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CLINICAL MICROBIOLOGY - RESEARCH PAPER

A fatal case of dengue hemorrhagic fever associated with dengue virus 4 (DENV-4) in Brazil: genomic and histopathological findings

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Abstract

Dengue infection is the most prevalent arthropod-borne viral disease in subtropical and tropical regions, whose primary vector is *Aedes aegypti* mosquitoes. The mechanisms of dengue virus (DENV) pathogenesis are little understood because we have no good disease models. Only humans develop symptoms (dengue fever, DF, or dengue hemorrhagic fever, DHF) and research has been limited to studies involving patients. Samples from serum, brain, cerebellum, heart, lungs, liver, and kidneys from a 13-year-old male patient that died with hemorrhagic manifestations were sent for differential diagnosis at Adolfo Lutz, using both classical virological methods (RT-qPCR, virus isolation, ELISA, and hemagglutination inhibition test) and immunohistochemistry (IHQ). A DENV serotype 4 was detected by a DENV multiplex RT-qPCR, and the C6/36 cell supernatant was used for NGS using Minion. Lesions were described in the heart, liver, lung, and kidney with positive IHQ in endothelial cells of the brain, cerebellum, heart, and kidney, and also in hepatocytes and Kuppfer cells. A whole genome was obtained, revealing a DENV-4 genotype II, with no evidence of secondary dengue infection.

Keywords Dengue virus · Immunohistochemistry · Whole-genome sequencing · Phylogeny

Introduction

Dengue virus serotypes 1–4 (DENV1-4) are members of the *Flavivirus* genus within the *Flaviviridae* family. DENV has a genome consisting of a single positive-polarity RNA strand with approximately 10.8 kb in length with a single open reading frame that encodes a single polyprotein that is further cleaved into 3 structural proteins, capsid (C), membrane (M), and envelope (E), and 7 non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, 2 K, NS4B, and NS5 [1].

Phylogenetic studies placed the origin of DENV in the forests of Malaysia, which later segregated into four main groups matching with the serotype and several genotypes within [2–4]. During the last decades, dengue's importance has dramatically changed, as it evolved from a sporadic disease to a major public health problem, mainly in tropical regions. It is believed that about 390 million people have dengue virus infections, with 96 million cases annually worldwide [5]. The virus is transmitted by *Aedes* (Stegomyia) spp., primarily *Aedes aegypti* and *Aedes albopictus*, which are globally distributed. Although the low fatality rate with most cases asymptomatic or presented by a mild disease with flu-like symptoms, dengue can progress to a severe hemorrhagic form (DH) and to dengue shock syndrome (DSS), which may lead to death [6]. It is believed that 1–2% of human infections may have hemorrhagic manifestations [7], such as petechiae, purpuric lesions, and ecchymoses [8].

Injuries associated with DENV may be a direct consequence of the virus itself or an exacerbation of the immune response after infection, namely antibody-dependent enhancement (ADE). In ADE, after a secondary dengue infection with a different serotype, there is a greater burden of infection that

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induces imbalanced pro-inflammatory and anti-inflammatory responses, causing vascular leakage and a possible hypovolemic shock [9, 10]. On the other hand, viral determinants may also contribute [11–14]. In Brazil, all serotypes co-circulate since 2010 after the reintroduction of DENV-4 in the North Region, which was further disseminated to other states within the country [15–18]. During the 2012 epidemic, a total of 1,162,998 cases had been recorded in the American continent, of which 565,510 in Brazil. Furthermore, the country reported 3774 severe cases, with 247 deaths according to the Brazilian Ministry of Health. Most histopathological reports indicate that infection targets the liver, spleen, heart, and lung [19–21]. However, reports of lesions caused by DENV-4 severe cases are scarce, and experimental animal models are limited considering dengue pathogenesis in humans. Here, we describe a complete genome of DENV-4 genotype II genome and its histopathological findings from a fatal case in São José do Rio Preto, Brazil, in 2012.

