BACTERIAL, FUNGAL AND VIRUS MOLECULAR BIOLOGY - RESEARCH PAPER

Genetic differences of dengue virus 2 in patients with distinct clinical outcome

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Abstract

The genetic diversity of the dengue virus is characterized by four circulating serotypes, several genotypes, and an increasing number of existing lineages that may have differences in the potential to cause epidemics and disease severity. Accurate identification of the genetic variability of the virus is essential to identify lineages responsible for an epidemic and understanding the processes of virus spread and virulence. Here, we characterize, using portable nanopore genomic sequencing, different lineages of dengue virus 2 (DENV-2) detected in 22 serum samples from patients with and without dengue warning signs attended at Hospital de Base of São José do Rio Preto (SJRP) in 2019, during a DENV-2 outbreak. Demographic, epidemiological, and clinical data were also analyzed. The phylogenetic reconstruction and the clinical data showed that two lineages belonging to the American/Asian genotype of DENV-2—BR3 and BR4 (BR4L1 and BR4L2)—were co-circulating in SJRP. Although preliminary, these results indicate no specific association between clinical form and phylogenetic clustering at the virus consensus sequence level. Studies with larger sample sizes and which explore single nucleotide variants are needed. Therefore, we showed that portable nanopore genome sequencing could generate quick and reliable sequences for genomic surveillance to monitor viral diversity and its association with disease severity as an epidemic unfolds.

Keywords Genomic surveillance · Epidemic · DENV-2 · Dengue severity · Sequencing · Brazil

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Introduction

Dengue virus (DENV) is an arbovirus that represents a classic example of reemerging disease, resulting in almost 400 million infections annually in the world [1], being endemic in more than 100 countries, with an incidence that increased in the last 50 years [2]. DENV belongs to the Flaviviridae family, including other clinically important arboviruses like Zika (ZIKV) and yellow fever (YFV). The flavivirus genome consists of a positive single-stranded RNA of approximately 11 kilobases (kb) in length, which encode three structural proteins (capsid (C), pre-membrane (prM), and envelope (E)) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [3].

The clinical manifestations of dengue are classified according to the levels of severity in (1) dengue without warning signs (patients presenting typical symptoms like nausea/vomiting, rash, headache, eye pain, muscle ache, or joint pain, leukopenia, and positive tourniquet test; (2) dengue with warning signs (patients presenting typical

symptoms more other one as abdominal pain, persistent vomiting, fluid accumulation, bleeding from the mucosa, lethargy, enlarged liver, increased hematocrit with decreased platelets); and (3) severe dengue (patients that show severe plasma leakage, severe bleeding, or organ failure) [4].

DENV is classified into four serotypes (DENV-1, 2, 3, and 4) that share approximately 70% of homology at an amino acid level, and within each DENV serotype, there is some degree of genetic diversity [5]. This genetic diversity provides heterogeneity in the strain's circulation, leading to the spread of serotypes and genotypes that differ in their potential to cause epidemics, representing a serious threat to the population. Thus, accurate identification of the genetic variability of viruses is essential for understanding and identifying a key mutation responsible for an epidemic [6].

In this study, we characterize, using a portable genomic approach [7, 8], the genetic diversity of DENV-2 detected in serum samples obtained from patients with infection classified as dengue without warning signs and dengue with warning signs attended at Hospital de Base of São José do Rio Preto, a Brazilian city located in the northeast of São Paulo State, that reported 65.7% (400,856) of all dengue cases identified in the country [9].

Material and methods

São José do Rio Preto is in the southwestern region of São Paulo State, with a population of around 469,173 inhabitants, a tropical climate, average annual rainfall of 1465 mm, and an altitude of 510 m asl [10, 11]. São José do Rio Preto is considered hyperendemic for DENV since these climatic conditions favor the proliferation of the mosquito *Aedes aegypti* [12] (the primary vector of DENV), whose reinfestation in São José do Rio Preto was detected in 1985 [13]. The first case of DENV in this city occurred in 1990, caused by DENV-1, and the first dengue epidemic occurred 4 years later. DENV-2, DENV-3, and DENV-4 were detected, in 1998, 2005, and 2011, respectively, causing new epidemics [14, 15]. In recent years, thousands of cases have been confirmed, highlighting 2013, 2015, and 2019 with 18,000, 22,000, and 33,000 cases, respectively [16].

