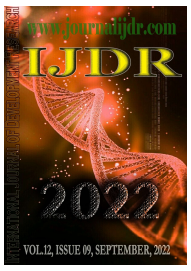
ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 12, Issue, 09, pp. 58936-58939, September, 2022
https://doi.org/10.37118/ijdr.25212.09.2022



RESEARCH ARTICLE OPEN ACCESS

MOLECULAR IDENTIFICATION OF DENGUE VIRUS SEROTYPES 1 AND 3 IN AMAZONAS STATE, BRAZIL DURING 2001–2002

Luís Felipe Alho da Silva, Ernandes Borges Reis Júnior and *Regina Maria Pinto de Figueiredo

Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (FMT-HVD), Manaus, AM, Brasil

ARTICLE INFO

Article History:
Received 10th August, 2022
Received in revised form
22nd August, 2022
Accepted 24th September, 2022
Published online 30th September, 2022

Key Words:
Dengue, serotype, Manaus, Amazonas, Brazil

Corresponding author:
Regina Maria Pinto de Figueiredo

ABSTRACT

Objective: Investigate DENV circulation in the serum of patients with febrile illness presented at the Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (FMT-HVD), Manaus - Amazonas between 2001–2002. Methods: 116 sera collected in the acute phase were tested by semi-nested multiplex PCR. Results: Among the 116 patients, 46 were positive for DENV: 6 DENV1 and 40 DENV3. Moreover, among the DENV3 samples collected in 2001, only two patients reported travel to communities near Manaus. Conclusions: This study reported the molecular detection of DENV1 and DENV3 and the circulation of DENV3 in autochthonous cases since 2001. These findings reinforce the importance of laboratory approaches in the surveillance of emerging and re-emerging diseases.

Citation: Luís Felipe Alho da Silva, Ernandes Borges Reis Júnior and Regina Maria Pinto de Figueiredo, 2022. "Molecular identification of dengue virus serotypes 1 and 3 in amazonas state, brazil during 2001–2002*", International Journal of Development Research, 12, (09),* 58936-58939.

INTRODUCTION

Dengue virus (DENV) belongs to the family Flaviviridae, genus Flavivirus, and has a single-stranded, positive polarity, 11 kb RNA genome containing an open reading frame (ORF), which encodes three structural proteins: capsid (C), membrane (M), and envelope (E), and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS51. Previous studies on the E gene have recognized five distinct genotypes for DENV1, six genotypes for DENV2, and four genotypes for DENV3 and DENV4 (Chen, 2011) (Harapan, 2020). The main vectors of DENV are Aedes aegypti and Aedes albopictus, which are important vectors in domestic and peridomestic areas (Moreli, 2013). The acute stage (0–6 days) of dengue can be diagnosed through the detection of the viral genetic material using molecular techniques derived from reverse transcription polymerase chain reaction (RT-PCR), which detects the DENV serotypes. Although these methods provide timely serotyping results and accurate diagnosis, they are not always available in in regions with dengue epidemic threat. In the diagnosis of dengue, serotypying is very important since severe cases are often related to DENV2 and DENV3 (Tsai, 2019). In Manaus, the capital of Amazonas state, the first dengue epidemic occurred in 1998–1999, in which DENV1 and DENV2 were detected[5]. In March 2002, DENV3 was isolated for the first time and, since then, with increased laboratory investigation, other cases were diagnosed through viral isolation in cell culture using continuous C6/36 cell lines (Ae. albopictus clone).

In 2005, DENV4 was detected in sera from autochthonous patients in Manaus using serological and molecular techniques (Figueiredo, 2008). Currently, the four serotypes have been detected in all Brazilian states, as confirmed using molecular techniques. In this study, we investigated the transmission of DENV among patients with undifferentiated febrile syndrome in the 2001–2002 epidemic in Manaus, Amazonas, contributing to a better understanding of arboviruses that have circulated in the region.

METHODS

Ethics: This study was approved by the Ethics Committee of the Tropical Medicine Foundation Doctor Heitor Vieira Dourado (FMT-HVD), number 0004.0.114.000-05.

