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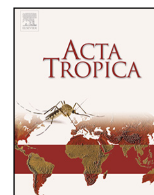

Dengue virus surveillance: Detection of DENV-4 in the city of São José do Rio Preto, SP, Brazil

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ABSTRACT

Dengue viruses are the most common arbovirus infection worldwide and are caused by four distinct serotypes of the dengue virus (DENV). In the present study, we assessed DENV transmission in São José do Rio Preto (SJRP) from 2010 to 2014. We analyzed blood samples from febrile patients who were attended at health care centers in SJRP. DENV detection was performed using multiplex RT-PCR, using flavivirus generic primers, based on the genes of the non-structural protein (NS5), followed by nested-PCR assay with species-specific primers. We analyzed 1549 samples, of which 1389 were positive for NS1 by rapid test. One thousand and eight-seven samples (78%) were confirmed as positive by multiplex RT-PCR: DENV-4, 48.5% (528/1087); DENV-1, 41.5% (449/1087); DENV-2, 9.5% (104/1087); and co-infection (5 DENV-1/DENV-4, 1 DENV-1/DENV-2), 0.5% (6/1087). Phylogenetic analysis of the DENV-4 grouped the isolates identified in this study with the American genotype and the showed a relationship between isolates from SJRP and isolates from the northern region of South America. Taken together, our data shows the detection and emergence of new dengue genotype in a new region and reiterate the importance of surveillance programs to detect and trace the evolution of DENV.

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1. Introduction

Dengue viruses (DENV) are the most important human arbovirus infection worldwide, transmitted by mosquitoes of the genus *Aedes*, the main vector being *Aedes aegypti* and at a lesser extent *Aedes albopictus*. Explosive epidemics have become a socially and economically significant public health problem, with great economic impact (Gubler and Meltzer, 1999; Gubler, 2002; Guzman et al., 2010; Shepard et al., 2014).

DENV, a member of the *Flaviviridae* family, is an enveloped virus with a positive sense, single stranded genomic RNA (ssRNA) (11 kb), which forms a nucleocapsid with multiple C protein copies (Freire et al., 2013). The four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) are closely related, yet antigenically distinct and contain a positive-sense RNA genome that is translated as a single polyprotein and post-translationally cleaved into three structural proteins, the capsid (C), the premembrane (prM) and the envelope (E), and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Lindenbach and Rice, 1999; Heinz et al., 2000; Acosta et al., 2011; Acosta-Bas and Gómez-Cordero, 2005; Fonseca and Figueiredo, 2005; Romanos, 2008).

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DENV-4 has not been detected in Brazil for 28 years after the first clinical and laboratory reports of dengue fever (DF) cases in Roraima State (Brazil) during 1981–1982 (Osanai et al., 1983). Since then, no additional cases have been reported in the country until the re-emergence of DENV-4 in Manaus city, Amazonas State (Brazil), in 2007 (Figueiredo et al., 2008). Following those first recent presentations of DENV-4 in Brazil, the virus was introduced in Salvador city, Bahia State (Nunes et al., 2012). In the Southeast, the first episode of the disease occurred in the state of Rio de Janeiro and São Paulo in 2011 (Nogueira and Eppinghaus, 2011; Souza et al., 2011).

Partial genomic studies have confirmed that the predominant virus in Brazil was directly associated with the Caribbean strains, belonging to genotype II. Genotype II has been responsible for several outbreaks in many countries in the Caribbean and South America (Forshey et al., 2009; Fernández et al., 2011; Nogueira and Eppinghaus, 2011; Souza et al., 2011; Temporão et al., 2011; Naveca et al., 2012; Campos et al., 2013; Villabona-Arenas et al., 2014). Phylogenetic analyses of different strains demonstrated the presence of two distinct genotypes (genotypes I and II) of DENV-4 in Brazil (De Melo et al., 2009; Shu et al., 2009; Temporão et al., 2011; Nunes et al., 2012; Martins et al., 2014). The re-introduction of a new serotype and a distinct DENV-4 genotype (Asian genotype I) into Brazil after nearly three decades of no circulation highlights the potential for future outbreaks of genotype I in this country. The presence of a serotype against which the population has no immunity indicates that the country is at risk for a sharp increase in the number of DENV infections, including severe cases (Temporão et al., 2011).