Methods

Case

Serum samples and fixed fragments of the brain, liver, lung, spleen, and kidney from a 13-year-old male patient from São José do Rio Preto, who died with a suspicion of hemorrhagic fever on June 1st 2012 (symptoms on May 20th) were sent to Adolfo Lutz Institute for differential diagnosis for dengue, yellow fever, leptospirosis, hantavirus, and Brazilian-spotted fever. Adolfo Lutz Institute is a reference laboratory belonging to the Secretary of Health, São Paulo state, Brazil. For instance, São José do Rio Preto is located in the northwestern region of São Paulo State and is endemic for DENV and YFV. Samples were collected on the same day the death has occurred.

Differential diagnosis

Serum sample was processed at Vector-Borne Diseases Laboratory. It was subjected to an in house capture-ELISA IgM for dengue virus and yellow fever virus [22], RNA extraction using QIAamp RNA Viral Mini Kit (QIAGEN, Hilden, Germany), according to manufacturer's instructions, and followed by a fourplex dengue RT-qPCR [23]; virus isolation in C6/36 cells (*Aedes albopictus* clone C6/36 ATCC CRL-1660) followed by immunofluorescence assay [24]; and hemagglutination inhibition test (HI), described by [25] and adapted for microplates by [26]. Antigens DENV 1–4 (Hawaii, New Guinea C, H-87, and H-241), yellow fever virus (YFV) H111, Rocio virus (ROCV) SPH34675, Saint Louis encephalitis virus (SLEV) An11916, and Ilheus virus (ILHV) BeH 7445 strains were used for HI.

Histopathology and immunohistochemistry

Samples from the brain, cerebellum, heart, right and left lungs, liver, and kidneys were placed in 10% neutral buffered formalin and sent to the Center of Pathological Anatomy of Adolfo Lutz Institute in São Paulo. The fragments were submitted for routine histological processing, embedded in paraffin, and stained in hematoxylin–eosin for histopathological analysis. Immunohistochemical techniques were performed for *Leptospira interrogans*, dengue virus, Rickettsia rickettsi, and hantavirus (Table 1).

Sequencing

A C6/36 isolate obtained from a sample named SPH329894 was extracted using QIAmp viral RNA mini kit (Qiagen, Hilde, Germany) according to manufacturer's instructions, followed by RT-qPCR described by [27]. In brief, cDNA was produced from viral RNA using random hexamers and the Protoscript II First Strand cDNA synthesis kit (NEB). The genome was amplified using a multiplex PCR scheme designed to produce overlapping 500 bp amplicons across the whole coding region of DENV4. PCR products were quantified, barcoded using the Oxford Nanopore Technologies (ONT) Native Barcoding Kit, and pooled in an equimolar fashion. Sequencing libraries consisting of 24 samples of DENV-4 were constructed using a Ligation Sequencing kit.

Table 1 Antigen, clone, clonality and species of origin, species target, working dilution, and source

Antigen	Clone	Clonality (species origin)	Species target	Dilution	Source
Leptospira interrogans	Polyclonal	Rabbit	Human	1:2000	DVDB CDC
Dengue	Polyclonal	Mouse	Human	1:10,000	DVDB CDC
Dengue NS1	4H2	Mouse	Human	1:500	Butantan Institute
Hantavirus	GB4-8F7	Mouse	Human	1:1000	DVDB CDC
Rickettsia	Polyclonal	Rabbit	Human	1:30,000	DVDB CDC

DVDB CDC, Division of Vector-Borne Diseases, Centers For Disease Control and Prevention, Fort Collins, CO, EUA

Phylogenetic analysis

In order to check DENV-4 genotype, we used Genome Detective tool [28]. The ORF sequence obtained was then aligned with full genomes of DENV-4 retrieved from Genbank (Sup) using MAFTT [29], visualized, and edited using Bioedit Sequence Alignment Editor [30]. The best model and phylogenetic analysis were performed using IQ-TREE [31] with an ultrafast bootstrap (1000 replicates), and the tree generated was edited using FigTree v.14.3 with a midpoint root. A sylvatic DENV-4 genotype IV was used as an outer group (GenBank accession number JF262779).