Study area: Amazonas is located in the center of the Northern Region of Brazil, bordered to the north by the State of Roraima, Venezuela, and Colombia, to the east by the State of Pará, to the southeast by the State of Mato Grosso; to the south by the State of Rondônia; and to the southwest by the State of Acre and Peru. The Amazonas has an estimated population of 3,332,330 inhabitants, 48% of whom are living in Manaus (03°06′07″ S, 60°01′30″ W) (https://viverde com.br). The economy is led by the activities of the industrial hub of Manaus, where the free trade zone is located. Vegetable extraction,

fishing, and cultivation of grains and vegetables also contribute to the economy (https://m.brasilescola.uol.com.br).

Sample collection : A total of 116 patients sought medical attention in 2001–2002 at Fundação de Medicina Tropical Doutor Heitor Vieira Dourado (FMT-HVD), the reference hospital for infection diseases in Manaus-AM–. The patients presented with undifferentiated febrile syndrome and at least three of the following signs and symptoms: fever, headache, eyeball pain, myalgia, arthralgia, prostration, and exanthema; associated or not with the presence of hemorrhages. Serum samples were initially tested for malaria using thick blood analysis. All negative sera for malaria were collected between 0–6 days of symptom onset and stored at -80° C. At this period, no molecular studies were performed; moreover, due to the large demand of samples, many sera were not processed using viral isolation in cell culture using continuous C6/36 cell lines (*Ae. albopictus* clone) [5,6]. In this study, samples from patients with suspected DENV that were collected in the acute phase and stored appropriately were selected for molecular testing**.**

Clinical and demographic characteristics of patients : Variants sex, age, residential address, commuting, and clinical characteristics were obtained from the patients' laboratory investigation forms completed at the time of collection, and subsequently tabulated in an Excel spreadsheet in order to report epidemiological and clinical data and compare them with positive results for DENV infection.

Extraction and amplification of the DENV RNA: Viral RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. After extraction, viral RNA was subjected to reverse transcription for complementary DNA (cDNA) synthesis using the AccessQuick™ RT-PCR system (Promega) kit, according to the manufacturer's recommendations, followed by the semi-nested multiplex PCR developed by Lanciotti et al.[8] for the detection and typing of dengue virus, using the primers designed for the conserved regions of the genes encoding the capsid/premembrane structural proteins (C/PrM) from DENV (Lanciotti, 1992).

The first round of PCR was performed using 12.5 µl of Hot Start Master Mix (Qiagen,), 0.5 µl of the forward primer D1 (5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′), 0.5 µl of the reverse primer D2 (5′-TTGCACCAACAGTC AATGTCTTCA GGTTC-3′), and 2 µL of the viral RNA. Amplification was carried out in a Veriti thermocycler (Applied Biosystems) under the following conditions: denaturation at 95 °C for 15 min, followed by 35 cycles of 91 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min[7]. Next, 10 µL each amplicon was diluted (1:100) and subjected to a second amplification cycle performed under the following conditions: denaturation at 95 °C for 15 min, followed by 28 cycles of the same cycling condition as that of the first cycle, and a final extension at 72 °C for 10 min. All nested-PCR products generated in the second round of amplification were electrophoresed on 1.5% agarose gel in 1X TBE buffer at 100 V for 60 min and analyzed under ultraviolet light.

RESULTS

Analysis of the 116 samples showed that 39.6% (46/116) were positive for DENV: 13.0% (6/46) for DENV1, and 86.9% (40/46) for DENV3 (Table 1). Children and adults of both sexes were infected, with a predominance in young adults (Figure 1). Fever, headache, myalgia, eyeball pain, arthralgia, bone pain, exanthema, and bleeding were the most frequent symptoms (Figure 2); no severe manifestations were recorded. Based on the analysis of the form filled out at the time of collection, all regions of the city had positive cases, with a predominance of the southern and central-western areas.

Regarding the displacement of patients, only three reported residences outside Manaus (in transit) and 11 reported travel 15 days before the onset of symptoms. Among the 11 patients with travel history, four traveled to other states. Moreover, among those positive for DENV3 collected in 2001, only two patients reported travel to communities near Manaus (Table 2).