São José do Rio Preto (SJRP), which is located in the northwestern region of São Paulo State, Brazil, has been presenting an endemic circulation of DENV for 10 years and all four serotypes have been detected in the city (Mondini et al., 2009; Souza et al., 2011). Studies on the genetic characterization of DENV in this region have been previously conducted (Drumond et al., 2012, 2013; Villabona-Arenas et al., 2013). As the distribution of serotypes and genotypes has been reported to be a major contributor towards the severity of the disease, in this study we carried out the surveillance of DENV, as well as the detection of serotype 4 in SJRP. This also provided an insight into the genetic variability of serotype 4 in SJRP, compared to the information available for DENV-4 in other areas of Brazil and around the world.

2. Materials and methods

2.1. Sample collection

Blood samples from febrile patients who sought treatment in the SJRP health care centers, were collected for diagnosis of dengue by the Public Health Authority. The NS1 antigen (present in the serum of infected individuals from the first day of the disease) was first detected by Epidemiological Surveillance, using the NS1 Ag rapid assay kit according to the manufacturer's recommendations. The samples with detected antigen (or some random chosen negative samples) were then investigated by the Research Laboratory of Virology, Faculty of Medicine of São José do Rio Preto (SP), for serotype detection and laboratory confirmation by PCR.

Serum was separated and RNA was extracted from 140 μL of each aliquot with the QIAamp Viral RNA mini kit (QIAGEN), according to the manufacturer's protocol. The extracted RNAs were immediately used for laboratory tests or stored at −70 °C.

2.2. Multiplex-nested-PCR assays

PCR was performed using Multiplex-Nested-PCR (M-N-PCR), to detect DENV 1-4 (Bronzoni et al., 2005). The first RT-PCR was performed using flavivirus generic primers, based on the sequence of a non-structural protein (NS5). In the second PCR, nested assays, based on multiplex or conventional systems, were used with species-specific primers to detect and identify Dengue viruses (DENV 1-4). M-N-PCR products were loaded onto a 1.5% agarose gel and visualized using ultraviolet light. Amplicon sizes were determined by comparison with a 100 bp DNA ladder (Invitrogen).

Table 1
Primers used in the sequencing reaction of the gene encoding the E protein of DENV-4.

Primer	Sequence (5′–3′)	Location in the genome[a]
664F	ACR TGG ATG TCA TCR GAR GG	765–784
865F	GAY TTT GTR GAA GGR GTC TC	966–985
REV382-400	GGA CAT CTT GTT GCC GTA G	1144–1162
1162F	GGR AAR GGA GGW GTT GTR AC	1263–1282
REV644-665	CAT GGC TGT AAC TCC ATG ATT G	1406–1427
1450F	ACR TGG CTY GTG CAY AAR CAA TG	1551–1573
REV926-947	TTC CTG AGA TCC CAG CAC TGT C	1688–1709
1721F	AGG GAA TGT CAT AYA CGA TG	1822–1841
REV1209-1226	ACG CCC AAC CAC TTT TTC	1972–1988
1997F	TRA CAC TCC ATT GGT TYA GR	2098–2117
D4a2	AAC YTG GTG YAC AGC CTT TCC C	2234–2255
FORW1555-1576	GGT TCT TAG TGT TGT GGA TTG G	2317–2333
2402R	TKG TAC TGY TCY RTC CAR G	2503–2522

[a] Established position in the genome after comparison with the sequence of the complete genome of DENV-4 (Dengue virus 4 strain H402276, complete genome, Genbank accession number JN559740.2).

2.3. Virus isolation

Samples of DENV-4 (NS1 and PCR Dengue positives) were randomly selected to infect C6/36 cells as previously described (Figueiredo, 1990). C6/36 cell culture confluent monolayer was infected, with 100 μL of serum samples diluted 1/10 in culture medium. After incubation, 5 mL of Leibovitz medium L-15 (Cultilab, Brazil) with 1% bovine fetal serum (Cultilab) was added to the cell monolayer. After 7 days, the cell culture was frozen at −80 °C and the infected cells were examined for the presence of virus by the specific PCR assay, as previously described (Bronzoni et al., 2005).

