Characterization of Dengue Virus Type 2: New Insights on the 2010 Brazilian Epidemic

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Abstract

Dengue viruses (DENV) serotypes 1, 2, and 3 have been causing yearly outbreaks in Brazil. In this study, we report the reintroduction of DENV2 in the coast of São Paulo State. Partial envelope viral genes were sequenced from eighteen patients with dengue fever during the 2010 epidemic. Phylogenetic analysis showed this strain belongs to the American/Asian genotype and was closely related to the virus that circulated in Rio de Janeiro in 2007 and 2008. The phylogeny also showed no clustering by clinical presentation, suggesting that the disease severity could not be explained by distinct variants or genotypes. The time of the most recent common ancestor of American/Asian genotype and the São Paulo and Rio de Janeiro (SP/RJ) monophyletic cluster was estimated to be around 40 and 10 years, respectively. Since this virus was first identified in Brazil in 2007, we suggest that it was already circulating in the country before causing the first documented outbreak. This is the first description of the 2010 outbreak in the State of São Paulo, Brazil, and should contribute to efforts to control and monitor the spread of DENVs in endemic areas.

Introduction

Dengue is the most prevalent arthropod-born viral disease in tropical and subtropical regions, infecting 50 to 100 million people annually [1]. Cases in Central and Latin America have increased almost five-fold in the last 30 years. In 2008, up to one million cases were reported in the Americas, with higher number of deaths occurring in the Southern Cone [2]. In the last two decades, Brazil has been responsible for more than 60% of the total reported dengue fever cases in the Americas [2]. The continuing occurrence in resource limited countries and the lack of novel therapeutic approaches or an effective vaccine have made dengue fever to be considered a neglected disease. Dengue surveillance is deprived in most countries and no existing model for predicting an outbreak in tropical and subtropical regions is available.

The mechanisms by which dengue virus (DENV) causes severe illness remain to be elucidated. Both viral and immune host factors seem to contribute to the level of the pathogenicity [3,4,5,6]. Immunity induced by natural infection is believed to provide serotype-specific lifelong protection. On the other hand, previous infection by a distinct serotype increases the risk for dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) [5,7].

In the Americas, DHF was first reported in Cuba in 1981 after the introduction of DENV2 in the coast of São Paulo State. Partial envelope viral genes were sequenced from eighteen patients with dengue fever during the 2010 epidemic. Phylogenetic analysis showed this strain belongs to the American/Asian genotype and was closely related to the virus that circulated in Rio de Janeiro in 2007 and 2008. The phylogeny also showed no clustering by clinical presentation, suggesting that the disease severity could not be explained by distinct variants or genotypes. The time of the most recent common ancestor of American/Asian genotype and the São Paulo and Rio de Janeiro (SP/RJ) monophyletic cluster was estimated to be around 40 and 10 years, respectively. Since this virus was first identified in Brazil in 2007, we suggest that it was already circulating in the country before causing the first documented outbreak. This is the first description of the 2010 outbreak in the State of São Paulo, Brazil, and should contribute to efforts to control and monitor the spread of DENVs in endemic areas.

Citation: Romano CM, de Matos AM, Araújo ESA, Villas-Boas LS, da Silva WC, et al. (2010) Characterization of Dengue Virus Type 2: New Insights on the 2010 Brazilian Epidemic. PLoS ONE 5(7): e11811. doi:10.1371/journal.pone.0011811

Editor: Laurent Renia, BMSI-A*STAR, Singapore

Received May 21, 2010; Accepted July 2, 2010; Published July 28, 2010

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Funding: The present work was supported by the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, Brazilian Ministry of Science and Technology (CNPq, grant #476088/2009-7 to EGK). AMM and EGK’s scholarships were supported by CNPq. KIC’s scholarship was supported by the Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior, Brazilian Ministry of Education. URL of CNPq: URL www.cnpq.br; URL of CAPES: www.capes.gov.br. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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coalescence approaches, we also estimated the time (in years) that this specific lineage entered in Brazil.

**Methods**

**Ethics Statement**

The current project was conducted after Hospital das Clínicas - University of São Paulo’s Institutional Review Board (CAPIESq) approval, under protocol #0652/09. Written informed consent was obtained from all participants.

