INTRODUCTION

Dengue viruses (DENV 1-4) belong to the Flaviviridae family and Flavivirus genus and exist in either sylvatic or human transmission cycles, most prevalently in tropical and subtropical areas worldwide. The disease has become a major public health problem with relevant social and economical impact due to the increased geographic extension, number of cases, and disease severity.¹

Key Clinical Message

In the 80s, dengue viruses type 1 and 4 (DENV-1 and 4) were isolated in North region of Brazil. However, it was only after the DENV-1 introduction in the state of Rio de Janeiro (RJ) in mid-1980s, that dengue became a nationwide public health problem. In 2009, this serotype re-emerged causing an explosive epidemic in the country. DENV-4 was first detected in RJ in 2011 and in 2012, DENV-1 and 4 were co-circulating and responsible for a high number of cases notifications. Here, we describe the detection and molecular characterization of a DENV-1/4 co-infection in sample of 2012 in RJ.

KEYWORDS
co-infection, dengue virus 1, dengue virus 4, genotype II, genotype V

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Roraima (RR), 28 years after its first isolation, arriving in the state of RJ in 2011, causing with DENV-1, a high number of cases in the following years, reviewed in. Here, we describe the detection and molecular characterization of a DENV-1/DENV-4 co-infection in a sample isolated in the epidemic occurred in RJ in 2012 and originally identified only as DENV-1.

**2 | MATERIALS AND METHODS**

**2.1 | Dengue case investigation and ethical aspects**

The dengue case analyzed here was received at the Flavivirus Laboratory (LABFLA), IOC/FIOCRUZ, Regional Reference Laboratory for Dengue and Yellow Fever Diagnosis. Cases are received as convenience sampling for diagnosis and investigation under approval by the resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05). The case was received accompanied by an investigation record and questionnaire containing the patient’s demographic (age, gender, date of birth, address) and clinical (onset of disease and sign and symptoms) information.

**2.2 | Viral isolation**

Viruses isolation was performed by inoculating the original serum into C6/36 *Aedes albopictus* cell line and identification by indirect fluorescent antibody test (IFAT) using serotype-specific monoclonal antibodies. The volume of 25 μL of the supernatant from the original isolate was passed in a new C6/36 cell monolayer in a 2 mL tube containing Leibovitz-15 medium with 2% fetal bovine serum and at 28°C.

**2.3 | Reverse transcription followed by the polymerase chain reaction (RT-PCR)**

The viral RNA was extracted from 140 μL of supernatant from the cultures isolated using the QIAGEN Viral Mini Kit (Qiagen, Inc., Hilden, Germany) according to the protocol described by the manufacturer and stored at −70°C. The detection of the viral serotypes was performed as described previously. This protocol detects all four DENV serotypes simultaneously, in a semi-nested procedure, generating amplification products with specific size in base pairs (bp) of each serotype.

**2.4 | Viral quantification**

The viral quantification of the isolated sample and the first passage in cell culture were measured by real-time RT-PCR according to the protocol described previously. The Taqman assay was performed in duplicate, using primers and probes for DENV-1 and DENV-4, separately.

**2.5 | Dengue virus genome amplification, sequencing, and phylogenetic analysis**

For the envelope (E) gene (1485 bp) sequencing of DENV-1 and DENV-4, two primers pairs for each virus were used to amplify overlapping fragments of approximately 1300 bp. The oligonucleotides primers forward (f) and reverse (r) used for the amplification of the E gene of Brazilian DENV-1 (D1) and DENV-4 (D4) were: fD1-2A 5’-tgacctatgggacgttgttctca-3’ and rD1-3B 5’-gggcccatcgctctctgtga-3’, fD1-3A 5’-gacgcgaactttggtgtc-3’ and rD1-4B 5’-cgaatggctgctgacagtctt-3’, fD4-3 5’-gtccacatgtatggcagatgcacc-3’ and rD4-6 5’-ctggaaccatggttgag-3’, fD4-5 5’-caccacgtacattggtggag-3’ and rD4-8 5’-cccctggtcttcatgat-3’. Five microliters of the extracted RNA was reverse transcribed into cDNA and amplified for sequencing using QIAGEN OneStep RT-PCR Kit (Qiagen, Inc., Germany) according to the protocol described by the manufacturer, in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). This amplicons were observed on 1% agarose gel and cut from the gel for purification a commercial gel extraction kit (Qiagen, Inc., Valencia, California, USA) according to the protocol described by the manufacturer.
PCR products were sequenced in both directions using the BigDye Terminator Cycle Sequencing Ready Reaction version 3.1 kit (Applied Biosystems) and six primers, separately, generating fragments of approximately 900 bp. The primers used for sequencing reaction of D1 and D4 were: fD1-2A 5’-tgacctatgggacgtgttctca-3’, rD1-2B
FIGURE 2  Maximum-likelihood tree of the envelope (E) gene of DENV-1 and DENV-4 sequences from a co-infection dengue case occurred in 2012, Rio de Janeiro, Brazil. The sequences analyzed are represented as black circles. DENV strains were named as follows: GenBank accession number/country/year. Only the aLRT support values of major clades are shown. The tree was rooted on the midpoint. All horizontal branch lengths are drawn to a scale of nucleotide substitutions per site as shown in the bar at the bottom.
rD4-8 5’-cccccatgtcttccatgaatat-3’. The thermocycling conditions consisted of 40 cycles of denaturation (94°C/1 minute), annealing (60°C/2 minutes), and extension (72°C/3 minutes). Sequencing was performed on an ABI 3730 DNA Analyzer, Applied Biosystems, and the sequences generated were deposited on GenBank (www.ncbi.nlm.nih.gov). The sequences analysis was performed using the Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), the sequences identity by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and alignments by CLUSTAL OMEGA (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Phylogenetic relationships for both serotypes were analyzed using a maximum-likelihood (ML) tree inferred with PhyML, under the TN93+G+Г4 model of nucleotide substitution as determined by automatic model selection by SMS: Smart Model Selection in PhyML, and the SPR branch-swapping heuristic tree search algorithm. The reliability of the phylogenies was estimated with the approximate likelihood-ratio (aLRT) SH-like test and trees were visualized with FigTree v1.4.2 program. Strains representative from the other DENV-1 and DENV-4 genotypes available in GenBank were also used for the analysis as outgroups.