2.4. Plaque reduction neutralization test

The serum sample (7 days after the onset of fever) for a supposedly index case of DENV-4 in SJRP (patient from a DENV-4 endemic area, Manaus, Brazil), was screened for specific antibodies in LLC-MK2 cells using a 90% plaque reduction criteria, as previously described (Russell et al., 1967).

2.5. Sequencing the envelope gene of DENV-4

Amplification for sequencing the envelope gene of the 33 serum samples of DENV-4 was performed, using the primers described in Table 1 and following a previously described protocol with some modifications (Christenbury et al., 2010). The sequences of all the strains reported in this paper has been deposited in GenBank under the accession numbers (Supplementary material S1).

The reverse transcription reaction was performed by adding 8 μL of RNA in a mixture containing 4 μL of buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1.4 μL of DTT at 0.1 M, 1.6 μL of dNTP mixture (2.5 mM each dNTP), 2.5 μL 2402R-D4 primer (10 μM, Table 1), 20 U ribonuclease inhibitor (RNaseOUT, Invitrogen, USA), 200 U reverse transcriptase enzyme (Superscript II, Invitrogen, USA), and sufficient water to adjust the reaction volume to 20 μL. The mixture was incubated at 50 °C for 50 min and at 70 °C for 15 min.

The amplification reaction was carried out with 8 μL of cDNA, diluted in 5 μL of buffer (500 mM Tris-HCl, 150 Ammonium Sulfate mM [pH 9.3 adjusted with NH_4OH], 25 mM $MgCl_2$, 1% TWEEN ® 20), 2.5 μL of dNTP mixture (2,5 mM each dNTP), 1 μL DMSO, 1 μL of each primer (D4-664 and D4-2402R, 10 μM, Table 1), 0.5 U of Accutaq LA DNA polymerase (Sigma) and sufficient ultrapure water to adjust the reaction volume to 50 μL. PCR was performed by an initial DNA denaturation step (98 °C, 30 s), followed by 30 cycles of denaturation (94 °C, 15 s), annealing (51 °C, 20 s) and extension (68 °C, 3 min). A final extension step was done at 68 °C for 10 min.

The fragments amplified were purified and sequenced using the BigDye v3.1 (Applied Biosystems, USA) in an ABI3130 automatic sequencer (Applied Biosystems, USA). The reaction consists of the amplified viral DNA, 4 μL of reaction buffer containing the fluorescent dideoxinucleotides ddATP, ddCTP, ddGTP, ddTTP (terminator reaction mix), 3.2 μM of each primer (Table 1) and Ultra-Pure Water (Invitrogen ™) to a final volume of 20 μL. The reactions were processed in an automatic thermocycler (Veriti Thermal Cycler, Applied Biosystems, USA), programmed to perform an initial step denaturation at 96 °C for 2 min, followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. Nucleotide sequences were analyzed using the DS Gene 2.0 Software (Accelrys, USA), and confirmed as envelope gene sequences of DENV-4.

2.6. Sequencing of the complete genome of DENV-4 isolates

Of the 33 serum samples sequenced with the envelope gene, 10 samples were submitted to Illumina GA sequencing platform using MiSeq Reagent kit for 300 cycles as a strategy to obtain the complete genome sequence. The accession number for the individual DENV-4 genome sequences, country of origin and date of collection for each sample used in this study (Supplementary material S2).

First, total RNA was extracted using the QIAamp Viral RNA kit (Qiagen®) according to manufacturer's instructions. The RNA obtained was quantified (2.0 Qubit® Fluorometer, Life Technologies) and then treated with DNAse (Sigma) according to manufacturer's standards.

Reverse transcription of RNA was performed by adding 10 μL of RNA in a mixture containing 1 μL Random Primer (50 ng), 1 μL mixture containing 10 mM dNTPs, and 1 μL of water. The mixture was incubated at 65 °C for 5 min. Next, the following reagents were added: 4 μL of 5 x concentrated buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 μL of DTT at 0.1 M, 40 U RNaseOUT, and 200 U reverse transcriptase enzyme (Superscript II, Invitrogen, USA). The mixture was incubated at 25 °C for 5 min, at 50 °C for 60 min and at 70 °C for 15 min. The cDNA was stored at −20 °C.

