
N = 130 mice	72 hpi	72 hpi	7 dpi	14 dpi	24 hpi	48 hpi	72 hpi
DENV-2 Lin I	10	10	10	10	5	5	5
DENV-2 Lin II	10	10	10	10	5	5	5
Negative control	5	10	5				
Total	25			70			35

Table 3. Number of mice used for histopathological, hematological and biochemical analysis and viral RNA quantification. hpi: hours post infection, dpi: days post infection, Lin: Lineage.

xylene and embedded in paraffin. Tissue Sects. (5 µm thick) were obtained using a microtome and stained with hematoxylin and eosin (H&E) for posterior analysis with a bright field microscope (AxioHome, Carl Zeiss). All procedures were performed in collaboration with Pathology Laboratory, IOC/FIOCRUZ (Table 3).

Histomorphometry. The morphometric analysis goal was to quantify the number of binucleated hepatocytes as well as the total number of hepatocytes in each group of mice. Fifteen glass slides containing histological sections of liver of BALB/c mice euthanized at 72 hpi and stained with H&E (five from noninfected mice, five from mice infected with each lineage) were analyzed. Thirty images of random areas were captured at 40 × magnification using a digital camera coupled to a bright field microscope (AxioHome, Carl Zeiss). Cells from a total of 450 areas were counted. The values obtained were then compiled by group, and the mean was calculated.

Statistical analysis. A database was constructed with the data collected during the experiment. T-tests were performed using SPSS 25 software (IBM) and graphics were constructed using GraphPad Prism 8.0.1 software. P values of $p \leq 0.05$ were considered statistically significant.

Received: 19 October 2020; Accepted: 12 April 2021
Published online: 06 May 2021

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Acknowledgements

The authors would like to thank Instituto Oswaldo Cruz (IOC) for providing us with its technology and facilities during the course of the research. We would also like to thank Laboratório de Flavivírus (IOC/FIOCRUZ), for providing the viral strains samples, Dr. Marcelo Pelajo and João Paulo Rodrigues, Laboratório de Patologia (IOC/ FIOCRUZ), for the support in sample processing and technical analysis. Dr. Felipe Anibal Carvalho Costa,

Laboratório de Epidemiologia e Sistemática Molecular (IOC/FIOCRUZ), for the support with statistical analysis and ICTB (IOC/FIOCRUZ) for the support in sample processing.

Author contributions

F.B.S. and D.F.B.V. conceived the study; F.C.J. designed and supervised the experiments; and was involved in data curation; D.D.C.S, A.P.C. and R.L. conducted the experiments; F.C.J., G.C.C , A.L.T.A. and A.C.R. analysed all images and data; F.C.J. drafted the manuscript; D.F.B.V., F.B.S.; and O.M.B reviewed the manuscript. O.M.B. and D.F.B.V were responsible for funding acquisition and resources.

Competing interests

The authors declare no competing interests.

Additional information

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3.2. Artigo 2: Envolvimento renal associado ao vírus dengue tipo 2 em um modelo murino: resultados após a infecção por duas brasileiras linhagens do genótipo Asiático/Americano

Revista: Pathogens

Resumo

O vírus dengue tipo 2 (DENV-2) é, tradicionalmente, o sorotipo mais estudado devido a sua associação com surtos explosivos e casos graves. No Brasil, quase 20 anos após a primeira introdução na década de 1990, uma nova linhagem (Linhagem II) do genótipo DENV-2 asiático/americano surgiu e causou uma epidemia com casos graves e hospitalizações. A dengue grave inclui falência de múltiplos órgãos, e o envolvimento renal pode estar potencialmente relacionado ao aumento da mortalidade. A fim de compreender melhor o papel da infecção por DENV na lesão renal, objetivamos investigar os resultados da infecção com duas linhagens distintas do genótipo Asiático/Americano DENV-2 no rim de um modelo murino. Camundongos BALB/c foram infectados com as linhagens I e II e os tecidos foram submetidos à estudos de histopatologia, imunohistoquímica, histomorfometria e análises ultraestruturais. Além disso, os rins destes camundongos foram pesados e os níves séricos de ureia (BUN) foram dosados. Foi observada uma tendência de aumento do peso dos rins em camundongos infectados com ambas as linhagens, mas os níveis de uréia, em média, aumentaram apenas em camundongos infectados com a Linhagem II. O antígeno DENV foi detectado no tecido de camundongos infectados com Linhagem II e as alterações morfológicas foram semelhantes às observadas em casos de dengue humana. Além disso, os parâmetros como peso do órgão, níveis de ureia e análise morfométrica, mostraram diferenças significativas entre as duas linhagens no BALB/c infectado, que se demonstrou ser um modelo experimental adequado para estudos de fisiopatologia da dengue em rins.

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Article

Brazilian Dengue Virus Type 2-Associated Renal Involvement in a Murine Model: Outcomes after Infection by Two Lineages of the Asian/American Genotype

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Abstract: Dengue virus type 2 (DENV-2) is, traditionally, the most studied serotype due to its association with explosive outbreaks and severe cases. In Brazil, almost 20 years after the first introduction in the 1990s, a new lineage (Lineage II) of the DENV-2 Asian/American genotype emerged and caused an epidemic with severe cases and hospitalizations. Severe dengue includes multiple organ failure, and renal involvement can be potentially related to increased mortality. In order to better understand the role of DENV infection in renal injury, here we aimed to investigate the outcomes of infection with two distinct lineages of DENV-2 Asian/American genotype in the kidney of a murine model. BALB/c mice were infected with Lineages I and II and tissues were submitted to histopathology, immunohistochemistry, histomorphometry and ultrastructural analysis. Blood urea nitrogen (BUN) was detected in blood sample accessed by cardiac puncture. A tendency in kidney weight increase was observed in mice infected with both lineages, but urea levels, on average, were increased only in mice infected with Lineage II. The DENV antigen was detected in the tissue of mice infected with Lineage II and morphological changes were similar to those observed in human dengue cases. Furthermore, the parameters such as organ weight, urea levels and morphometric analysis, showed significant differences between the two lineages in the infected BALB/c, which was demonstrated to be a suitable experimental model for dengue pathophysiology studies in kidneys.

Keywords: dengue 2; Asian/American lineages; BALB/c mice; kidney; histopathology and transmission electron microscopy

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Citation: Jácome, F.C.; Caldas, G.C.; Rasinhas, A.d.C.; de Almeida, A.L.T.; de Souza, D.D.C.; Paulino, A.C.; da Silva, M.A.N.; Barth, O.M.; dos Santos, F.B.; Barreto-Vieira, D.F. Brazilian Dengue Virus Type 2-Associated Renal Involvement in a Murine Model: Outcomes after Infection by Two Lineages of the Asian/American Genotype. *Pathogens* **2021**, *10*, 1084. https://doi.org/10.3390/pathogens10091084

Academic Editor: Juarez Antônio Simões Quaresma

Received: 30 June 2021
Accepted: 29 July 2021
Published: 26 August 2021

1. Introduction

Dengue virus serotypes 1 to 4 (DENV-1–4) are arboviruses belonging to the genus *Flavivirus* of the family *Flaviviridae* [1]. Transmission occurs in over 125 countries and around 4 billion people are at risk of infection annually [2]. Dengue poses a major threat to urban populations in Asia and Latin America, mainly due to its increased incidence in the last 50 years [3–5]. In Brazil, Lineage I of DENV-2 Asian/American genotype has been circulating since the 1990's [6], when the first cases of dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS) were reported [7]. After 17 years, the emergence of Lineage II of DENV-2 Asian/American genotype was associated with increased disease

severity and a high mortality rate, especially in children [6,8–10]. However, despite belonging to the same lineage from the 2007–2008 epidemic, DENV-2 strains circulating in 2019 are phylogenetically distant from any Brazilian strain, probably originating in Puerto Rico [11].

Dengue symptoms range from a mild flu-like syndrome to a severe and, sometimes, fatal disease, classified by the World Health Organization (WHO) as severe dengue (SD) which may affect multiple organ systems [12]. The DENV has been detected in a number of organs [13–16] and, although the liver is the most commonly affected one [17,18], gastrointestinal, hepatic, respiratory, cardiac, neurological and renal manifestations during DENV infection have already been reported [19–26].

The presence of DENV in the kidney has already been demonstrated through the detection of the viral antigen in the tissue cells and in macrophages and monocytes circulating in kidney blood vessels [13,15,17,26,27]. Furthermore, the observation of microtubule reticular structures and dilatation of endoplasmic reticulum in necrotic cells and dense virus-like particles in glomeruli in a transmission electron microscope (TEM), suggested viral infection [17,28,29].

Kidney damage induced by DENV infection can result from a direct viral cytopathic effect, inflammatory mediators released in response to the infection, hemodynamic instability, rhabdomyolysis, hemolysis or acute glomerular injury [17,30,31]. Increased levels of urea, creatinine, proteinuria, hematuria, glomerulonephritis and acute DENV infection have been related to dengue [32–35].

Acute kidney injury (AKI) and acute renal failure (ARF) are significant complications of dengue, and patients presenting SD are more likely to develop them [25,34,36–40]. Rates of mortality due to AKI are 1% for classic dengue, 12–40% for DHF and 60% for DSS [41].

Analysis of kidney samples from DENV-infected human cases revealed parenchyma and circulatory damage [17,27]. Tubular necrosis, evidenced by the presence of pyknotic nuclei of epithelial cells, thickening of the glomerular basement membrane, mesangial proliferation, glomerular congestion and hyalinosis, interstitial area with focal fibrosis, diffuse mononuclear infiltrate and hemorrhage foci in the cortical and medullary regions and the increase in populations of CD68+ and CD4+ cells have been reported [27,42–44].

The pathophysiological basis for SD is yet to be fully understood and likely to be multifactorial [45], involving the host's genetic background and immunological status, sequence of serotypes in secondary infections, serotype and virulence of infectious viral strains [46,47]. In fact, DENV infecting strain can contribute to detrimental progression of severe disease and death [48,49].

An ideal experimental model for studying dengue should recapitulate the disease progression as it occurs in humans [50]. To date, there are no experimental models that fulfill this requirement [51,52], which hinders the thorough comprehension of DENV pathogenesis mechanisms as well as drug and vaccine development [53]. It is believed that immunocompetent mice are less susceptible to DENV infection [52]. However, studies have shown that BALB/c mice present thrombocytopenia, increased levels of hepatic enzimes alanine aminotrasferase (ASL) and aspartate aminotransferase (ALT), anorexia, weight loss, anemia and even develop severe disease and paralysis, when infected with neuroadapted strains [54–57]. Viral replication and dissemination have been observed in this experimental model infected with DENV-1, -2 and -4 and viral genome or antigen have been detected in heart, lungs spleen, brain, liver, kidneys and saliva samples [53–55,58–63]. In kidney tissue of DENV infected BALB/c mice, increased glomerular volume and mesangial cellularity have been reported [58].

Since the DENV infection outcome can be affected by the virulence of different strains [47], and dengue related histopathological data on kidney is still scarce, here we aimed to investigate the renal involvement of BALB/c mice after infection with two distinct DENV-2 Asian/American lineages. Moreover, as this report is a part of a project whose goal is to present BALB/c mice as a suitable non-severe dengue experimental model for studies on the pathogenesis of dengue in different organs, we have recently demonstrated

the susceptibility of the aforementioned murine model to both lineages and showed that the changes induced by those strains in the liver were similar to those observed in human cases, but it was observed that some parameters were manifested at different times of infection [63].

2. Results

2.1. Evaluation of Kidney Weight

A slight increase in the mean weight of the kidney of infected mice was observed when those were infected by both Lineages of DENV-2 Asian/American genotype, when compared to the uninfected mice (mean = 0.423 g/standard deviation (±) = 0.06). The mean weight of kidneys of mice infected by Lineage I presented its highest value at 72 hpi and subsequent weight loss at 7 dpi and 14 dpi (from 0.45 ± 0.05 g to 0.426 ± 0.05 g and 0.428 ± 0.05 g, respectively). In individuals infected by Lineage II, there was an increase in the mean organ weight at all times of infection: at 72 hpi, the mean weight was 0.43 ± 0.06 g, at 7 dpi, 0.468 ± 0.04 g and at 14 dpi, 0.477 ± 0.04 g. The difference between the means of individuals infected with Lineage I and Lineage II was statistically significant at 7 and 14 dpi ($p = 0.03$ and 0.048, respectively) and, the difference between Lineage II and uninfected mice was statistically significant at 7 and 14 dpi ($p = 0.035$ and 0.034, respectively) (Figure 1A–C). Likewise, the means of the ratio of the kidney weight to the total body weight of infected mice by both Lineages of DENV-2 Asian/American genotype, increased, on a small scale, when compared to the uninfected controls (mean = 1.496 ± 0.17%) at all times of infection. In Lineage I infected mice, the average percentage (1.621 ± 0.01%) was significantly higher than the observed for the uninfected control group at 72 hpi ($p = 0.013$), decreasing after the 7 dpi (1.522 ± 0.11%) and rising again, on the 14 dpi (1.576 ± 0.09%). In Lineage II infected mice, the highest ratio between the kidney and the body weight (mean = 1.663 ± 0.19%), was also statistically significant ($p = 0.005$) when compared to the uninfected control group, but it was observed at 7 dpi. Mice infected with this Lineage, and euthanized at 72 h and 14 dpi, also had higher means than those for the uninfected mice (1.624 ± 0.17% and 1.593 ± 0.12%, respectively) (Figure 1D–F). In addition, there was a statistically significant difference between the uninfected control group and the groups infected by Lineages I at 72 hpi ($p = 0.013$) and II at 72 hpi ($p = 0.005$) and at 7 dpi ($p = 0.005$); and between the infected groups at 7 dpi ($p = 0.005$) (Figure 1D–F).

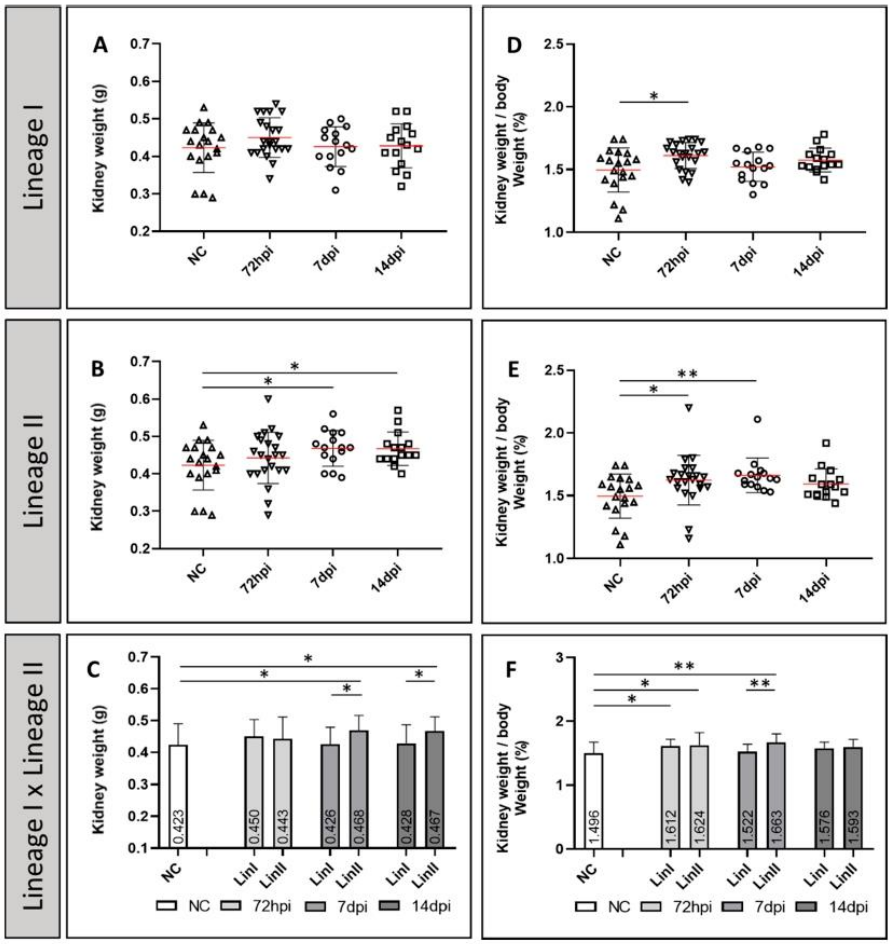

Figure 1. Mean kidney weight (**A**–**C**) and weight/body weight ratio (%) (**D**–**F**) of BALB/c mice uninfected and infected with DENV-2 Strains 72 hpi, 7 and 14 dpi. CN: (n = 19); LinI: 72 hpi (n = 22), 7 dpi (n = 15), 14 dpi (n = 15); LinII: 72 hpi (n = 22), 7 dpi (n = 15), 14 dpi (n = 15). NC: negative control, n: number of mice, hpi: hours post-infection, dpi: days post-infection, Lin: Lineage, *: $p < 0.05$, **: $p < 0.01$.

2.2. Evaluation of Urea (BUN) Levels

The concentration of BUN in serum of uninfected mice control group was 47.6 mg/dL and interquartile range (IR) = 41.9–44.8). The medians presented for Lineage I were 48.5 mg/dL (IR = 40.1–49.7) at 24 hpi; 41.8 mg/dL (IR = 37.4–48.3) at 48 hpi and 43.4 mg/dL (IR = 39–49) at 72 hpi. In Lineage II infected mice, the level of the organic compound present in the serum, rose on the first day of infection (median = 54.1 mg/dL, IR = 52.5–54.9) and peaked at 48 hpi (median = 58.6 mg/dL, IR = 51.9–67.7). At 72 hpi, BUN concentration (median = 43.4 mg/dL, IR = 41.1–46.4) was lower than that of the uninfected mice control group. The differences between the medians were statistically significant between negative control and Lineage II at 48 hpi (p = 0.028) and between the two DENV-2 Lineages at 24 and 48 hpi (p = 0.009 and 0.011, respectively) (Figure 2).

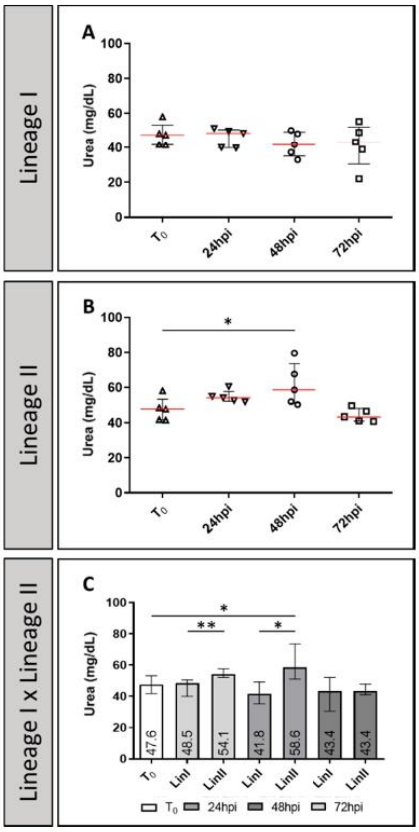

Figure 2. Urea levels (mg/dL) of BALB/c mice uninfected and infected with DENV-2 Lineages 24 hpi, 48 hpi and 72 hpi. CN: (n = 5); LinI: 24 hpi (n = 5), 48 hpi (n = 5); 72 hpi (n = 5); LinII: 72 hpi (n = 5); 7 dpi (n = 5); 14 dpi (n = 5). N: number of mice, hpi: hours post-infection. NC: negative control, Lin: Lineage, *: $p < 0.05$, **: $p < 0.01$.

2.3. Evaluation of Histopathological Alterations, Histomorphometry and Antigen Detection

Kidney tissues of uninfected mice presented well preserved Malpighian corpuscles (Mc), composed by Bowman capsule and glomeruli. There were no signs of atrophy, glomerulitis or bleeding. In addition, the interstice, proximal and distal contorted tubules and collecting tubules showed no signs of exudate, inflammatory infiltrates or hemorrhage (Figure 3A). The images displayed herein are representative of alterations observed in tissues of BALB/c mice infected with either Lineage I or II and euthanized at 72 hpi.

The morphological changes observed in kidney tissue of BALB/c mice infected by the two DENV-2 Lineages were focal and did not differ qualitatively. Mononuclear inflammatory cells infiltrate (Figure 3B), peritubular congestion (Figure 4C) and tubular necrosis (Figure 3C–E) were the most common histopathological changes observed among samples. In addition to desquamation of necrotic cells and loss of microvilli of cubic epithelium that constitutes the convoluted tubules (Figure 3C), high chromatin (Figure 3E) and cytoplasmic (Figure 3D) loss was observed. Those apparently vacuolated cells, when present in increased amount and clustered, gave the cortical parenchyma, a translucid appearance. Moreover, a number of tubular cells presented regular cytoplasmic inclusions (Figure 4A).

The commonest alteration observed in renal corpuscle was glomerular atrophy (Figure 4B), presented as an apparent reduction in the number of cells that constitute the glomerulus (Figure 4B') or with the glomerulus forming a compact and reduced cell

mass (Figure 4B″). In the latter, it was possible to notice the loss of integrity of the parietal layer of Bowman's capsule, and in both cases, an increase in Bowman's space was noted. Furthermore, in some renal cortex regions, it was not possible to distinguish the Bowman's space from the renal corpuscle due to enlargement of glomerular area caused by apparent cellularity increase (Figure 4D). Finally, focal hemorrhage (Figure 4E), a common DENV infection feature, was observed tissues from infected BALB/c with both DENV-2 lineages. Table 1 shows the number of mice in which each alteration was observed.