3 RESULTS AND DISCUSSION

The 46-year-old female patient (3 days of illness), presented fever, prostration, headache, myalgia, anorexia, nausea/vomiting, diarrhea, and platelet count of 106 000/mm³ and a positive dengue NS1 analyzed by the Platelia© Dengue NS1 Ag-ELISA (Bio-Rad Laboratories, Marnes-La-Cotquette, France).

The case analyzed here was originally misdiagnosed only as DENV-1, either by RT-PCR or viral isolation. However, when performing the RT-PCR from the cell culture inoculated with the patient’s serum during a retrospective study, it was possible to identify the amplification of both fragments corresponding to the DENV-1 and DENV-4 genomes. In order to verify whether the result would be repeated, the isolated sample was submitted to a single passage on cell culture for further analysis by conventional and real-time RT-PCR techniques. The viral passage from the original isolate presented alterations in cell morphology, characterized by an initial cytopathic effect (CPE) after 6 days of incubation period at 28°C (data not shown). DENV-1 and DENV-4 genomes were recovered and detected by RT-PCR using the mixture of the four DENV primers and by reactions containing only DENV-1 and DENV-4 type-specific primers, separately (TS1 and TS4, respectively), Figure 1A.

Both DENV-1 and DENV-4 genomes were also amplified by real-time RT-PCR, and the viral titers detected were 1,16E+06 copies/mL and 1,46E+06 copies/mL in the original isolate for DENV-1 and DENV-4, respectively, and 8,93E+03 copies/mL and 2,58E+04 copies/mL in the first passage (Figure 1C-F, respectively). It may be possible that DENV-4 may not have been identified from the patient’s serum in the first analysis performed in 2012, due to low a viral load, which may have been increased in cell culture.

The amplicons generated for sequencing of the E gene of the two viral strains (Figure 1B) were used for genetic characterization and phylogenetic analysis. DENV-1 genotype V and DENV-4 genotype II were identified and, both are circulating in country (Figure 2). DENV-1 and DENV-4 genome sequences obtained here were deposited in GenBank under accession numbers MH311981 to MH311984.

In 2012 year, DENV-1 and 4 were prevalent throughout the country, including the Southeast region, in particular the state of RJ.

Considering the current co-circulation of different arboviruses in the country, cases of co-infections may occur. In fact, co-infections by DENV-1 and 4 and other DENV serotypes have been previously reported in Brazil, but until now, we were unaware of DENV serotypes co-infections reported during epidemics occurred in RJ.

Due to the emergence of major arboviruses, such as zika virus (ZIKV) and chikungunya virus (CHIKV) worldwide, co-infections have been recently reported. Despite rare events, DENV/ZIKV and ZIKV/CHIKV co-infections have been reported in humans.

In Brazil, a recent study conducted by during a dengue and zika epidemic in the state of Mato Grosso do Sul in 2016, identified patients co-infected with DENV/ZIKV. Another study by the same group, investigating the triple epidemic (dengue, zika, and chikungunya) occurred in RJ in 2016, also reported distinct arboviral co-infections: CHIKV/ ZIKV, CHIKV/DENV, and DENV/ZIKV (manuscript in preparation).

It is unknown the consequences of a co-infection in the course of the disease for the patient, but the characterization of co-infections in a same patient with more than one virus circulating in a single viremia period, reinforces the role of virological and entomological surveillance. Whether the patient was bitten by a single mosquito carrying multiple viruses or by distinct mosquitoes, is unclear; however, it has been shown that co-infections, such as ZIKV/DENV, may strongly influence the vector competence.

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CONFLICT OF INTEREST

The authors declare no conflict of interest exists.

AUTHORSHIP

FBN and FBS: designed the study. FBN, NRCF, and PCGN: implemented the study. FBN, NRCF, PCGN, and FBS: analyzed the data. FBN and FBS: wrote the manuscript. FBS, RMRN and AMBF: sponsored the experiments. All authors read and approved the final version of the manuscript.

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