For each sequencing reaction, 10 μL of cDNA were added to a mixture containing 10 μL of 10 x concentrated buffer (300 mM Tris-HCl, pH 7.8 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP), 0.4 μL of dNTP (10 mM), 1 μL of RNAse H (5 U/mL), 0.2 μL of T4 DNA/pol (5u/μL), 0.3 μL of T4 DNA Ligase (5 u/μL; Invitrogen), 78.1 μL of water and 10 μL of reverse transcriptase enzyme. The mixture was incubated at 12 °C for 60 min and at 22 °C for 60 min.

Quantification of the amplified and purified samples (Agencourt AMpure XP) was performed using the Qubit® Fluorometer 2.0 and the DNA High Sensitivity kit (Life Technologies). The samples were prepared according to the manufacturer's recommendations (0.2–100 ng of DNA double strand) and then stored for construction of the DNA library (Ullmann et al., 2015).

Upon completion of the sequencing run in Illumina MiSeq, the assembly of contigs was done using the Geneious program, as reference to the complete genome of DENV-4 (Dengue virus 4, complete genome − GenBank accession number: NC002640).

2.7. Data sets and phylogenetic analyses

Nucleotide sequences were analyzed using algorithms of the BioEdit Sequence Alignment software (Hall, 1999). Maximum likelihood phylogenetic tree was constructed in Mega 6.06 software. Bootstrapping with 1000 replicates was done to show support for each node in the tree (Tamura et al., 2013).

Nucleotide sequences from envelope gene (Supplementary material S3 and S4) were retrieved from GenBank and used for phylogenetic and evolutionary analyses. Nucleotide sequences were aligned using Clustal W program implemented in MEGA6 (Tamura et al., 2013). Using the software MEGA 6, the nucleotide substitution model that best fit the data was chosen (Tamura-Nei (TN93 + G)) (Tamura and Nei, 1993) and then it was used to estimate the evolutionary distance between sequences. Phylogenetic trees were reconstructed based on envelope gene sequences, using the nucleotide substitution model TN93 + G, the Maximum Likelihood method, and 1000 bootstrap replicates (Felsenstein, 1981; Kumar et al., 2008).

2.8. Statistical analyses

For data analysis, descriptive calculations were initially performed, using central tendency and dispersion measures. The inferential statistical analysis was conducted using the chi-square test for comparison of frequencies. For quantitative data, we applied the Kolmogorov-Smirnov normality test followed by the ANOVA test. The data correlation analyses were performed using the Spearman method. The significance level was $\alpha = 5\%$ and the software used were the Epi Info™ 7.1.3.9 and Prism 6.0®.

2.9. Ethics statement

This study was conducted in samples collected regularly for public health purposes by public health authority and since no specific procedure was required for this study the Ethical Review Board waived the contentment form. All data was anonymized for analysis. This protocol was approved by the Ethical Review Board of the Faculdade de Medicina de São José do Rio Preto (Protocol 02078812.8.0000.5415).

3. Results

Out of a total of 1549 serum samples from symptomatic patients (0–3 days after onset of fever), 1389 were positive for NS1, in the NS1 Ag rapid assay (Panbio® Dengue Early ELISA), and a total of 1.087 (78%) were confirmed by Multiplex-Nested PCR. The DENV serotypes were as follows: 48.5% (528/1087) DENV-4, 41.5% (449/1087) DENV-1, 9.5% (104/1087) DENV-2 and 0.5% (6/1087) co-infection (5 DENV-1/DENV-4, 1 DENV-1/DENV-2).