To assess the genetic diversity of DENV-2 circulating in São José do Rio Preto, we randomly selected 22 RT-qPCR positive samples of DENV-2 from the 2019 bank, collected from 1 to 25 days after the onset of symptoms. Samples were provided by the Hospital de Base of São José do Rio Preto in a study submitted and approved by the Ethical Review Board (process number 28309320.3.0000.5415) from Faculdade de Medicina de São José do Rio Preto (FAMERP), São Paulo, Brazil.

The samples collected during the 2019 dengue epidemic, between February and May, were submitted to total viral RNA extraction using QIAmp Viral RNA Kit (Qiagen®), following the manufacturer's recommendations. Subsequently, the total RNA was submitted to RT-qPCR Singleplex-DENV [17] to check the CT (cycle threshold) values. DENV-2 whole-genome sequencing was performed using a multiplex PCR primer scheme to amplify the DENV-2 genome [18]. The extracted RNA was transcribed to cDNA using Superscript IV First-Strand Synthesis System (Thermo Fisher Scientific, MA, USA) and random hexamer oligonucleotide (Invitrogen®). Then, multiplex PCR was performed to generate overlapping amplicons from the entire viral genome. The amplification of the DENV-2 genome consisted of 35 PCR cycles according to the reaction and thermocycling mixture described by Quick and collaborators [19]. Magnetic beads (AmpureXP (Beckman Coulter, High Wycombe, UK)) were used to purify the PCR products, which were then quantified with Qubit dsDNA High Sensitivity on a Qubit 3.0 device (Life Technologies).

Sequencing libraries were generated using the Genomic DNA Sequencing Kit SQK-LSK108 (Oxford Nanopore Technologies), combining, in equimolar proportions, a total of 250 ng of PCR products using the Native Barcoding Kit (NBD103, Oxford Nanopore Technologies, Oxford, UK). The libraries were loaded into an Oxford Nanopore R9.4 Flow cell (FLO-MIN106). The generated reads were analyzed using the Guppy Basecalling Software version 3.0.3.2 [20], in which the electrical signal of the MinION run is analyzed and converted into nucleotide bases. Basecalled reads were then analyzed in Porechop version 0.2.3 [21] software which removes the adapters and recognizes the sequences of the two barcodes in each final portion of the reads, thus demultiplexing the reads. Subsequently, demultiplexed reads were aligned with the reference sequence of the DENV-2 complete genome (EU920833.1) using bwa-bwa (Alignment via Burrows-Wheeler transformation) version 0.7.17-r1188 [22]. Consensus sequence generation and variant calling were performed using the Nanopolish version 0.11.1 [23], which also checks the original sequencing data generated using Variant Calling. Finally, a consensus sequence was generated for all genomic sites with a depth of at least 20 reads for each site. In cases below this value, "N" bases were added to these positions in the consensus sequence.

We constructed a maximum likelihood (ML) phylogeny to identify the genotypes of the samples. The ML phylogenetic analysis was based on comparing the study sequences with the sequences stored in a database belonging to the different DENV-2 genotypes and lineages originating in different regions of the world in different years of circulation. Sequences were aligned using MAFFT v 7.450 [23] and edited on AliView version 1.23 [24]. Phylogenetic reconstruction was performed using IQ-TREE multicore software version 2.0-rc2 [25], using TIM3 + F + I + G4 as the nucleotide substitution model. The reliability of the branches

was tested through ultrafast bootstrapping and approximate likelihood-ratio test (SH-aLRT), 1000 runs each. After that, we inferred time-scaled trees by using TreeTime [26]. The final tree generated was edited and visualized using FigTree v.1.4.4 [27]. Demographic, epidemiological, and clinical data (symptoms, radiologic, and laboratory findings) were obtained from the Hospital de Base of São José do Rio Preto database. Confidentiality was ensured by de-identifying all questionnaires and samples before data entry and analyses. The data collected were entered into a spreadsheet using Excel® software (Microsoft, Redmond, WA, USA) and imported to SPSS software for IOS (version 25, SPSS, Inc; Chicago, IL, USA). Pearson's chi-square test was used to determine whether the expected frequency in the groups was met. When the chi-square criteria were not achieved, Fisher's test was chosen. A significance level of 0.05 (5%) was adopted for all tests employed.