Structural analysis

In order to visualize differences in the electrostatic interactions due to the mutations found in this case, we proceeded to create a model of the protein based on homology to others using the software I-TASSER [32] with default options and no ligand binding site prediction (LBS). We used as input sequences the virus described in the paper and two other reference sequences from related DENV4 2012 viruses from São José do Rio Preto, Brazil (accession number KP188562), as well as a canonical sequence. After the homology model was constructed, we performed a series of energy minimization steps using CHIMERA [33] to achieve a stabilized structure. Clashes and bumps were removed in CHIMERA following directions given by Molprobity [34] evaluations of the generated structures. The output structures were then submitted to the APBS server to produce electrostatic maps for the whole set. Structure and maps were then uploaded to PyMol. Visualizations were made using in house scripts in PyMol (available at https://pymol.org/2/support.html).

Results

Laboratory findings

Serum analysis for dengue virus detection was positive for ELISA IgM and DENV-4 serotype (RT-qPCR and IFA). HI revealed titers for YFV (1:40) and DENV-4 (1:40), and negative results for the other flavivirus tested. The main results for histopathological and IHQ analyses are depicted in Table 2.

Briefly, microscopic examinations of the brain and cerebellum not revealed any histopathological changes. Immunohistochemistry for NS1-dengue virus protein was focally positive in blood capillary endothelial cells in the heart. Bilaterally, the lung parenchyma showed marked vascular congestion, edema, and a discrete infiltrate of lymphomononuclear inflammatory cells in the interstitium, characterizing a mild, interstitial pneumonia. In addition, extensive areas of alveolar and bronchial hemorrhage were observed. Immunohistochemistry for DENV was negative in the lung. The liver parenchyma revealed areas of hepatocellular necrosis in the mid-zone and centrilobular areas (zones 2 and 3), with sparse apoptotic hepatocytes resembling Councilman-Rocha Lima bodies. There was also moderate hepatocellular and intracanalicular cholestasis, multifocal macrogoticular steatosis, sinusoidal congestion, and discrete infiltrate of lymphomononuclear cells in portal spaces. Positive immunostaining with anti-dengue NS1 antibody was observed in Kuppfer cells and sinusoidal endothelial cells. The kidney showed areas of hydropic degeneration and acute tubular necrosis, associated with mild intracytoplasmatic brown pigment in proximal convoluted tubules. The endothelial and mesangial cells of glomerular capillary

Table 2 Histopathological findings and immunohistochemistry results of a positive DENV-4 fatal case

Tissue	Histological findings	Immunohistochemical reactivity
Brain	No changes	Endothelium and leukocytes
Cerebellum	No changes	Endothelium and leukocytes
Heart	Discrete edema and rare interstitial lymphocytes	Endothelium and leukocytes
Lung	Marked vascular congestion, edema, and discrete infiltrate of lymphomononuclear cells in the interstitium Extensive area of alveolar and bronchial hemorrhage Interstitial pneumonitis with alveolar hemorrhage	(-)
Liver	Necrosis in midzonal and centrilobular topography (Zones 2 and 3) Rare Coulciman corpuscles Moderate hepatocellular and intracanalicular cholestasis Focal macrogoticular steatosis Discrete infiltrate of portal lymphomononuclear cells	Hepatocytes Liver Kuppfer cells
Kidney	Hydropic degeneration and tubular necrosis Pigment in the lumen of the proximal convoluted tubules	Endothelial cells of glomerular capillaries and arterioles Distal convoluted tubule epithelial cells

loops and the endothelial cells of small-caliber arterioles were immunostained for dengue NS1 antigen (Fig. 1).

