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Communication

Emergence of a New Strain of DENV-2 in South America: Introduction of the Cosmopolitan Genotype through the Brazilian-Peruvian Border

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Abstract: Dengue virus 2 (DENV-2) have been seriously contributing to dengue-related mortality. It includes five non-sylvatic genotypes, being the Cosmopolitan the most widespread with a significant contribution in global parameter to the total number of DENV-2 cases. In South America, the genotype was first recorded in 2019, in Madre de Dios, Peru, and then in Goiás (Midwest Brazil) in November 2021. Here, we tested 163 human sera samples from Acre (Northern Brazil) collected during a DENV outbreak between 2020 and 2021, to all DENV by RT-qPCR. From the 163 samples, 139 were positive to DENV-2 and 5 to DENV-1. Five DENV-2 positive samples from early 2021 were sequenced, and the sequences clustered with the three other DENV-2 Cosmopolitan genotype sequences already recorded on the continent. These results create a geographical link, suggesting the possible route of introduction of the DENV-2 Cosmopolitan genotype into Brazil through the border with Peru, from which may have dispersed to Midwest Brazil.

Keywords: arboviruses; dengue virus serotype 2; Cosmopolitan genotype

1. Introduction

Dengue virus (DENV) is a major cause of morbidity and mortality worldwide, causing recurrent epidemics in urban and rural areas. DENV includes four antigenically distinct and genetically similar serotypes, which contain about 65% identity of their genome [1].

The most recent epidemiological reports on epidemics in South America have described a wide circulation of serotypes DENV-1 and DENV-2, with DENV-2 seriously contributing to dengue-related mortality [2]. In Brazil, the dengue viruses circulate in all regions, causing outbreaks and epidemics alternately among Brazilian states [3]. According to Brazilian Ministry of Health reports, 2,956,149 suspected cases of dengue fever were registered from 2020 to 2022, with 1798 deaths. The co-circulation of serotypes DENV-1 and DENV-2 was also observed with a high frequency, such as in other South American countries [2-7]. In this period, the outbreaks occurred simultaneously with the SARS-CoV-2 pandemic, which contribute to a less detailed investigation and a consequent underreport of epidemiological surveillance data in several Brazilian states.

DENV-2 includes five non-sylvatic genotypes: American – Genotype I (restricted to Central and South America and already extinct); Asian I and Asian II – Genotypes IV and V (Asian continent countries); Asian/American – Genotype III (Central and South America, and Southeast Asia), and; Cosmopolitan – Genotype II (Asia-Pacific, Middle East, and Africa), this being the most widespread, with a significant contribution in global parameter to the total number of DENV-2 cases [8,9].

Much has been discussed about genomic diversity, evolution, and transmission dynamics in regions favorable for virus dissemination. The high geographic circulation creates potential conditions for the proliferation of viral agents and their genotypes and lineages. This determinant added to the high growth rate of spatial distribution set the favorable conditions for the dissemination of vectors, modifications of evolutionary dynamics, and remodeling of the enzootic amplification of DENV [10].

From the introduction of DENV-2 in Brazil in the 1990s to 2020, only the genotype III was detected in the country [3,11]. A new genotype, the Cosmopolitan (genotype II) was detected for the first time in Brazil from a human case reported in the state of Goiás (Midwest region) in November 2021 [12], being the second official record of this genotype in the Americas, following an outbreak in Peru in 2019 [9]. The increased dengue severity on the continent may be related to the shift from single serotype endemicity to hyperendemicity from the co-circulation of multiple serotypes and the introduction of new genotypes [9,13].

Based on the understanding of the evolutionary dynamics and global dispersion of DENV-2 in the Americas, we can presume that there has been a more expressive heterogeneity of the Cosmopolitan genotype when compared to the other DENV-2 genotypes. Despite the phylogenetical relationship between the single case of the cosmopolitan genotype in the Midwest region of Brazil and the cases described in Madre de Dios, Peru [9,12], no geographic link between these regions, which do not share borders, has been established [12,14,15].

Here we describe cases of the Cosmopolitan genotype in Brazil reported during a DENV-2 outbreak in the state of Acre (Northern region) in early 2021. These cases create a closer geographic link between the findings from Peru and the Midwest region of Brazil, clarifying the most likely route of introducing this genotype in Brazil from the border with Peru at the Madre de Dios region.

