

**ISOLATION AND IDENTIFICATION OF DENGUE VIRUS SEROTYPE 3 IN  
RONDONIA, BRAZIL**

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## **ABSTRACT**

Dengue Fever is a serious public health problem in tropical and subtropical regions of the world. The state of Rondônia has 52 municipalities, of which 32 are found inside *Amazonia Legal* and 20 in border areas. In the period of January 2004 until May 2006, 160 blood samples were collected from patients suspected of having DENV disease during outbreaks of dengue fever in the municipalities of Ariquemes, Jaru, Ouro Preto do Oeste, Cacoal, Colorado do Oeste, Vilhena and Porto Velho. Samples were submitted for viral isolation following RNA viral extraction and RT-PCR, in order to identify the dengue virus. Afterwards, hemi-nested-PCR was used to identify the viral serotype. Seventy eight samples were characterized as DENV-3. The amplicons characterized by hemi-nested-PCR were sequenced for similar research with nucleotide sequences of DENV-3 deposited in Genbank, using the BLAST program.

## **INTRODUCTION**

Arboviruses represent a serious public health problem. They are frequently associated with epidemics that have great economic and social impact in tropical and subtropical regions of the world. Dengue virus (DENV) causes a highly infectious illness and is transmitted to humans by mosquitos of the *Aedes* family causing high rates of morbidity and mortality (Rice 1996, Monath 1990, Gubler 1987). DENV belongs to the genus *Flavivirus* and the *Flaviviridae* family. It is an enveloped virus and contains a single-stranded positive sense RNA of about 11kb and is classified in four serotypes designated as DENV-1, DENV-2, DENV-3 and DENV-4, based on its antigenic characteristics (Rice 1996, Chambers et al. 1990). The genome of DENV contains 10 genes in an open reading

frame (ORF), the translation of the ORF results in a polyprotein which is processed by a signal-peptidase of the host cell into 3 structural proteins (C, E and pr M) and 7 non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) (Rice et al. 1985). Infection by any of the above serotypes causes clinical manifestations that vary and are both nonspecific and benign, until more serious stages which sometimes have fatal consequences in the form of dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Taulil 2001).

There are 52 municipal districts within the state of Rondônia of which 32 are to be found in *Amazonia Legal* and 20 in border areas. The territorial limits are: the north, northeast and northwest of the state of Amazonas; the east and southeastern with the state of Mato Grosso and the south and southwestern border with the Republic of Bolivia. Between 1999 and 2005, 29,395 cases of dengue were reported by the state health department (SESAU-RO) and Federal Health Secretary (SVS) (SIVAN/AGEVISA/SESAU/RO, 2006). Among the states of northern Brazil, Rondonia presented the highest number of reported cases of dengue between the months of January and May 2006 with a total of 5,577 cases (SVS 2006).

In order to establish an epidemiological surveillance of DENV, as well as the immunological techniques (MAC-ELISA, HI, fixation of complement, etc.) used in the detection of anti-dengue antibodies, separation is necessary through viral serotyping, and that is accomplished using molecular techniques with specific primers already described (King et al. 1991, Guzman et al. 1996). This study had as its objective the isolation, identification and sequencing of samples of DENV circulating in several municipalities of the state of Rondonia between January 2004 until May 2006.

## **MATERIALS AND METHODS**

### **Biological samples.**

Biological blood samples were collected between January 2004 and May 2006 by a team from the virology laboratory of the Institute for Research of Tropical Pathology (IPEPATRO), based in the city of Porto Velho, which responded to requests made by several municipal councils along the length of the BR 364 highway, namely: Ariquemes, Jaru, Ouro Preto do Oeste, Cacoal, Colorado do Oeste and Vilhena.

One hundred sixty blood samples were collected for viral isolation from patients that presented clinical symptoms of dengue between 1 and 5 days of onset. All patients were clinically examined by a physician of the CEPEM/IPEPATRO staff. Patients that showed symptoms compatible with those of dengue, and with negative results for malaria in the microscopical diagnosis of *Plasmodium*, were included in this study. Blood samples were collected after the patient signed a consent form approved by the Ethics in Research Commission from CEPEM/IPEPATRO.

### **Virus isolation.**

For virus isolation, C6/36 cells of *Aedes albopictus* were used (Figueiredo 1990). Cells were cultivated in Leibovitz L-15 medium (Gibco BRL, USA) containing 10% of bovine fetal serum (Gibco BRL, USA) and 50 µg/ml of gentamicine (Gibco BRL, USA) on 24-well plates (Corning, USA) incubated at 28°C in a BOD incubator until cellular monolayers were formed. The samples of serum were diluted 1:10 and inoculated onto the

monolayer of C6/36 cells and incubated for 7 days (Tesh 1979). The confirmation of viral infection was made by RT-PCR.

**Extraction of RNA.**

On the seventh day after inoculation, all C6/36 cell samples were submitted to extraction of viral RNA, by the method of Trizol® - LS (Invitrogen, USA) adapted from the original method described by Chomczynski et al. (1987).

**cDNA.**

To obtain cDNA, we incubated 5µl of RNA extracted with 3µg of random primer pd (N)<sub>6</sub> (Gibco® BRL, USA), 1µl of dNTP 10mM (Gibco® BRL, USA), 4µl of buffer, 10U/µl inhibitor of RNase and 200U/µl SuperScript™ (Gibco® BRL, USA) at 42°C for 1 hour.