**Clinical samples and virus Isolation**

Serum samples were obtained from patients with clinical suspected dengue fever at the Ana Costa Hospital located in the city of Santos, State of São Paulo. DENV2 was successfully isolated from 18 serum samples by inoculation into monolayers of C6/36 Aedes albopictus cell line (ATCC number CRL-1660). Supernatants from cell cultures presenting cytopathic effect compatible with DENV infection were assayed by reverse transcriptase polymerase chain reaction (RT-PCR) to amplify serotype-specific viral genome.

**RNA extraction and RT-PCR**

Viral RNA was isolated directly from 140 µl of cell culture supernatants using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, USA) following the manufacturer’s instructions. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). A total reaction volume of 20 µl was made with 10 µl of RNA and 10 µl of mix, containing 2.0 µl 10× buffer, 0.8 µl dNTP Mix (100 mM), 2.0 µl 10× random hexamer primers, 1.0 µl reverse transcriptase and 4.2 µl nuclease-free water.

**PCR amplification and sequencing**

A 665 bp of envelope region corresponding to nucleotides 1857 to 2522 of DENV2 complete genome (GenBank reference sequence DQ181801) was amplified by PCR using following primers specific for DENV2: D2_EnvF -5'-AARRTYGRAAR-GAAATAGCAGAA-3' and D2_EnvOutR- 5'-CTGTCCTGAY-TRTGYAGCTTCT-3'. The thermal profile for amplification was 38 cycles of 45 seconds of denaturation at 94°C, 1 minute of annealing at 56°C, and 1 minute of extension at 72°C. The PCR products were purified for elimination of primer dimers and excess oligonucleotides with the PureLink Quick Gel extraction Kit (Invitrogen), with few adaptations for direct purification of PCR products in solution. Sequencing reactions were performed with BigDye Terminator kit v.3.0 (Applied Biosystems, Bedford, MA) and one of each primer used in sequencing reaction. The electroforeograms were analyzed and consensus sequences were achieved with CodonCode Aligner v.3.0 (available at http://www.codoncode.com/).

**Phylogenetic and coalescent analysis**

The 18 sequences generated were aligned together with reference sequences downloaded from GenBank using the ClustalX program [18]. An initial dataset including our sequences and reference sequences from all four serotypes (n = 93) was used to confirm that our samples were truly DENV2. A second dataset included our samples and reference sequences of all DENV2 genotypes known to cause outbreaks around the word (n = 91). The phylogenetic trees were reconstructed using a Bayesian Markov Chain Monte Carlo (MCMC) framework implemented in BEAST v1.5.3 [19]. The GTR+G substitution model was used as the best model obtained in MODELTEST [20]. Also, the previously estimated substitution rate for DENV2 of 6.5×10^{-4} s/s/y [21] was used to obtain the time of the most recent common ancestor (TMRCA) for the American/Asian genotype and also for the Brazilian DENV2. The Bayesian Skyline plot (BSL) was performed under strict and relaxed uncorrelated lognormal molecular clock. The molecular clock that best fitted the data was chosen by Bayes factor (BF) comparison. Convergence of parameters during the MCMC was inspected with Tracer v.1.4 [19], with uncertainties addressed as 95% highest probability density (HPD) intervals. In all cases, ten million chains were sufficient to achieve the convergence of all parameters (ESS>200).

The trees were sampled at each 1000 steps, resulting in a final file of 10,000 trees. These trees were summarized in a maximum clade credibility (MCC) tree using TreeAnnotator (part of the BEAST package), and visualized in FigTree v.1.2.2 (http://beast.bio.ed.ac.uk/FigTree).

**Results**

An initial phylogenetic tree including all DENV serotypes confirmed, with high posterior probability, that our samples belong to serotype 2 (supplementary Figure S1). The maximum clade credibility (MCC) tree (Figure 1) showed that the DENV2 isolates circulating in 2010 in Guarujá and Santos clustered within the American/Asian genotype and were closely related to viruses that caused the epidemics in Rio de Janeiro during 2007 and 2008 [15]. Although this study included only two patients with severe dengue (patients 28 and 42, Table 1), the phylogenetic tree showed no segregation by clinical manifestations, since mild and severe dengue fever cases samples clustered together. Moreover, we found no differences at aminoacid level in the envelope region among patients with distinct clinical presentations. However, it must be taken into account that differences in clinical manifestation may not be evident by our analysis, since they included a limited number of patients and encompassed only partial envelope gene. Age, gender and further clinical data are described in Table 1.