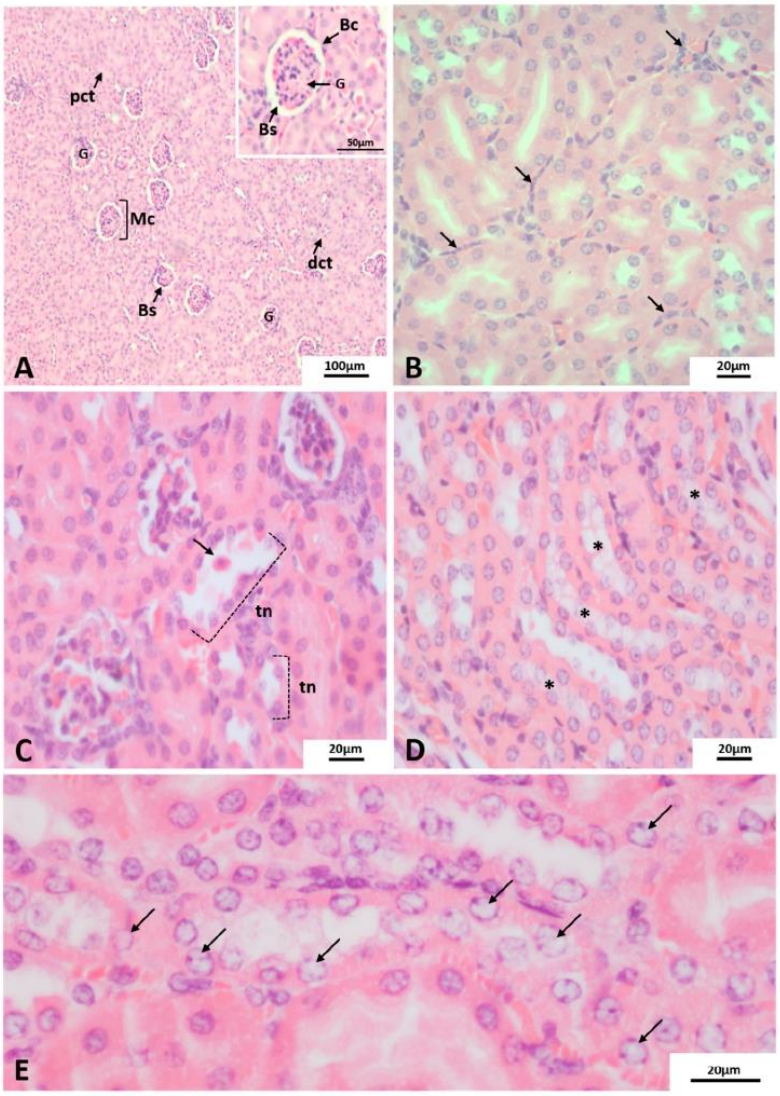

Figure 3. Histopathological alterations of renal cortex of BALB/c mice. H and E staining. Euthanasia; 72 hpi. (**A**) non-infected mice. (**B**–**D**) mice infected with DENV-2 Lineages. (**B**) mononuclear cell infiltrate, (**C**) tubular necrosis (tn), desquamation of necrotic cells (arrow). (**D**) areas of cytoplasmic loss (*) (**E**) chromatin loss [arrows]. Mc: Malpighian corpuscle, G: glomerulus, dct: distal convoluted tubules pct: proximal convoluted tubules. Bs: Bowman's space, Bc: Bowman capsule. Experimental infection: (**C**,**E**) Lineage I, (**B**,**D**) Lineage II.

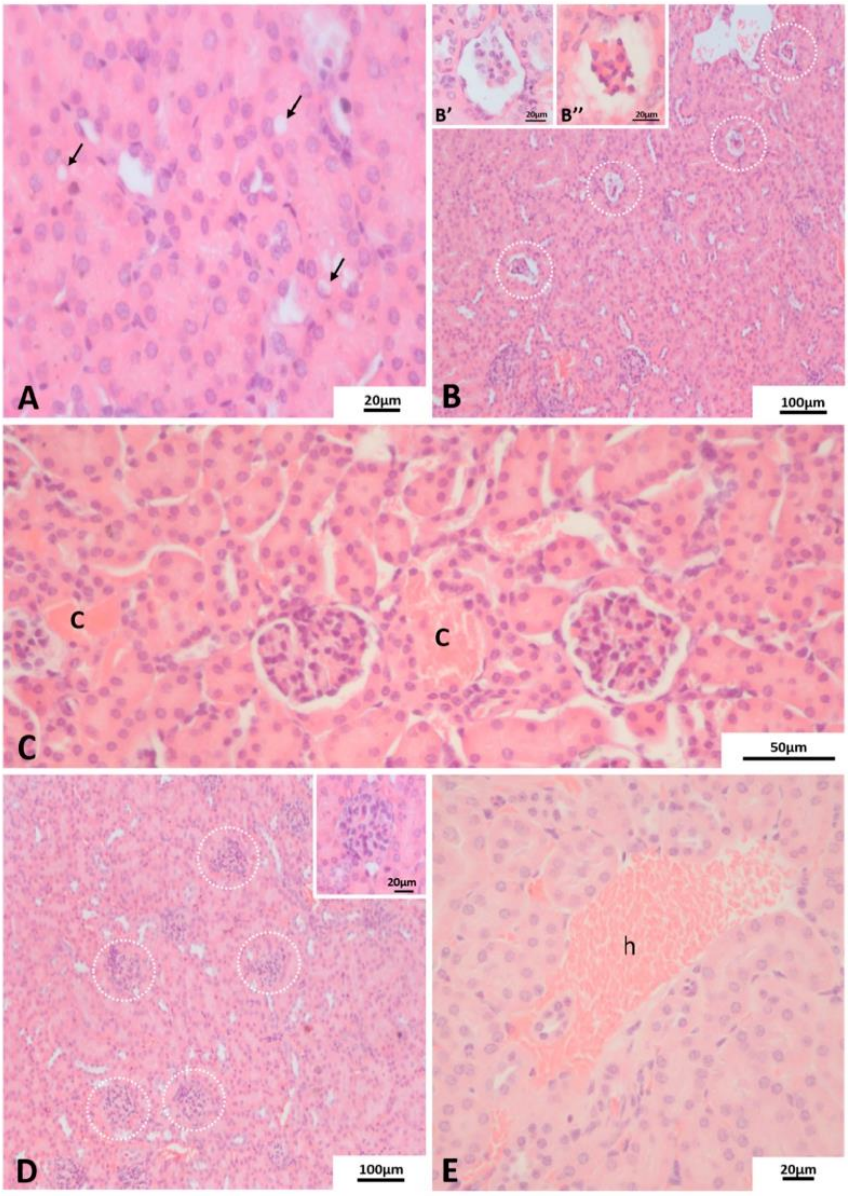

Figure 4. Histopathological alterations of renal cortex of BALB/c mice. H and E staining. Euthanasia; 72 hpi. (**A**) cytoplasmic inclusions (arrows). (**B**) glomerular atrophy (circled/insets). (**C**) congestion (C), (**D**) enlargement of glomerular volume (circled area/inset), (**E**) focal hemorrhage (h). Experimental infection: (**A**,**B**,**B′**,**B″**) Lineage I, (**C**–**E**) Lineage II.

Table 1. Histopathological alterations observed in kidney samples of BALB/c infected with DENV-2 Lineages I or II and euthanized at 72 hpi. Number of mice whose livers presented the alteration/total number infected mice.

Alterations	DENV-2		
	Lineage I (%)	Lineage II (%)	Total (%)
Tubular necrosis	10/10 (100)	10/10 (100)	20/20 (100)
Mononuclear cell infiltrate	9/10 (90)	10/10 (100)	19/20 (95)
Cytoplasmic loss	10/10 (100)	8/10 (80)	18/20 (90)
Capillary congestion	8/10 (80)	9/10 (90)	17/20 (85)
Glomerular atrophy	8/10 (80)	8/10 (80)	16/20 (80)
Chromatin loss	8/10 (80)	7/10 (70)	15/20 (75)
Enlargement of glomeruly	9/10 (90)	6/10 (60)	15/20 (75)
Cytoplasmic inclusions	3/10 (30)	3/10 (30)	6/20 (30)
Haemorrhage	1/10 (10)	3/10 (30)	4/20 (20)

As expected, no DENV antigen staining was observed in the kidney tissue of the uninfected mice control group (Figure 5A). However, samples from Lineage II infected BALB/c, viral staining was observed in epithelial cells in the medullary area (Figure 5B) and endothelial cells in the cortical area (Figure 5C).

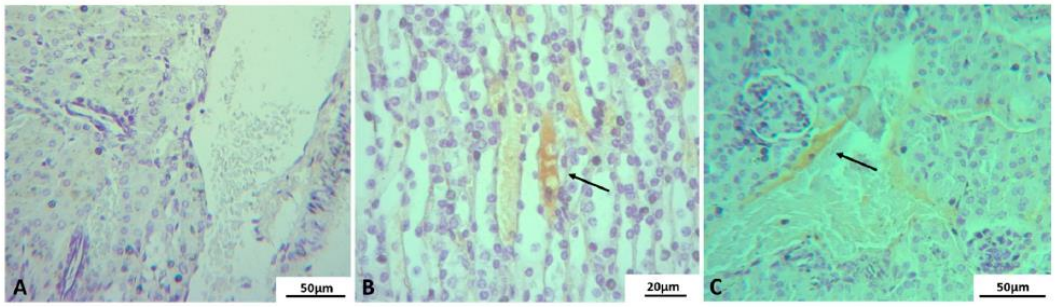

Figure 5. DENV antigen detection in kidney of BALB/c mice infected with DENV-2 Lineage II. Euthanasia: 72 hpi. (**A**) Negative control showing no peroxidase reactive cells, (**B**) peroxidase reactive epithelial cells from the loop of Henle (arrow), (**C**) peroxidase reactive endothelial cells (arrow). Experimental infection: Lineage II.

At 72 hpi, a statistically significant decrease in the number of corpuscles in mice infected with both Lineage I ($p = 0.016$) and Lineage II ($p \leq 0.001$) was observed when those were compared to uninfected BALB/c. In the latter, it was possible to observe 3.89 ($\pm$1.8) Mc per analyzed area. For mice infected with DENV-2 Lineages I and II, the averages were 3.61 ($\pm$1.6) and 3.15 ($\pm$1.6) Mc per analyzed area, respectively. The difference in the number of renal corpuscles between the two DENV-2 Lineages was also statistically significant ($p \leq 0.001$) (Figure 6A). The mean area occupied by glomeruli was measured and, despite the decrease in the number of Mc and glomerular atrophy observed in the infected mice, the mean area occupied by glomeruli in mice infected with DENV-2 exceeded the control group ($p \leq 0.001$ for both strains) (Figure 6B).

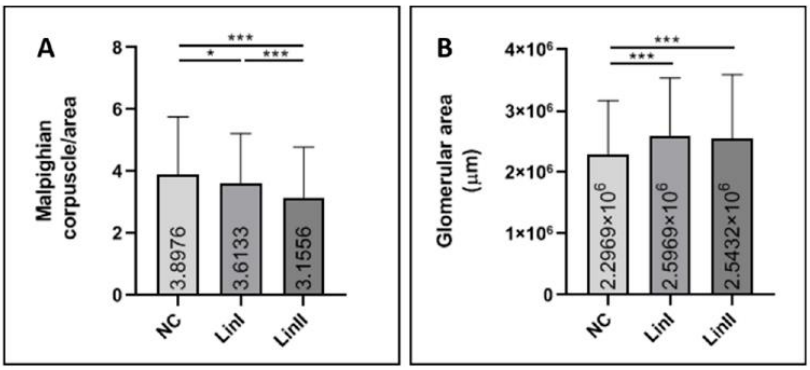

Figure 6. Malpighian corpuscles count (**A**) and mean area occupied by glomeruli (**B**) of BALB/c mice infected with DENV-2 Lineages. Euthanasia; 72 hpi. Glomerular area standard deviation: NC ($\pm 8.69 \times 10^5$), Lin I ($\pm 9.41 \times 10^5$), Lin II ($\pm 1.04 \times 10^6$). NC: negative control, Lin: Lineage. *: $p < 0.05$, ***: $p < 0.00$.

2.4. Evaluation of Ultrastructural Alterations

The ultrastructural analysis of kidney tissue of uninfected and infected BALB/c mice euthanized at 72 hpi, corroborates histopathological findings. As expected, tissues from uninfected control mice showed no damage to the renal parenchyma. Epithelial cells presented round nuclei with regular looking chromatin pattern and no cytoplasmic rarefaction or pyknotic nuclei were observed (Figure 7A).

The renal tissue of DENV-2 infected mice showed cytoplasmic rarefaction (Figure 7B–D), pyknosis (Figure 7B,C) and death of convoluted tubule's epithelial cells (Figure 7B), which are consistent with tubular necrosis. Additionally, a number of epithelial cells presented altered distribution and amount of chromatin in the nucleus, which could be caused by a process of karyolysis (Figure 7D).

Mononuclear inflammatory cells infiltrates were present both in renal interstitium (Figure 7E) and within glomeruli (Figure 8A,B). Some glomeruli appeared to be congested due to increase in cellularity and edema, and as observed in our histological sections, Bowman's space could not be distinguished (Figures 7F and 8A). Moreover, focal areas in the renal cortex were congested (Figure 8D) and small vesicles (Figure 8E) and inclusions of unknown nature were observed within epithelial cells (Figure 8F) and in the edematous area of a glomerulus (Figure 8A).

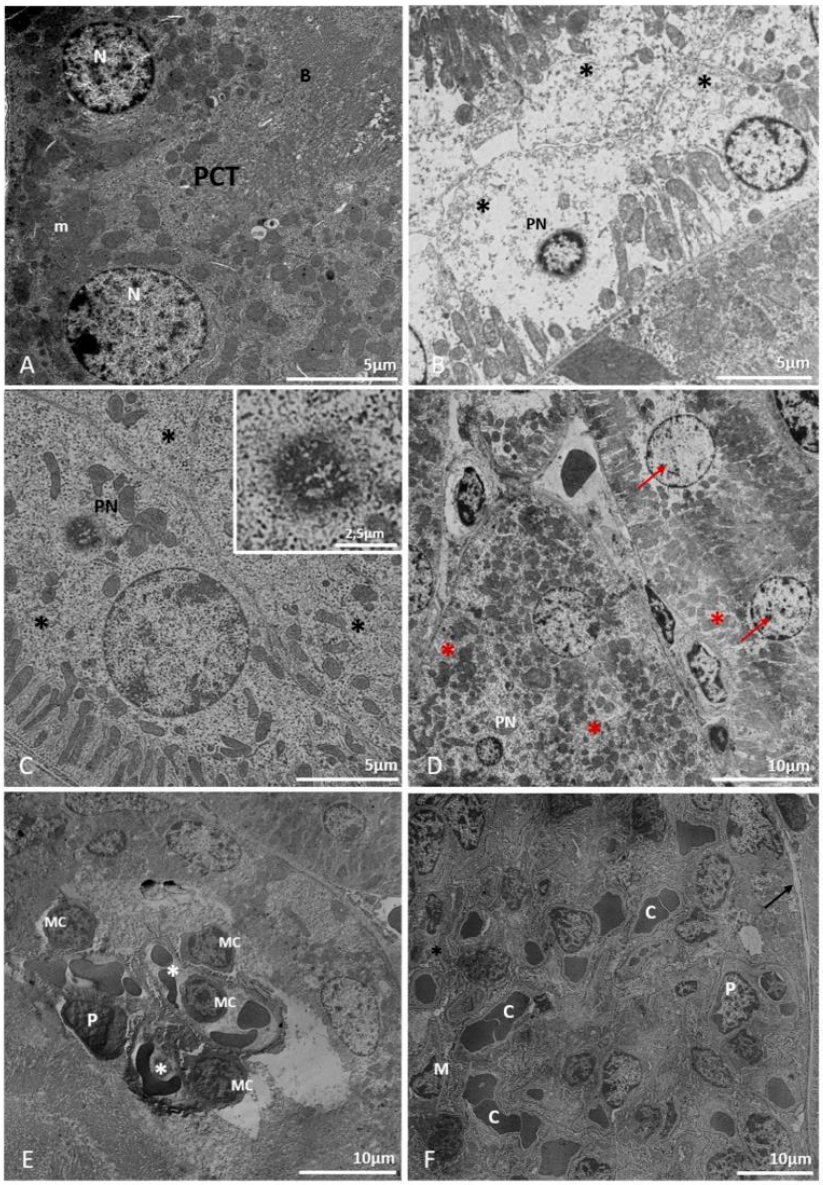

Figure 7. Electron micrography of kidney samples of BALB/C mice. (Noninfected: A; infected with DENV-2 Lineage I or II: (**B**–**E**)). (**A**): proximal convoluted tubule (PCT), brush border (B), nucleus (N), mitochondria (m). (**B**): pyknotic nuclei (PN), massive cytoplasmic loss (*). (**C**): pyknotic nuclei (PN/arrow), cytoplasmic rarefaction (*). (**D**): pyknotic nuclei (PN), chromatin loss (arrow). cytoplasmic rarefaction (*), (**E**): glomerular mononuclear infiltrate. Mononuclear cell (MC), podocyte (P), capillary (*), (G) glomerulus, (**F**): capillary congestion. (**C**), podocyte (P), mesangial cell (M), mononuclear cell (MC), reduced Bowman space [arrow]. Experimental infection: Lineage I (**C**,**E**), Lineage II (**B**,**C**,**F**).

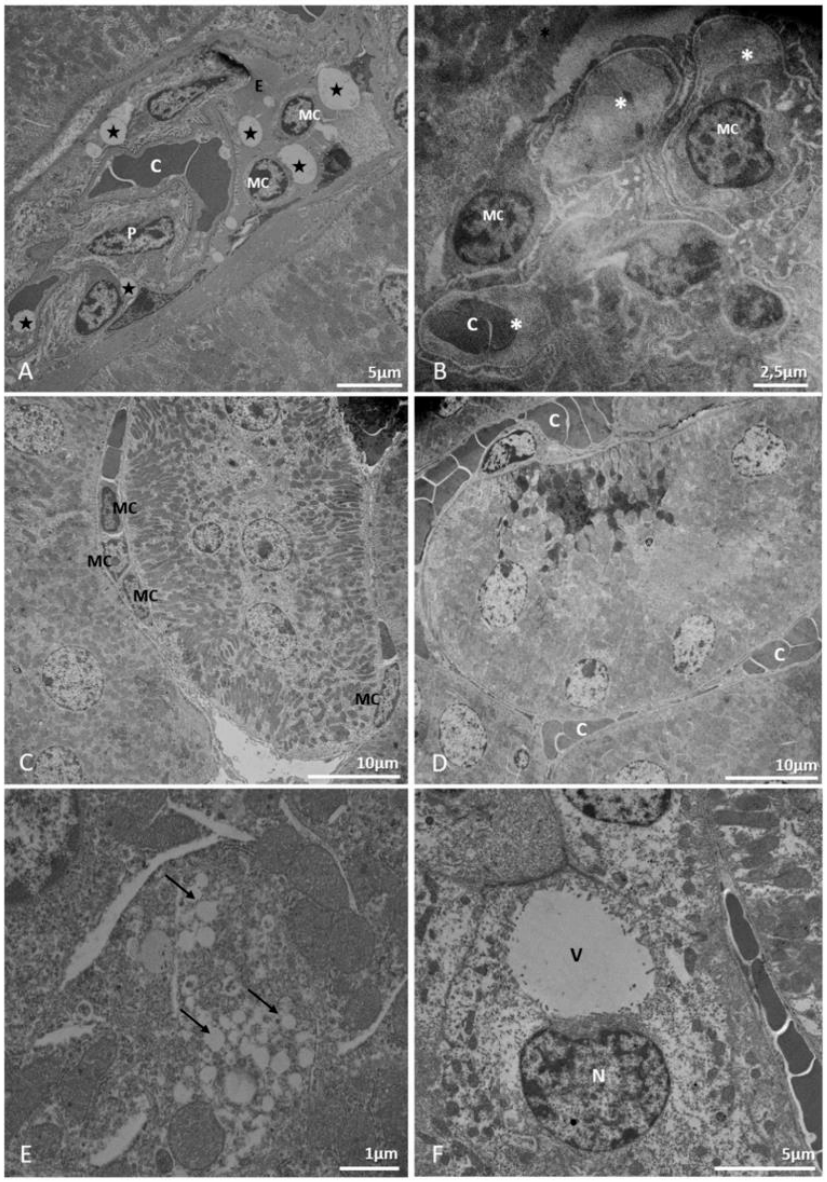

Figure 8. Electron micrography of kidney samples of BALB/C mice infected with DENV-2 Lineage I or II. (**A**,**B**): congested glomeruli. Capillary congestion (C), edema [E], vesicles within glomerulus [star], capillary (*), mononuclear cell (MC). (**C**): mononuclear cell infiltrate (MC). (**D**): capillary congestion. (**E**): lipid-like cytoplasmic inclusions (arrows). (**F**): nucleus (N) dislocated by vesicle (V). Experimental infection: Lineage I (**B**,**C**), Lineage II (**A**,**D**–**F**).

3. Discussion

In Brazil, the emergence of the Lineage II DENV-2 in 2007–2008 resulted in major outbreaks with a new epidemiological profile and number of severe cases, hospitalizations and deaths, especially in children 15 years old and under [6,8–10,64]. Moreover, it has been

shown that genetic variations are important determinants of viral fitness, virulence and tropism [47,59,65].

Although some authors suggest that the kidney is not a target organ of DENV due to the lack of evidence of viral replication [13,17], renal involvement during dengue is fairly well documented. Proteinuria, hematuria and glomerulonephritis have been reported during or shortly after acute DENV infection. Moreover, AKI and ARF are somewhat common features among patients with DHF/DSS [22,34,35,43,66–69]. Because data on renal manifestations induced by DENV infection are not as abundant as data concerning target organs, in this study, we sought to characterize and compare the alterations induced by two distinct DENV-2 Lineages of the Asian/American genotype in the kidney of BALB/c mice.

Our results showed that there was a tendency of kidney weight increase in some infected mice when compared to uninfected control group. To ensure that the increase in kidney weight was not solely a result of body weight gain, the kidney weight/body weight ratio (%) was calculated, and means of the infected group were also higher than control group. Despite that, to our knowledge, there are no reports of kidney weight increase due to DENV infection, liver, spleen and pancreas weight increases during DENV infection, and hepatomegaly, splenomegaly and pancreatic enlargement have been associated with dengue [70–73].

Altered vascular permeability, accompanied by plasma and albumin leakage, is a common feature of dengue pathophysiology [5], organ enlargement or increase in weight could be a consequence of fluid accumulation in the interstice. We did not observe interstitial edema in the renal tissue analyzed here, only fluid leakage inside glomeruli; however, another study on the same mice model infected by DENV-3, also carried out by our group, showed transudate in the kidney's cortical area and reported statistically significant increase in kidney weight [74]. Furthermore, histopathological findings on autopsies of human fatal cases also showed edema, although mostly in the medullary region [17,75].

For each strain, the weight means reached higher values at distinct times of infection. Lineage I peaked at 72 hpi, while Lineage II peaked at 7 dpi. Difference between the two Lineages was statistically significant at 7 and 14 dpi. A possible explanation for such difference is that Lineage II takes longer to manifest its signs.

Since urea is reabsorbed by the kidney, its altered levels in the blood may indicate renal dysfunction. Increased levels of BUN are often reported is renal involvement during DENV infections [22,34,37,76,77], and can be a consequence of glomerular injury or hypotension [37]. Mice blood samples were collected at 24, 48 and 72 hpi, and BUN levels were measured. While the median of the Lineage I infected mice was higher than the control group median only at 24 hpi, Lineage II infected mice presented higher BUN levels when compared to those of the uninfected group, at 24 and 48 hpi. Increased levels of BUN in the sera of BALB/c mice infected by DENV-3, supports our findings [74]. Furthermore, difference between Lineage II and control group was statistically significant at 48 hpi and between Lineages I and II infected mice, at 24 and 48 hpi, and this difference may be due to the different viral strains or host genetic factors.