Table 1. Demographic characteristics and molecular testing of patients positive for DENV

	DENV1		DENV3	
YEAR OF COLLECTION	N=6	%	N=40	%
2001	3	6.5%	12	26%
2002	3	6.5%	28	60%
GENDER				
Male	2	33.3%	3	50%
Femele	4	66.7%	19	47.5%
NR	NA	NA	1	2.5%
TRAVELED ABROAD +/- 15 DAYS				
YES	1	16.60%	10	25%
NO	5	83.5%	30	75%

NR: Not reported

Table 2. Displacement of patients to neighboring communities and/or other states

ID MAO	TRANSIT	SOROTYPE	TRAVELED ABROAD +/- 15 DAYS	YEAR COLLECTION
103	-	DENV1	BR 174 kM10[c]	2002
13766	-	DENV3	SÃO PAULO	2001
14	-	DENV3	KM 28[c]	2001
37	-	DENV3	KM22[c]	2001
117	-	DENV3	RJ[a]	2002
123	-	DENV3	KM AM010[c]	2002
162	YES	DENV3	RJ[a]	2002
167	YES	DENV3	NÃO	2002
173	-	DENV3	Paraná	2002
202	-	DENV3	CNSF[b]	2002
204	-	DENV3	PRESIDENTE FIGUEIREDO[b]	2002
231	YES	DENV3	NÃO	2002
233	-	DENV3	KM 38[c]	2002

CNSF= Nossa Senhora de Fátima Community; MAO, Manaus; RJ, Rio de Janeiro; [a] Brazilian state; [b] Municipalities of Amazonas; [c] State highways.

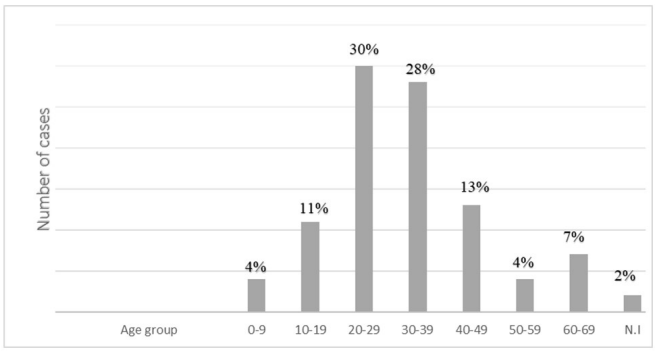


Figure 1. Distribution of the number of dengue cases by age group in Manaus, Amazonas in 2001–2002

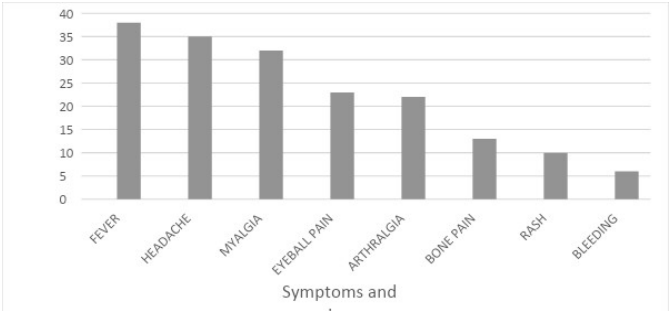


Figure 2. Comparison of the most frequent signs and symptoms caused by DENV reported by patients in the years 2001–2002 in Manaus-AM