Alignment and phylogenetic findings

Phylogenetic analysis clustered DENV-4 isolate SPH329894 (Genbank accession number OM641997) within genotype II (Fig. 2) (best-fit model according to BIC: TIM + F + G4). The highest nucleotide identity found compared to other DENV-4 isolates was 99.8% (isolate Br246RR/10 from Boa Vista, Roraima State). Considering DENV-4 genomes, we found 2 non-synonymous mutations at positions I759M (position 480 of E protein, stem) and S2910N (NS5 protein), being the first one unique within all DENV-4 sequences analyzed (Sup. 1). Isolate KP188564, from São José do Rio Preto, and LC410196, from Thailand, also shows the same NS5 mutation. Both were conservative.

Electrostatic maps

To also evaluate the possible perturbations in the structure of the viral Envelope protein, we proceeded to generate a model by homology based on the sequence derived from the de novo assembly of the next-generation sequencing produced. RMSD deviation maps were generated for three experimental conditions in silico, in addition to the evaluation of loads and dihedrals formed. We used models for comparison from the outbreaks that occurred in 2012 and 2013 to evaluate the three-dimensional characteristics of protein E and infer interference in intermolecular interactions compared with the

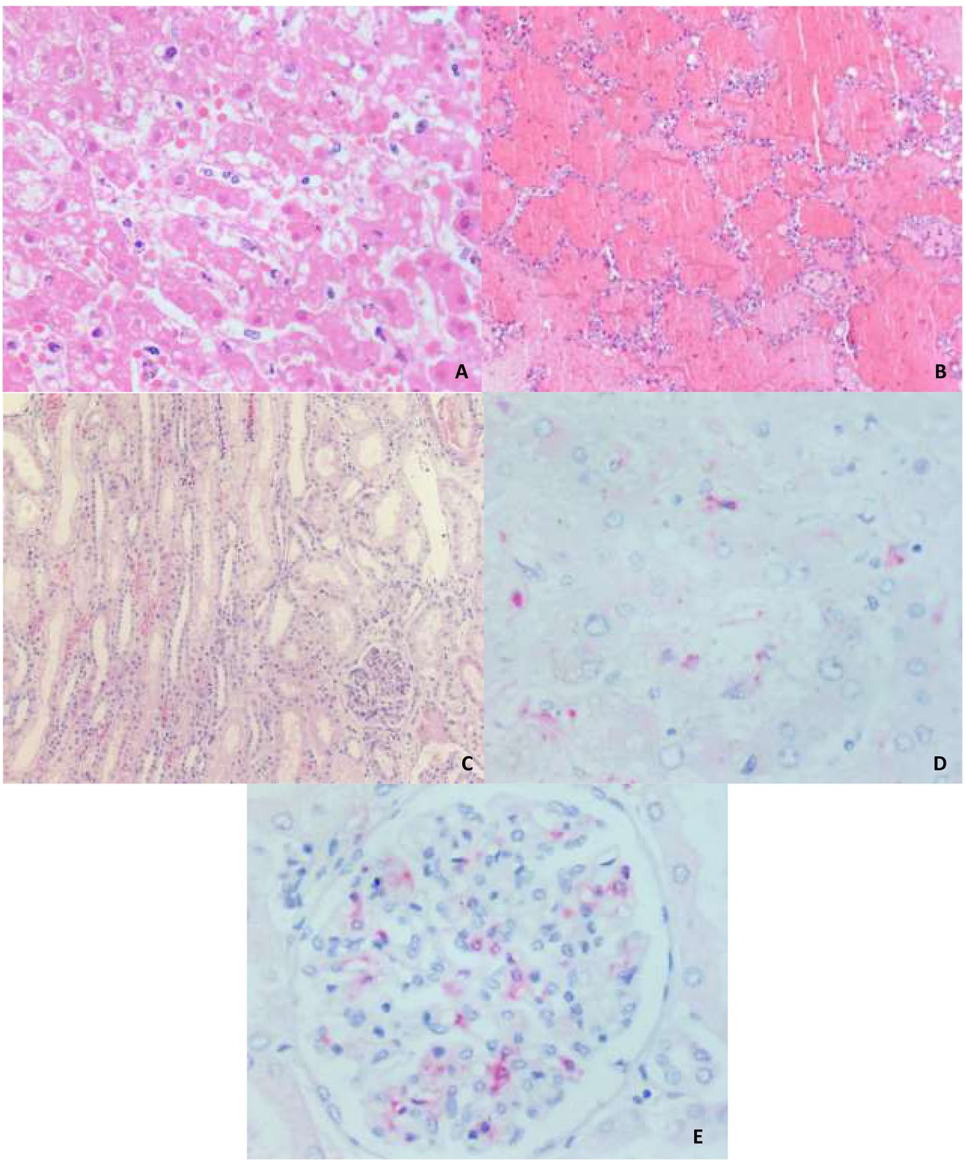

Fig. 1 **A** Photomicrography of the liver. Hepatocellular necrosis and apoptosis with Councilman-Rocha Lima resembling bodies. Hematoxylin and eosin (×400). **B** Photomicrography of the lung. Marked intra-alveolar hemorrhage and edema. Hematoxylin and eosin (×100). **C** Photomicrography of the kidney. Mutifocal acute tubular necrosis. Hematoxylin and eosin (×100). **D** Photomicrography of the liver. Kupffer and endothelial cells have cytoplasmic immunolabeling for dengue virus antigen. IHC (×400). **E** Photomicrography of the kidney. Dengue virus antigen in mesangial and endothelial cells of the glomerulus. IHC (×400)