2. Materials and Methods

2.1. Study samples

The study samples were selected within an epidemiological context, in the period 2020-2021, during an outbreak in the state of Acre, northern Brazil, and totalize 163 sera samples from humans clinically suspected of dengue fever.

2.2. RNA extraction

For RNA extraction, 140 µL of cell culture supernatant was used using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden Germany), following the manufacturer's recommendations. After purification, the RNA was quantified using Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) together with the Qubit 3.0 equipment (Thermo Fisher Scientific), according to the manufacturer's recommendations. They were subsequently stored in a -70 ºC freezer (under a range of -60 ºC to -80 ºC) until use.

2.3. Molecular detection

The assay was performed using the SuperScript III Platinum One-Step Quantitative RT-PCR Kit (Thermo Fisher Scientific), and included a set of oligonucleotide primers and dual-labeled fluorescent (Taqman) probes for in vitro, qualitative detection of DENV-1–4. Target amplification was recorded as increase and accumulation of fluorescence over time in contrast to background signal. The 25 µL reaction was composed of 12.5 µL of a 2X SuperScript III Platinum One-Step RT-PCR Master Mix, 2,2 µL of nuclease-free water, 1,0 µL of forward and reverse primers for DENV-1 (F – 5′, CAAAAGGAAGTCGYGCAATA, 3′; R – 5′, CTGAGTGAATTCTCTCTGCTRAAC, 3′), 0.5 µL for

DENV-2 (F – 5′, CAGGCTATGGCACYGTCACGAT, 3′; R - 5′, CCATYTGCAGCARCACCATCTC, 3′), 1,0 for DENV-3 (F – 5′, GGACTRGACACACGCACCCA, 3′; R - 5′, CATGTCTCTACCTTCTCGACTTGYCT, 3′), 0,5 for DENV-4 (F – 5′, TTGTCCTAATGATGCTRGTCG, 3′; R - 5′, TCCACCYGAGACTCCTTCCA, 3′), 0.25 µL of DENV-1-DENV-4 probes, and 5 µL of extracted RNA [16].

In a 7500 Fast Real-Time PCR system (Thermo Fisher Scientific), the RT-qPCR assays were performed under the following cycling conditions: an initial RT step at 50 °C for 30 min, a denaturation step at 95 °C for 2 min, 45 cycles of 15 s at 95 °C and a final extension step of 1 min at 60 °C. Each sample was analyzed in duplicate and considered as positive when the average cycle threshold (Ct) value was less than 37. In total, 163 samples were tested. Positive samples for DENV-2 with Ct values less than 20 were selected for viral isolation and subsequent genomic analysis of the supernatants. The assay was validated by positive (DENV-1-4 lyophilized antigens) and negative (nuclease-free water) controls.

2.4. Viral isolation

To obtain the isolates, we used an *Aedes albopictus* derived cell line, clone C6/36 (ATCC: CRL1 660), which were seeded in 10 mL culture tubes containing 1.5 mL of glutamine-modified Leibowitz medium (L-15), plus 2.95% tryptose phosphate, non-essential amino acids, antibiotics (penicillin and streptomycin), and 2% fetal bovine serum. They were inoculated in a 1:10 ratio of serum in glutamine-modified Leibowitz medium (L-15) and were observed daily for a period of 7 days or until cytopathogenic effect (CPE) was verified. Upon completion they were forwarded for genomic sequencing.

2.5. Sequencing

Genomic analysis using Next-Generation Sequencing (NGS) was performed on five DENV-2 isolates of human sera samples screened by RT-qPCR. They were prepared for sequencing by synthesizing first and second strands of complementary DNA, which were obtained with the cDNA Synthesis System Kit (Roche Diagnostics, Basel, Switzerland) and 400 µM Roche random primer. Agencourt AMPure XP Reagent Kit (Beckman Coulter, Brea, CA, USA) magnetic beads were used for cDNA purification and library preparation was performed by amplicon using the DNAprep kit (Illumina, San Diego, CA, USA) adapted to the DENV-2 oligonucleotide set. Quantification of cDNA was assessed using Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and fragments size range was evaluated using 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed using the MiniSeq sequencing system (Illumina) [17].