DISCUSSION

In Brazil, epidemics caused by DENV have been continuously registered with the simultaneous circulation of four serotypes (Duarte, 2019). The first dengue epidemic in Brazil with laboratory confirmation was in 1982 in the city of Boa Vista-Roraima, caused by DENV1 and DENV4, wherein approximately 11,000 people were infected (Osanai, 1983). In Manaus, DENV1 was identified during the first DENV epidemic in the period 1998–1999 using viral isolation, sensitizing a large part of the population, determined to be the serotype with the highest prevalence, and sporadically identified in the following year (Figueiredo, 2004; Figueiredo, 2013). Meanwhile, DENV2 was isolated for the first time in 2001 from stored samples collected in 1999 (Figueiredo, 2013). DENV2 and DENV3 were introduced in Rio de Janeiro in 1990 and 2001, respectively, with the detection of DENV3 in Roraima in 2001 (Barreto, 2011; Bezerra, 2021) since is located on the international border in Venezuela and Guyana, countries that serve as a link to the Caribbean, where dengue has been endemic since 1968 and 1970, where the four serotypes are circulating (Barreto, 2011; Salles, 2018). In 2002, DENV3 was identified for the first time in Manaus using viral isolation (Figueiredo, 2013). Furthermore, a retrospective study, in which the C/prM and E genes were sequenced in 27 strains from different geographical areas in Brazil including a sample from the State of Amazonas (BRBeH657637-AM-2002), confirmed that DENV3 was present in Amazonas in 2002 (Cruz, 2010).

In this study, we detected DENV3 in samples from 2002, corroborating the findings of previous studies (Salles, 2018). Moreover, we identified DENV3 in samples collected in 2001 from patients with no history of travel to other states where this serotype had been isolated, confirming the autochthonous presence of DENV3 in Amazonas. The Amazonas is near other states and countries where this serotype had been identified at the time when the Amazonas received visitors from different regions due to the developing ecological tourism and the industrial complex that attracted investors from various parts of the world. In this study, we emphasized that DENV4 was identified in Brazil after 20 years of its first identification in the state of Roraima in autochthonous samples from Manaus-AM 15. (Figueiredo, 2021). Among the infected patients, there was no predominance between genders, which was observed from 1998–1999, probably most cases appeared in the city center where the male population had tendencies to bring the virus into their home environment. In the end, men and women were equally affected (Figueiredo, 2004) . The dissemination of DENV observed since the first epidemic in Manaus (1998-1999) (Figueiredo, 2004) is proven in 2001-2002, when we analyze the residential address of the patients we find the virus in all areas of the city, with predominance in the southern zone that includes the traditional commercial center near the black river and neighborhoods near it and the central-western zone composed of neighborhoods with commercial centers in expansion, confirming the analyzes carried out in 1998-1999 (Figueiredo, 2004) . The analysis by age group indicated that the highest number of cases occurred in adults aged 20–29 and 30–39 years, and this pattern was observed in the states of Pará in 1999 (Araújo, 1999) and Manaus during 1998–1999, indicating that, in this period, the most vulnerable groups were those with higher productive activity. As mentioned previously, the city center presented the highest number of cases, because it was where the largest active human population was concentrated during the day, and a high frequency of Ae. aegypti was observed (Figueiredo, 2004).

Despite the history of dengue epidemics in our city and the circulation of two serotypes (DENV1 and DENV2) since the first epidemic (Figueiredo, 2004), with much of the population immunized, analysis of epidemiological records revealed the absence of more severe clinical manifestations. Several factors must be considered in the development of severe dengue fever (Wang, 2020; Harapan, 2020). Among the epidemiological factors are high vector density, susceptible population, and high virus circulation intensity (Halstead, 2019) Individual risk factors include chronic diseases, preexistence of antibodies to dengue, and individual host response (.Ranawaka, 2021). Moreover, the viral factors include the virulence of the infecting strain, serotype, and variant or genotype within each serotype (Wang, 2020; Pang, 2017). This study recorded the molecular detection of DENV1 and DENV3 in samples from 2001–2002 in Manaus-Amazonas, the presence of DENV3 in 2001 in autochthonous cases, and epidemiological information from samples collected after the peak of the first epidemic in Manaus. Our results highlight the importance of epidemiological studies to better understand the circulation of arboviruses in a given period, geographic space, and laboratory surveillance in the prevention of emerging and re-emerging diseases.

Conflict of interest statement: The authors declare that there are no conflicts of interest.

Acknowledgments: The authors are thankful to financial support: Council for Scientific and Technological Development - CNPq (http://www.cnpq.br, grant 475740/2011-4, Universal announcement 2011).

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