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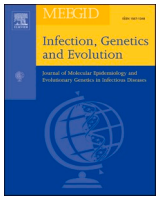

Short communication

Genome sequencing of dengue virus serotype 4 in a bat brain sample (*Platyrrhinus helleri*) from the Brazilian Amazon

Luciano Chaves Franco Filho [a,*], Rafael Ribeiro Barata [a], Mônica Silva Coelho [b], Jedson Ferreira Cardoso [a], Poliana da Silva Lemos [c], Herald Souza dos Reis [a], Joana da Felicidade Ribeiro Favacho [a], Nuno Rodrigues Faria [d], Marcio Roberto Teixeira Nunes [a]

[a] *Evandro Chagas Institute, Ananindeua, Pará, Brazil*
[b] *Federal University of Pará, Belém, Pará, Brazil*
[c] *Faculty of Public Health, University of São Paulo, São Paulo, São Paulo, Brazil*
[d] *Department of Zoology, University of Oxford, Oxford, UK*

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ABSTRACT

The existence of sylvatic transmission of dengue virus in communities of neotropical bats remains uncertain. In this work we present a near-complete genome of dengue virus serotype 4 obtained from the brain sample of a bat from *Platyrrhinus helleri* specie collected in the Brazilian Amazon region. The presence of the virus in the brain sample may indicate a possible tropism for the central nervous system in bats, which may justify negative results in previous studies that focused on analysis of other tissues, such as liver and spleen. Besides the duration of dengue virus circulation in the Americas (circa 40 years) may be too short for an implementation of a sylvatic dengue virus cycle. Our findings suggest that continued monitoring is needed to confirm with the neotropical bats could potentially act as a natural reservoir of dengue in the region.

1. Introduction

Dengue virus (DENV) is a single stranded RNA virus from the Flavivirus genus, that also includes yellow fever, Zika and West Nile viruses (Lefkowitz et al., 2017). DENV is one of the most important zoonosis worldwide and causes approximately 390 million infections per year. There are around 500,000 severe dengue cases yearly, most of them in tropical and subtropical regions (Bhatt et al., 2013; Pierson and Diamond, 2020). There are four serotypes of dengue virus. Serotype 1 (DENV-1) can be further classified as genotypes I, II, III, IV and V; serotype 2 (DENV-2) as genotype I (American), II (Cosmopolitan), III (Southern Asian-American), IV (Asian II), V (Asian I) and VI (Sylvatic); Serotype 3 (DENV-3) as genotype I, II, III, V; and serotype 4 (DENV-4) as genotype I, II, III and IV (Fonseca et al., 2019). In Brazil, circulation of dengue serotypes has been a major problem for at least three decades, with cases of DENV serotypes 1 (genotypes III and V), 2 (genotype III) and 3 (genotypes I, III, IV and V) increasing since the 1990s (Ramos-Castañeda et al., 2017). In 2010, a large outbreak of DENV serotype 4 genotype II reemerged in Brazil, 29 years after its first detection in 1982 in state of Roraima (Osanai et al., 1983; Nunes et al., 2012). Genetic analysis indicates that this serotype was circulating cryptically years before its first detection in Brazil (Nunes et al., 2012).

DENV sylvatic cycles in Southeast Asia and West Africa have non-human primates as natural reservoirs (Turell et al., 2019; Valentine et al., 2019). However, the establishment of a dengue virus sylvatic cycle has not yet been confirmed in the American continent. Some studies have investigated the possibility of neotropical non-human primates playing a role in establishing a sylvatic cycle by acting as reservoirs in the Americas, however existing data does not support DENV sylvatic maintenance in neotropical non-human primates (Turell et al., 2019). Other studies suggest that neotropical bats could be likely reservoir or amplifying hosts in the DENV sylvatic cycle transmission in the Americas, differently from what is observed in Southeast Asia and West Africa (Calderón et al., 2019; Olival et al., 2017; Moratelli and Calisher, 2015).