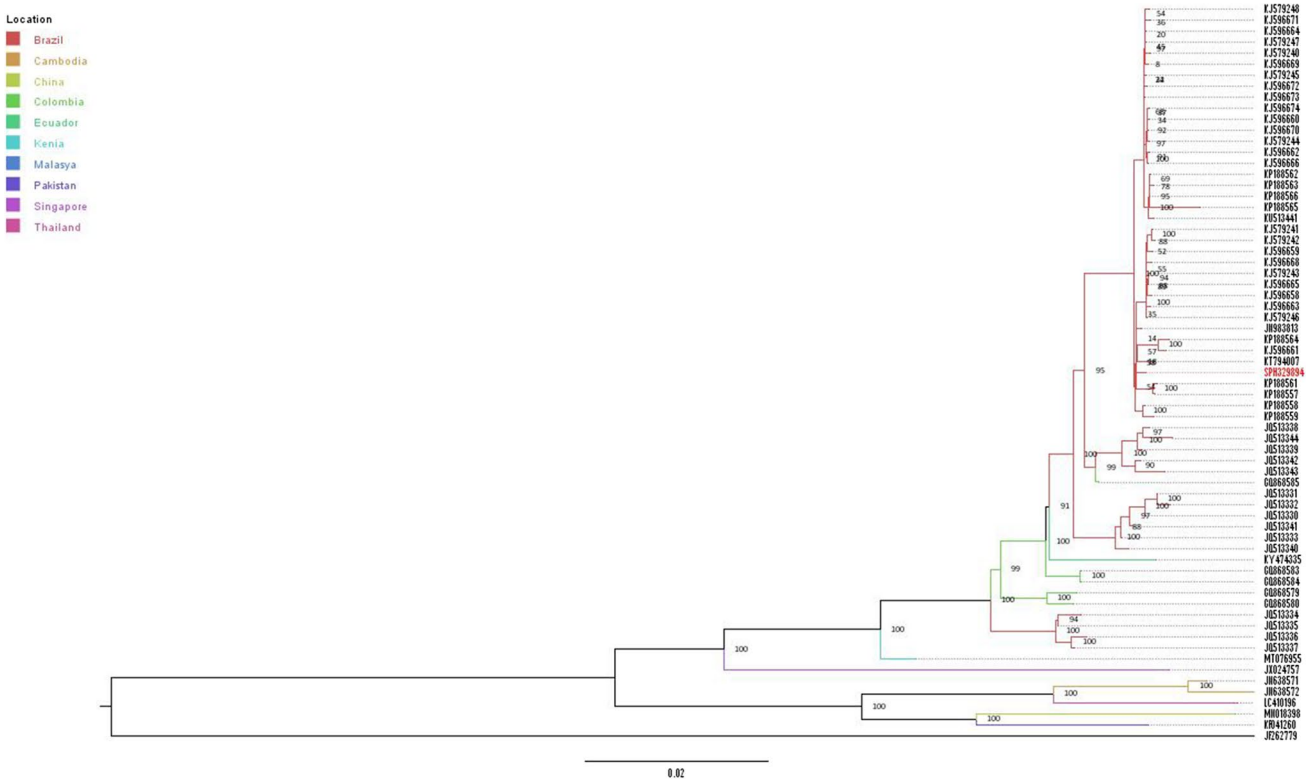

Fig. 2 ML Phylogenetic tree of DENV-4 genotype II isolate SPH329894 (depicted in red). Branches are colored according the country. Sequences of DENV-4 whole genomes were aligned using MAFFT. Best model and ML tree were generated using IQ-Tree [22]. The tree was constructed using FigTree v.1.4.3 with an automatic scale and mid-point root. Scale in nucleotide substitutions per site. Numbers at nodes indicate bootstrap values. Sylvatic DENV-4 was used as outgroup. Sequences are represented by Genbank accession numbers

reference structures of previous outbreaks. The Ramachandran qualifiers for the modeled proteins showed good agreement in Phi and Psi, both for the model of this work and for the models generated for the 2012–2013 cases (S2). *Z*-scores were compared to the protein bank structures in the non-redundant set ($1 < |Z\text{-score}| < 2$). The observed electrostatic changes were not significant in the steam region (345–449). The electrostatic surfaces are represented in supplementary 3.

Discussion

Here, we report a whole genome of a fatal case caused by a DENV-4 genotype II showing hemorrhagic lesions in a 13-year-old male patient from the southeastern region, Brazil, with a positive immunostaining on reticuloendothelial or mononuclear phagocytic system. Although this is a hyperendemicity area for DENV in the country, there was no evidence of a secondary infection, which may lead to antibody-dependent enhancement (ADE), one of the causes for