Importantly, the monophyletic cluster of the recent Brazilian DENV2 was closely related to viral strains from Cuba and the Dominican Republic, with small genetic distance and high posterior probability, suggesting that this lineage was imported from the Caribbean.

The coalescent analysis based on the dataset containing the five DENV2 genotypes revealed that the lineage causing the epidemics in the last four years in Brazil was introduced in the country at least at 5.5 years ago (median value of 9.8 years ago, and upper value of 16 years ago). Importantly, Figure 1 also showed that our estimates for the time of the most recent common ancestor (TMRCA) of the key nodes covered essentially the same range as those estimated by previous reports [21,22], further supporting our results. The TMRCA data described here were obtained under relaxed uncorrelated molecular clock. Although the relaxed clock was the best model by Bayes Factor comparison, the coalescent dates obtained under the strict clock were similar (Supplementary Files S1 A and B).

The sequences generated in this work were submitted to GenBank database under accession numbers HM185186 to HM185292.

**Discussion**

Our phylogenetic analysis based on the partial DENV2 envelope gene sequencing showed that viruses causing the current epidemic in the cities of Guarujá and Santos, as well as surrounding areas, were sister taxa of those sampled two years
ago in the cities of Rio de Janeiro and Ribeirão Preto. The presence of Aedes aegypti and the frequent traveling amongst such regions are the necessary ingredients for viral spread through viremic patients moving between these cities.

It was interesting to find that the lineage causing outbreaks in Brazil in the end of 90’s, the isolates from Northeast Brazil between 2001 and 2006, and the viruses representing more recent epidemics (2007 to 2010) in São Paulo and Rio de Janeiro States segregated into temporal sub-clades (Figure 1). Several longitudinal studies have also observed temporal clustering of dengue, as well as marked patterns of geographical structure [23,24,25]. The circulation of distinct lineages of DENV2 in Brazil had already been observed before [15] and was ascribed either to independent introductions in different time points or as a result of local evolution.

The sequences distribution in the phylogenetic tree indicated that the oldest Brazilian DENV2 are more related to viruses from South American countries (Paraguay and Venezuela), and the more recent isolate appears to be a result of a re-introduction from the Caribbean (Martinique, Cuba, and the Dominican Republic). In fact, migration of DENV2 from the Caribbean islands into America’s mainland was reported to be relatively frequent [26]. However, due to the low availability of sequences from other South American countries, we could not precisely determine the location of such introduction.

It is well known that DENV is characterized by frequent clade replacements (i.e., the replacement of the successful lineage present in one sampling period to the next) [25]. Also, this “lineage turnover” phenomenon appears to be a consequence of the stochastic forces and host immunity pressures, which ultimately determines the prevalence of a specific clade or serotype in a particular season [25,27]. This model agrees to both conditions observed during the recent epidemics in Brazil: (i) the shift from DENV3 to DENV2 prevalence in the Southern region and, (ii) the emergence of a genetically distinct lineage of DENV2 in 2007, possibly caused by the re-introduction of viruses imported from the Caribbean. Although phylogenetic data show that some strains can persist and locally evolve, our data on DENV2 in Brazil suggest that the re-introduction may be important for the dynamics of the epidemics.

The evolutionary history of dengue virus is recent and DENV2 is estimated to have emerged between 120 and 215 years ago [21,22,28], which is in line with our prediction of 123 years for this serotype. Additionally, we estimated that the American/Asian genotype emerged about 40 years ago, which is also in accordance to previous data of 39 years for the origin of this genotype [21]. However, it was intriguing that the viruses causing the outbreaks in São Paulo and Rio de Janeiro States during the last three years share a common ancestor few years before the first reported

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**Table 1. Characteristics of the 18 patients sampled in Santos (2010) with DENV2 disease.**