Several studies have evaluated the presence of DENV in Neotropical bats in the wild. In 2000, Platt et al. detected antibodies for DENV-1, DENV-2 and DENV-3 serotypes in bats from 3 different genera collected in 1998 in Costa Rica (Platt et al., 2000). Other studies in

* Corresponding author at: Evandro Chagas Institute (IEC-SABMI-LABIST) SVS, MS - 2007-2013 Rodovia Br-316 Km 7 S/N – Levilândia, 67030-000, Ananindeua, Pará, Brazil.
E-mail address: lucianofilho@iec.gov.br (L.C. Franco Filho).

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Mexico detected DENV-2 RNA in several bat species using the RT-PCR technique (Aguilar-Setién et al., 2008; Sotomayor-Bonilla et al., 2014). But only in 2007, the first partial sequence of a genome fragment (419 bp) of DENV-1 was detected in a neotropical bat, from a sample of *Carollia perspicillata* collected in French Guiana (Thoisy et al., 2009). Since then, another twenty partial sequences have been deposited in the NCBI genomic database: two strains of the DENV-2 identified in Colombia in 2016, ten DENV-2 and eight DENV- 4 strains identified in Costa Rica. Although these data were deposited between 2007 and 2016 year, these studies remain unpublished.

To better understand the possible role of neotropical bats in the sylvatic cycle of dengue in the Brazilian Amazon, we present a nearly complete DENV-4 genome sequence recovered from a brain sample of *Platyrrhinus helleri*.

2. Methods

2.1. Sample collection

A total of 40 bats comprising 16 species (supplementary Table 1), were obtained in November 2015 and February and May 2016. The collection area is located nearby the city of Canaã dos Carajás (state of Pará, Brazil), an area of preserved forest next to a mining field (Supplementary Table 2).

For the morphological classification of bats, the identification key Morcegos do Brasil (Reis, Pedro and Lima; 2007) was used. For molecular classification of bats, the mitochondrial cytochrome oxidase subunit 1 (COI) of each bat was compared with the sequences in the Barcode of life database (BOLD SYSTEMS - https://www.boldsystems.org/index.php).

2.2. Sample preparation

From each animal the brain, intestine, liver, lungs and kidney were removed and processed separately. The viral particles were released from the cells using a stainless bead with a TissueLyser II (Qiagen). Subsequently, the samples were preenriched using 0.45-μm-pore filters and an enzymatic treatment (Benzonase; 25 U/l). RNA was extracted using the iPrep PureLink virus kit (Thermo Fisher) following the manufacturer's guidelines. The extracted RNA samples were quantified by the Qubit 2.0 fluorometer, using the Qubit RNA high-sensitivity (HS) assay kit (Thermo Fisher). The RNA samples were subjected to reverse transcription using the cDNA synthesis system kit (Roche, Branford, CT) according to the manufacturer's guidelines. All procedures up to the extraction stage were performed in a Biosafety Level 3 Laboratory (BSL3).

2.3. qPCR assay

The samples were tested with two rounds of an RT-qPCR protocol using specific primers for Dengue virus detection and typing (Santiago et al., 2013). For the first round of qPCR assays, the RNA samples of each tissue from the same individual were combined and processed as a single sample (pool) for each bat. For the positive samples from the first round, a second qPCR assays were performed using the samples from each tissue. To ensure that no contamination occurred during the extraction and qPCR procedure two Vero Cells samples (Thermo Fisher Scientific) were included.

2.4. Genome sequencing and assembling

The RNA samples, positives for DENV-4, were subjected to reverse transcription using the cDNA synthesis system kit (Roche, Branford, CT) according to the manufacturer's guidelines. The complete DENV-4 genome was amplified with a multiplex PCR Protocol adapted from Quick et al. (Quick et al., 2017) using a DENV-4 specific set of primers designed by the Primal Scheme open-source software available at https://primalscheme.com (Supplementary Table 3). The amplicons were sequenced using the Ion Torrent PGM platform (Thermo Fisher Scientific) and applying the 400-bp fragment library through the Ion Xpress Plus fragment library kit and the AB library builder system (Thermo Fisher Scientific), according to the manufacturer's recommendations. After the libraries' construction, sequencing was performed with the Ion 318 chip kit v.2 BC.