Results

To explore the genetic diversity of DENV in patients with different clinical forms of dengue fever, we sequenced 22 samples from São José do Rio Preto with collection dates from February to May 2019. The 22 DENV-2 sequences are available in GenBank (https://www.ncbi.nlm.nih.gov;

GenBank accession numbers can be found in Table S1 in Supplementary Material).

Epidemiological data of DENV-2 samples from patients at São José do Rio Preto are presented in Table 1. About the patients, the age range was $24-82$ years, and the median age was 51 years. The frequency of males and females was 36.7% and 63.3%, respectively. All samples were positive for DENV-2 by RT-qPCR, with CT values ranging from 18.82 to 30.72, presenting an average of 25.51 and a median of 25.58. All samples were positive for DENV-2 by RT-qPCR, and CT values with an average of 25.51 (Table 1). These samples were submitted to the amplicon-based sequencing protocol adapted to MinION nanopore sequencing [19]. The generated consensus genomes had a mean coverage of 82% of the genome at $20\times$ minimum sequencing depth.

The 22 newly generated genomes from São José do Rio Preto were subtyped using the Dengue Virus Typing tool [28] and identified as belonging to Genotype III. The consensus sequences were analyzed and aligned with 595 other DENV-2 sequences from the Americas from 1986 to 2019 (Accession numbers can be found in Table S2 in Supplementary Material).

During the sequence analysis, we detected that the genome coverage is not complete. For this reason, we decided to apply the phylogenetic reconstruction only to the envelope, a region that presented complete coverage and that has already been widely used for the detection of DENV

Table 1 Epidemiological data of DENV-2 samples from patients at São José do Rio Preto. *CT*, cycle threshold; *d*, days between the onset of symptoms and data collection

	ID study	Lineage	*d*	Age, sex	Ct RT-qPCR
Dengue without warning signs	636	BR4L2	4	66, F	21.5
	454	BR4L2	3	24, F	18.82
	2491	BR4L1	15	61, M	21.68
	1392	BRL1	6	49, M	23.33
	3589	BR4L1	2	28, F	24.38
	3594	BR4L1	1	33, F	19.3
	1402	BR4L1	4	34, F	22.98
	3588	BR4L1	4	66, F	26.49
Dengue with warning signs	2195	BR3	1	36, M	28.37
	3758	BR4L1	2	53, F	20.1
	3744	BR4L2	15	66, F	28.5
	3449	BR4L1	2	71, M	23.07
	688	BR3	3	23, F	28.34
	3429	BR4L1	0	65, M	24.67
	715	BR4L1	4	30, M	30.76
	1388	BR4L1	1	63, F	21.46
	3055	BR4L2	2	67, F	29.59
	689	BR4L1	2	31, F	27.26
	3295	BR4L1	2	27, M	30.45
	3294	BR4L1	5	82, F	30.23
	3596	BR4L2	4	73, M	29.26
Severe dengue	571	BR4L1	26	39, F	30.72

strains [29]. Genotyping analyses (Fig. 1) showed that all Sao José do Rio Preto sequences were classified as DENV-2 Genotype III (DENV-2-III, Asian-American genotype).

Most samples were classified as DENV-2-III BR4 lineage ($n = 20$, 91%), and only two samples (9%) belonged to the DENV-2-III BR3 lineage. DENV-2-III BR4 lineage can be further subdivided into DENV-2-III BR4L1 and DENV-2-III BR4L2, according to their clustering patterns. In our dataset, we found that 75% of the samples ($n = 15/20$) were identified as DENV-2-III BR4L1 and the remaining 25% ($n = 5/20$) as DENV-2-III BR4L2.