In the period between October 2010 to January 2011, 31 positive cases (97% DENV-1 and 3% DENV-2), February to July 2011, 263 positive cases (91.5% DENV-1, 6.5% DENV-2 and 2% DENV-4), August 2011 to January 2012, 15 positive cases (93.5% DENV-1, 6.5% DENV-4); February to July 2012, 64 positive cases (62.5% DENV-1, 23.5% DENV-4, 12.5% DENV-2, 1.5% DENV-1/DENV-4 co-infection); August 2012 to January 2013, 221 positive cases (71.5% DENV-4, 22.5% DENV-2, 5.5% DENV-1, 0.5% DENV-1/DENV-4 co-infection); February to July 2013, 370 positive cases (90% DENV-4, 6.5% DENV-1, 3% DENV-2, 0.5% DENV-1/DENV-4 co-infection); August 2013 to January 2014, four positive cases (75% DENV-4, 25% DENV-1), and February to June 2014, 119 positive cases (74% DENV-1, 13.5% DENV-2, 11% DENV-4, 1.5% DENV-1/DENV-2 co-infection) (Fig. 1).

DENV-4 was first detected in the city in February 2011 (4 months after the beginning of the study), simultaneously by our study and

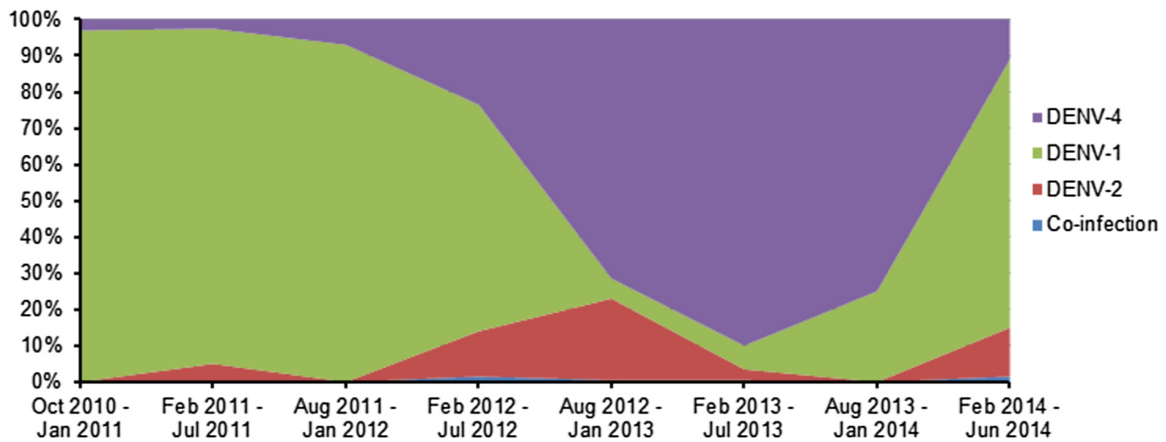

Fig. 1. Percentage of each serotype of Dengue virus identified in the SJRP, 2010–2014.

by the state public health authority in the same sample (Souza et al., 2011).

After the detection of an autochthonous case of DENV-4 infection, the Public Health Authority performed a field investigation and one imported case from a DENV-4 circulation area was identified. The patient was from Manaus, Brazil and arrived in the city by airplane, in the 3rd day of the fever in January 2011 (1 month before the detection of DENV-4 cases in the city). The patient sought medical attention in a public outpatient clinic on the 7th day after the onset of fever. At that time, the NS1 was negative, but both IgM and IgG were positive and the serum was kept in the freezers of the Public Health laboratory. $PRNT_{90}$ confirmed DENV-4 (titer of 1: 960) and DENV-1 (titer of 1: 480) positivity, suggesting a secondary infection by DENV-4 in a patient previously exposed to DENV-1.

The mean age of the infected patients was 36.74 ± 17.21 (ranging from 1 to 86 years) and 53.38% of them were females, no significant difference at $p \leq 0.05$. Statistical analysis of the mean ages of DENV-1 (38.12 ± 18.82), DENV-2 (38.06 ± 18.78), DENV-4 (36.57 ± 16.69) and coinfection (29.66 ± 18.94) cases showed that there was no significant difference ($P = 0.4470$, $F = 0.8876$) at $p \leq 0.05$.

The correlation between the gender of DENV patients and the infecting serotype also showed that there was no significant difference, DENV-1 (39.47 ± 20.37 male, 36.40 ± 16.92 female, $P = 0.4868$), DENV-2 (36.86 ± 18.51 male, 33.92 ± 14.43 female, $P = 0.5634$), DENV-4 (36.43 ± 16.91 male, 36.68 ± 16.55 female, $P = 0.7781$) and coinfection (42.33 ± 10.59 male, 17 ± 17.43 female, not been possible to compare the means).