The morphological changes described in this study were from kidney of mice infected with each one of the DENV-2 Lineages and euthanized 72 hpi. The alterations observed in renal tissues were focal and did not differ qualitatively. On the other hand, quantitatively, the difference between Lineages I and II, regarding enlargement of glomeruli (90% and 60% of infected mice, respectively) was noteworthy. In accordance with these results, morphometric analysis revealed that, on average, glomerular area in mice infected with the Lineage I slightly exceeded the glomerular area in mice infected with by Lineage II. Nonetheless, the difference was not significant.

Glomerular changes affecting the kidney are often reported in dengue human cases [31,34,43] and, DENV inducing glomerulopathies is well documented [34,67,77,78]. Deposition of immuno complexes has been suggested as a mechanism of glomerular injury in AKI induced by dengue [28]. Moreover, it has been suggested that glomerulonephritis results from an autoimmune mediated glomerular damage triggered by the virus [79].

In this study, renal tissue of DENV-2 infected BALB/c mice presented both glomerular atrophy and enlargement of glomeruli. Indeed, the morphometric analysis showed that, while, on average, the glomerular area of infected mice exceeded that of the uninfected control group, Mc/glomeruli on uninfected mice kidneys outnumbered the ones in infected groups. Our findings on Mc counting could suggest that the atrophy is an early stage of necrosis, since, in a similar study, Caldas that observed glomeruli in different stages of atrophy, as well as, areas free of glomeruli in the renal cortex of BALB/c mice infected with DENV-3. Difference of Mc number was statistically significant among all groups.

We observed areas with enlarged glomeruli, due to increased cellularity, to the point of Bowman's space obliteration. By TEM, it was possible to observe that the enlargement was a result of mesangial proliferation and mononuclear cells migration. Analysis of tissues from dengue patients and BALB/c mice experimentally infected has associated mesangial proliferation with deposition of immuno complexes in glomeruli [33,58,80,81]. The increase in the cellularity resulted in congested capillaries and edema. Nunes [27] and Pagliari [43] also observed congestion in capillaries in glomeruli; however, no mesangial proliferation was reported.

One of the hallmarks of dengue pathogenesis is the involvement of the endothelium [82]. Vascular permeability plays an important role in SD pathogenesis [83,84]. Our ultrastructural studies showed some fluid leakage in the glomeruli, but we did not observe any endothelial damage. The capillary permeability could be due to the release of inflammatory mediators from the mononuclear cell present, both inside capillaries and among mesangial cells, in the glomeruli. This hypothesis is supported by authors who believe that altered vascular permeability in dengue is caused by immunological host response, rather than by infection of endothelial cells or cell death [84–86]. Moreover, our histopathological findings showed hemorrhage foci and small mononuclear cells infiltrates in the cortical area of kidney of the DENV-2 infected mice and corroborates studies carried out with human cases of DENV-3 and -4 infection, and BALB/c mice infected with DENV-3 [17,27,74,75,87].

Studies on human autopsy tissues have described tubular necrosis on dengue cases [17,27,43]. Mohsin [76] reported a case of ARF with tubular necrosis in a dengue patient who did not present signs of hemorrhagic fever, only classic dengue symptoms. Moreover, it has been suggested that necrosis results from ischemic processes due to severe hypovolemic shock, hypoprofusion and hypoxia which leads to decreased kidney perfusion, interstitial edema and mononuclear infiltration, and acute glomerulonephritis [17,76,88,89]. In a BALB/c model, Caldas [74] observed mitotic figures, indirect signs of hyperplasia, which may be related to tubular injury.

In this study, all kidneys of DENV-2 infected mice presented tubular necrosis, mostly in proximal convoluted tubules. The tissues showed desquamation of epithelial cells and loss of the border brush. Conversely, Caldas [74] observed the thickening of the brush boarder. Autopsy data described loss of basement membrane, pyknotic nuclei and dilation of endoplasmic reticulum in necrotic cells; tubular hemorrhage and atrophy with discrete mononuclear inflammatory infiltrate. Besides that, IL18 and IL6, both proinflamatory citokines, were detected in tubular cells [17,27,43,75]. Here, some nuclei were pyknotic, an alteration seen in the same murine model infected by DENV-3 [74]. Others, presented massive chromatin loss. There were areas of cytoplasmic rarefaction and some tubular epithelial cells were lightly stained, even though the plasmatic membrane looked intact. Upon ultrastructural analysis, it was possible to see that those cells had lost most of its contents and cytoplasm was almost completely absent.

Additionally, histological analysis revealed some dislocated nuclei due to fairly large unstained round cytoplasmic inclusions resembling lipid droplets. These inclusions were also seen by Caldas [74]. In fact, samples presented small lipid-like inclusions, however, the structures responsible for nuclei dislocation were, actually, vesicles that, to our knowledge, has not been described as an alteration in renal tissue during dengue.

DENV-like particles have been observed in ultrathin sections of BALB/c mice infected by DENV-3 and tissues of renal biopsy [28,74], but they were not observed here. At 72 hpi,

two out of 10 kidneys of the infected mice were positive for the viral antigen, both of them, from mice infected by Lineage II, and morphological changes compatible with those reported both in human cases and animal models of infection [17,58,69,74,75,90] were also observed in this study. The detection of anti-DENV antigen, in tubular cells and infiltrate macrophages and monocytes, has been reported in studies on human cases and experimental models [13,15,17,27,90,91], however, no virus RNA negative strand was detected and, NS3 was only detected by Nunes [27] in mesangial cells and macrophages. Even though some studies report viral RNA detection in kidneys of human biopsies and animal models tissues [15,27,74,90], some authors hypothesize that DENV antigens detected in the kidney are from reabsorbed immuno complexes, and that it is more likely that damage to renal parenchyma is caused by immuno-mediators released as a host response to DENV infection [17,43,44], or secondary to other dengue complications, such as rhabdomyolysis, myositis, hypoperfusion and hypoxia [37,42,66,92–94].

Several studies have reported DENV infection leading to kidney injury, especially in SD. Mortality rates among DHF patients who develop AKI can reach up to 60% [89]. Therefore, the mechanisms behind renal involvement in dengue must be better understood. Our results show that BALB/c mice infected with two distinct Lineages of DENV-2 Asian/American genotype presents renal alterations that are commonly observed in human cases and may be a suitable experimental model for studies of pathophysiology and immunopathogenesis of dengue in kidney.

4. Material and Methods

4.1. Ethical Statement

All experiments with mice were conducted in compliance with Ethical Principles in Animal Experimentation stated in the Brazilian College of Animal Experimentation and approved by the Institute's Animal Use Ethical Committee (L-023/2018) and the Human Research Ethic Committee (274/05) from the Oswaldo Cruz Institute (IOC), Oswaldo Cruz Foundation (FIOCRUZ).

4.2. DENV-2 Viral Strains

DENV-2 strains BR/RJ66985/2000 (GenBank #HQ012518) and BR/RJ0337/2008 (GenBank #HQ01253), representative of Lineage I and Lineage II from the Asian/American genotype [6], were isolated from patient sera at the Flavivirus Laboratory, IOC, FIOCRUZ, during the epidemics of 2000 and 2008, respectively, and kindly provided. Serotype was confirmed by indirect immunofluorescence, using DENV-type-specific monoclonal antibody (3H5), and RT- PCR [95,96]. Viral stocks were prepared by inoculating 100 µL of each strain into 175 cm^2 cell culture bottles containing mosquito Aedes albopictus cell line (C6/36) at a concentration of 5×10^5 cells/mL. Titers of both strains (BR/RJ66985/2000: $10^{6.66}$ $TCID_{50}$/0.1 mL and BR/RJ0337/2008: 10^9 $TCID_{50}$/0.1 mL) were calculated by the Reed and Muench method [97]. The viruses did not undergo any passages through mice brain for neuroadaptation.

4.3. BALB/c Experimental Infection

For experimental infection, two-month-old, male BALB/c mice, provided by Institute of Science and Technology in Biomodels (ICTB)-Fiocruz, were used. During experimentation period, the animals were kept under controlled temperature, photoperiod, nutrition and hydration conditions, as previously described [26]. Briefly, for infection with both Lineages I and II of DENV-2, BALB/c mice were inoculated by the intravenous route (IV) through the caudal vein. Inocula volume was 100 µL and viral concentration, 10,000 $TCID_{50}$/0.1 mL. The mice were anesthetized [ketamine = 150 mg/kg, xylazine = 10 mg/kg and tramadol = 10 mg/kg] and euthanized 24, 48 and 72 h post-infection (hpi), at 7 or 14 days post-infection (dpi), according to their experimental group. In order to collect blood samples, cardiac puncture was performed before euthanasia. Kidney samples, destined to morphological and immunohistochemistry analysis, were

fixed in Millonig buffered formalin. All kidney samples were weighted immediately after harvesting at 72 hpi, seven and 14 dpi. Non-infected mice were used as negative controls. Table 2 shows the number of mice used in the study.

Table 2. Number of mice used for histopathological, immunohistochemical and biochemical analysis and measuring of kidneys' weight.

n = 123 Mice	Histopathology/IHQ	TEM	Biochemical Analysis			Kidney Weight		
	72 hpi	72 hpi	24 hpi	48 hpi	72 hpi	72 hpi	7 dpi	14 dpi
DENV-2/Lineage I	10/5	5	5	5	5	22	15	15
DENV-2/Lineage II	10/5	5	5	5	5	22	15	15
Negative control	5/5	5			5			19
Total [samples]	25	15		35			123	

IHQ: immunohistochemistry, TEM: transmission electron microscopy, hpi: hours post infection, dpi: days post infection.

4.4. Biochemical Analysis

For each DENV-2 Lineage, a total of 15 mice were infected. The mice were divided into three groups of five animals, and each group euthanized at different times after infection (24, 48 and 72 hpi). After the determined periods of infection, the mice were anesthetized and blood was collected by cardiac puncture. Blood samples were then centrifuged for 10 min, at 5000 rotations per minute, to separate the serum from the cellular components. Non-infected mice (n = 5) blood was collected at the same day as the 72 hpi group. Blood levels of urea were measured by dry chemistry testing using the Vitros 250 equipment (Ortho clinical Diagnostics, Jonhson & Jonhson, New Brunswick, NJ, USA) in collaboration with ICTB.

4.5. Bright Field Microscopy

For each DENV-2 Lineage, 10 mice were infected. Five non-infected mice were used as negative control. Seventy-two hpi, the mice were euthanized, kidney samples were collected and fixed in Millonig buffered formalin (formalin P.A.: 100 mL, distilled water: 900 mL, NaH_2PO_4: 18.03 g, NaOH: 4.2 g). The samples were then dehydrated in decreasing concentrations of ethanol, clarified in xylene and embedded in paraffin. Tissue sections 5 µm thick were obtained using a microtome (Leica 2025) and stained with hematoxylin and eosin (H and E) and analyzed using a bright field microscope (AxioHome, Carl Zeiss, Oberkochen, Germany). All procedures were performed in collaboration with the Pathology Laboratory, IOC, FIOCRUZ.

4.6. Immunohistochemistry

For DENV antigen detection in kidney tissue an immunohistochemistry assay was performed. Briefly, five slides containing histological sections of kidney infected with each DENV-2 lineage were heated at 60 °C for one hour, de-paraffinized in xylene and rehydrated with alcohol. Antigen retrieval was performed by heating the tissue in the presence of citrate buffer. Next, tissues were blocked for endogenous peroxidase with 3% hydrogen peroxidase in methanol for 10 minutes and rinsed in tris-HCl (pH 7.4). To reduce non-specific binding, sections were incubated in Protein Blocker solution (Spring Bioscience, Pleasanton, CA, USA) for 10 min at room temperature. Tissues were incubated with rabbit anti-dengue 4G2 antibody (1:200), used as the primary antibody, and afterwards, with a rabbit anti-mouse IgG-HRP conjugate (REVEAL polyvalent HRP, Spring Bioscience, Pleasanton, CA, USA). Finally, the slides were counterstained with Harris hematoxylin, and analyzed using a bright field microscope (AxioHome, Carl Zeiss, Oberkochen, Germany). Samples from non-infected mice were used as negative control.

4.7. Histomorphometry

Morphometrical analysis goals were quantifying the number Malpighian corpuscles (Mc) and measuring the area occupied by glomeruli in kidney samples of each group of

mice. Fifteen histological sections of kidney of BALB/c mice euthanized at 72 hpi stained with H and E (5 from non-infected mice as negative control, 5 from mice infected with Lineage I of DENV-2 and 5 from mice infected with Lineage II of DENV-2) were analyzed. For each section, 30 images of random areas were captured at a 200× magnification using a digital camera coupled to a bright field microscope (AxioHome, Carl Zeiss, Oberkochen, Germany). The analyses were performed with the aid of the image processing program Image J. Aiming at the recognition and calculation of the area occupied by the structure, glomerular area was delimited and colored differently from the colors present in the studied field. The values obtained were then compiled by group and the mean was calculated.

4.8. Transmission Electron Microscopy (TEM)

Kidney tissues were processed as described by Barreto-Vieira [98]. Briefly, samples were fixed by immersion in 2% glutaraldehyde diluted in sodium cacodylate buffer (0.2 M, pH 7.2), cut into smaller fragments (~1 mm^3), post-fixed in 1% osmium tetroxide and dehydrated in increasing concentrations of acetone. Subsequently, samples were embedded in Epoxy resin (Electron Microscopy Sciences, Hatfield, PA, USA). For light microscopy, semithin sections (0.5 µm) were stained with methylene blue and azure II and analyzed using a Zeiss PrimoStar light microscope (Carl Zeiss, Oberkochen, Germany). Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed using a Hitachi HT 7800 transmission electron microscopy (Hitachi, Tokyo, Japan).

4.9. Statistical Analysis

A database with data related to organ weight, histomorphometry and biochemical analysis was created in Microsoft Excel. The graphs were created using the GraphPad Prism software version 8.0.1. For statistical analysis, a *t*-test was performed when groups presented normal distribution and a Mann–Whitney test was performed when groups presented non-normal distribution, using the SPSS Statistics software version 25. Results of $p \leq 0.05$ were considered statistically significant.

Author Contributions: D.F.B.-V. and F.B.d.S. conceived the study; F.C.J. designed and supervised the experiments; and was involved in data curation; D.D.C.d.S., A.C.P. and M.A.N.d.S. conducted the experiments; F.C.J., G.C.C., A.L.T.d.A. and A.d.C.R. analyzed all images and data; F.C.J. drafted the manuscript; D.F.B.-V., F.B.d.S. and O.M.B. reviewed the manuscript. O.M.B. and D.F.B.-V. were responsible for funding acquisition and resources. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by Laboratory of Viral Morphology and Morphogenesis, Instituto Oswaldo Cruz, Fiocruz, FIOTEC to D.F.B.-V. [grant number IOC-023-FIO-18-2-58], Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro [FAPERJ] to F.B.d.S. [grant number E-26/202.003/2016], Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 302462/2018-0 to F.B.d.S.; grant number: 301992/2017-7 and to O.M.B. [grant number: 301992/2017-7], Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and CNPq for the students fellowships. The funders had no role in the study design, data collection, analysis and decision to publish or preparation of the manuscript.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Instituto Oswaldo Cruz (L-023/2018; 11 July 2018 and 274/05; 7 April 2014).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We would like to thank to the Flavivirus Laboratory, IOC/FIOCRUZ, for providing the viral strains, to Marcelo Pelajo and João Paulo Rodrigues, Pathology Laboratory, IOC/FIOCRUZ, for the support in sample processing and technical analysis. Felipe Anibal Carvalho Costa, Epidemiology and Systematics Laboratory, IOC/FIOCRUZ, for the support with statistical analysis, Raphael Leonardo, Laboratory of Viral Morphology and Morphogenesis, IOC/FIOCRUZ, for technical support and ICTB, IOC/FIOCRUZ, for the support in sample processing.

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

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3.3. Artigo 3: Camundongos imunocompetentes infectados por duas linhagens do vírus dengue tipo 2: observações sobre a patologia do pulmão, coração e músculo esquelético

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Resumo

A infecção pelo vírus da dengue (DENV) por um dos quatro sorotipos (DENV-1 a 4) pode resultar em um amplo espectro de manifestações clínicas, com evolução imprevisível e envolvimento de órgãos. Devido à sua associação com epidemias graves e manifestações clínicas, o DENV-2 tem sido frequentemente investigado. De fato, o primeiro surgimento de uma nova linhagem do genótipo Asiático/Americano de DENV-2 no Brasil (Linhagem II) em 2008, foi associado a casos graves e aumento da mortalidade relacionada ao envolvimento de órgãos. Um grande desafio para os estudos da patogênese da dengue é a falta de um modelo animal adequado. Entretanto, o uso de camundongos imunocompetentes, embora às vezes controverso, tem se mostrado útil, pois observações histológicas em animais infectados revelam alterações teciduais consistentes com as observadas em casos humanos de dengue. Aqui, nosso objetivo foi investigar os resultados causados por duas linhagens distintas do genótipo Asiático/Americano de DENV-2 em tecidos do pulmão, coração e músculo esquelético de camundongos BALB/c infectados. Os tecidos de camundongos infectados com uma Linhagem ou outra, foram submetidos à análise histopatológica, imunohistoquímica, de histomorfometria e de microscopia eletrônica de transmissão (MET). Ademais, a creatina quinase (CK) foi dosada em amostras de soro destes animais. O genoma viral foi detectado em amostras de coração e músculo esquelético e o antígeno viral, em cardiomiócitos e células endoteliais de tecido cardíaco. Amostras de tecido cardíaco e pulmonar apresentaram alterações morfológicas comparáveis às observadas em casos humanos de dengue. Os níveis séricos de creatina quinase foram maiores em camundongos infectados com ambas as linhagens de DENV-2. Além disso, diferenças estatisticamente significativas, em relação ao espessamento dos septos alveolares e peso do coração, foram observadas entre os camundongos BALB/c infectados com ambas as linhagens de DENV-2, o que demonstrou que o BALB/c é modelo experimental apropriado para estudos de patogênese da dengue no pulmão, coração e músculo esquelético.

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[Microorganisms] Manuscript ID: microorganisms-1395218 - Accepted for Publication

1 mensagem

Microorganisms Editorial Office <microorganisms@mdpi.com> 28 de setembro de 2021 09:53
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Type of manuscript: Article
Title: Immunocompetent mice infected by two Lineages of dengue virus type 2: observations on the pathology of the lung, heart and skeletal muscle
Authors: Fernanda Cunha Jácome *, Arthur da Costa Rasinhas *, Gabriela Cardoso Caldas, Ana Luisa Teixeira de Alameida, Daniel Dias Coutinho de Souza, Amanda Carlos Paulino, Marcos Alexandre Nunes da Silva, Derick Mendes Bandeira, Ortrud Monika Barth, Flávia Barreto dos Santos, Débora Ferreira Barreto-Vieira
Received: 9 September 2021
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Article

Immunocompetent mice infected by two Lineages of dengue virus type 2: observations on the pathology of the lung, heart and skeletal muscle

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Abstract: Dengue virus (DENV) infection by one of the four serotypes (DENV-1 to 4) may result in a wide spectrum of clinical manifestations, with unpredictable evolution and organs involvement. Due to its association with severe epidemics and clinical manifestations, DENV-2 has been commonly investigated. In fact, the first emergence of a new Lineage of DENV-2 Asian/American genotype in Brazil (Lineage II) in 2008, was associated to severe cases and increased mortality related to organs involvement. A major challenge for dengue pathogenesis studies has been a suitable animal model, but the use of immune-competent mice, although sometimes controversial, has proven to be useful, as histological observations in infected animals reveal tissues alterations consistent to those observed in dengue human cases. Here, we aimed to investigate the outcomes caused by two distinct Lineages of DENV-2 Asian/American genotype in the lung, heart and skeletal muscle tissues of infected BALB/c mice. Tissues were submitted to histopathology, immunohistochemistry, histomorphometry and transmission electron microscopy (TEM) analysis. The viral genome was detected in heart and skeletal muscle samples and the viral antigen, in cardiomyocytes and endothelial cells of heart tissue Heart and lung tissue samples presented morphological alterations comparable to those seen in dengue human cases. Creatine kinase serum levels were higher in mice infected with both lineages of DENV-2. Additionally, statistically significant differences, concerning alveolar septa thickening and heart weight, were observed between both DENV-2 Lineages infected BALB/c mice, which was demonstrated to be an appropriate experimental model for dengue pathogenesis studies on lung, heart and skeletal muscle tissues.

Keywords: Dengue virus serotype 2; lineages; BALB/c mice, lung, heart; skeletal muscle

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Microorganisms* **2021**, *9*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

1. Introduction

The incidence of dengue has grown dramatically around the world in recent decades and currently, more than half of the global population lives in areas with risk of DENV transmission [1, 2]. Infection by any of the four serotypes may result in a wide spectrum of clinical manifestations with unpredictable evolution and outcome, varying from a self-limited flu-like illnesses to a severe form of the disease, characterized by thrombocytopenia, coagulopathy, increased vascular permeability that may lead to multiple organ impairment, hemorrhage, hypovolemic shock and death [3, 4, 5, 6]. Moreover, during infection, organs involvement such as hepatic, cardiac, renal, pulmonary, neurological, muscular, splenic, gastrointestinal and even ocular injury can be observed [5, 7, 8, 9].

Heart, lung and skeletal muscle are not considered the main DENV targets. Regarding the lung, a study has suggested that pulmonary involvement during DENV infection are mild to moderate and is more likely to be observed in patients presenting severer symptoms [10]. Respiratory complications vary from dyspnea to acute respiratory failure and/or acute respiratory distress syndrome [9, 11]. Since its first report in 1926 [12], cardiac involvement during dengue has been increasingly reported and it is mostly associated with severe cases [13-18]. Several arboviruses have tropism for muscle cells and can cause pathological alterations of the skeletal muscle [19]. Myalgia is a common feature in dengue patients and muscle pain can persist beyond the acute phase of dengue [20]. Moreover, some studies have reported muscle alteration in human samples [21-26].

The pathophysiological mechanisms involved in severe cases are still to be fully elucidated (Guzman, 2015), but do consider viral strains virulence [5, 27]. DENV-2 has traditionally been the most studied serotype due to its association with large epidemics and severe clinical manifestations [28]. Moreover, the DENV-2 Asian/American genotype, introduced in Brazil in the 90's and currently circulating in the country [29-32], has been associated with a higher fitness in both humans and mosquitoes [33]. The emergence of a new Lineage (Lineage II) of the DENV-2 Asian/American genotype in 2008, different of that introduced in the 90's (Lineage I), was associated with increased pathogenicity, reflected by the high number of severe cases, hospitalizations and deaths in Brazil [28, 34, 35]. Replacements of DENV-2 Lineages over outbreaks has been described as a common phenomenon in American countries [31].

The establishment of experimental models for DENV infections studies and their impact on disease severity is of particular relevance. Immunocompetent mice, including BALB/c, have been used for DENV pathogenesis and tropism studies by using different infection routes and clinical signs as well as tissue alterations similar to those observed in dengue human cases were observed, even using epidemic non adapted DENV strains. Viral genome and antigen have been detected in the spleen, liver, brain, heart, lung, kidney and saliva [36-49]. Moreover, BALB/c has been recently shown to be useful in a therapeutic approach to treat DENV infection with improved tissue repair and regeneration [50].