The nucleotide composition of all dengue sequences obtained in this work was analyzed by structural (capsid, pre-membrane/membrane, and envelope) and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins (Fig. 2). We observed different proportions of nucleotides when comparing the three lineages. In the membrane, for example, we found that 44% of BR3 genome samples correspond to G, but 29% and 28% of BR4L1 and BR4L2, respectively, correspond to this mentioned nucleotide. However, these differences did not result in many amino acid changes, except for the presence of a Serine in the BR3 lineage and an Alanine in both BR4 lineages, in 697 positions. Some amino acid differences were noted in some samples of each lineage, including D23N in the BR3 samples, A121T in six BR4L1 samples, A32T in two BR4L1 samples, E558K in eleven BR4L1 samples, A120T in three BR4L2 samples, E558K in four BR4L2 samples, and I109L in two BR4L2 samples.

Concerning the dengue clinical classification defined by WHO 2009 [4], eight cases were classified as dengue without warning signs, 14 as dengue with warning signs and/or severe dengue (with neurologic impairment). We did not find a relation between the clinical outcome and the lineage and sublineage classification, as the phylogenetic analyses showed clustering of both clinical forms within different lineages and sublineages. In addition to clinical forms, the frequency of signs and symptoms was analyzed separately. Fever was the most common symptom reported at hospital admission (21/22; 95.45%), followed by myalgia (17/22; 77.27%), headache (14/22; 63.63%), exanthema (7/22; 31.81%), ocular pain (7/22; 31.81%), arthralgia (6/22; 27.27%), and vomits (4/22; 18.18%). The most frequent warning signs were abdominal pain (6/12; 50%), hemoconcentration (6/12; 50%), fluid accumulation (4/12; 33.33%), and bleeding (3/12; 25%). The severe form case evolved with seizures, hemodynamic dysfunction, fluid accumulation, and brain bleeding. The frequency of signs and symptoms according to lineage classification is presented in Tables 2 and 3.
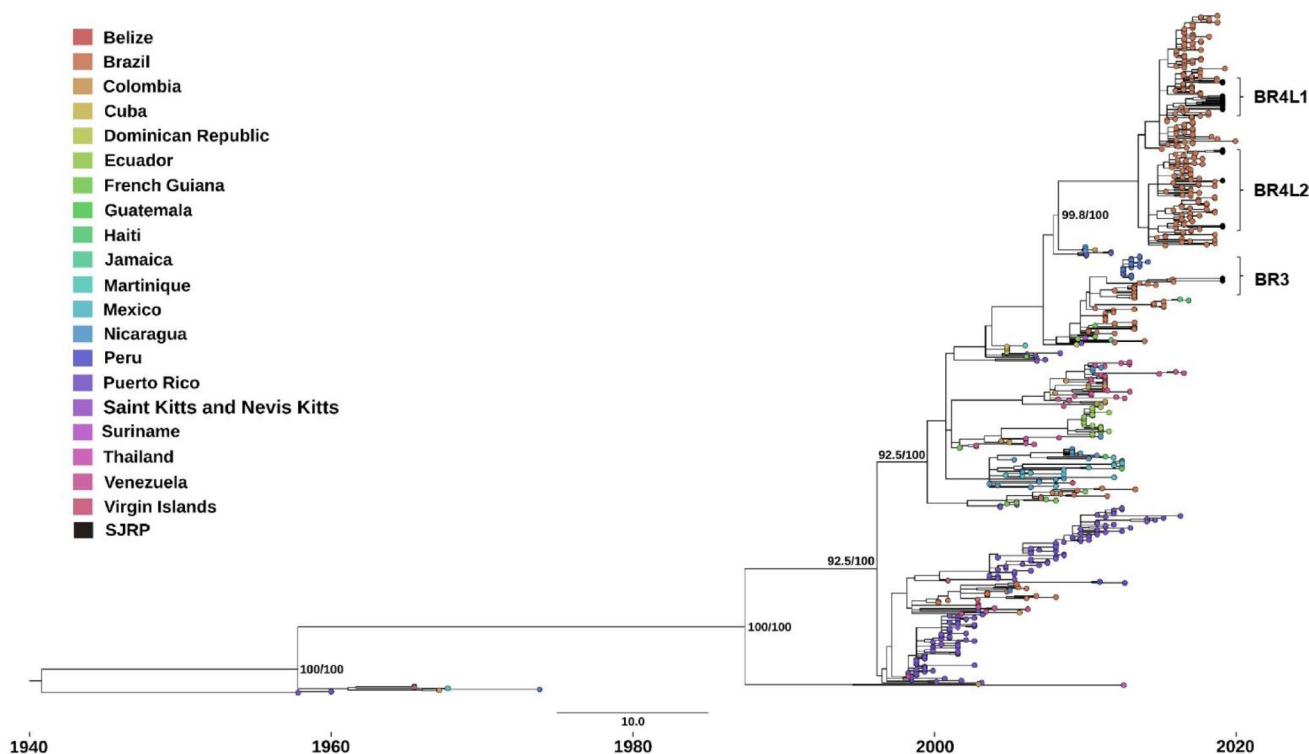