DENV-4 sequencing reads were mapped against the closest DENV-4 genome available (KP188560) in the NCBI database (Blastx Result - NR database) using Geneious V9 (Kearse et al., 2012). After confirmation of the DENV-4 sequence in the bat sample, to better understand the dynamic of the infection between the bats and human hosts, six DENV-4 RT-PCR positive samples (Supplementary Table 2) collected from human hosts from Pará state, north Brazil, between 2014 and 2016 were sequenced using the same protocol.

2.5. Phylogenetic analysis

For the estimation of phylogenetic trees, all the complete DENV-4 sequences available until July 2020 were selected for comparison with the complete sequences recovered from the bats and human samples. Sequences with missing data (country and year of collection) were removed. The maximum likelihood (ML) analysis and the selection of the best fit model were performed using the IQtree V1.6.12 (Hoang et al., 2018).

3. Results

For the first round of qPCR assays, four samples tested positive for DENV-4 (supplementary Table 1). For the second round, only the brain samples tested positive for DENV-4. The Vero Cells samples and PCR negative controls were clean (Table 1).

For the four DENV-4 positive brain samples, only M11CE sample showed satisfactory results in the Next Generation Sequencing using a DENV-4 genome multiplex PCR protocol. The sequencing generated a total of 656 DENV-4 reads for the M11CE sample. After the assembly process, the consensus sequence comprised 8409 of 10,649 nucleotides (79% with 11.8× coverage), presenting 99.26% nucleotide identity and 99,55% amino acid identity to Dengue virus 4 isolate BR/SJRP/514/2012 from Brazil (KP188560), which belongs to DENV-4 genotype 2. The reads were recruited for all open reading frames (ORFS) present in the reference genome. However, some gaps (21% of the genome) were still present owing to limitations in the volume of data generation.

To investigate the evolutionary relationships between bat and human DENV sequences, we conducted a ML phylogenetic approach (Fig. 1). The M11CE strain (*P. helleri* – Brazil-2016) clustered with other sequences from serotype 4, confirming that M11CE belongs to serotype 4. The M11CE strain obtained in the Northern region of Brazil, Pará state, is more closely related to human sequences from Pará. Particularly, the M11CE strain is most closely related the A1-AB human strain obtained from Santa Isabel do Pará collected in June 26, 2014. The two strains form a monophyletic clade with 100 of Bootstrap. More generally, 60% ($n = 6$ of the 10) sequences analyzed from Pará state form a well-supported monophyletic clade (Bootstrap = 97.

4. Discussion

We recovered the first nearly complete DENV-4 genome from the brain sample of a *P. helleri* bat from Pará state in the Brazilian Amazon region collected in 2016. The genomic approach undertook here was based on highly sensitive and specific primers. Considering the all procedures tacked to avoid the contamination, the low coverage and high CT-value could indicate a low viremia in the host, suggesting that neotropical bats may be acting as dead-end hosts. There are, indeed, high chances of DENV is unable to replicate efficiently in a neotropical

Table 1
Cycle threshold (CT) values for each tissue of bat samples positive for DENV-4.

Sample		Info	Cycle Threshold Value (Ct)			
			Replicate 1	Replicate 2	Replicate 3	CT Mean
M11CE	*Platyrrhinus helleri*	Brain sample	39,34	36,82	35,38	37,18
M11CE	*Platyrrhinus helleri*	Intestine sample	ND	ND	ND	–
M11CE	*Platyrrhinus helleri*	Liver sample	ND	ND	ND	–
M11CE	*Platyrrhinus helleri*	Lungs sample	ND	ND	ND	–
M11CE	*Platyrrhinus helleri*	Kidney sample	ND	ND	ND	–
C04A1	*Phyllostomus elongatus*	Brain sample	38,69	40,87	39,36	39,62
C04A1	*Phyllostomus elongatus*	Intestine sample	ND	ND	ND	–
C04A1	*Phyllostomus elongatus*	Liver sample	ND	ND	ND	–
C04A1	*Phyllostomus elongatus*	Lungs sample	ND	ND	ND	–
C04A1	*Phyllostomus elongatus*	Kidney sample	ND	ND	ND	–
C10A2	*Artibeus cinereus*	Brain sample	38,89	38,47	38,67	38,68
C10A2	*Artibeus cinereus*	Intestine sample	ND	ND	ND	–
C10A2	*Artibeus cinereus*	Liver sample	ND	ND	ND	–
C10A2	*Artibeus cinereus*	Lungs sample	ND	ND	ND	–
C10A2	*Artibeus cinereus*	Kidney sample	ND	ND	ND	–
P2A1	*Phyllostomus discolor*	Brain sample	38,46	38,12	39,88	38,82
P2A1	*Phyllostomus discolor*	Intestine sample	ND	ND	ND	–
P2A1	*Phyllostomus discolor*	Liver sample	ND	ND	ND	–
P2A1	*Phyllostomus discolor*	Lungs sample	ND	ND	ND	–
P2A1	*Phyllostomus discolor*	Kidney sample	ND	ND	ND	–
NC1	Vero Cells	Negative DENV4 sample	ND	ND	ND	–
NC2	Vero Cells	Negative DENV4 sample	ND	ND	ND	–
DENV-4	*Homo sapiens*	Positive DENV4 sample	24,01	24,95	25,24	24,74
NRC	H_20	Negative reaction control	ND	ND	ND	–