We previously showed the BALB/c susceptibility to Lineages I and II of DENV-2 of the Asian/American genotype and its impact in the kidney and liver [47, 48], however, considering the systemic profile DENV infections may present, here, we aimed to further investigate the impact of those Lineages in the lung, heart and skeletal muscle tissues of infected mice.

2. Materials and Methods

2.1. Ethical Statements

All procedures performed in this study were approved by the Animal Ethic Committee (protocol L-023/2018) and the Human Research Ethics Committee (protocol 274/05) of Oswaldo Cruz Institute (IOC), Oswaldo Cruz Foundation (FIOCRUZ).

2.2. Viral strains

Strains BR/RJ66985/2000 and BR/RJ0337/2008 (GenBank #HQ012518 and #HQ01253, respectively), representative of Lineages I and II of DENV-2 Asian/American genotype [30], were isolated from patient sera at Flavivirus Laboratory, IOC, FIOCRUZ, during the epidemics of 2000 and 2008, respectively and kindly provided. The DENV-2 infecting

serotype was confirmed by indirect immunofluorescence, using DENV type-specific monoclonal antibody (3H5) and RT- PCR [51, 52]. Virus stock was prepared by inoculating 100µl of each strain into cell culture bottles containing mosquito *Aedes albopictus* C6/36 cell line at a concentration of $5x10^5$ cells/ml. Titers (BR/RJ66985/2000: $10^{6.66}$ $TCID_{50}$/1ml and BR/RJ0337/2008: 10^9 $TCID_{50}$/1ml) were calculated by the Reed & Muench method [53]. The viruses did not undergo any passages through mouse brain for neuroadaptation.

2.3. Study Design

For experimental viral infection, BALB/c mice were inoculated by the intravenous route (IV) through the caudal vein. Inocula volume was 100µl and viral concentration, 10,000 $TCID_{50}$/0,1ml. The mice were anesthetized (0,2ml of ketamine, xylazine and tramadol solution) and euthanized 24, 48, 72, hours post-infection (hpi), seven days post-infection (dpi) or 14 dpi, according to their experimental group.

Histopathological, morphometric and ultrastructural analysis, viral genome and antigen detection were performed with organ samples of mice euthanized at 72 hpi. Lung, heart and skeletal muscle samples destined to morphological analysis and immunohistochemistry assay were fixed with proper fixatives. Those destined to qRT-PCR assay were stored at -80°C. Mice lungs and hearts were weighted immediately after harvesting (72 hpi, seven and 14 dpi). Non-infected mice were used as negative controls. Table 2 shows the number of mice used in this study.

Table 1: Number of BALB/c used for histopathological, immunohistochemical and CK levels analysis, genome detection and measuring of organs' weight after experimental with Lineages I and II of DENV-2 Asian/American genotype.

N = 123	**Histopathology / qRT-PCR / IHQ**	**TEM**	**CK level analysis**			**Organ weight**		
	72 hpi	**72 hpi**	**24 hpi**	**48 hpi**	**72 hpi**	**72 hpi**	**7 dpi**	**14 dpi**
DENV-2/Lineage I	10/10/5	5	5	5	5	22	15	15
DENV-2/Lineage II	10/10/5	5	5	5	5	22	15	15
Negative control	5/5/5	5			5			19
Total [samples]	25	15	35			123		

IHQ: immunohistochemistry, TEM: transmission electron microscopy, hpi: hours post infection, dpi: days post infection.

2.4. Histopathology

For each DENV-2 Lineage, 10 mice were infected. At 72 hpi, the mice were euthanized, lung, heart and skeletal muscle samples were collected and fixed in Millonig buffered formalin. The samples were then dehydrated in decreasing concentrations of ethanol, clarified in xylene and embedded in paraffin. Tissue sections 5 µm thick were obtained using a microtome (Leica 2025, Germany), stained with hematoxylin and eosin (H&E) and analyzed using a brigh-tfield microscope (AxioHome, Carl Zeiss, Oberkochen, Germany). Five noninfected mice were used as negative control. All procedures were carried out in collaboration with Pathology Laboratory, IOC, FIOCRUZ.

2.5. Immunohistochemistry

For immunohistochemistry assays, five slides containing histological sections of lung, heart and skeletal muscle of BALB/c mice euthanized at 72 hpi were selected. The slides were heated to 60°C and immersed in xylene for removal of paraffin. The samples

were then dehydrated in decreasing concentrations of ethanol, prior to antigen retrieval, immersed in Dako buffer, in a pressure cooker. After cooling, the sections were incubated with anti-4G2 antibody produced in rabbit (1:200), used as the primary antibody, and later with the secondary anti-rabbit antibody conjugated with horseradish peroxidase (Spring Bioscience, Pleasanton, CA, USA). Finally, the slides were counterstained with Harris' hematoxylin and analyzed under a bright-field microscope (AxioHome, Carl Zeiss, Oberkochen, Germany). Slides containing histological sections from noninfected mice were used as a negative control.

2.6. Transmission Electron Microscopy (TEM)

For ultrastructural studies, organ samples from 15 mice (five from the control group and five from the groups infected with both DENV2 strains), euthanized at 72, hpi were processed as described by Barreto-Vieira [54]. Briefly, samples were fixed by immersion in glutaraldehyde (Electron Microscopy Sciences, USA) at 2% diluted in sodium cacodylate buffer (0.2 M, pH 7.2), cleaved into smaller fragments (~1 mm3), post-fixed in 1% osmium tetroxide and dehydrated in increasing concentrations of acetone. Subsequently, the samples were embedded in Epoxy resin (Electron Microscopy Sciences, USA). Ultrathin sections (50-70 nm thick) were obtained with the aid of an ultramicrotome (Leica, Germany), placed on copper grids and counterstained with uranyl acetate and lead citrate. Finally, the samples were observed under the Hitachi HT 7800 TEM (Hitachi, Japan).

2.7. Histomorphometry

To perform the histomorphometric analysis, a total of 15 slides containing histological sections of lungs, from mice euthanized at 72 hpi, stained with H&E were used. For each slide, 20 images of random areas were captured with the aid of a camera coupled to the AxioHome bright-field microscope (Carl Zeiss, Germany), using a 40x objective lens for images, result in in 300 areas. Analysis were performed using the public domain image processing program Image J. For each lung image, the thickness of 20 alveolar septa was measured. Alveolar septal thickness values were compiled by experimental group and a simple mean was calculated.

2.8. CK levels analysis

For each DENV-2 Lineage, 15 mice were infected. The mice were divided into three groups of five animals and each group euthanized at different times after infection (24, 48 and 72 hpi). After the determined periods of infection, the mice were anesthetized and blood was collected by cardiac puncture. The samples were then centrifuged for 10 minutes, at 5000 rotations per minute, to separate the serum from the cellular components. Non-infected mice (n=5) blood was collected on the same day as the 72 hpi group. Blood levels of CK were measured by dry chemistry testing using the Vitros 250 equipment (Ortho clinical - Jonhson & Jonhson) in collaboration with Instituto de Ciências e Tecnologia de Biomodelos (ICTB), Fiocruz. The assay was carried out immediately after sampling.

2.9. Viral genome extraction and quantitation

For the extraction of the viral genome, lung, heart and skeletal muscle fragments from 10 mice infected with DENV-2 Lineages, euthanized at 72 hpi. were macerated in 500 µl of Leibovitz culture medium (Invitrogen, USA) and centrifuged for 15 minutes at 10,000 RPM at a temperature of 4°C. RNA was extracted from 140 µl of the macerated organ supernatant using the QIAmp Viral RNA mini kit (Qiagen, Germany) according to the manufacturer's recommendations. For the detection and quantification of the viral genome, a standard curve was constructed from a serial dilution of RNA extracted from a DENV-2 sample (strain S16083), with known titer (8.7X106 PFU/ml). The protocol used

was described by Johnson et al. [55], using the primers DENJ2-R (5'-CCATCTGCAG-CAACACCATCTC-3') and DENJ2-F (5'-CAGGTTATGGCACTGTCACGAT-3'), designed from a fragment of the 3' non-coding region, and the probe DENJ2-P (CY5 5'-CTCTCCGA-GAACAGGCCTCGACTTCAA-3' BHQ-1). The reaction was performed according to the protocol of the commercial kit SuperScript III Platinum One-Step Quantitative RT-PCR (Invitrogen, USA), with ideal concentrations of the primers and probe determined by optimization assays. In a 96 microwell optical-bottom plate (Applied Biosystems, USA), 1 µl of each primer (50 µM), 12.5 µl of reaction mix (0.4 µM of each dNTP and 6 µM of MgSO4) were added; 0.5 µl SuperScript III RT enzyme, 3.5 µl DNase / RNase free water; 1 µl MgSO4 (5 mM) and 0.75 µl probe (9 µM), totaling a volume of 20 µl per well. Soon after, 5ml of extracted RNA was added, thus obtaining a final volume of 25ml/reaction. Each sample and control were applied in duplicate. The plates were placed on the 7500 FAST platform (Applied Biosystems, USA) for the qRT-PCR reaction, according to the following cycling parameters: reverse transcription (50°C, 15', 1 cycle), enzyme activation (95 °C, 2', 1 cycle), denaturation (95°C, 15", 40 cycles) and annealing/extension (60°C, 1', 40 cycles).

3. Results

3.1. Organ weight

A slight increase in the mean variation in lung weight of mice infected with DENV-2 Lineage I was observed when compared to noninfected mice (mean = 0.203g) at 72 hpi and 7 dpi (means: 0.210g and 0.214g); however, the mean value decreased at 14dpi (0.199g). For animals infected with Lineage II, on the other hand, the variation in lung weight was different, as the mean of infected mice was greater than that of the negative control at all times of infection (means: 72hpi = 0.217g; 7dpi = 0.223g; 14dpi = 0.233g).

The proportion between lung weight and body weight, on average, was higher in infected mice when compared to the noninfected group (mean = 0.704%). When analyzing mice infected with Lineage I, we observed that, on average, the ratio between lung weight and body weight was slightly higher on the 7dpi (mean = 0.766%). In mice infected with Lineage II, although the means did not vary during the entire period of infection, they were all higher when compared to Lineage I. There was a statistically significant difference between the infected groups that were euthanized 14 dpi in relation to the total organ weight and the organ weight/body weight ratio ($p < 0.001$ and $p = 0.035$, respectively). Figure 1 shows the lung weight variation (A-C) and lung weight/body weight ratio (D-F).

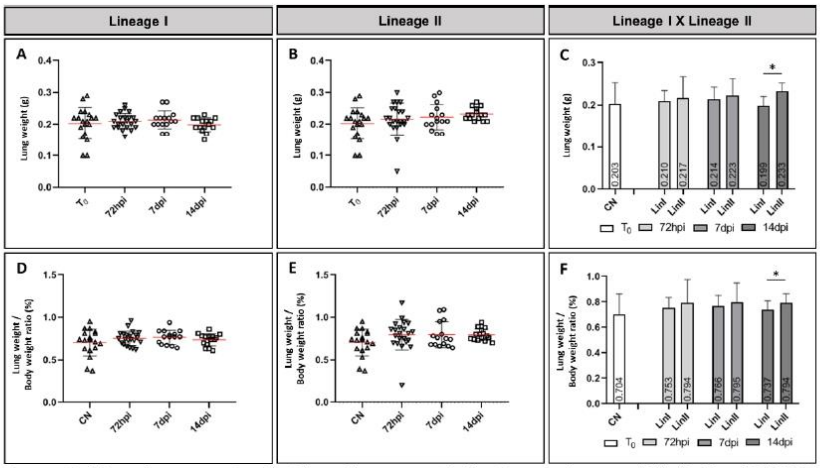

Figure 1: Mean lung weight (A-C) and lung weight/body weight ratio (%) (D-F) of BALB/c mice noninfected and infected with DENV-2 Lineages at 72 hpi, 7 and 14 dpi. CN: (N=19); LinI: 72 hpi (N = 22), 7 dpi (N= 15), 14 dpi (N= 15); LinII: 72 hpi (N= 22), 7dpi (N= 15), 14 dpi (N= 15). NC: negative control, N: number of mice, hpi: hours post-infection, dpi: days post-infection, Lin: Lineage, *: $p < 0.05$, **: $p < 0.01$.

The variation in heart weight of mice infected with Lineages I and II of DENV-2 presented distinct profiles. The mean heart weight of mice infected with Lineage I increased at all times of infection (mean: 72 hpi = 0.166g; 7 dpi = 0.173g; 14 dpi = 0.183g) when compared to the mean of the negative control (mean = 0.158g). The mean heart weight of animals infected with the other strain was slightly lower than the mean of the noninfected group at 72 hpi (mean = 0.156g). Finally, mice euthanized on the 7 dpi and 14 dpi showed an increase in mean heart weight (means = 0.163g and 0.183g, respectively)

On average, heart weight/body weight ratio of mice infected with DENV-2 Lineages also increased compared to noninfected mice (mean = 0.562%). In mice infected with Lineages I and II, the ratio increased gradually, with a higher mean on the 14 dpi (means = 0.647% and 0.621, respectively). The difference between the means of the control group and the group infected with Lineage I euthanized at 14 dpi was significant ($p = 0.031$). Figure 2 shows the heart weight variation (A-C) and heart weight/body weight ratio (D-F).

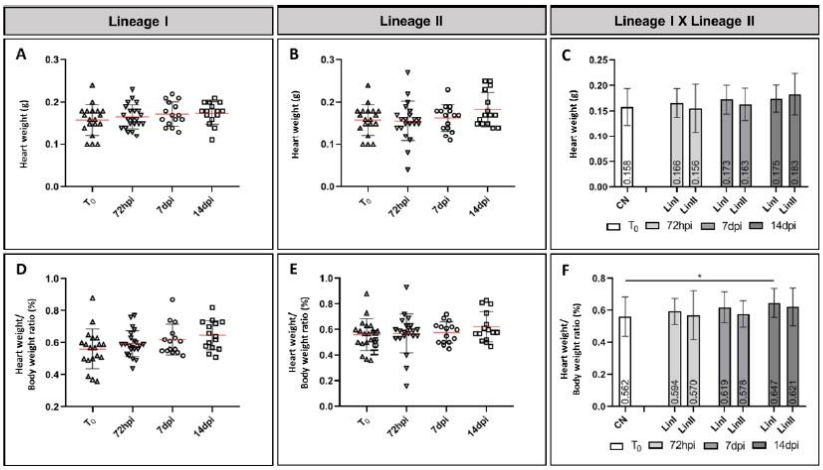

Figure 2: Mean heart weight (A-C) and heart weight/body weight ratio (%) (D-F) of BALB/c mice noninfected and infected with DENV-2 Lineages at 72 hpi, 7 and 14 dpi. CN: (N=19); LinI: 72 hpi (N = 22), 7dpi (N= 15), 14 dpi (N= 15); LinII: 72 hpi (N= 22), 7dpi (N= 15), 14 dpi (N= 15). NC: negative control, N: number of mice, hpi: hours post-infection, dpi: days post-infection, Lin: Lineage, *: $p < 0.05$, **: $p < 0.01$.

3.2. Morphology

Lung tissues from noninfected BALB/c mice showed bronchioles, alveolar sacs, alveoli, no signs of rupture, edema, hemorrhage or other morphological changes (Figure 3A). Alveolar septa, however, presented some areas of mild thickening. The histopathological changes induced by the infection of the two DENV-2 Lineages in the lung of BALB/c mice were focal and similar. The thickening of the alveolar septum (Figures 3B and 3D), collapse of the alveolar space, due to the migration of inflammatory cells to the interstitium (Figure 3C) was the most observed alteration. Areas of alveolar hyperinflation (Figures 3B and 3D) and vascular congestion (Figure 3D) were also observed. Furthermore, edema and focal areas of mild alveolar hemorrhage (Figure 3E and 4F), also observed in the tissues of the infected mice, suggest that there was an alteration in vascular permeability.

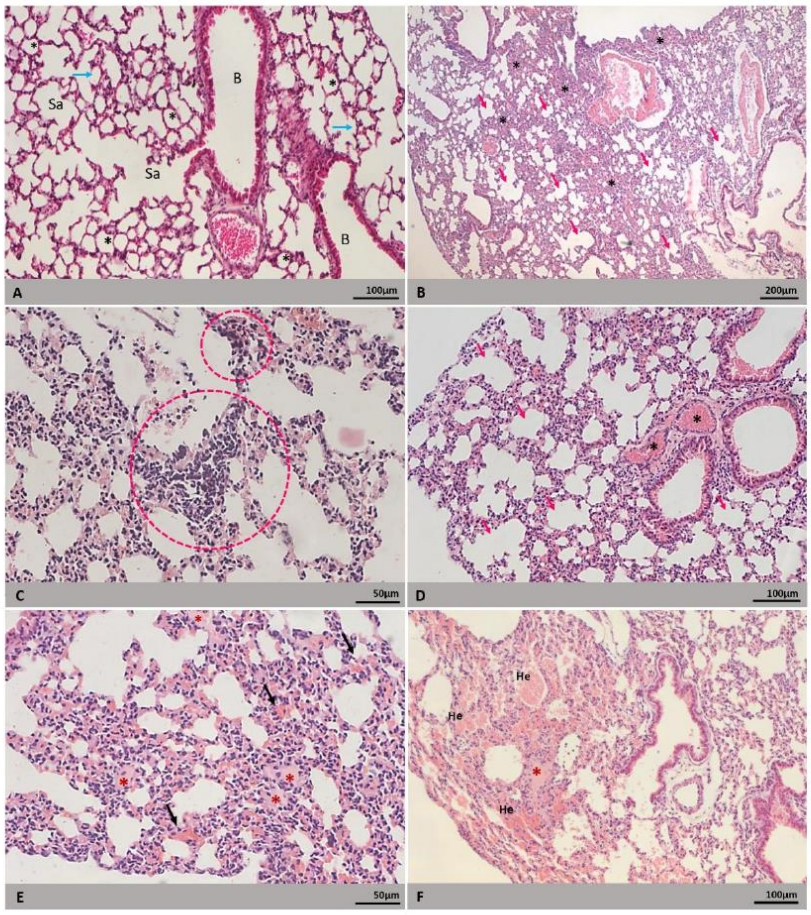

Figure 3: Histopathological alterations of lung of BALB/c mice. H&E staining. Euthanasia; 72 hpi. **(A)** noninfected mouse. Alveolar sac (Sa), alveolar septum (arrow), alveolus (*), bronchiole (B); **(B-D)** mice infected with DENV-2 Lineages. **(B)** alveolar septa thickening (*), alveoli hyperinflation (arrow); **(C)** inflammatory infiltrate (circled area); **(D)** vascular congestion (*), alveoli insufflation (arrow) **(E)** alveolar edema (*), vascular congestion (arrow); **(F)** hemorrhage (He), edema (*). Experimental infection: (B/C/E) Lineage I, (D/F) Lineage II.

Heart tissues from noninfected BALB/c mice showed fusiform cardiomyocytes arranged in parallel bundles. Signs of edema, vascular congestion, hemorrhage and inflammatory cell infiltrate were not observed (Figure 4A and 5A). The heart from BALB/c mice infected with both DENV-2 Lineages showed fairly well-preserved areas. The histopathological changes observed in both groups were the presence of mostly mild inflammatory infiltrates (Figure 4C), although some samples presented an apparent increase in cellularity due to mononuclear infiltrates, (Figure 4D) and apparent cytoplasmic rarefaction of cardiomyocytes (Figure 4B). Some of those cells presented lightly stained nuclei, as if some nuclear content was lost (Figure 4B).

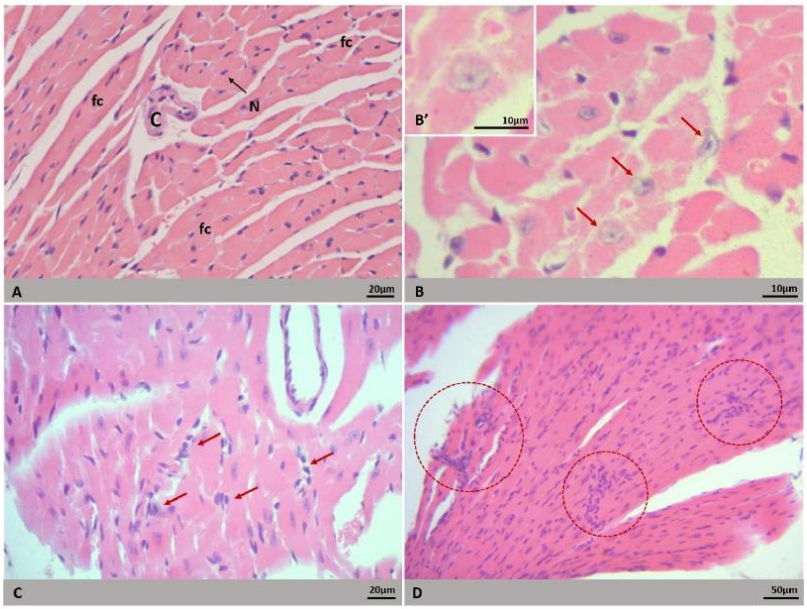

Figure 4: Histopathological alterations of heart of BALB/c mice. H&E staining. Euthanasia; 72 hpi. **(A)** noninfected mouse. Cardiac fiber (fc), capillary (C), nucleus (N); **(B-D)** mice infected with DENV-2 Lineages. **(B)** rarefaction of cytoplasm and nuclear content (arrow); **(C)** inflammatory infiltrate (arrow); **(D)** increased interstitial cellularity. Experimental infection: (C) Lineage I, (B/D) Lineage II.

Ultrastructural analysis of mice heart samples corroborated the histopathological findings. There were mononuclear cells infiltrate in the interstitium (Figures 5B, 5D and 5E) and areas of apparent loss of cytoplasmic content showed myofilament degeneration (Figure 5C). In addition, congested capillaries (Figure 5E) and alteration of mitochondria, characterized by apparent enlargement and a less electron dense appearance (Figure 5F), were observed. Table 1 shows the number of infected mice whose lungs or heart showed the aforementioned histopathological changes.

277 Table 2: Histopathological alterations observed in lung and heart samples of BALB/c infected
278 with DENV-2 Lineages I or II and euthanized at 72 hpi. Number of mice whose livers pre-
279 sented the alteration/total number infected mice.

Histopathological alterations	DENV-2		
	Lineage I (%)	Lineage II (%)	Total (%)
Alveolar septum thickening	10/10 (100)	9/10 (90)	19/20 (95)
Inflammatory infiltrate	10/10 (100)	9/10 (90)	19/20 (95)
Vascular congestion	8/10 (80)	6/10 (60)	14/20 (70)
Alveolar hemorrhage	6/10 (60)	6/10 (60)	12/20 (60)
Edema	4/10 (40)	4/10 (40)	8/20 (40)
Inflammatory infiltrate	7/10 (70)	9/10 (90)	17/20 (85)
Cytoplasmic rarefaction	7/10 (70)	4/10 (40)	11/20 (55)
Increased cellularity	2/10 (20)	4/10 (40)	6/20 (30)

280 hpi: hours post infection, dpi: days post infection.