Phylogenetic analyses, based on the envelope gene, demonstrate that the samples are located in the same clade, grouped into DENV-4 genotype II (American) and are closely related to other samples in circulation in the country, as well as in other countries of South America (Fig. 2), including Manaus, AM, which is source of the virus, as suggested by our investigation.

The analysis of the complete genome of ten isolates of the DENV-4 circulating in SJRP (Supplementary material S2) showed that all isolates studied, belonging to the American genotype, are phylogenetically related to isolates in northern South America (Supplementary material S5).

4. Discussion

A total of 1549 serum samples from Dengue-suspected patients were analyzed in this study. Of this, 1389 were positive for NS1 Ag rapid assay, suggesting that Dengue places a considerable burden in the community. Molecular detection revealed the presence of DENV in 1087 (78%) patients' sera. Results from serotyping identified the presence of DENV-1, DENV-2 and DENV-4 serotypes in SJRP, with DENV-4 as the predominant serotype, followed by DENV-1 and DENV-2. Simultaneous circulation of the three serotypes in SJRP in this period is clear evidence hyperendemicity (Pessanha et al., 2010; Rodriguez-Barraquer et al., 2011; Bastos et al., 2012; Melo et al., 2012; Martins et al., 2014).

The introduction of a new serotype (DENV-4) in a city (like in our study), raises extreme concern. It is noteworthy that in 2011 there was co-circulation of DENV-1, DENV-2 and introduction of DENV-4 for the first time in the state (Souza et al., 2011). Almost a year and a half after the introduction of DENV-4, this serotype was found to be the main one circulating, since there is evidence that the introduction of a given serotype of DENV in a particular location, is followed by an epidemic blast with high incidence rates, and a large number of cases (Zeller et al., 2013).

High incidence of infection in young adults was observed to all DENV serotype in SJRP. Studies in Rio de Janeiro, Rio Grande do Sul, Pará and Amazonas states reported similar findings (Araújo et al., 2002; De Simone et al., 2004; Baroni and Oliveira, 2009; Figueiredo et al., 2011). However, some studies have signaled the severe disease in much younger populations, as already occurs in Asian countries, with consequent increase in hospitalizations and deaths in this population (Rodriguez-Barraquer et al., 2011; Libraty et al., 2009; Rocha and Tauil, 2009).

In this study, we detected a higher prevalence of women who were infected with DENV than of men. These data are similar to rates reported in other studies (Cordeiro et al., 2007; França et al., 2011; San Martín et al., 2010; Souza et al., 2013; Teixeira et al., 2013). This prevalence may be linked to the vector *Aedes aegypti* is most frequently found in urban and sub-urban environments, with high concentrations of human beings and houses (Lima-Camara et al., 2016).

The risk of becoming infected with any dengue serotype, according to the scenario found in SJRP between 2010 and 2014, independent of gender and age, is a different situation from that observed in another study, which emphasizes that with increasing length of time of co-circulation of multiple dengue serotypes in Brazil, adults have a lower probability of remaining susceptible to infection, due to the accumulation of multitypic immunity in the adult population (Rodriguez-Barraquer et al., 2011).

Our data show that all the isolates from SJRP/SP group within the genotype II, as previously demonstrated for other Brazilian isolates (Souza et al., 2011; Naveca et al., 2012; Campos et al., 2013). Countries in South and Central America, and the Caribbean have reccuring DENV-4 outbreaks assumed to involve genotype II (Foster et al., 2003; Forshey et al., 2009; Acosta et al., 2011; Villabona-Arenas and Zanotto, 2011; Nunes et al., 2012). The intro-