ND: No detection.

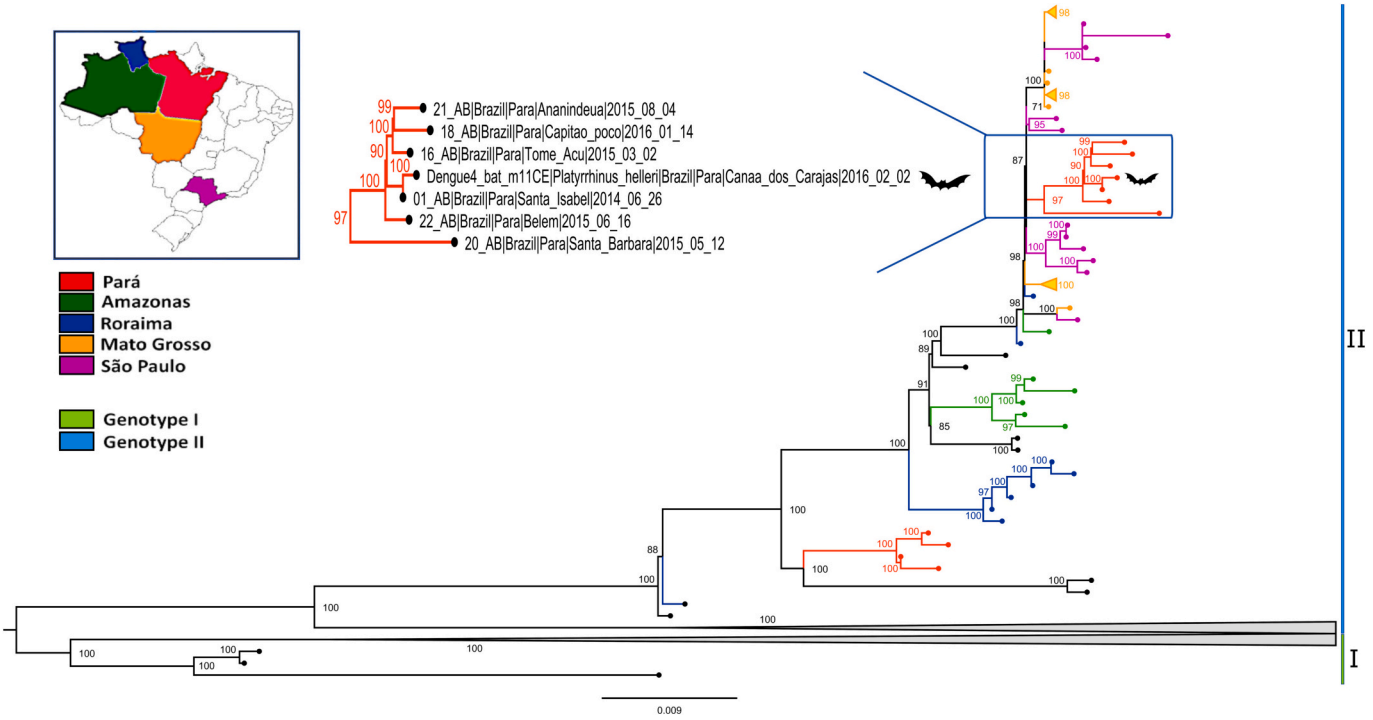

Fig. 1. Maximum Likelihood (ML) analysis of the DENV-4 estimated from complete genome sequences. The sequence from *P. helleri* is represented by black bat icon.

bats, based in previously works (Vicente-Santos et al., 2017), however, viremia could vary during the period of infection and qPCR assay has a small window of detection. Experimental infections are necessary to confirm the possibility of the Dengue virus to replicate in *P. helleri* cells.