Fig. 1 Phylogenetic analysis of 22 DENV-2 sequences from dengue cases with warning signs and without warning signs sequenced in this study (black) and 595 DENV-2-III from Latin America
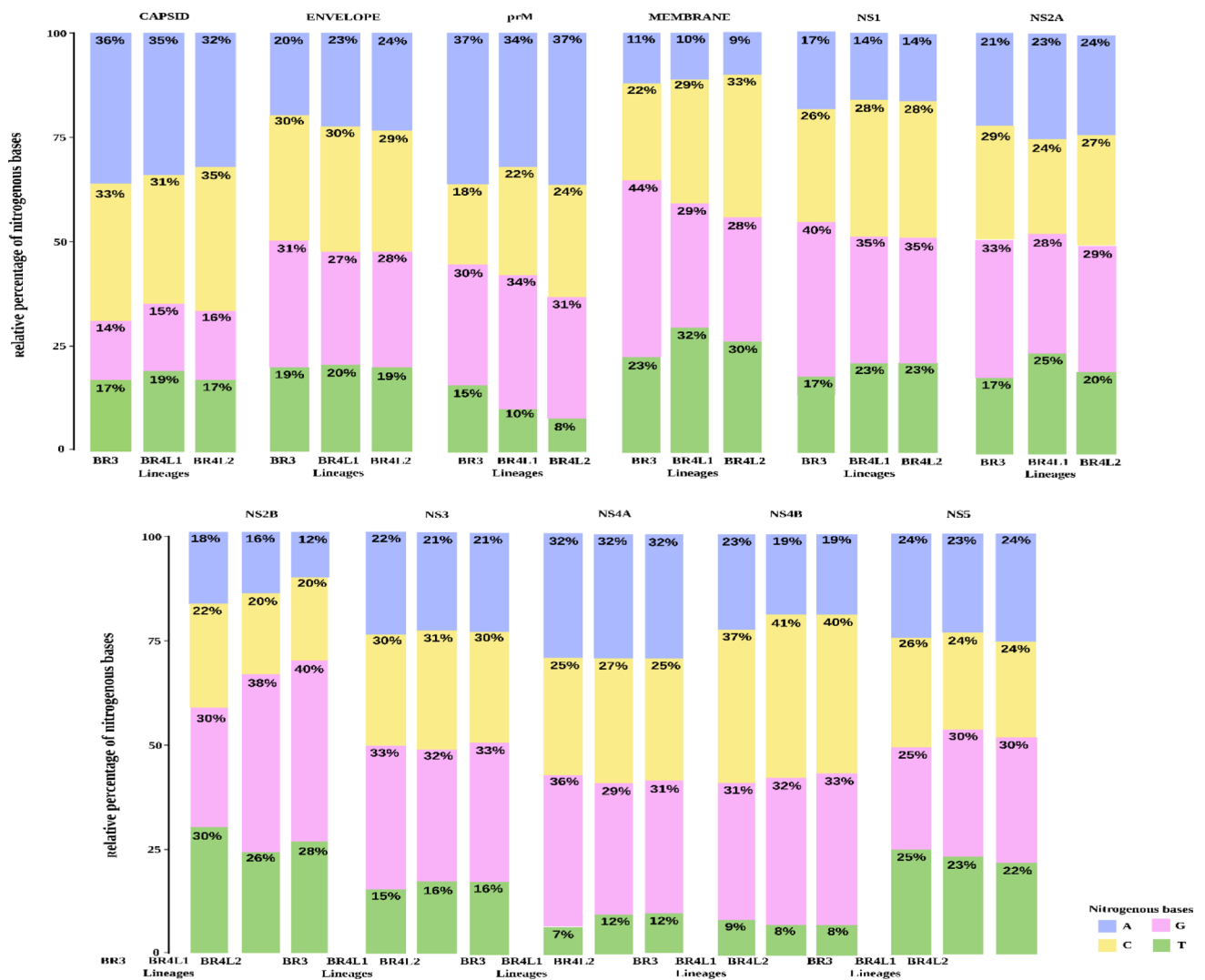
Fig. 2 Nucleotide composition of all DENV-2 sequences obtained