| Patient ID | City       | Gender | Age (years) | Platelets (1000/µl) | Clinical presentation* | Death |
|------------|------------|--------|-------------|---------------------|------------------------|-------|
| 05         | São Vicente| Female | 25          | 174                 | Dengue fever           | No    |
| 07         | Santos     | Female | 34          | 253                 | Dengue fever           | No    |
| 10         | Guarujá    | Female | 37          | 152                 | Dengue fever           | No    |
| 11         | Santos     | Male   | 53          | 179                 | Dengue fever           | No    |
| 13         | São Vicente| Female | 74          | 120                 | Dengue fever           | No    |
| 16         | Santos     | Female | 22          | 200                 | Dengue fever           | No    |
| 17         | Santos     | Male   | 47          | 237                 | Dengue fever           | No    |
| 18         | ND**       | Female | 16          | 39                  | ND**                   | Yes   |
| 25         | ND**       | Male   | 50          | 48                  | Dengue fever           | No    |
| 28         | Santos     | Male   | 79          | 17                  | Severe (DSS)           | Yes   |
| 42         | Santos     | Female | 86          | 20                  | Severe (DHF)           | No    |
| 44         | São Vicente| Female | 29          | 216                 | Dengue fever           | No    |
| 45         | Santos     | Male   | 31          | 230                 | Dengue fever           | No    |
| 47         | Santos     | Female | 31          | 147                 | Dengue fever           | No    |
| 49         | Santos     | Male   | 51          | 158                 | Dengue fever           | No    |
| 51         | Santos     | Female | 53          | 135                 | Dengue fever           | No    |
| 52         | Santos     | Female | 46          | 132                 | Dengue fever           | No    |
| 327        | Santos     | Female | 0***        | ND**                | Dengue fever           | No    |

*The patients presenting severe dengue must present at least one of the follow clinical findings: plasma leakage (leading to DSS and/or respiratory distress); severe bleeding and severe organ involvement. This has been defined by the World Health Organization Guideline, 2009.

**ND not determined.

***One week.

doi:10.1371/journal.pone.0011811.t001
outbreak (median of 9.8 years ago, and lower value of 5.5 years ago), suggesting that this lineage was already circulating in Brazil before its detection in 2007.

In fact, a “silent” introduction of a new DENV into a naïve population has already been documented for DENV4 in Brazil [29,30]. In 2008, DENV4 was identified in three patients in Manaus, without international travelling history. Based on these data, we can envisage the possibility that the DENV2 found São Paulo and Rio de Janeiro States has maintained a cryptic circulation in such regions or elsewhere (as well as DENV4 in Manaus), until meets favourable conditions to trigger an outbreak.

Because the introduction of a new serotype in an endemic area may imply in more severe clinical presentations of dengue disease, our findings shed lights on the importance of a more systematic surveillance of the dengue viruses in endemic regions.

In summary, this work described the molecular and phylogenetic characteristics of DENV2 during the current outbreak in the coastal region of São Paulo State, Brazil. The present epidemic in the cities of Guarujá, Santos and surrounding areas, was caused by the same DENV2 lineage that circulated in Rio de Janeiro in 2007 and 2008, but distinct from viruses that circulated during more ancient DENV2 epidemics in Brazil. Also, since the introduction of a new serotype is considered to increase the risk for severe dengue, our data on the silent introduction of DENV2 claims for the risks of cryptic introduction and circulation of new viruses into endemic areas.

This work supports the importance of dengue surveillance improvement and may help developing dengue-control programs. Finally, it is important to remember that the infectious disease dynamic is more than a local problem, involving neighboring regions and the population migration rate. Surveillance programs during non-epidemic seasons and in non-endemic regions may be crucial for early identification of new dengue virus introduction.

Supporting Information

File S1 A. TMRC of the DENV2 obtained by Strict and Relaxed Uncorrelated Lognormal Molecular Clock. B. Bayes factor values obtained for molecular clock comparison of DENV2. Found at: doi:10.1371/journal.pone.0011811.s001 (0.03 MB DOC)

Figure S1 Phylogenetic tree of the four DENV serotypes. The maximum clade credibility (MCC) tree was inferred from 93 partial envelope dengue viruses sequences obtained from patients from the cities of Guarujá and Santos (collapsed blue clade) and representative sequences of all DENV serotypes. The posterior probabilities of the key nodes are depicted above the respective node. Found at: doi:10.1371/journal.pone.0011811.s002 (2.95 MB TIF)

Acknowledgments

We are in debt with Maria Candida Souza Dantas and Islder Moraes Silva for the grant’s administrative support, and Bianca Natali Santos and Fernanda Romano Bruno for the laboratory support at the LIM-60.

Author Contributions

Conceived and designed the experiments: CMR CSP. Performed the experiments: AMdM LSVB KJC MCdMS CLR. Analyzed the data: CMR EGK. Contributed reagents/materials/analysis tools: ESA WCdS OMO.

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