The ML tree (Fig. 1) show that the M11CE strain obtained from *P. helleri* in Canaã dos Carajás, Pará state, in February 2016, is more closely related to the human strain 01-AB from Santa Izabel, Pará state, collected in late April 2014. Both the M11CE strain and the 01-AB strains were recovered in or around the peak of dengue season in Pará state (Wunderlich et al., 2018). Some virus lineage can be associate to a particular reservoir host, this genetic differentiation between strains probably occurs due to adaptation of the virus to a susceptible host, been this adaptation and differentiation unlikely to occur in non-susceptible host, reinforce the idea the neotropical bats may be acting as dead-end hosts for dengue virus (Vicente-Santos et al., 2017). However, the introduction of serotype 4 in Brazil occurred through multiple entries, probably the first in the North, around 1978, from the Caribbean, and later with other entries from Venezuela and Colombia in the 90s.

Nevertheless, the circulation of DENV-4 in this period was quite low, gaining strength only around 2010 (Nunes et al., 2012). Considering the circulation time of serotypes 4 in northern Brazil is quite recent, in evolutionary terms, it is a very short time for an establishment of an effective wild cycle, since factors such as geographic or ecological isolation are important barriers that hinder a transmission event between hosts (Wasik et al., 2019).

The low number of reads and high CT vales (Table 1) can be indicated that DENV is unable to replicate efficiently in a neotropical bats suggest that neotropical bats are acting as dead-end host. Nevertheless, the detection of RNA only in the brain samples can suggesting tropism for the central nervous system, which may explain the lack of positive results in other studies focused on blood and other tissue analysis (Cabrera-Romo et al., 2016). Also is unwise not take in consideration the high diversity of neotropical bats and the possibility of just a few ones are able to act as a reservoir or transeunt host. The lack of conclusive results regarding the capacity of neotropical bats to harbor or not the dengue virus, reinforces the necessity for more studies. Especially in actual Covid-19 pandemic scenario, the maintaining of studies that involve both the understanding of human health as well animal and environmental health, is one of best options to get more accurate data to creation of effective methods and strategies for public health.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2023.105407.

Ethics and biosafety measures

The project received approval from the Animal Ethics Committee (CEUA / IEC-031/2014) and the Biodiversity Authorization and Information System (SISBIO –47592-1 and 57210). All procedures were carried out in accordance with institutional biosafety standards. The laboratories are certified by ISO 15189 (molecular biology and serology laboratory I and II) and WHO Collaborating Centre for Emerging and *Re*-emerging Arboviruses and other Emerging Zoonotic Viruses (https://apps.who.int/whocc/Detail.aspx?HjIEEe+gYCdRLdRl+NbnpA==).

CRediT authorship contribution statement

Luciano Chaves Franco Filho: Methodology, Investigation, Formal analysis, Writing – original draft. **Rafael Ribeiro Barata:** Formal analysis. **Mônica SilvaCoelho:** Methodology, Investigation. **Jedson Ferreira Cardoso:** Formal analysis. **Poliana da Silva Lemos:** Investigation, Writing – review & editing. **Herald Souza dos Reis:** Data curation, Visualization. **Joana da Felicidade Ribeiro Favacho:** Funding acquisition, Supervision. **Nuno Rodrigues Faria:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Marcio Roberto Teixeira Nunes:** Conceptualization, Funding acquisition, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

We know of no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome. As Corresponding Author, I confirm that the manuscript has been read and approved for submission by all the named authors.

Data availability

Data will be made available on request.

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Dr. Luciano Chaves Franco Filho is a research fellow in Evandro Chagas Institute, Ananindeua, Pará, Brazil. His primary research interests include metagenomic analysis, microbial ecology and epidemiology of emerging infectious diseases with zoonotic origins.