281

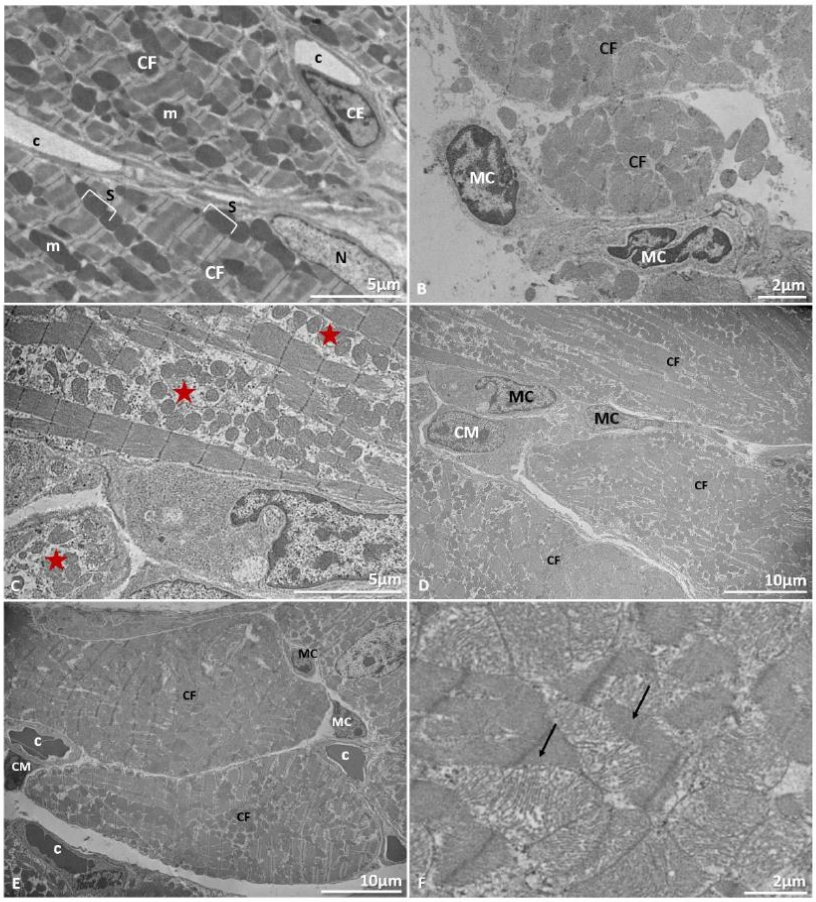

Figure 5: Ultrastructural alterations of heart of BALB/c mice. Euthanasia; 72 hpi. **(A)** non infected mouse. Cardiac fibers (CF), nucleus (N), mitochondrion (m), sarcomere (s), endothelial cell (CE), capillary (c); **(B)** mononuclear cells (MC); **(C)** degeneration of myofilaments (star); **(D)** mononuclear cells infiltration (MC); **(E)** capillary congestion (C) mononuclear cells (MC); **(F)** mitochondrial tumefaction (arrow). Experimental infection: (B) Lineage I, (C-F) Lineage II.

3.3. Morphometry

In order to compare the magnitude of alveolar septum thickening in infected and control mice, the thickness of the structure was measured. Histomorphometric analysis showed that the mean thickening observed in lungs of mice infected with both DENV-2 strains (means = 24.82 μm and 26.61 μm, respectively) was greater than in the control group (mean = 12.14 μm). The difference between the three experimental groups was statistically significant ($p < 0.001$) (Figure 6).

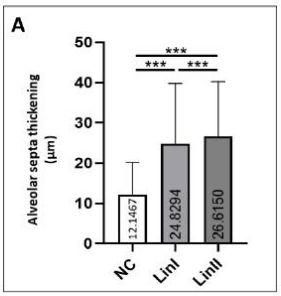

Figure 6: Morphometrical analysis of alveolar septum of infected and noninfected BALB/c mice. Euthanasia: 72 hpi. NC: negative control, Lin: lineage, hpi: hours post infection.

3.4. Viral genome and antigen detection

Viral genome or antigen was not detected in lung samples. However, in mice infected with Lineage I, viral RNA was detected in three heart tissues ($1.56x10^{-1}$; $6.7x10^{3}$ and $2.52x10^{7}$ RNA copies/μl) and two skeletal muscle tissues ($1.59x10^{0}$ and $3.23x10^{4}$ RNA copies/μl). In mice infected with Lineage II, the viral genome was also detected in three heart tissue samples ($3.5^{8}x100$, $3.79x10^{7}$, and $3.37x10^{-2}$ copies of RNA/μl) and two skeletal muscle samples ($1.97x10^{4}$ and $2.7x10^{2}$ RNA copies/μl). Moreover, the viral antigen was detected in endothelial cells and cardiac fibers (Figure 7B) in one heart sample infected with Lineage II. Noninfected samples did not show peroxidase reactive cells (Figure 7A).

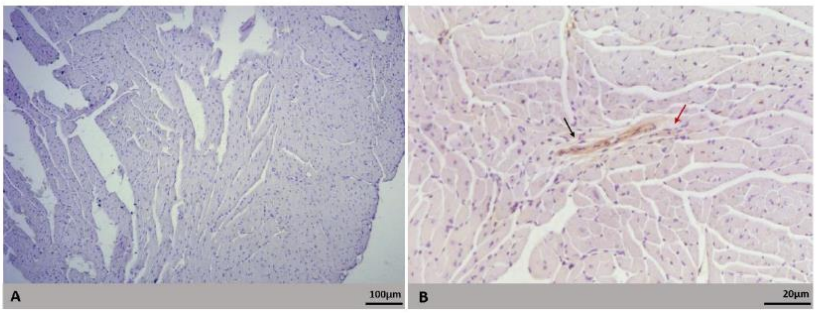

Figure 7: DENV antigen detection in heart of BALB/c mice infected with DENV-2 Lineage II. Euthanasia: 72 hpi. (A) negative control showing no peroxidase reactive cells; (B) peroxidase reactive endothelial cells (black arrow) and cardiomyocyte (red arrow).

3.5. Creatine kinase (CK) levels

The mean concentration of CK present in the serum of noninfected mice was 830 U/L. In mice infected with DENV-2 Lineage I, the average at 24 hpi, 813 U/L, was lower than the control group, but increased at 48 and 72 hpi, with averages 933.2 U/L and 677.4 U/L, respectively. In mice infected with Lineage II, an increase in the enzyme level was observed one dpi and two dpi (means: 1059.4 U/L and 1307.4 U/L, respectively). On the third dpi, the mean (459 U/L) observed was lower than in the control group. Figure 7 shows CK levels variation in serum samples from infected and noninfected mice. The difference between the means of the control group and the group infected with Line II of DENV-2 was statistically significant at 72 hpi ($p = 0.023$).

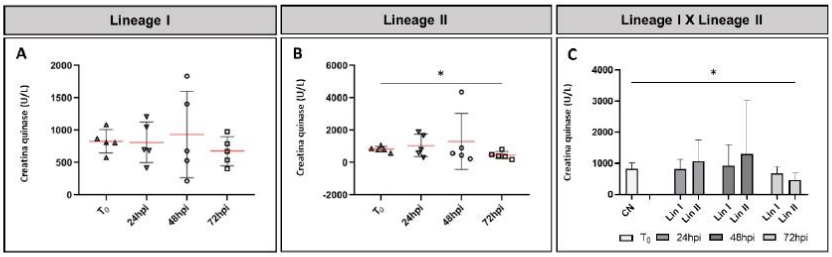

Figure 8: Creatine kinase (CK) levels present in serum from BALB/c mice noninfected (T0) and infected with DENV-2 strains I or II 24, 48 and 72 hpi. (A) Lin I of DENV-2; (B) Lin II of DENV-2; (C) comparison of mean creatine kinase levels. CN: negative control, Lin: Lineage, hpi: hours post infection, *: $p \leq 0.05$.

4. Discussion

Dengue is an acute febrile disease caused by one of the four DENV serotypes, which cause similar clinical manifestations, although variations in intensity may occur due to the host genetic factors, heterophile immunity, and infective strain characteristics [4, 27, 56]. Most patients are asymptomatic or develop nonsevere dengue, presenting mild symptoms such as fever, headache, retro-orbital pain, gastrointestinal symptoms, myalgia, arthralgia, and rash [57]. Bleeding manifestations include petechiae, ecchymosis, epistaxis, gingival hemorrhage and metrorrhagia, appearing at the end of the febrile period [58]. However, there are cases in which the disease can progress to severe dengue, a potentially lethal complication due to plasma leakage, fluid accumulation, respiratory distress, severe bleeding, and multi-organ involvement [59].

The most frequently affected organ during DENV infection is the liver (Póvoa, 2014). Nonetheless, DENV has already been detected in different tissues [7, 60, 61] and neurological, renal, muscular, cardiac, and pulmonary manifestations and disorders during severe dengue cases have been reported [8, 9, 21, 26]. Unusual manifestations may be underreported and, since the majority of studies concern autopsy samples, data on different tissues involvement during nonsevere cases of dengue are scarce [6, 62-64]. Therefore, here, we aimed to evaluate alterations induced by the infection of two DENV-2 Lineages in lung, heart, and skeletal muscle using the immunocompetent BALB/c as an experimental model.

Pulmonary manifestations during dengue are not common [11]. Pleural effusion is a common finding in dengue patients who present with respiratory symptoms, and is the most frequent cause of dyspnea among patients. Ground-glass opacity and consolidation are seen less frequently and may represent pulmonary edema or hemorrhage [10, 11, 16,

353 61, 65, 66]. A study that evaluated lung involvement during dengue using computed to-
354 mography scans reported that these changes are more frequent in dengue severe patients
355 and suggested that this condition is related to plasma leakage [10]. In this study, an in-
356 crease in lung weight in infected mice when compared to the control group was observed.
357 Except for Lineage I/14 dpi group, the means of infected animals were slightly higher.
358 Regarding the lung weight/body weight ratio, the means of the infected groups were
359 higher throughout the entire course of infection analyzed here. Furthermore, the means
360 of mice infected with Lineage II were higher than those infected with Lineage I, with a
361 statistically significant difference on the 14dpi. Although there is a tendency in organ in-
362 crease in infected mice, imaging exams were not performed in this study, therefore, it is
363 not possible to assess whether the increase in lung weight was a result of pleural effusion.
364 However, in our histological analysis, alveolar edema and hemorrhage was observed, as
365 described in both human fatal cases and experimental models [6, 10, 45, 67], and it would
366 not be unjustified to associate such alteration with the mild increase in the average weight
367 of this organ.

368 Few cases of severe pulmonary manifestation due to DENV infection has been re-
369 ported [68-70] and a study, carried out by Rodrigues et al (2014), showed that there is a
370 tendency of mild to moderate lung involvement. Neither the genome nor the antigen of
371 DENV was detected in our lung tissue samples. Nonetheless, there were alterations in
372 lung of infected mice that were not present in noninfected ones. Here, we observed alve-
373 olar septum thickening and alveolar collapse due to migration of inflammatory cells to
374 the interstice. As a compensatory mechanism, some alveoli were hyperinflated. These
375 finds are in accordance with fatal cases investigation data and experimental model studies
376 [6, 10, 36, 38, 42, 45, 67]. As mentioned above, some samples presented focal hemorrhage
377 and edema as seen in BALB/c mice infected with DENV-3 [45]. In human cases, lung hem-
378 orrhage is reported in fatal cases [6, 10, 67] and some patients with severe symptoms pre-
379 sented blood-tinged secretion coming from both lungs [69, 70]. To our knowledge, no lung
380 hemorrhage/edema concerning non severe dengue has been reported in human cases,
381 most likely due to the fact that pulmonary involvement is not investigated in such cases.

382 Although data on the cardiac involvement in dengue is scarce, lately, heart involve-
383 ment has been increasingly reported [14-18] and it is mostly associated with severe cases
384 [14, 15, 18]. Main finds include atrioventricular conduction disorders, supraventricular
385 arrhythmias, myocarditis, bradycardia, tachycardia, pericardial effusion, diastolic dys-
386 function, and increased levels of cardiac enzymes [14, 16, 18, 71]. Cardiomegaly has been
387 reported in cases of DENV infection [13, 15, 16, 72] and it can be caused by coronary artery
388 disease, hypertension, valvulopathies, pulmonary diseases and myocarditis, among other
389 complications [73]. In this study, the mean heart weight and the heart weight/body weight
390 ratio increased in infected mice. Only one group (Lineage II/72h) presented a mean lower
391 than the control group. In a study carried out with 10 patients with a positive diagnosis
392 for arboviruses (dengue or chikungunya), nine had increased heart volume and another
393 case study reported thickening of the ventricular wall [13, 15]. Viral infections are the most
394 common cause of myocarditis, which can result in dilated cardiomyopathy with conse-
395 quent enlargement of the heart [16]. In this study, no apparent difference between the
396 heart volume of infected and noninfected mice was observed. Histopathological analysis
397 did not reveal edema or hemorrhage foci as occurred in other organs, therefore, we cannot
398 confirm that the accumulation of fluid in the interstitium resulted in an increase in the
399 average weight of the heart and further investigation must be carried out to better under-
400 stand the causes of the weight change.

401 Most cardiac manifestations are attributed to myocarditis due to viral infection [9,
402 16]. The presence of DENV in heart has been demonstrated in fatal human cases [6, 14, 60,
403 71]. In this study, the viral genome and antigen were detected in heart samples of mice

infected with both Lineages of DENV-2. And, although it is not clear whether tissue damage is caused by direct viral infection or host's immune cells response [14, 74], a number of histopathological alterations have been reported [6, 62, 72, 74].

The tissues showed mild histopathological and ultrastructural alterations. Inflammatory infiltrates are commonly found in DENV-infected tissues and have been observed in both human cases and experimental models [6, 62, 71, 74, 75]. Miranda [71] reported that mononuclear infiltrate consisted mainly of CD68+ macrophage-type cells. Apparent cytoplasmic rarefaction and nuclear content loss of cardiomyocytes were observed in histological samples. Upon ultrastructural analysis, we could see that the rarefaction was a result of myofilaments degeneration, also seen by Miranda [62] and Póvoa [6]. Mitochondrial swelling was also observed. Póvoa [6] associated mitochondrial and nuclear alterations with apoptotic process of cardiac fibers. As observed by Caldas [45] and Rasinhas [42] in mice infected with DENV-3 and -4, respectively, our samples showed small areas of vascular congestion. The changes present in our samples were moderate; however, studies carried out with BALB/c mice infected with serotypes 3 and 4 revealed changes in the morphology of intercalated discs, increase in heart rate due to reduced blood pressure and increased plasma leakage in the heart [42, 45, 49]. The differences observed in the alterations induced by different serotypes may indicate that a particular DENV serotype has greater or lesser tropism for a particular organ [76].

Myalgia and muscle weakness are manifestations presented by dengue patients. It has been reported that acute renal failure in dengue can be caused by rhabdomyolysis, a medical condition resulting in the dissolution of damaged or injured skeletal muscle [21-23, 25, 26]. The disruption of skeletal muscle integrity leads to the release of intracellular muscle components, such as CK into the bloodstream and extracellular space [77]. Analysis of skeletal muscle biopsies from dengue patients revealed perivascular inflammatory infiltrate, mitochondrial proliferation, foci of necrosis with or without myofagocytosis, and interstitial hemorrhage [21-24]. The presence of edema, hemorrhage foci, and metabolic alterations may be responsible for transient muscle weakness [24]. In this study, morphological changes were not observed in the histological sections of the infected mice. However, viral RNA was detected in skeletal muscle samples from mice infected with both Lineages of DENV-2. This finding is in agreement with a study of experimental infection of human myotubes culture, that showed that DENV is able to infect and replicate in muscle cells [14].

Elevated CK levels are indicative of muscle, heart, or brain damage. As CK-MB levels are more appropriate to assess cardiac damage, the most likely hypothesis is that the altered levels of the enzyme are due to muscle damage. On average, at 24 and 48 hpi, CK levels were higher in mice infected with both DENV-2 Lineages, mainly in the Lineage II group. The individual analysis showed that six infected mice had CK levels well above the mean of each group (Lin I: 1,209 U/L, 1,837 U/L and 979 U/L and Lin II: 1,897 U/L, 4,354 U/L and 822 U/L). It is noteworthy that the mean CK level of Lineage II group peaked at 48 hpi due to one individual (4354 U/L). This individual was not paired with the experimental group for histopathological studies, so tissue alteration could not be assessed. Our results are in agreement with studies of human dengue cases that report increase in the CK levels. However, these studies also report functional and histopathological changes in skeletal muscle that were not observed here [21, 24-26].

Dengue has a wide spectrum of clinical manifestations, with unpredictable evolution and involvement of different organs. Thus, the establishment of animal experimental models is of great relevance to provide a better understanding of pathogenesis mechanisms. BALB/c mice were shown to be an appropriate experimental model for dengue

pathogenesis studies on lung, heart and skeletal muscle tissues as we reported viral detection in heart and skeletal muscle and described tissue alterations in lung and heart of mice infected with two distinct epidemic and nonadapted DENV-2 Lineages.

Author Contributions: D.F.B.-V. and F.B.d.S. conceived the study; F.C.J. designed and supervised the experiments; and was involved in data curation; D.D.C.d.S., A.C.P. and M.A.N.d.S. con-ducted the experiments; F.C.J., G.C.C., A.L.T.d.A. and A.d.C.R. analyzed all images and data; F.C.J. drafted the manuscript; D.F.B.-V., F.B.d.S.; and O.M.B. reviewed the manuscript. O.M.B. and D.F.B.-V. were responsible for funding acquisition and resources. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by Laboratory of Viral Morphology and Morphogenesis, Instituto Oswaldo Cruz, Fiocruz, FIOTEC to D.F.B.-V. [grant number IOC-023-FIO-18-2-58], Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro [FAPERJ] to F.B.d.S. [grant number E-26/202.003/2016], Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) [grant number 302462/2018-0 to F.B.d.S.; grant number: 301992/2017-7 and to O.M.B. [grant number: 301992/2017-7], Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and CNPq for the students fellowships. The funders had no role in the study design, data collection, analysis and decision to publish or preparation of the manuscript.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Instituto Oswaldo Cruz (L-023/2018; July 11, 2018 and 274/05; April 07, 2014).

Acknowledgments: We would like to thank to the Flavivirus Laboratory, IOC/FIOCRUZ, for providing the viral strains, to Marcelo Pelajo and João Paulo Rodrigues, Pathology Laboratory, IOC/FIOCRUZ, for the support in sample processing and technical analysis. Felipe Anibal Carvalho Costa, Epidemiology and Systematics Laboratory, IOC/FIOCRUZ, for the support with statistical analysis, Raphael Leonardo, Laboratory of Viral Morphology and Morphogenesis, IOC/FIOCRUZ, for technical support and ICTB, IOC/FIOCRUZ, for the support in sample processing.

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

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Tabela 4: Resultados obtidos através de avaliações histopatológicas e histomorfométricas de diferentes órgãos de camundongos BALB/c infectados com as Linhagens I ou II de DENV-2.

	Avaliação histopatológica e histomorfométrica	DENV-2	
		Linhagem I	Linhagem II
Fígado	Infiltrado inflamatório	7/10	8/10
	Balonização de hepatócitos	7/10	8/10
	Aumento da área nuclear	7/10	8/10
	Congestão vascular	6/10	8/10
	Dilatação do capilar sinusóide	9/10	1/10
	Atipias nucleares	7/10	3/10
	Necrose	0/10	2/10
	Esteatose macrovesicular	0/10	1/10
	Hemorragia	0/10	1/10
	Hepatócitos multinucleados (%)	25,41	28,1
	Hepatócitos (/área)	99.967	105.444
Rim	Necrose tubular	10/10	10/10
	Infiltrado inflamatório	9/10	10/10
	Perda de citoplasma	10/10	8/10
	Congestão capilar	8/10	9/10
	Atrofia glomerular	8/10	8/10
	Perda de cromatina	8/10	7/10
	Aumento do volume glomerular	9/10	6/10
	Inclusões citoplasmáticas	3/10	3/10
	Hemorragia	0/10	1/10
	Corpuscúlos renais (/área)	3,613	3.155
	Área glomerular (μm^3)	$2,59x10^6$	$2,54x10^6$
Pulmão	Espessamento de septo alveolar	10/10	9/10
	Infiltrado inflamatório	10/10	9/10
	Congestão vascular	8/10	6/10
	Hemorragia alveolar	6/10	6/10
	Edema	4/10	4/10
	Espessamento de septo alveolar (µm)	24,8	26,6
Coração	Infiltrado inflamatório	7/10	9/10
	Rarefação de citoplasma	7/10	4/10
	Aumento da celularidade	2/10	4/10

Tabela 5: Resultados obtidos através de avaliações clínicas de camundongos BALB/c infectados com as Linhagens I ou II de DENV-2 e detecção viral em diferentes órgãos deste modelo.

	Avaliação clínica e detecção viral		DENV-2					
			Linhagem I			Linhagem II		
			72 hpi	7 dpi	14 dpi	72 hpi	7 dpi	14 dpi
Peso	Variação de peso corporal (g)		0,709	0,673	1,02	0,311	0.703	1,339
	Peso do órgão (g) - Peso do órgão/Peso corporal (%)	Fígado	1,54 - 5,5	1,49 -5,3	1,44 - 5,3	1,49 - 5,4	1,56 - 5,5	1,6 - 5,4
		Rim	0,45 - 1,6	0,45 - 1,5	0,42 - 1,5	0,44 - 1,6	0,46 - 1,6	0,46 - 1,9
		Pulmão	0,21 - 0,75	0,21 - 0,76	0,19 - 0,73	0,21 - 0,79	0,22 - 0,79	0,23 - 0,79
		Coração	0,16 - 0,59	0,17 - 0,61	0,17 - 0,64	0,15 - 0,57	0,16 - 0,57	0,18 - 0,62
Hemograma	Plaquetas (milhares/mm^3)		1304,4	1308,7	1103,3	1308,57	1217,4	1002,4
	Leucócitos (milhares/mm^3)		3,143	2,34	2,75	4,2	3,66	3,08
	Hematócrito (%)		55,1	51,3	49,9	48,6	48,8	50,4
Bioquímica			**24 hpi**	**48 hpi**	**72 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
	AST (U/L)		221	174	176	185	324	143,2
	ALT (U/L)		144,6	86,4	118,2	61	204	107
	Ureia (mg/dl)		48,5	41,8	43,4	54,1	58,6	43,4
	Citoquina quinase (U/L)		813	933,2	677,4	1059,4	1307,4	459
Vírus	Genoma viral (72 hpi)		fígado / coração / músculo esquelético			fígado / coração / músculo esquelético		
	Antígeno viral (72 hpi)		-			coração / rim		

g: gramas, AST: aspartato aminotransferase, ALT: alanina aminotransferase, U/L: unidades por litro, mg/dl: miligramas por decilitros, hpi: horas pós-infecção, dpi: dias pós-infecção.