disease severity. The former DHF/DSS (dengue hemorrhagic fever/dengue shock syndrome) classification was recognized as a clinical syndrome of dengue infection during the second half of the XX century. Since then, DENV has spread to new areas of the world, including the American region. The first DHF outbreak occurred in Cuba, where 10,000 cases were reported in 1981, which was caused by DENV-2. Similarly, the first cases of DHF in Brazil were detected in the 1990–1991 epidemic, when DENV-2 was introduced in Rio de Janeiro, and later, in 2000, after DENV-3 introduction [35]. Other evidences suggest that certain DENV genotypes have a greater virulence, such as the DENV-2 Southeast Asian genotype [36–38]. On the other hand, infectious caused by DENV-4 have generally been associated with mild disease and sporadically with DHF [39]. In adults, primary DENV-1 and DENV-3 infections result in high rates of classic dengue fever, while DENV-2 and DENV-4 infections cause milder disease [40, 41]. In children of all ages, primary infection by DENV-2 and DENV-4 are frequently inapparent [42]. However, in Indonesia, where multiple serotypes and genotypes co-circulate, DENV-4 was responsible for 6 cases among 79 positive cases, 4 classified as hemorrhagic fever. However, all of these cases were secondary infection [43]. Phylogenetic analysis classified DENV-4 as a genotype II in a cluster with other Asian isolates, while isolate SPH 329,894 clustered in a monophyletic group with other Brazilian sequences.