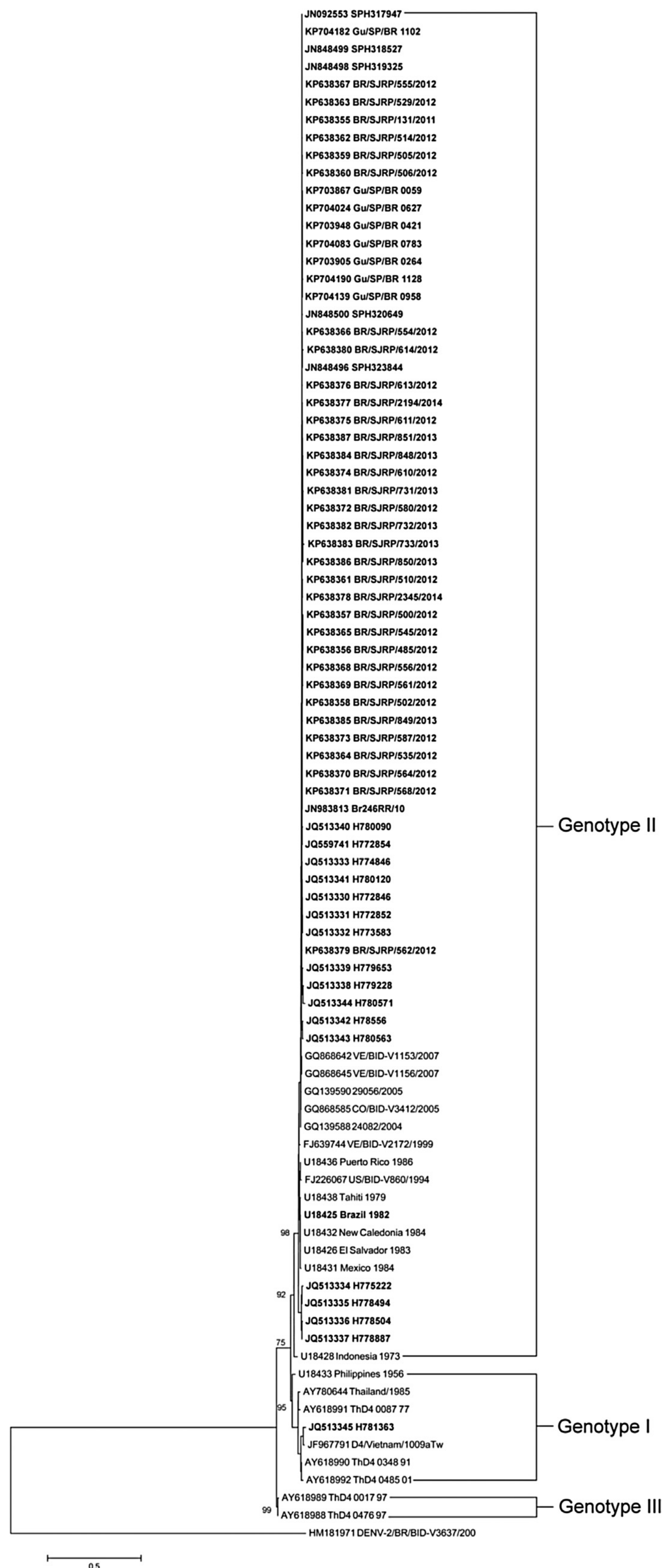

Fig. 2. Phylogenetic analysis of DENV-4, based on the envelope gene sequence. The phylogenetic tree was constructed by the Maximum Likelihood method with bootstrap 1000 replications. The 33 isolates DENV-4 were aligned with 53 reference sequences global from GenBank. Representative of DENV-2 was used as outgroup. Identification of DENV-4 samples in the present study: Country/City/sample identification/year of isolation.

duction of genotype I, from an Asian country (De Melo et al., 2009), has been documented in northern and northeast Brazil (Figueiredo et al., 2008; Martins et al., 2014; Pinho et al., 2015) but no other circulating genotypes of DENV-4 have been detected in SJRP so far.

The phylogeny of the complete genome of DENV-4 shows the relationship between isolates from SJRP with isolates collected from the northern South America, which corroborates with what was shown in another study suggesting that DENV-4 genotype II emerged and reemerged in Brazil from at least three distinct origins (Southeast Asia, the Caribbean region, and Venezuela), and demonstrated a dispersal pattern in Brazil that is far more complex than expected from standard epidemiologic data (Nunes et al., 2012).

5. Conclusion

Our data shows the detection and emergence of new dengue serotype in a new region and reiterate the importance of surveillance programs to detect and trace the evolution of DENV.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2016.09.004.

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