4. Discussão

A dengue é uma doença febril aguda causada por um dos quatro sorotipos do DENV, que apresentam manifestações clínicas semelhantes, embora variações na intensidade possam ocorrer devido a fatores genéticos do hospedeiro, imunidade heterofílica e características da cepa infecciosa (Rothman *et al.*, 1999; Edelman *et al.* 2008; Halstead, 2015).

A maioria dos pacientes desenvolve a FD, apresentando sintomas leves como febre, cefaleia, dor retro-orbitária, sintomas gastrointestinais, mialgia, artralgia e erupção cutânea. As manifestações hemorrágicas incluem petéquias, equimoses, epistaxe, hemorragia gengival e metrorragia, surgindo no final do período febril (Nogueira *et al.*, 2005; Souza *et al.*, 2008; Dalrymple *et al.*, 2012). No entanto, existem casos em que a doença pode progredir para DG, uma complicação potencialmente letal devido a extravasamento de plasma, acúmulo de líquido nas cavidades pleurais e peritoneais, dificuldade respiratória, sangramento grave e envolvimento de vários órgãos. (OMS, 2018).

Manifestações incomuns podem ser subnotificadas e, uma vez que a maioria dos estudos diz respeito a amostras de autópsia, são escassos os dados sobre o envolvimento de diferentes tecidos durante casos não graves da doença (Miranda *et al.*, 2013; Póvoa *et al.*, 2014; Kadam, 2016; Nunes, 2019B). Neste trabalho nós avaliamos e comparamos as alterações induzidas pela infecção de duas Linhagens brasileiras epidêmicas do genótipo Asiático/Americano de DENV-2 em diferentes órgãos utilizando os camundongos imunocompetentes BALB/c como modelo experimental.

Pacientes de dengue podem manifestar febre aguda que persiste por três a sete dias após o período de incubação do vírus (Souza *et al.*, 2008). Neste estudo, independente da Linhagem de DENV-2 infectante, a variação média da temperatura nos camundongos BALB/c, foi muito pequena. Curiosamente, apenas o grupo controle mostrou um ligeiro aumento na temperatura em 7 e 14 dpi. Contudo, valores individuais demonstram que apenas camundongos infectados apresentaram variação de temperatura acima de 1,5°C. Em estudos realizados pelo nosso grupo utilizando camundongos BALB/c infectados com DENV-3 e DENV-4, foi observado um aumento da temperatura em 72 hpi (Caldas *et al.*, 2019; Rasinhas *et al.*, 2017). A ausência de aumento de temperatura e outros sinais clínicos neste mesmo modelo animal infectado com DENV-2 foram relatados anteriormente (França *et al.*, 2010; Sakinah *et al.*, 2017) e podem estar relacionados à dengue assintomática, já que apenas cerca de 25% dos casos da doença apresentam sintomas (WHO, 2009).

Em média, o ganho de peso entre os camundongos infectados foi inferior ao do grupo controle, o que pode estar associado à perda de apetite, conforme observado em casos de dengue em humanos e modelos imunocompetentes infectados com DENV-2 (Gubler *et al.*, 1998; Atrasheuskaya *et al.*, 2003; Sirivichayakul *et al.*, 2012; Hotchandani *et al.*, 2014; Chen *et al.*, 2016). Este resultado está de acordo com achados de Tan *et al.* (2010), que observou perda de peso entre camundongos AG129 infectados com o DENV-2. Um estudo de coorte realizado com pacientes de dengue em uma clínica geriátrica relatou anorexia e perda de peso (Chen *et al.*, 2016). Em análises de casos entre crianças, anorexia foi relatada em 78% dos casos de DC e em 91,2% dos pacientes com FHD (Sirivichayakul *et al.*, 2012). Em contraste com estes achados, estudos anteriores realizados pelo nosso grupo revelaram ligeiro aumento na média do peso corporal em camundongos infectados com DENV-3 (Caldas *et al.*, 2019).

Manifestações neurológicas podem ocorrer durante a infecção pelo DENV. Casos de encefalite associados à dengue, além de outras manifestações menos frequentes, têm sido relatadas. Estudos já reportaram o isolamento do DENV a partir do líquido cefalorraquidiano de crianças diagnosticadas com encefalite associada à doença (Solomon *et al.*, 2000; Malik *et al.*, 2014). Hematoma subdural e hemorragia intracraniana também já foram reportados. (Jayasinghe *et al.*, 2016; Chang *et al.*, 2021). Nesse estudo, nenhum sinal clínico associado à alteração neurológica foi observado nos camundongos infectados pelas duas linhagens do DENV-2 durante o período entre a infecção e eutanásia. Entretanto, durante a coleta de órgãos, observamos acúmulo focal de sangue na entre o cérebro e o crânio em 24% dos camundongos infectados com a Linhagem I e 8%, infectados com a Linhagem II no início da infecção (72 hpi), enquanto que, no sétimo dia de infecção, 8% dos camundongos infectados com as Linhagens I e II apresentaram esta alteração (dados não demonstrados).

Hemorragia intracraniana associada a dengue é resultante de fatores como doenças vasculares e disfunções plaquetárias. A trombocitopenia grave ($<50{,}000/mm^3$) desempenha papel importante em sangramentos espontâneos durante a dengue (Attavinijtrakarn *et al.*, 2000). Nossos resultados demonstram apenas uma pequena queda do número de plaquetas de camundongos infectados. Da mesma forma, Jayasinghe *et al*. (2016), reportou um caso de hemorragia intracraniana e hematoma subdural em uma paciente com trombocitopenia moderada. Atrasheuskaya *et al.* (2003), descreveu extravasamento vascular caracterizado por edema perivascular discreto em amostras de cérebro de camundongos BALB/c infectados com DENV-2, que pode indicar alteração da permeabilidade vascular. Entretanto, tal fato pode estar relacionado à via de inoculação invasiva escolhida.

Visto que o aumento da permeabilidade vascular, acompanhada de extravazamento de plasma e albumina, é uma característica comum da fisiopatologia da dengue (Guzman *et al.*, 2016), o aumento de volume ou peso de órgãos pode ser uma consequência do acúmulo de líquido no interstício. Neste estudo, fígado, rim, pulmão, coração e baço de camundongos infectados e do grupo controle foram pesados imediatamente após serem removidos. Para garantir que o aumento do peso dos órgãos não foi apenas um reflexo do ganho de peso corporal, a relação peso do órgão/peso corporal (%) foi calculada.

O peso médio do fígado de camundongos infectados com ambas as linhagens de DENV-2 aumentou ligeiramente e excedeu o do grupo controle no início da infecção (72 hpi) em camundongos infectados com a Linhagem I e, mais tardiamenre (7 e 14 dpi) em camundongos infectados com a Linhagem II. O aumento do fígado é comumente observado em dengue, principalmente em casos de DG (Gubler *et al.*, 1998; Ferreira *et al.*, 2018), e pode resultar de edema devido à alteração da permeabilidade vascular ou acúmulo de gordura nos hepatócitos (Singer *et al.*, 2014; Guzman *et al.*, 2015). Bhamarapravati *et al.* (1967), em um estudo com amostras de necrópsia, relatou figado aumentado em 58% dos casos e observou alterações gordurosas e hemorragia ao analisar os cortes histológicos. Outro estudo, realizado com pacientes com DG, mostrou uma alta prevalência (72,7%) de hepatomegalia e associou hepatomegalia dolorosa a níveis elevados de ALT (Ferreira *et al.*, 2018; Bhattacharya *et al.*, 2019). Não houve diferença aparente entre o volume dos fígados de camundongos infectados e não-infectados. Porém, ao analisarmos a relação peso do fígado/peso corporal, observamos que ela variou de forma semelhante ao peso do fígado, sendo essa relação maior, em média, às 72 hpi nos camundongos infectados com a Linhagem I e em todos os pontos de infecção do grupo infectado com a Linhagem II.

Nossos resultados mostraram que as médias de peso dos rins e da relação peso do rim/peso corporal dos camundongos infectados foram maiores do que as do grupo controle em todos os momentos da infecção. Embora não haja relatos de aumento do volume do rim devido à infecção por DENV, hepatomegalia, esplenomegalia e aumento do pâncreas têm sido associados à dengue (Setiawan *et al.*, 1998; Vabo *et al.*, 2004; 2015; Fernando *et al.*, 2016; Ferreira, 2018). Não observamos edema intersticial em nossas amostras, apenas extravazamento de líquido em glomérulos; no entanto, outro estudo com o mesmo modelo de camundongos infectados com DENV-3, realizado por nosso grupo, mostrou transudato na área cortical do rim e aumento estatisticamente significativo no peso do rim (Caldas *et al.*, 2019). Além disso, as amostras de autópsia de caso fatal humano também mostraram edema principalmente na região medular (Basílio-de-Oliveira *et al.*, 2005; Póvoa *et al.*, 2014). Para

cada cepa, os valores de variação de peso mais elevados foram observados às 72 hpi, no que diz respeito à Linhagem I, e 7 dpi quando os camundongos foram infectados com a Linhagem II. A diferença entre as duas linhagens foi estatisticamente significativa em 7 e 14 dpi. Uma possível explicação para tal diferença seria que a Linhagem II manifesta seus sinais em um tempo mais tardio da infecção.

Derrame pleural é um achado comum em pacientes de dengue que apresentam sintomas respiratórios, e é a causa mais frequente de dispneia. Opacidade de vidro fosco e consolidação, são observados com menos frequência e podem representar edema ou hemorragia pulmonar (Vabo, 2004 *et al.*; Rodrigues *et al.*, 2014; Tahir *et al.*, 2015; Almeida *et al.*, 2017; Marchiori *et al.*, 2020; Cunha *et al.*, 2021). Um estudo que avaliou o envolvimento pulmonar durante a dengue através de exames de tomografia computadorizada relatou que estas alterações são mais frequentes em pacientes com DG e sugeriu que tal condição está relacionada ao extravasamento de plasma (Rodrigues *et al.*, 2014). Assim como observado em fígado e rim, houve aumento do peso do pulmão em camundongos infectados quando comparados ao grupo controle. Com exceção do grupo Linhagem I/14 dpi, as médias dos animais infectados foram mais altas. Com relação à proporção peso corporal/peso do pulmão, as médias dos grupos infectados foram mais altas durante toda a infecção. Ademais, as médias dos camundongos infectados com a Linhagem II foram superiores àquelas dos infectados com a Linhagem I, havendo diferença estatisticamente significativa no décimo quarto dia de infecção. Exames de imagens não foram realizados neste estudo, desta forma, não é possível avaliar se o aumento do peso do pulmão foi resultado de derrame pleural. Entretanto, em nossas amostras histológicas, pudemos observar edema alveolar e hemorragia, nos permitindo inferir que tais alterações estejam relacionadas ao aumento de peso médio deste órgão.

D e maineira geral, as médias do peso do coração e da proporção peso do coração/peso corporal também se elevaram em camundongos infectados. Apenas um grupo (Linhagem II/72h) apresentou média inferior ao grupo controle. Cardiomegalia já foi reportada em casos de infecção por DENV (Obeyesekere & Hermon, 1973; Madhavan *et al.*, 2014; Pareda *et al.*, 2015; Tahir *et al.*, 2015). Pode ser causada por doença arterial coronariana, hipertensão, valvulopatias, doenças pulmonares e miocardite, entre outros (Amin & Siddiqui, 2020). Em um estudo realizado com dez pacientes com diagnóstico positivo para arboviroses (dengue ou chikungunya), nove apresentavam a alteração e outro estudo de caso reportou espessamento da parede ventricular (Obeyesekere & Hermon, 1973; Pareda *et al.*, 2015). Infecções virais são a causa mais comum de miocardite, que pode resultar em cardiomiopatia dilatada (Tahir *et al.*, 2015). Apesar de termos observado um aumento de peso, nenhuma diferença aparente entre o

volume dos corações de camundongos infectados e não infectados foi observada. As análises histopatológicas também não revelaram edema ou focos de hemorragia como ocorreu em outros órgãos, logo, não podemos afirmar que acúmulo de fluidos no interstício resultou no aumento do peso médio do coração e uma investigação mais aprofundada deve ser feita para melhor entendermos as causas da alteração de peso.

Durante a infecção pelos DENV pode haver envolvimento do baço. Esplenomegalia e ruptura esplênica espontânea já foram verificadas em pacientes com dengue. O baço encontra-se frequentemente congestionado em casos de FHD e hematoma subcapsular é observado em 15% dos casos de óbito (Vabo *et al.*, 2004; Gulati *et al.*, 2007; De Moura Mendonça *et al.*; 2011; Mukhopadhyay *et al.*, 2014). Neste estudo, não foram observados sinais de hematoma subcapsular ou ruptura, entretanto, o peso médio (em todos os todos os tempos de infecção) e a média da razão entre o peso deste órgão e o peso corporal (em todos os todos os tempos de infecção para a Linhagem I e às 72 hpi e aos 14 dpi para a Linhagem II) em camundongos infectados foi maior quando comparados às médias do grupo não infectado. Apesar do aumento médio observado no peso do baço não configurar esplenomegalia, esta condição foi vista em um camundongo infectado com a Linhagem II de DENV-2 72 hpi, cujo baço apresentava 0,21g, e era 25% maior que os baços do grupo controle (dados não demonstrados). Ademais, Schul *et al.* (2007) e Tan *et al.* (2010) também observaram esplenomegalia em camundongos infectados experimentalmente com DENV-2.

Levando em consideração o menor ganho de peso corporal observada em camundongos infectados,em relação ao observado em animais controle, estes resultados sugerem que o aumento da média do peso dos órgãos estudados, maior nos grupos infectados, não está relacionado apenas ao ganho de peso e pode ser uma consequência da infecção.

Durante a infecção por DENV, alterações nas contagens de componentes do sangue são comumente observadas e indicam o prognóstico da doença. Entre as alterações, a trombocitopenia (contagem de plaquetas abaixo de 100.000/mm^3) é uma característica das formas leve e grave de dengue. As plaquetas desempenham papel importante na imunopatogênese da dengue, secretando mediadores inflamatórios e formando agregados com leucócitos que induzem respostas inflamatórias (Hottz *et al.*, 2013; Quirino-Teixeira *et al.*, 2020). A trombocitopenia pode resultar da agregação plaqueta-leucócito, da infecção de populações de células hematopoéticas da medula óssea (o que reduz sua capacidade proliferativa), da deposição de plaquetas no leito microvascular, ou da destruição plaquetária no sangue periférico (Basu *et al.*, 2008; de Azeredo *et al.*, 2015, Hottz *et al.*, 2018). Neste

estudo, às 72 hpi, o número de plaquetas de camundongos infectados foi maior do que no grupo controle. Nos momentos subsequentes da infecção, este número diminuiu e atingiu valores ligeiramente inferiores aos do grupo controle. Uma diminuição na contagem de plaquetas é esperada e foi observada em estudos anteriores com camundongos imunocompetentes infectados com DENV-2 (Atrasheuskaya *et al.*, 2003). A leve trombocitose às 72 hpi, que também foi observada em camundongos quando infectados com DENV-3 (Caldas *et al.*, 2019), pode estar associada a uma resposta a fatores desencadeados pela infecção que aumenta a produção de trombopoietina (Pluthero *et al.*, 2018).

Outra alteração observada na dengue é a hemoconcentração, que pode ser resultado do extravasamento de plasma (Gubler, 1998). Um aumento do HCT de 20% sobre a linha de base é um sinal de FHD, e sua elevação máxima coincide com o choque (Torres, 2008). O HCT aumentou significativamente em camundongos BALB/c infectados com DENV-3 (Caldas *et al.*, 2019). Os camundongos infectados com a Linhagem I de DENV-2 apresentaram um aumento muito leve (menos de 2%) no HCT às 72 hpi e aos 7 dpi. Já o grupo infectado com a outra Linhagem apresentou média acima do grupo controle apenas aos 14 dpi. A pequena diferença do HCT entre grupos infectados e não-infectados, de certa forma, está de acordo as análises histopatológicas, uma vez que poucas amostras apresentavam extravazamento de plasma. Entretanto, as amostras utilizadas para análises de morfologia e hemograma não foram as mesmas.

A contagem leucocitária durante a dengue é variável e, embora leucopenia seja relatada com maior frequência, há casos de leucocitose associada à DG (Eu-Ahsunthornwattana *et al.*, 2008). Nosso estudo, em média, mostrou um aumento inicial na contagem de leucócitos de camundongos infectados com ambas as Linhagens de DENV-2. Essa alteração foi mais proeminente em camundongos infectados com a Linhagem II. Após atingir o pico (72 hpi), o número de leucócitos começou a diminuir e alguns indivíduos infectados em ambos os grupos apresentaram valores muito abaixo da média. Outro modelo de camundongo infectado com DENV-2 também mostrou leucocitose seguida por leucopenia (Rajmane *et al.*, 2013), e camundongos BALB/c infectados com DENV-3 apresentaram uma diminuição no número de leucócitos às 72 hpi, aos7 e 14 dpi (Caldas *et al.*, 2019).

Como a dengue é uma doença dinâmica, suas manifestações podem variar ao longo do tempo de infecção. A gravidade pode aumentar abruptamente durante, ou imediatamente após o período de defervescência (Torres *et al.*, 2008; Martina, 2009) e apenas um entre 20 pacientes que desenvolvem a doença vão apresentar sinais graves (Stanaway *et al.*, 2016). Quando

analisamos os valores individualmente, três camundongos infectados (Linhagem I: 24mil/mm^3 e Linhagem II: 63 e 148 mil/mm^3) apresentaram número de plaquetas muito abaixo da média de seus respectivos grupos aos 14 dpi. Para o HCT o número de valores discrepantes foi maior: quatro indivíduos infectados com a Linhagem I e seis infectados com a Linhagem II também apresentaram valores bem acima da média de seus grupos. Já em relação à contagem de leucócitos, havia "*outliers"* tanto entre valores que tendiam à leucopenia (Linhagem I: 1,8; 0,6 e 1,2 mil/mm^3 e Linnhagem II: 1,9; 2,1 e 2,3 mil/mm^3) quanto aos que tendiam à leucocitose (Linhagem II: 8,1; 6,6 e 4,8 mil/mm^3). Estes valores discrepantes podem indicar que, apesar de o BALB/c ser um modelo experimental proposto para estudos de FD, alguns indivíduos podem apresentar sinais que tendem à DG.

Os DENV entram na célula por ligação específica da proteína E com receptores celulares. Várias proteínas envolvidas na adsorção do vírus já foram identificadas, incluindo o heparan sulfato, DC-SIGN (ICAM-3), as "heat shock protein" (Hsp) 70, Hsp 90, receptores de manose, e lectinas (Chen *et al.*, 1997; Lozach *et al.*, 2005; Reyes-Del Valle *et al.*, 2005 Miller *et al.*, 2008; Hoving *et al.*, 2014; Lo *et al.*, 2016; Laureti *et al.*, 2018). Em humanos, o genoma e antígenos de DENV já foram detectados em diversos órgãos em diferentes tipos celulares (Jessie *et al.*, 2004; Lima *et al.*, 2011; Póvoa *et al.*, 2014; Nunes, *et al.*, 2019B; Cunha *et al.*, 2021). Neste estudo o genoma do DENV foi detectado em amostras de coração, músculo esquelético e fígado e o antígeno viral, em CE de coração e rim e em cardiomiócitos de camundongos infectados. Tais dados apontam que houve disseminação do vírus pelo organismo dos camundongos que foram inoculados pela via intravenosa, e estão de acordo com outros estudos que detectaram o RNA viral em diferentes tecidos após infecção experimental pelas vias intravenosa ou peritoneal (Shresta *et al.*, 2006; Tan *et al.*, 2010; Rasinhas *et al.*, 2017; Caldas *et al.*, 2019).

O resultado da infecção e o tropismo do DENV por determinado tecido podem ser influenciados pelo sorotipo ou pela virulência da cepa infecciosa (Tuiskunen *et al.*, 2011; Halstead, 2015; Dissanayake *et al.*, 2018). Um estudo realizado com soros de pacientes relatou que a viremia induzida por amostras da Linhagem II era duas ordens de magnitude maior do que a viremia induzida pela Linhagem I e um aumento no número de casos de DG ocorreu após a emergência da nova Linhagem de DENV-2, em 2007 (Nunes *et al.*, 2016). Entretanto, em nossos estudos, apenas entre amostras de fígado, houve uma diferença notável entre os títulos resultantes da infecção das duas linhagens ($1,2x10^{-1}$ cópias de RNA/µl para Linhagem I e $3,93x10^{8}$ cópias de RNA/µl para a Linhagem II).

As anormalidades da função hepática induzidas pela infecção por DENV variam de um leve aumento nos níveis de transaminase e bilirrubina até a insuficiência hepática aguda, que pode levar à morte (Seneviratne *et al.*, 2006; Dalugama *et al.*, 2018; Kularatne *et al.*, 2018). ALT e AST são consideradas indicadores de disfunção hepática, pois são liberadas na corrente sanguínea após lesão das células hepáticas (Ozer *et al.*, 2008). Níveis elevados (geralmente, leve ou moderadamente) dessas enzimas são um marcador precoce de dengue. Os níveis de transaminases são mais elevados em pacientes com FHD ou SCD (Nguyen *et al.*, 1997; Kularatne *et al.*, 2018). Um estudo revelou um aumento superior à 10 vezes nos níveis destas enzimas em pacientes que vieram a óbito (Dissanayake *et al.*, 2018;). O aumento dos níveis das transaminases hepáticas também foi observado em camundongos imunocompetentes infectados com DENV-2 (Paes *et al.*, 2005; 2009; França *et al.*, 2010) assim como neste estudo, que mostrou um aumento médio nos níveis de AST e ALT em todos os momentos de infecção em camundongos infectados com a Linhagem I, enquanto as amostras infectadas com a Linhagem II apresentaram valores mais elevados nos primeiros dias de infecção (24 e 48 hpi) (AST) e às 48 e 72 hpi (ALT). Ademais, ambos os grupos infectados apresentaram indivíduos com os níveis de AST e ALT bastante acima da média de seus grupos. Essas diferenças, como já foi mencionado, podem indicar uma tendência a manifestações mais graves.