2.6. Bioinformatic Analysis

The generated reads were initially mapped against the DENV-2 reference genome (NC_001474) available in the National Center for Biotechnology Information (NCBI) database to obtain the consensus sequences using the Geneious v.9.1.8 [18] software. The coding region of five obtained sequences (two complete and three partial) were aligned to other 124 sequences of different genotypes of DENV-2 available in GenBank, using Mafft v.7 [19] software. Then, a Maximum Likelihood (ML) phylogenetic inference was built [20] with 1000 bootstrap iterations using the IQ-TREE v.1.6.12 program [21]. The GTR+F+I+G4 was selected as the best nucleotide substitution model, according to ModelFinder embedded in IQ-TREE [22]. The ML tree was rooted on the midpoint with FigTree v.1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/) and edited with Inkscape v.1.1 (https://inkscape.org/release/inkscape-1.1/).

3. Results

We analyzed 163 sera samples from individuals clinically suspected to all four den-gue serotypes using a multiplex RT-qPCR and obtained DENV-2 detection in 144 (88%) sera, of which 139 were positive for DENV-2 and 5 for DENV-1. The results showed an in-crease in the number of cases for DENV-2 between January and March 2021. From the 139 positive samples to DENV-2, five presented Ct value less to 20 and were inoculated in C6/36 cells, from which we obtained five isolates.

The five isolates were sequenced and clustered in a monophyletic clade, including the sequence from the state of Goiás, Brazil, and two Peruvian sequences. Furthermore, these genomes belong to lineage 5 of the DENV-2 Cosmopolitan genotype (Figure 1). The sequences generated in this study were deposited in GenBank under the following accession numbers: OQ511271 to OQ511275. All sequences included in the phylogenetic inference are listed in the Supplementary Materials (Table S1).

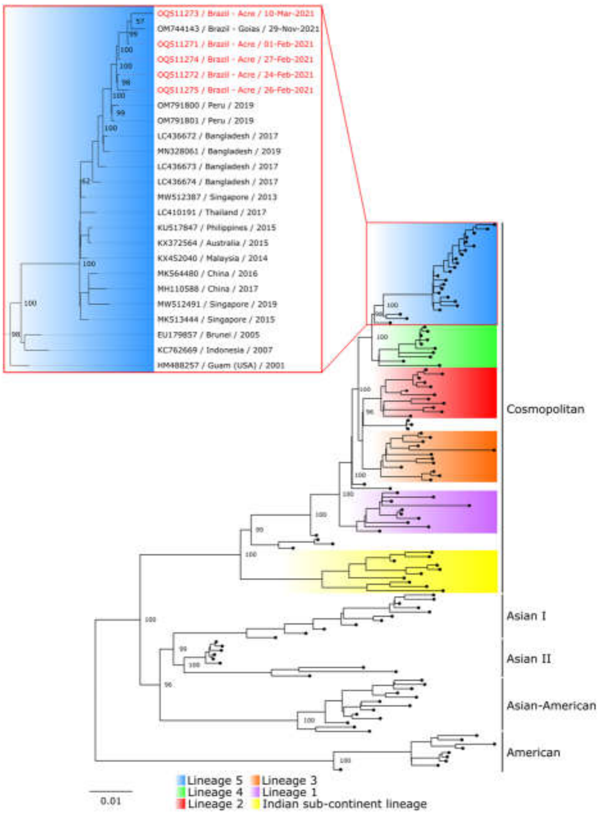
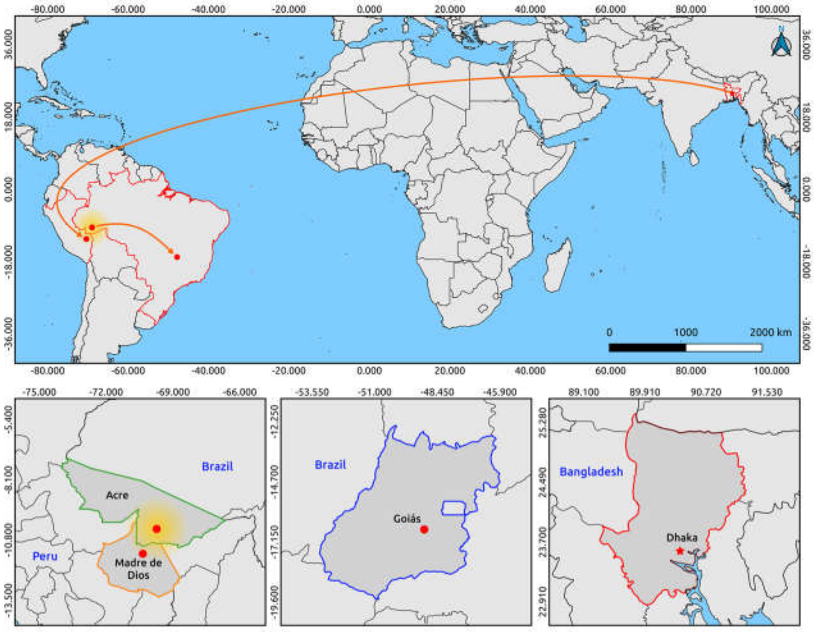