Discussion

Globalization, climate change, and increased human mobility have facilitated the increased incidence and co-circulation of multiple DENV serotypes, genotypes, and lineages in the same region [30]. In endemic and hyperendemic regions, the epidemiological dynamics of the DENVs are typically characterized by clade replacement from time to time [31–34]. The replacement of circulating DENV lineages is usually associated with an increase in the number of total cases and the number of severe cases. Thus, the early identification of new lineages must be carried out to rapidly track virus diversity during an outbreak. Genomic surveillance can help guide measures to contain the emergence and spread of new DENV outbreaks and decrease the number of severe dengue cases, as well as deaths [28, 29]. Here, we sequenced DENV-2 clinical samples and demonstrate that

the use of Oxford Nanopore technology in the diagnosis of DENV could be helpful to identify and describe the circulating lineages.

In 2019, the predominant serotype in São José do Rio Preto was DENV-2 [35]. This serotype can be classified into six genotypes, named I-VI. Until 2019, three genetic lineages of the DENV-2 Genotype III (DENV2-III) had been reported in Brazil. These three circulating lineages were named lineages 1–3 [31] or BR1-BR3 [29]. However, Jesus et al. [36] demonstrated, through phylogenetic analysis, the introduction of another lineage of DENV-2-III in São Paulo State, called DENV-2-III BR-4.

In this study, we sequenced 22 DENV-2 samples, obtained for routine diagnosis in a Hospital of São José do Rio Preto, and showed the co-circulation of two lineages belonging to the DENV-2 Genotype III: DENV-2-III BR3 and DENV-2-III BR4 (BR4L1 and BR4L2). The findings

Table 2 Frequency of signs and symptoms of 22 patients from Hospital de Base of São José do Rio Preto where the samples were obtained

		BR3 (n=2)			BR4L1 (n=15)			BR4L2 (n=5)			p-value
		N responses	N positive response	%	N responses	N positive response	%	N responses	N positive response	%	
WHO clinical classification	DwWS	2	0	0	15	6	40	5	2	40	0.96
	DWS+SD	2	2	100	15	8	53	5	3	60	
	SD	2	0	0	15	1	7	5	0	0	
Sign and symptoms	Fever	2	2	100	15	15	100	5	4	80	0.16
	Myalgia	2	1	50	15	11	73	5	5	100	0.29
	Headache	2	1	50	15	11	73	5	2	40	0.37
	Arthralgia	2	1	50	15	4	27	5	1	20	0.72
	Nausea	2	1	50	15	7	47	5	1	20	0.55
	Ocular pain	2	1	50	15	5	33	5	1	20	0.72
	Exanthema	2	0	0	15	6	40	5	1	20	0.42
	Vomits	2	0	0	15	3	20	5	1	20	0.78
	Leukopenia	2	0	0	15	5	33	5	3	60	0.30
	Abdominal pain	2	1	50	15	2	13	5	3	60	0.09
	Persistent vomits	2	0	0	15	1	7	5	0	0	0.78
	Lethargy	2	0	0	15	1	7	5	0	0	0.78
Alarm signs*	Liver enlargement	2	0	0	15	2	13	5	0	0	0.59
	Fluid accumulation	2	0	0	15	3	20	5	1	20	0.78
	Bleeding	2	0	0	15	2	13	5	1	20	0.78
	Hemoconcentration	2	0	0	15	6	40	5	0	0	0.14