Alterações histopatológicas induzidas pela infecção por DENV podem ser observadas em fígados de camundongos imunocompetentes experimentalmente infectados, bem como em amostras de autópsia de casos graves (Atrasheuskaya *et al.*, 2003; Barreto *et al.*, 2004; Basílio-de-Oliveira *et al.*, 2005; Paes *et al.*, 2005; Gulati *et al.*, 2007; França *et al.*, 2010; Póvoa *et al.*, 2014; Sakinah *et al.*, 2017). Embora apenas duas amostras de fígado tenham testado positivo para o genoma viral (Linhagem I: 1/10 e Linhagem II: 1/10), uma série de alterações foram observadas em nossas amostras. As alterações foram focais, conforme relatado em outros estudos (Barreto *et al.*, 2004; Barth *et al.*, 2006). A alteração mais frequentemente observada foi infiltrado de células inflamatórias, o que corrobora achados anteriores em camundongos BALB/c infectados com DENV-2, -3 e -4 (Atrasheuskaya *et al.*, 2003; Barreto *et al.*, 2004; Paes *et al.*, 2005; Rasinhas *et al.*, 2017; Caldas *et al.*, 2019). Após a infecção por DENV, as células de Kupffer e macrófagos liberam citocinas e quimiocinas que ativam as células inflamatórias. A liberação de citocinas pró-inflamatórias pelas células Th1 pode, então, resultar em vasodilatação (Dissanayake *et al.*, 2018). A dilatação dos capilares sinusóides foi observada com maior frequência nas amostras infectadas com a Linhagem I (90%), enquanto a frequência entre as amostras infectadas com a outra linhagem foi de 10%. Essa alteração foi observada em

outros estudos usando o mesmo modelo de camundongo (Barreto *et al.*, 2004; Barth *et al.*, 2006; Caldas *et al.*, 2019).

Outra alteração observada no fígado foi a balonização das células hepáticas, que é presumivelmente causada pelo influxo de fluidos na célula devido ao dano à membrana citoplasmática (Saxena *et al.*, 2018). Essa alteração estava presente em 70% e 80% das amostras infectadas com a Linhagem I e II, respectivamente, e já foi relatada como resultado de infecção viral (Chau *et al.*, 2004; Sanyal *et al.*, 2005). Sabe-se que a infecção por DENV pode alterar o metabolismo lipídico (Randall *et al.*, 2018). Esteatose macrovesicular foi observada em uma amostra infectada com a Linhagem II. Essas alterações foram observadas em estudos realizados por nosso grupo, em camundongos BALB/c infectados com DENV-3 e -4 (Rasinhas *et al.*, 2017; Caldas *et al.*, 2019). O acúmulo intracelular de gordura ocorre em diferentes células humanas infectadas com DENV, bem como na linha de células C6/36 de *Aedes. albopictus*, e pode desempenhar um papel na produção viral (Samsa *et al.*, 2009; Zhang *et al.*, 2018; Ambroggio *et al.*, 2021).

Neste estudo, também foi observado o aumento do volume do núcleo de hapatócitos. A cariocitomegalia pode ser causada pela poliploidia de hepatócitos. Não há na literatura menção de tal alteração em dengue; entretanto, o aumento do número de células hepáticas com núcleos aumentados foi associado a danos ao fígado (Dumková *et al.*, 2017; Rabelo *et al.*, 2018). Outros núcleos, de aparência atípica, apresentavam inclusões e padrões alterados de cromatina, que também foram observados em fígados de camundongos BALB/c infectados com DENV-3 (Caldas *et al.*, 2019). As inclusões nucleares acumulam, na matriz nuclear, substâncias que não são encontradas no núcleo em circunstâncias normais e podem ser causadas por infecção viral. Nos hepatócitos, podem ser resultado do acúmulo de glicogênio (Ip *et al.*, 2010). Paes *et al.* (2005) também observou inclusões nucleares em amostras de fígado de camundongos BALB/c e as descreveu como semelhantes a lipídeos. Porém, uma investigação mais aprofundada é necessária para identificar a natureza das inclusões observadas neste estudo.

A poliploidização e binucleação dos hepatócitos são características do crescimento e da fisiologia do fígado ou podem estar associadas a doenças. Podem ser favoráveis para patógenos ou induzidas por eles para melhorar a sobrevida (Grizzi *et al.*, 2007). Por outro lado, as células bi/multinucleadas são mais capazes de responder a uma grande demanda de síntese de proteínas (Toyoda *et al.*, 2005; Austin *et al.*, 2014; Wang *et al.*, 2017). Nossas amostras infectadas apresentaram um aumento significativo na bi/multinucleação. A mesma alteração foi observada em camundongos BALB/c por Sakinah *et al.* (2017) e por nosso grupo em um estudo sobre

reinfecção por sorotipo heterólogo de DENV (Almeida *et al.*, 2018). As células binucleadas podem ser formadas por divisão nuclear ou fusão celular (Hedgecock *et al.*, 1985; Glotzer *et al.*, 2005) e vários vírus têm atividade fusogênica (Duelli & Lazebnik, 2007; Gentric *et al.*, 2014). Grizzi *et al.* (2007) sugeriu que a binucleação é a resposta da célula à doença hepática porque aumenta com a progressão do estado necroinflamatório. Além disso, pode ser um sinal de regeneração do tecido, pois o aumento de bi/multinucleação após hepatectomia parcial também já foi reportado (Miyaoka *et al.*, 2012). A binucleação observada neste estudo pode ter aumentado em resposta à infecção. Além disso, uma diminuição no número total de hepatócitos foi observada em camundongos infectados com ambas as Linhagens, o que pode indicar morte celular. De fato, algumas de nossas amostras apresentaram necrose, como observado também em outros estudos com DENV-2 (Atrasheuskaya *et al.*, 2003; Barreto *et al.*, 2004; Paes *et al.*, 2005) e DENV-4 (Rasinhas *et al.*, 2017).

A DG está associada ao extenso envolvimento do endotélio (Basu *et al.*, 2008). A alteração da permeabilidade vascular desempenha um papel importante na patogênese da mesma (Gluber *et al.*, 1998; Puerta-Guardo *et al.*, 2016). Estudos demonstraram que o aumento do número de células endoteliais hepáticas infectadas coincide com o início da DG (Zellweger *et al.*, 2010). Entretanto, postula-se que a permeabilidade vascular é causada por mediadores inflamatórios e não por dano ao endotélio ou morte celular (Aye *et al.*, 2014; Puerta-Guardo, 2016; Malavige, 2017). Embora o nosso modelo não seja de DG, hemorragia focal foi observada em uma amostra de fígado, sugerindo permeabilidade vascular alterada. Este achado está de acordo com relatos de casos humanos (Basílio-de-Oliveira *et al.*, 2005; Póvoa *et al.*, 2014) e estudos desse mesmo modelo experimental infectado com DENV-3 e DENV-4 (Rasinhas *et al.*, 2017; Caldas *et al.*, 2019).

Alguns autores sugerem que o rim não seja um alvo do DENV devido à falta de evidências de replicação viral nesse órgão (Jessie *et al.*, 2004; Póvoa *et al.*, 2014). Entretanto, o envolvimento desse órgão durante a dengue é documentado. Proteinúria, hematúria e glomerulonefrite foram relatadas durante ou logo após a infecção aguda por dengue. Além disso, insuficiência renal aguda (IRA) e lesão renal aguda são características comuns entre pacientes com FHD / SCD (Acharya *et al.*, 2010; Laoprasopwattana *et al.*, 2010; Bhagat *et al.*, 2012; Mehra *et al.*, 2012; Oliveira *et al.*, 2015; Vachvanichsanong *et al.*, 2015; Pagliari *et al.*, 2016; Vakarani *et al.*, 2017; Nunes, 2019B).

Como a ureia é reabsorvida pelos rins, níveis alterados desse metabólito no sangue podem indicar disfunção renal. Níveis elevados de ureia são frequentemente mencionados

quando há envolvimento renal durante a dengue (Mohsin *et al.*, 2009; Laoprasopwattana *et al.*, 2010; Oliveira *et al.*, 2015; Mallhi *et al.*, 2015; Tansir *et al.*, 2017), e pode ser uma consequência de lesão glomerular ou hipotensão (Mallhi *et al.*, 2015). Para a análise fa função renal dos camundongos, amostras de sangue foram coletadas às 24, 48 e 72 hpi, e os níveis de ureia (BUN) foram medidos. Enquanto as médias do grupo infectado com a Linhagem I não ultrapassaram as médias do grupo controle, os camundongos do grupo Linhagem II apresentaram maiores níveis do metabólito quando comparados ao grupo controle negativo às 24 e 48 hpi. Um estudo prévio também aponta o aumento dos níveis de ureia (BUN) em soros de camundongos BALB/c infectados com DENV-3 (Caldas *et al.*, 2019). A diferença entre os grupos infectados com as Linhagens I e II foi estatisticamente significativa em 24 e 48 hpi. Os resultados distintos podem ser um reflexo das diferentes cepas virais ou fatores genéticos do hospedeiro.

As alterações observadas nas amostras de rins foram focais e não diferiram qualitativamente entre as linhagens. Por outro lado, quantitativamente, a diferença entre as Linhagens I e II quanto ao aumento do volume dos glomérulos (90% e 60% dos camundongos infectados, respectivamente) foi notável. De acordo com esses resultados, a análise morfométrica revelou que, em média, a área glomerular em camundongos infectados com a Linhagem I excedia ligeiramente a área glomerular em camundongos infectados com a outra linhagem. No entanto, a diferença não foi significativa. Alterações glomerulares são frequentemente relatadas em casos de dengue em que os rins estão afetados (Oliveira *et al.*, 2015; Pagliari *et al.*, 2016; Lim *et al.*, 2019). Neste estudo, as amostras de rim de camundongos infectados apresentaram tanto atrofia glomerular quanto aumento do volume de glomérulos. De fato, a análise morfométrica mostrou que, enquanto, em média, a área glomerular dos camundongos infectados excedia a do grupo controle, o número de corpúsculos de Malpighi (cM) nos rins dos camundongos não infectados superava os dos grupos infectados. Nossos achados morfométricos relativos aos cM podem sugerir que a atrofia é um estágio inicial de necrose, pois, em um estudo semelhante, Caldas *et al.* (2019) observou glomérulos em diferentes estágios de atrofia, bem como áreas livres de glomérulos no córtex renal de camundongos BALB/c infectados com DENV-3. A diferença do número de cM foi estatisticamente significativa entre todos os grupos. Em relação ao aumento do volume glomerular, havia, em nossas amostras, áreas onde os glomérulos estavam aumentados devido ao aparente aumento de celularidade, a ponto de obliterar o espaço de Bowman. Após observação sob microscópio eletrônico de transmissão, foi possível verificar que tal aumento foi resultado da proliferação mesangial e migração de células mononucleares. Análises de

amostras de pacientes com dengue e camundongos BALB/c infectados experimentalmente associou a proliferação mesangial com a deposição de imunocomplexos em glomérulos (Boonpucknavig *et al.*, 2003; Barreto *et al.*, 2004; Lizzaraga, 2014; Upadhaya, 2010). Como resultado do aumento da celularidade, os glomérulos apresentaram capilares congestionados e edema. Nunes *et al.* (2019B) e Pagliari *et al.* (2016) também observaram congestão em capilares em glomérulos; entretanto, não houve proliferação mesangial em suas amostras.

Em nossas análises ultraestruturais, observamos acúmulo de líquido no interior de glomérulos, no entanto, não observamos nenhum dano endotelial. A permeabilidade capilar pode ser decorrente da liberação de mediadores inflamatórios da célula mononuclear presente, tanto no interior dos capilares quanto infiltradas entre as células do folheto parietal da cápsula de Bowman. Essa hipótese é corroborada por autores que acreditam que a permeabilidade vascular alterada na dengue é causada pela resposta imunológica do hospedeiro, e não pela infecção de células endoteliais ou morte celular (Aye *et al.*, 2014; Puerta-Guardo *et al.*, 2016; Malavige & Ogg, 2017). Além disso, algumas de nossas amostras apresentaram focos de hemorragia e pequenos infiltrados de células mononucleares na área cortical de rim, o que está de acordo com estudos realizados com casos humanos de infecção DENV-3 e -4, e camundongos BALB/c infectados com DENV-3 (Basílio-de-Oliveira *et al.*, 2005; Limonta *et al.*, 2012; Póvoa *et al.*, 2014; Nunes *et al.*, 2019B; Caldas *et al.*, 2019).

Análises a partir de amostras de autópsia associaram necrose tubular à dengue (Póvoa *et al.*, 2014; Pagliari *et al.*, 2015; Nunes *et al.*, 2019B). Mohsi *et al.* (2009) relatou um caso de IRA com necrose tubular em um paciente com dengue que apresentava apenas sintomas clássicos da dengue. Alguns autores sugerem que a necrose resulta de processos isquêmicos devido a choque hipovolêmico grave, hipoprofusão, hipóxia, edema intersticial, infiltrado mononuclear, glomerulonefrite aguda ou rabdomiólise (Wiwanitkit *et al.*, 2005; Lee *et al.*, 2009; Mohsin *et al.*, 2009; Póvoa *et al.*, 2014; Tansir *et al.*, 2017). Análises de rim de modelo murino BALB/c demonstraram figuras mitóticas, sinais indiretos de hiperplasia, que podem estar relacionados à lesão tubular (Caldas *et al.*, 2019). Todos os rins de camundongos infectados apresentaram áreas focais de necrose tubular, principalmente em túbulos contorcidos proximais. As amostras apresentaram descamação das células epiteliais e perda das vilosidades que constituem a borda em escova, assim como observado por Tansir *et al* (2017) em amostra de biópsia. Por outro lado, Caldas (2019) reportou espessamento da planura estriada. Dados de autópsias descreveram perda de membrana basal, núcleos picnóticos e dilatação do RE em células necróticas, hemorragia e atrofia tubular com infiltrado inflamatório mononuclear discreto. Além disso, IL-18 e IL-6, ambas citocinas pró-inflamatórias, foram detectadas em

células tubulares (Basilio-de-Oliveira *et al.*, 2005; Póvoa *et al.*, 2014; Pagliari *et al.*, 2015; Nunes *et al.*, 2019B). Em nossas amostras, alguns núcleos apresentavam-se picnóticos, alteração observada neste mesmo modelo infectado com DENV-3 (Caldas *et al.*, 2019). Outros, apresentaram grande perda de cromatina. Havia áreas de rarefação citoplasmática e algumas células epiteliais dos túbulos contorcidos estavam palidamente coradas. As análises ultraestruturais revelaram que essas células estavam esvaziadas, com perda da maior parte do seu conteúdo citoplasmático. Ademais, os cortes histológicos revelaram alguns núcleos deslocados devido a inclusões citoplasmáticas arredondadas e de grande volume e semelhantes a gotículas de lipídios. Essas inclusões também foram vistas por Caldas *et al.* (2019). Análises ultraestruturais demonstraram que, na verdade, estas estruturas eram vesículas, o que, até o presente momento não foi descrito como uma alteração no tecido renal durante a infecção pelos DENV.

Há na literatura poucos relatos de manifestação pulmonar grave devido à infecção por DENV (Sharma *et al.*, 2007; Marchiori *et al.*, 2009; 2012; Koyama, 2021), e um estudo realizado por Rodrigues *et al.* (2014) mostrou que há uma tendência leve à moderada de envolvimento do pulmão. O genoma ou antígeno do DENV não foram detectados em nossas amostras deste órgão. Contudo, havia alterações no pulmão de camundongos infectados, tais como espessamento do septo interalveolar e colapso alveolar devido à migração de células inflamatórias para o interstício. Análises histomorfométricas demonstraram que a diferença entre a espessura média de septos de camundongos do grupo controle e infectados foi estatisticamente significativa, assim como foi observado neste mesmo modelo experimental infectado com DENV-3 (Caldas *et al.*, 2019). A diferença entre os dois grupos infectados também foi significativa. Como mecanismo compensatório, alguns alvéolos estavam hiperinsuflados. Estas constatações estão de acordo com dados de investigação de casos fatais e estudos experimentais com camundongos (Barreto *et al.*, 2004; Basílio-de-Oliveira *et al.*, 2005; Barth *et al.*, 2006; Rodrigues *et al.*, 2014, Póvoa *et al.*, 2014; Rasinhas *et al.*, 2017, Caldas *et al.*, 2019).

Conforme mencionado acima, algumas amostras de pulmão apresentavam hemorragia focal e edema, também observados em camundongos BALB/c infectados com DENV-3 (Caldas *et al.*, 2019). Em humanos, hemorragia pulmonar é relatada em casos fatais (Basílio-de-Oliveira *et al.*, 2005; Rodrigues, *et al.*2014, Póvoa *et al.*, 2014), e alguns pacientes com sintomas mais graves apresentaram secreção tingida de sangue provenientes de ambos os pulmões (Marchiori *et al.*, 2009; 2012). Até onde se sabe, nenhum caso de hemorragia pulmonar relacionado à FD

foi relatado em humanos, talvez pelo fato de que o envolvimento pulmonar não é frequentemente investigado em tais casos.

Embora os dados sobre manifestações cardíacas na dengue sejam escassos, ultimamente, o envolvimento do coração tem sido cada vez mais relatado (Salgado *et al.*, 2010; Pareda *et al.*, 2015; Tansir *et al.*, 2017; Bhatt *et al.*, 2020; Janakiraman Abhinayaa *et al.*, 2021) e está principalmente associado a casos graves (Salgado *et al.*, 2010; Pareda *et al.*, 2015; Janakiraman Abhinayaa *et al.*, 2021). Os principais achados incluem distúrbios de condução atrioventricular, arritmias supraventriculares, miocardite, bradicardia, taquicardia, derrame pericárdico, disfunção diastólica e níveis aumentados de enzimas cardíacas (Salgado *et al.*, 2010; Miranda *et al.*, 2013B; Tahir *et al.*, 2015; Janakiraman Abhinayaa *et al.*, 2021). A maioria das manifestações cardíacas é atribuída à miocardite, causada por infecção viral (Tahir *et al.*, 2015; Estofolete *et al.*, 2019).

A presença do DENV no coração já foi demonstrada em casos humanos fatais e em modelo experimental (Lima *et al.*, 2011; Salgado *et al.*, 2010; Miranda *et al.*, 2013B; Póvoa *et al.*, 2014, Rasinhas *et al.*, 2018; Cladas *et al.*, 2019). Neste estudo, o genoma e o antígeno viral foram detectados em amostras de coração de camundongos infectados com ambas as linhagens de DENV-2. Embora não seja claro se o dano tecidual é causado por infecção viral direta ou resposta celular do sistema imunológico do hospedeiro (Salgado *et al.*, 2010; Oliveira *et al.*, 2017, Kangussu *et al.*, 2021), uma série de alterações histopatológicas já foram relatadas (Miranda *et al.*, 2013A; 2013B; Póvoa *et al.*, 2014; Oliveira *et al.*, 2017, Rasinhas *et al.*, 2017; Caldas *et al.*, 2019; Kangussu *et al.*, 2021).

As amostras de tecido cardíaco de murinos analisadas aqui apresentaram alterações histopatológicas e ultraestruturais focais e comuns entre as duas linhagens de DENV-2. Infiltrado inflamatório é encontrado em coração de modelos murinos infectados experimentalmente com DENV e também já foi observado em casos humanos (Miranda *et al.*, 2013A; 2013B; Póvoa *et al.*, 2014; Oliveira *et al.*, 2017, Jácome *et al.*, 2019, Rasinhas *et al.*, 2018; Kangussu *et al.*, 2021). Miranda e colaboradores (2013B) relataram que o infiltrado mononuclear consiste principalmente em células do tipo macrófago $CD68^{+}$. Rarefação citoplasmática e do conteúdo nuclear dos cardiomiócitos foi observada em nossas amostras histológicas. As análises por MET demonstraram que a rarefação é decorrente da degeneração de miofilamentos, que também foi observada por Miranda (2013A) e Póvoa (2014). Também foi observada alteração de mitocôndrias, que em estudos em humanos foi associada ao processo apoptótico das fibras cardíacas (Póvoa *et al.*, 2014). Assim como observado por Caldas *et al.*

(2019) e Rasinhas *et al.* (2017) em camundongos infectados com os DENV-3 e DENV-4, nossas amostras apresentaram pequenas áreas de congestão vascular (Rasinhas *et al.*, 2017; Caldas *et al.*, 2019). As diferenças observadas nas alterações induzidas por sorotipos diferentes podem indicar que um determinado sorotipo de DENV tenha maior ou menor tropismo por determinado órgão (Dissanayake *et al.*, 2018). Neste estudo, as alterações observadas em coração foram moderadas; entretanto, estudos realizados com camundongos BALB/c infectados com os DENV-3 e DENV-4 revelaram alterações na morfologia de discos intercalares, redução da pressão sanguínea e consequente aumento da frequência cardíaca e aumento do extravasamento de plasma no coração (Rasinhas *et al.*, 2017; Kangussu *et al.*, 2021).

Mialgia e fraqueza muscular são manifestações apresentadas por pacientes de dengue. De acordo com um estudo realizado por Misra *et al* (2012), o envolvimento muscular durante a dengue é benigno e auto-limitante (Malheiros *et al.*, 1993; Davis *et al.*, 2004; Acharya *et al.*, 2010; Tansir *et al.*, 2017; Gulati *et al.*, 2020). Análises de biópsias de músculo esquelético revelaram infiltrado inflamatório perivascular, proliferação mitocondrial, focos de necrose com ou sem miofagocitose e hemorragia intersticial (Malheiros *et al.*, 1993; Davis *et al.*, 2004; Acharya *et al.*, 2010; Misra *et al.*, 2012). Presença de edemas, focos de hemorragia e alterações metabólicas podem ser responsáveis pela fraqueza muscular transiente (Misra *et al.*, 2012). Não obeservamos alterações morfológicas neste tecido. Entretanto, o vRNA foi detectado em amostras de músculo esquelético de camundongos infectados com ambas as linhagens de DENV-2. Este achado está de acordo com um estudo de infecção experimental de cultura de miotubos humanos, que mostrou que o DENV é capaz de infectar e replicar em células musculares (Salgado *et al.*, 2010).

A elevação dos níveis de CK é indicativo de dano no tecido muscular, no coração ou no cérebro. A hipótese mais provável é que o dano causado seja muscular. Para avaliar dano cardíaco, CK-MB é mais apropriada. Nossos resultados mostraram que, em média, 24 e 48 hpi, os níveis de CK estavam mais elevados em camundongos infectados com ambas as linhagens de DENV-2, principalmente no grupo Linhagem II. A análise individual mostrou que seis camundongos infectados apresentaram níveis de CK muito acima da média de cada grupo (Linnhagem I: 1.209 U/L, 1.837 U/L e 979 U/L e Linhagem II: 1.89 U/L 7, 4.354 U/L e 822 U/L). Nossos resultados estão em concordância com estudos de casos humanos de dengue em que há elevação dos níveis desta enzima. Porém, estes estudos também relatam alterações funcionais e histopatológicas do músculo esquelético que não foram observados neste trabalho (Malheiros *et al.*, 1993; Misra *et al.*, 2012; Tansir *et al.*, 2017; Gulati *et al.*, 2020).