It was once proposed that severe disease pathogenesis was also due to viral virulence [44], and some markers are described. For example, a conservative mutation in NS1 (T164S) was suggested to be responsible for enhancing clinical severity in DENV-2 [45, 46]. However, T164 is found on 98% of DENV-4 genomes [46], including isolate SPH329894. In Singapore, Hapuarachchige et al. [47] reported a DENV-4 genotype II fatal case in a 28-year female with encephalitis, cardiomyopathy, elevated transaminases, and coagulopathy. The E protein sequenced in this study showed isoleucine amino acid at position 480. Also, this case was associated with a secondary infection. Limonta et al. [48] described a DENV-4 fatal case in a 41-year-old female from Cuba with necrosis on the kidney and liver. Also, focal areas of hemorrhage were found in the interstitial myocardial tissues. DENV-4 was detected by IHQ in the brain in a 17-year-old male from Mexico with the diagnosis of dengue hemorrhagic fever. The brain showed slight meningeal opacity, vein congestion, and generalized edema with bilateral uncus herniation [49], but there was no genome information from both cases. In children, DENV neurologic complications are also described as a severe outcome. Acute disseminated encephalomyelitis, encephalitis, and acute hypoxic injury have already been reported [50, 51].

Although some studies state no evidence of DENV infecting endothelial cells, reports suggest that these cells are activated during the infection [52]. Here, we describe a focal positive IHQ on capillary endothelial cells in the liver and kidneys, corroborating this last hypothesis. Dengue antigens in Kupffer cells, sinusoidal endothelial cells, and in circulating mononuclear cells of glomerular capillary loops by IHQ have already been described [53]. We have also found apoptotic cells resembling Councilman-Rocha Lima bodies in the liver, which is also frequently described for yellow fever virus [54]. However, the hepatocellular necrosis in dengue infection is primarily paracentral compared to mid-zonal in yellow fever [51]. Circulatory lung lesions, such as pleural petechiae, pulmonary edema, and scattered hemorrhages, were also associated with dengue infection cases. And a degree of tubular injury and casts are common described [51].

In a study from Vietnam, among 5 children with hemorrhagic manifestation, Councilman bodies were observed in four cases, generally midzonal in distribution, in and around the foci of necrosis [55]. IHQ for these cases was positive for DENV-3, and four patients were classified as secondary DENV infection. However, antigens were not significantly detected in endothelial cells. In Brazil, analysis of 4 patients with a fatal outcome (aged 21–63 years) caused by DENV revealed hemorrhage and edema in the liver, lung, heart, kidney, and spleen [56]. All cases were positive for DENV-3, and 2 patients had co-morbidities (diabetes and obesity). Histopathological analysis of the liver showed circulatory and parenchyma damages, with detection of antigens in hepatocytes and in a lesser extend in Kupffer cells and in the endothelium. The lungs presented diffuse areas with hemorrhage and edema, presenting virus antigens in alveolar macrophages. Kidneys presented acute tubular necrosis and focal areas with hemorrhage and edema, with DENV antigens in circulating macrophages and monocytes into blood vessels.

To check if the unique E mutation could enhance viral binding, we have performed an electrostatic map survey. The results show no significant alterations in the profile of the charges distributed on the surface of the E protein, thus suggesting that other factors may be at play. Previous articles have also found that structural perturbations in the E protein can cause cryptic epitopes to be exposed and vice versa, depending upon the region of the mutations that occurred [57]. As a limitation of our study, using structural dynamics could potentially expose such subtleties. Protein–protein interactions could also evaluate a number of solutions to this problem. More, we do not have information regarding patient's laboratory findings and history of comorbidities, although the HI test suggests a primary dengue infection.

Conclusions

Here, we report a whole genome of a fatal case caused by a DENV-4 genotype II showing hemorrhagic lesions in several organs in a 13-year-old male patient from an endemic region in Brazil, with positive immunohistochemistry on endothelial cells, liver, and kidney, with no signs of a secondary dengue infection.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42770-022-00784-4.

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Author contribution Manuscript preparation: MSC, JMG; phylogenetic analysis: MSC; obtained funding and study supervision: ECS; sequencing: MSC, TMC, IMC, FS; histopathology and IHC: CCP, JMG, NCCRS, RR; protein analyses: DFLN. All authors reviewed, contributed to, and approved the final version of the manuscript.

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Declarations

Ethics approval CADDE project GB04-BF07 (for etiological research).

Conflict of interest The authors declare no competing interests.

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