*Including one case of severe dengue

Table 3 Laboratory findings at the admission of 22 patients from Hospital de Base of São José do Rio Preto where the samples were obtained

Lab findings (at admission)	BR3 (n = 2)			BR4L1 (n = 15)			BR4L2 (n = 5)		
	N total	Mean or N positive	% or (s.d.)	N total	Mean or N positive	% or (s.d.)	N total	Mean or N positive	% or (s.d.)
Hematocrit	2	38.2		15	37		5	36.53	
Leukocytes	2	4210		15	6		5	2.66	
Platelets	2	166,500		15	114.93		5	130.6	
Chest X-ray	2	0	0%	14	5	36%	6	5	83%
Fluid accumulation	-	-	-	5	0	0%	5	2	40%
Abdominal US	2	0	0%	14	2	14%	5	0	0%
Liver enlargement	-	-	-	2	2	100%	-	-	-
Abdominal fluid	-	-	-	2	2	100%	-	-	-
Thickened gallbladder wall	-	-	-	2	2	100%	-	-	-

suggest no evidence of phylogenetic clustering of cases with warning signs, indicating that it may be challenging to distinguish patients who will evolve to more severe clinical forms using only information at the consensus sequence level. It is important to highlight that sample size is a limitation of our study; thus, our results should be interpreted with caution and as a preliminary analysis. Studies with larger sample sizes and which explore single nucleotide variants are needed.

Chin-inmanu et al. sequenced the variants of 40 samples from patients diagnosed with DENV 1, 2, 3, or 4. They showed that the comparison between the data of the dengue fever (dengue without warning signs) and dengue hemorrhagic fever (dengue with warning signs) specimens was conserved between their major populations with the consensus sequences sharing 99% similarity [37]. Although our study and that of Chin-inmanu et al. have different objectives, both studies were unable to differentiate the three clinical dengue classifications (dengue without warning signs, dengue with warning signs, and severe dengue) by analyzing only sequence variability and phylogenetic relationships.

In 2009, the WHO reclassified dengue disease, differing significantly from the previous clinical classification [4] newest classification correlated clinical findings associated with the natural history of plasma leakage and organ failure and aimed to identify severe dengue [38]. Since then, dengue has been classified as dengue without warning signs, dengue with warning signs, and severe dengue, reflecting its intended use as a case-clinical management tool [4]. Severe dengue may occur by all four DENV serotypes; however, each genotype presents different virulence, which may be noted historically (reviewed by Bhatt et al. [39]). DENV-2 has previously been associated with dengue epidemics, especially when variants emerge subsequently [40]. The model of antibody-dependent enhancement (ADE) has been the most accepted to justify the severe forms of the disease, which occur mainly in heterotypic secondary DENV infection (a distinct DENV serotype from the primary infecting serotype) [39]. However, several other factors play a role in the pathogenesis of severe forms, including non-structural protein 1 (NS1) viral antigen [41], cytokine levels [42], memory cross-reactive T cells [43], age [44], viral serotypes [45, 46], genotypes [47], and lineages [44].

In conclusion, our phylogenetic reconstruction showed that in São José do Rio Preto, two lineages belonging to the American/Asian genotype of DENV-2: BR3 and BR4 (BR4L1 and BR4L2) are co-circulating. Our preliminary results suggest no specific association between DENV-2 clinical form and phylogenetic clustering at the virus consensus sequence level. In this work, we demonstrate that a protocol of genome sequencing using the portable nanopore MinION platform can be used as a methodology for genomic surveillance to monitor viral lineages during an epidemic and that this approach was able to produce quick and reliable sequences. Given our sample size limitation, larger studies are needed to confirm our findings, but the data does encourage us to follow this approach.

Declarations

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