Desde a introdução do DENV-2 no Brasil, três diferentes linhagens deste sorotipo foram descritas (Drummond *et al.*, 2013; Faria *et al.*, 2015; Torres *et al.*, 2019). A segunda Linhagem a ser descrita ficou restrita ao nordeste do país (Drummond *et al.*, 2013) e não foi associada a nenhuma grande epidemia. Já a primeira e a terceira linhagem, aqui referidas como Linhagem I e Linhagem II, respectivamente, foram responsáveis por dois grandes surtos com perfis epidemiológicos distintos: a epidemia provocada pela Linhagem II resultou em maior número de casos graves, hospitalizações e óbitos e acometeu um número maior de jovens abaixo de 15 anos de idade (Nogueira *et al.*, 2007; Siqueira *et al.*, 2011; Rodriguez-Barraquer *et al.*, 2011).

A cocirculação de diferentes sorotipos, a emergência de novas cepas virais do genótipo Asiático e a teoria de que variações genéticas do DENV podem resultar em diferentes graus de virulência ressaltam a importância da investigação do papel destes fatores na ocorrência de casos graves e fatais. Ademais, o evidente acometimento de múltiplos órgãos durante a dengue torna necessário o melhor entendimento da infecção sistêmica e sua potencial contribuição na evolução para a DG. Ficou demonstrado, neste estudo, que em modelo experimental BALB/c, a infecção pelas Linhagens de DENV-2 não induz alterações diferentes qualitativamente. Contudo, para alguns parâmetros, houve diferença significativa na magnitude/intensidade e tempo com que tais alterações se manifestaram.

5. Perspectivas

- Avaliar o envolvimento do baço e do sistema nervoso central, órgãos de importante relevância na patogênese das arboviroses.
- Avaliar o perfil citocínico e a viremia resultantes da infecção dessas Linhagens no modelo murino.

6. Conclusões

- O modelo experimental BALB/c demonstrou-se susceptivél a ambas as Linhagens de DENV-2
- De uma forma geral, independente da Linhagem de DENV-2 infectante, não foi observado aumento na temperatura média dos camundongos infectados durante o curso da infecção.
- Camundongos infectados com a Linhagem II ganharam, significativamente, menos peso que aqueles infectados com a Linhagem I, no entanto, com exceção do coração, os pesos dos órgãos de camundongos do grupo Linhagem II foram significativamente maiores aos 14 dpi.
- Nos aninais infectados pela Linhagem I, os níveis de AST, foram ligeiramente elevados durante todo o curso da infecção, e naqueles infectados com a Linhagem II, somente às 24 e 48 hpi. Já os níveis de ALT, em camundongos infectados pela Linhagem I encontraram-se mais elevados mais precocemente (24 hpi) do que aqueles infectados pela Linhagem II (48 hpi).
- Níveis de ureia (BUN) foram significativemante mais elevados em camundongos infectados pela Linhagem II e o HCT, mais elevado no grupo infectado com a Linhagem I.
- Animais infectados com a Linhagem II apresentaram níveis séricos de CK significativamente mais elevados que em camundongos infectados com a Linhagem II 24 e 48 hpi.
- As alterações morfológicas não apresentaram diferenças qualitativas entre as linhagens na maioria dos órgãos, no entanto, no fígado, necrose, esteatose macrogoticular e focos de hemorragia só estavam presentes em camundongos infectados com a Linhagem II.
- Apesar de camundongos infectados com a Linhagem I apresentaram maior redução na população de hepatócitos, aqueles infectados com a Linhagem II apresentaram mais bi/multinucleação nestas células, menor número de corpúsculos renais e septos interalveolares mais espessados.
- A análise individual dos camundongos infectados com a Linhagem II indicou uma maior tendência destes aniamais a exibir sinais mais graves da doença.
- Com base nos tempos de infecção pré-estabelecidos, os dados sugerem que os sinais de infecção induzidos pela Linhagem II se manifestam com maior intensidade em momentos mais tardios quando comparados à Linhagem I.
- Camundongos BALB/c, mostraram-se um modelo experimental adequado para estudos de fisiopatologia da dengue nos órgãos avaliados.

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8. Anexos

8.1. Artigos publicados durante o desenvolvimento da tese

viruses

MDPI

Article

Morphological Aspects and Viremia Analysis of BALB/c Murine Model Experimentally Infected with Dengue Virus Serotype 4

Arthur da Costa Rasinhas [1,*], Fernanda Cunha Jácome [1], Gabriela Cardoso Caldas [1], Ana Luisa Teixeira de Almeida [1], Marcos Alexandre Nunes da Silva [1], Daniel Dias Coutinho de Souza [1], Amanda Carlos Paulino [1], Derick Mendes Bandeira [1], Raphael Leonardo [1], Priscila Conrado Guerra Nunes [2], Ronaldo Mohana-Borges [3], Ortrud Monika Barth [1], Flavia Barreto dos Santos [2] and Debora Ferreira Barreto Vieira [1]

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Abstract: Ever since its brief introduction in the Brazilian territory in 1981, dengue virus serotype 4 (DENV-4) remained absent from the national epidemiological scenario for almost 25 years. The emergence of DENV-4 in 2010 resulted in epidemics in most Brazilian states. DENV-4, however, remains one of the least studied among the four DENV serotypes. Despite being known as a mild serotype, DENV-4 is associated with severe cases and deaths and deserves to be investigated; however, the lack of suitable experimental animal models is a limiting factor for pathogenesis studies. Here, we aimed to investigate the susceptibility and potential tropism of DENV-4 for liver, lung and heart of an immunocompetent mice model, and to evaluate and investigate the resulting morphological and ultrastructural alterations upon viral infection. BALB/c mice were inoculated intravenously with non-neuroadapted doses of DENV-4 isolated from a human case. The histopathological analysis of liver revealed typical alterations of DENV, such as microsteatosis, edema and vascular congestion, while in lung, widespread areas of hemorrhage and interstitial pneumonia were observed. While milder alterations were present in heart, characterized by limited hemorrhage and discrete presence of inflammatory infiltrate, the disorganization of the structure of the intercalated disc is of particular interest. DENV-4 RNA was detected in liver, lung, heart and serum of BALB/c mice through qRT-PCR, while the NS3 viral protein was observed in all of the aforementioned organs through immunohistochemistry. These findings indicate the susceptibility of the model to the serotype and further reinforce the usefulness of BALB/c mice in studying the many alterations caused by DENV.

Keywords: DENV-4; BALB/c mice; liver; lung; heart; histopathology; ultrastructure

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Citation: da Costa Rasinhas, A.; Cunha Jácome, F.; Cardoso Caldas, G.; Teixeira de Almeida, A.L.; Nunes da Silva, M.A.; Dias Coutinho de Souza, D.; Carlos Paulino, A.; Mendes Bandeira, D.; Leonardo, R.; Conrado Guerra Nunes, P.; et al. Morphological Aspects and Viremia Analysis of BALB/c Murine Model Experimentally Infected with Dengue Virus Serotype 4. *Viruses* **2021**, *13*, 1954. https://doi.org/10.3390/v13101954

Academic Editors: Charu Kaushic and Amy Gillgrass

Received: 26 August 2021
Accepted: 24 September 2021
Published: 29 September 2021

PLOS ONE

RESEARCH ARTICLE

Structural investigation of C6/36 and Vero cell cultures infected with a Brazilian Zika virus

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Citation: Barreto-Vieira DF, Jácome FC, da Silva MAN, Caldas GC, de Filippis AMB, de Sequeira PC, et al. (2017) Structural investigation of C6/36 and Vero cell cultures infected with a Brazilian Zika virus. PLoS ONE 12(9): e0184397. https://doi.org/10.1371/journal.pone.0184397

Editor: Zheng Xing, University of Minnesota College of Veterinary Medicine, UNITED STATES

Received: April 24, 2017

Accepted: August 23, 2017

Published: September 12, 2017

Abstract

Zika virus (ZIKV) is a member of the flavivirus genus, and its genome is approximately 10.8 kilobases of positive-strand RNA enclosed in a capsid and surrounded by a membrane. Studies on the replication dynamics of ZIKV are scarce, which limits the development of antiviral agents and vaccines directed against ZIKV. In this study, *Aedes albopictus* mosquito lineage cells (C6/36 cells) and African green monkey kidney epithelial cells (Vero cells) were inoculated with a ZIKV sample isolated from a Brazilian patient, and the infection was characterized by immunofluorescence staining, phase contrast light microscopy, transmission electron microscopy and real-time RT-PCR. The infection was observed in both cell lineages, and ZIKV particles were observed inside lysosomes, the rough endoplasmic reticulum and viroplasm-like structures. The susceptibility of C6/36 and Vero cells to ZIKV infection was demonstrated. Moreover, this study showed that part of the replicative cycle may occur within viroplasm-like structures, which has not been previously demonstrated in other flaviviruses.

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 10^9 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

Virus Research 260 (2019) 163–172

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Different aspects of platelet evaluation in dengue: Measurement of circulating mediators, ability to interact with the virus, the degree of activation and quantification of intraplatelet protein content

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ARTICLE INFO

Keywords:
Platelets
Dengue
Activation markers

ABSTRACT

Platelets play a role in hemostasis, coagulation, angiogenesis, inflammation and immune response is one of the most affected cells in dengue. Here we describe some aspects of platelets by observing their specific circulating mediators, the ability to interact with the virus and morphological consequences of this interaction, activation markers and intraplatelet protein contents in dengue. We conducted this study using dengue-patients as well as healthy donors. Immunoenzymatic assay, flow cytometry, transmission electron microscopy and intraplatelet proteins expression assays were carried out. Briefly, we found an increase in sCD62L, NO or TBX2 ratio in platelet count, mostly in patients with the worse clinical outcome. After in vitro DENV infection or during natural infection, platelets underwent morphological alteration with increased expression of platelet activation markers, particularly in natural infections. Analysis of intraplatelet protein contents revealed different angiogenic and inflammatory profiles, maintaining or not extracellular matrix integrity between DF and DFWS patients. Thus, platelets are frequently affected by dengue, either by altering their own functionality, as "carrier" of the virus, or as an antiviral and mediator-secreting effector cell. Thus, strategies aimed at recovering platelet amounts in dengue seem to be essential for a better clinical outcome of the patients.

Acta virologica **63**: 292 – 300, 2019 doi:10.4149/av_2019_309

Secondary dengue infection in immunocompetent murine model leads to heart tissue damage

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Received October 10, 2018; accepted November 23, 2018

Summary. – Dengue, considered the most important arthropod-borne viral disease affecting humans, is transmitted by the bite of mosquitoes of the genus Aedes and caused by one of the four distinct serotypes of dengue virus (DENV-1, -2, -3 and -4). Infection with one of the four serotypes provides lifelong homotypic immunity. However, immunity against the heterologous serotypes is transient. As a consequence, secondary infection may lead to severer manifestations due to cross-reactivity of antibodies and T-cells. Over 500,000 people are hospitalized every year and around 2,5 million, living in endemic areas, are at risk of infection. Given the background, the development of vaccines and anti-DENV drugs is of the utmost importance, as is the characterization of an animal model for testing them. The purpose of this study was to investigate ultrastructural alterations caused by DENV secondary infection in BALB/c mice heart. To achieve our goal, six BALB/c mice were infected with DENV-1 and, 4 months later, reinfected with DENV-2. Uninfected mice were used as negative controls. Heart samples were collected and processed for ultrastructural and histopathological analysis. Our results showed edema, endothelium activation characterized by the presence of transport vesicles, free platelets in interstitium, mitochondria presenting rarefied matrix and degenerated cristae, and disorganization of muscle fibers. These results point not only to BALB/c mice susceptibility to DENV infection, but also to the fact that, although it is not an often reported occurrence, dengue can lead to heart damage.

Keywords: dengue; experimental model; reinfection; BALB/c mice

Dengue, Yellow Fever, Zika and Chikungunya epidemic arboviruses in Brazil: ultrastructural aspects

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BACKGROUND The impact of arbovirus cocirculation in Brazil is unknown. Dengue virus (DENV) reinfection may result in more intense viraemia or immunopathology, leading to more severe disease. The Zika virus (ZIKV) epidemic in the Americas provided pathogenicity evidence that had not been previously observed in flavivirus infections. In contrast to other flaviviruses, electron microscopy studies have shown that ZIKV may replicate in viroplasm-like structures. Flaviviruses produce an ensemble of structurally different virions, collectively contributing to tissue tropism and virus dissemination.

OBJECTIVES AND METHODS In this work, the *Aedes albopictus* mosquito cell lineage (C6/36 cells) and kidney epithelial cells from African green monkeys (Vero cells) were infected with samples of the main circulating arboviruses in Brazil [DENV-1, DENV-2, DENV-3, DENV-4, ZIKV, Yellow Fever virus (YFV) and Chikungunya virus (CHIKV)], and ultrastructural studies by transmission electron microscopy were performed.

FINDINGS We observed that ZIKV, the DENV serotypes, YFV and CHIKV particles are spherical. ZIKV, DENV-1, -2, -3 and -4 presented diameters of 40-50 nm, and CHIKV presented approximate diameters of 50-60 nm. Viroplasm-like structures was observed in ZIKV replication cycle.

MAIN CONCLUSIONS The morphogenesis of these arboviruses is similar to what has been presented in previous studies. However, we understand that further studies are needed to investigate the relationship between viroplasm-like structures and ZIKV replication dynamics.

Key words: arboviruses - transmission electron microscopy - viroplasm - flaviviruses

Clinical aspects and detection of Zika virus RNA in several tissues of experimentally infected BALB/cAn mice

Aspectos clínicos e detecção de RNA do vírus Zika em diferentes tecidos de camundongos BALB/cAn infectados experimentalmente

DOI:10.34119/bjhrv4n1-160

Recebimento dos originais: 21/12/2020
Aceitação para publicação: 25/01/2021

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ABSTRACT
Our group infected BALB/cAn mice with Zika virus to evaluate clinical signs and viral load in several tissues at three different kinetic points. We inoculated fifteen mice with a 100µl of a viral solution to collect nine different tissues, from each animal, for RNA extraction and quantification. Infections caused no lethality. Some of them, however, showed great agitation, hair bristling, and itchy skin. Viral RNA was detected in one sample of heart, eight of the spleen, and two of skeletal muscle. Seven positive detections were from the third day after infection. Only spleen yielded positive results at a later time.

Keywords: Zika virus, BALB/cAn mice, qRT-PCR.

RESUMO
Nosso grupo infectou camundongos BALB/cAn com o vírus Zika para avaliar sinais clínicos e a carga viral em diferentes tecidos em três pontos distintos de uma cinética. Inoculamos 15 camundongos com 100µl de uma solução viral para coletar, em cada animal, nove diferentes tecidos para a extração de RNA e quantificação. As infecções não foram letais. Alguns deles, no entanto, mostraram grande agitação, eriçamento de pelos e coceira intensa. O RNA viral foi detectado em uma amostra de coração, oito de baço e duas de músculo esquelético. Sete das detecções positivas ocorreram em três dias após a infecção. Apenas no baço resultados continuaram positivos em momentos mais tardios.

Palavras-chave: Vírus Zika, BALB/cAn, qRT-PCR.

8.2. Capítulo de livro publicado durante o desenvolvimento da tese

Novel Trends in Microbiology and Biotechnology
ISBN: 978-81-947154-2-9
First Edition; Volume – 1; 2020
Chapter – 7, Page: 104 - 140

7

EXPERIMENTAL ANIMAL MODELS OF DENGUE VIRUS INFECTION

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1. Introduction

Dengue and dengue virus

Dengue (DEN) is the most prevalent Arbovirosis among humans. It is classified by the World Health Organization (WHO) as the fastest spreading viral disease transmitted by vectors. DEN has an enormous potential to cause major epidemics throughout the world and recent estimates suggest that from 390 million people that are infected annually, about 96 million develop clinical manifestations, with approximately 500 thousand episodes of severe dengue and more than 20 thousand deaths related to the disease (WHO, 2013; Bhatt *et al*, 2013, Murray *et al*, 2013). According to the Center for Disease Control and Prevention, 40 % of the world's population lives in endemic areas and is at risk of contracting DENV (CDC, 2019).

8.3. Parecer do comitê de ética em pesquisa

Ministério da Saúde
Fundação Oswaldo Cruz
COMITÊ DE ÉTICA EM PESQUISA-CEP/FIOCRUZ

Rio de Janeiro, 07 de abril de 2014.

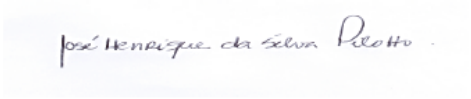
Solicitação de extensão do prazo de execução de projeto

Título do Projeto: *"Dengue no Brasil: vigilância virológica, epidemiologia molecular e padronização de método sorológico utilizando antígenos recombinantes".*
Protocolo de pesquisa: 274/05
Pesquisador Responsável: Dra. Rita Maria Ribeiro Nogueira.
Instituição: Laboratório de Flavivírus do IOC/Fiocruz

Foi submetido à apreciação do CEP Fiocruz/IOC e APROVADO, relatório e solicitação de extensão do prazo de execução do projeto supracitado, datada de 15 de fevereiro de 2014, e recebido no CEP Fiocruz/IOPC em 11 de março de 2014, que tem como objetivo o monitoramento contínuo de casos de dengue, procurando identificar o sorotipo envolvido. A confirmação de casos suspeitos de dengue repercute diretamente no diagnóstico precoce, tratamento e recuperação dos pacientes, nas medidas de combate ao vetor, e no melhor conhecimento da epidemia da dengue no nosso país.

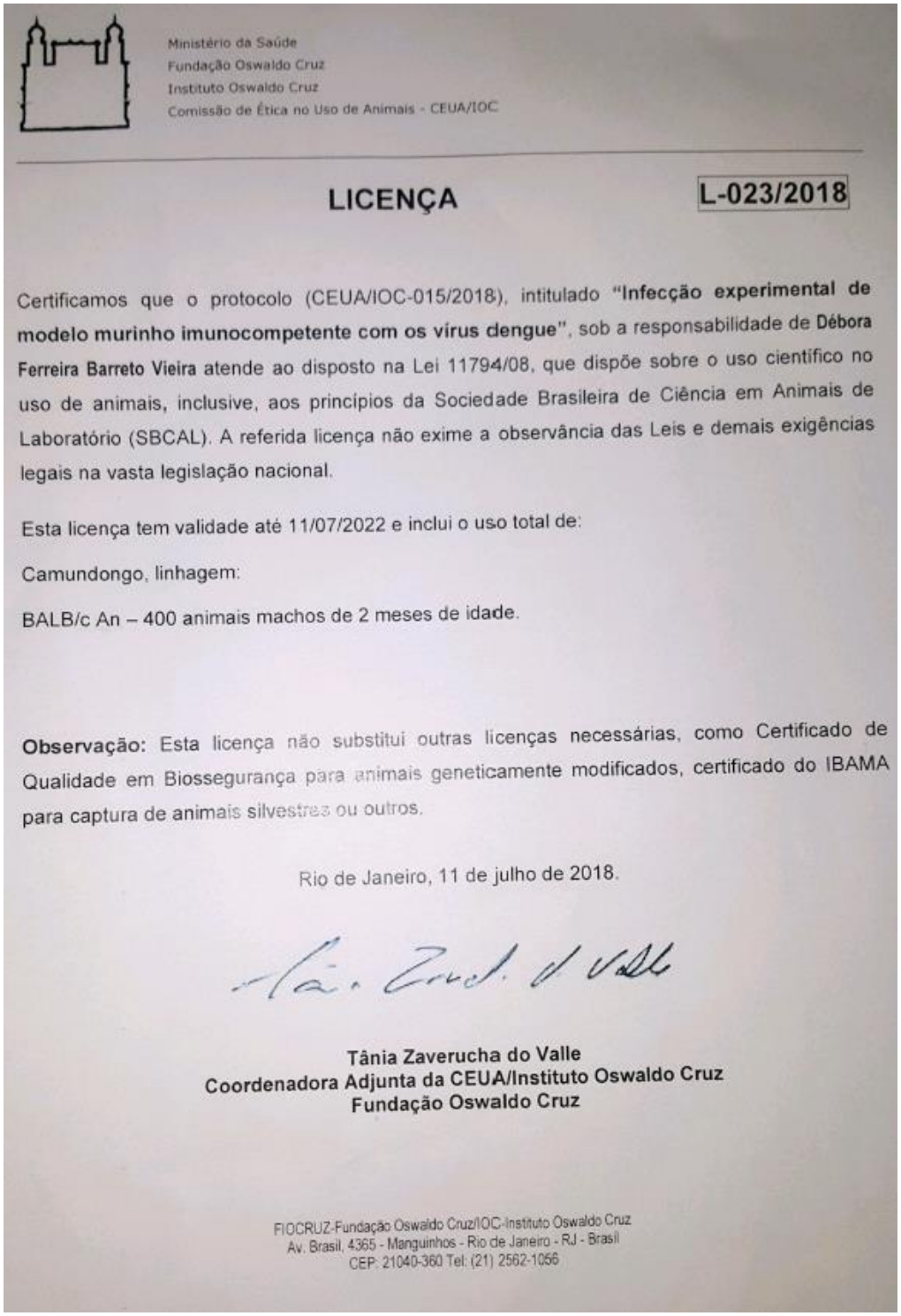
Diante do exposto, o Comitê de Ética em Pesquisa do Instituto Oswaldo Cruz (CEP FIOCRUZ/IOC), de acordo com as atribuições definidas na Resolução CNS 466/12, manifesta-se pela aprovação da solicitação de extensão do prazo de execução do projeto supracitado.

Informamos que deverão ser apresentados relatórios parciais e relatório final do projeto de pesquisa. Além disso, qualquer modificação ou emenda ao protocolo original deverá ser submetida para apreciação do CEP/Fiocruz.



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

8.4. Parecer do comitê de ética no uso de animais



Ministério da Saúde
Fundação Oswaldo Cruz
Instituto Oswaldo Cruz
Comissão de Ética no Uso de Animais - CEUA/IOC

LICENÇA **L-023/2018**

Certificamos que o protocolo (CEUA/IOC-015/2018), intitulado **"Infecção experimental de modelo murinho imunocompetente com os vírus dengue"**, sob a responsabilidade de **Débora Ferreira Barreto Vieira** atende ao disposto na Lei 11794/08, que dispõe sobre o uso científico no uso de animais, inclusive, aos princípios da Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). A referida licença não exime a observância das Leis e demais exigências legais na vasta legislação nacional.

Esta licença tem validade até 11/07/2022 e inclui o uso total de:

Camundongo, linhagem:

BALB/c An – 400 animais machos de 2 meses de idade.

Observação: Esta licença não substitui outras licenças necessárias, como Certificado de Qualidade em Biossegurança para animais geneticamente modificados, certificado do IBAMA para captura de animais silvestres ou outros.

Rio de Janeiro, 11 de julho de 2018.

Tânia Zaverucha do Valle
Coordenadora Adjunta da CEUA/Instituto Oswaldo Cruz
Fundação Oswaldo Cruz

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