Figure 1. Maximum Likelihood phylogenetic tree based on the alignment of the coding region of a DENV-2 dataset. The five sequences presented in the study (highlighted in red) and 124 others belonging to the five non-sylvatic genotypes of the virus. The nucleotide substitution model selected was GTR+F+I+G4. The genotypes are identified on the right side of the tree, and a different color represents each lineage of the Cosmopolitan genotype. Each branch of the highlighted lineage 5 clade contains the accession number, country, and collection date of the sequence.

4. Discussion

The first report of a DENV-2 cosmopolitan strain described in the Americas occurred in the late 1990's in the Yucatan Peninsula of Mexico, but only one viral isolate was studied [9]. This genotype is circulating in other regions of the world and has been replacing the genotypes predominantly circulating in several regions of Asia [23]. We can infer from recent reports that there is probably a large-scale introduction of the cosmopolitan genotype of DENV-2 from Asian regions to Madre de Dios, Peru, which is derived from the strains reported in Dhaka, Bangladesh. The Cosmopolitan genotype has recently been reported in Madre de Dios and is circulating worldwide, presenting a wide dispersal pattern of its lineages (Figure 2) [9,12].



DATUM: WGS 84 - EPSG 4326 | CARTOGRAPHIC BASE: IBGE 2021/NATURAL EARTH 2023 | ELABORATION: BIOINFO/BIOMOL - SAARB/IEC

Figure 2. Map illustrating the introduction route of DENV-2 suggested in this study. The orange arrows indicate the routes of entry of DENV-2 based on reports from the last five years (Dakha/Bangladesh - Acre/Brazil - Goiás/Brazil). The colors delineate the states and the intersections between the country regions. The circle in yellow gradient indicates the occurrence of a possible dispersion of the lineage to other regions of Brazil and border countries. The map was built using the Qgis v.3.28 software, available at https://qgis.org/pt_BR/site/.

Until 2020, the Asian-American genotype was the only DENV-2 genotype circulating in Brazilian territory [3]. The single evidence of circulation of Cosmopolitan genotype was obtained from a patient from Goiás in November 2021 [12]. The five sequences obtained in this study clustered into a South American clade, well supported by high bootstrap values, with the two Peruvian sequences and the only previously sequence described from Brazil. The data presented here represents the first evidence of the detection of the DENV-2 Cosmopolitan genotype in the Northern region of Brazil and, more specifically, in the Acre state, months before the detection from Goiás.

The human sera samples were collected in February and March 2021, implying the circulation of this genotype in Brazil before the case reported in Goiás [12] and two years after the outbreak in Madre de Dios, Peru [9]. The phylogenetic inference also revealed that the South American clade was positioned among the sequences belonging to lineage 5 of the genotype [8], also described in the literature as Lineage C [24] or clade A [25]. These findings indicate a possible dispersal route of the DENV-2 cosmopolitan genotype in South America from Peru, with a feasible introduction into Brazil through the border of Acre and Madre de Dios, from where it may have spread to the Brazilian Midwest.

5. Conclusions

This study may contribute to filling gaps about the dispersion of the Cosmopolitan genotype from Peru [9] into Brazilian territory, highlighting the importance of strengthening the genomic surveillance of DENV-2 and conducting retrospective studies in Brazil and South America to monitoring the genotype dispersion and the precise time of its introduction into the continent and in Brazil.

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