**PCR and Hemi-nested-PCR.**

The universal primers for flavivirus, FG1 and FG2, that amplify a fragment of 958 bp of the gene NS5 which identifies the dengue virus, were used for RT-PCR (Bronzoni et al. 2005). The sequences of primers are shown in Table 1. The products of PCR were submitted to an electrophoresis field in 2% agarose gel (GIBCO® BRL, USA), with the addition of ethylene bromide. Results were observed through an ultraviolet light transilluminator and the size of amplicons was determined through comparison of the migration line with a marker molecular weight of 100bp (Invitrogen, USA). Afterwards, hemi-nested-PCR for molecular characterization of the four serotypes of dengue virus was carried out. The primers were FG1 with nden1- (DENV 1) generating fragment 472bp; nden2- (DENV 2) generating fragment 316bp; nden3- (DENV 3) generating fragment of 659bp and nden4- (DENV 4) generating fragment of 222bp (Bronzoni et al. 2005). The

amplicons produced by this test were submitted to an eletrophoretic field in 2.5% agarose gel. Sequences of the primers used for hemi-nested-PCR are shown in Table 1 (GIBCO® BRL, USA) to observe results comparing with marker bands of a molecular weight of 100 bp (Invitrogen, USA).

**Table 1.** Sequence of primers to identify and characterize DENV.

<b>Primers</b>	<b>Sequence</b>	<b>Size of amplicon pb</b>
FG1	5'TCAAGGAACTCCACACATGAGATGTACT 3	958bp
FG2	5'GTGTCCCATCCTGCTGTGTCATCAGCATACA 3	
nden1-	5' CGTTTTGCTCTTGTGTGCGC 3'	472bp
nden2-	5'GAACCAGTTTGGTTDRTTTCATCGCTGCC 3	316 bp
nden3-	5' TTCCTCGTCCTCAACAGCAGCTCTCGCACT 3	659bp
nden4-	5' GCAATCGCTGAAGCCTTCTCCC 3'	222 bp

**Purification.**

After the confirmation of viral RNA amplification, the amplicons were sectioned from the agarose gel and purified by QIAquick ® Gel Extraction kit (QIAGEN-USA) in conformity with the manufacturer's instructions.

**Sequencing of DENV.**

Hemi-nested-PCR amplicons that were obtained and characterized as isolated DENV-3 of samples analyzed were sequenced at the Center for Virology Research of the

Faculty of Medicine of the University of São Paulo, with the Sequencer Personal Seq 4X4 (Amersham-Pharmacy-Biotech, USA) using the kit Thermo Sequence Cy5.5 dye terminator cycle sequencing (Amersham-Pharmacy-Biotech, USA).

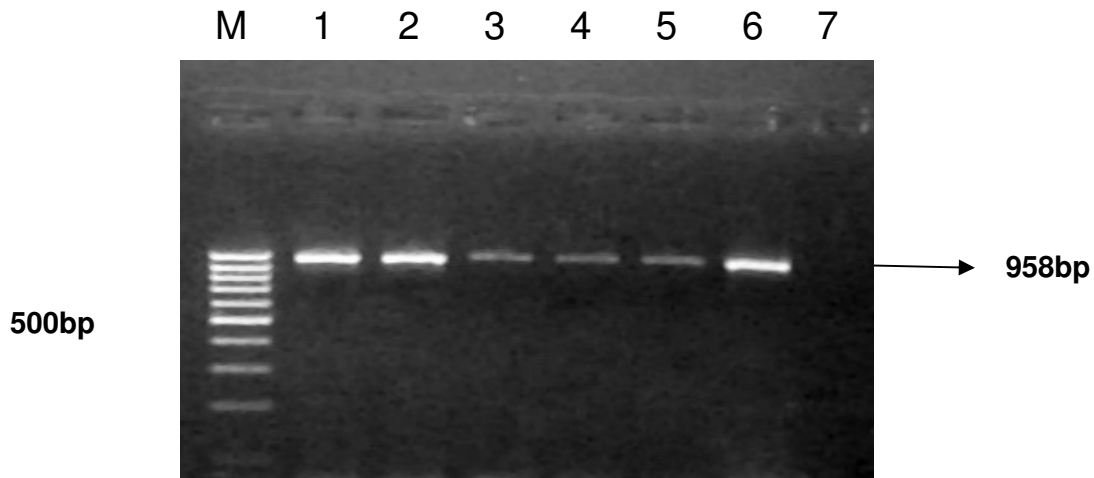
## **RESULTS**

### **Identification of biological samples.**

One hundred sixty samples of serum were inoculated onto C6/36 cells, submitted to viral RNA extraction and RT-PCR; 43.75% were confirmed positive, see Table 2, generating fragments of 958 bp, Figure 1.

**Table 2.** - Results of samples analyzed by municipal district in first PCR, using primers FG1 and FG2.

<b>Municipalities</b>	<b>Positive</b>	<b>Negative</b>	<b>Total</b>
Ariquemes	01	03	04
Cacoal	02	04	06
Colorado do Oeste	08	06	14
Jaru	02	04	06
Ouro Preto do Oeste	16	22	38
Porto Velho	45	37	82
Vilhena	04	06	10
Total	78.	82	160



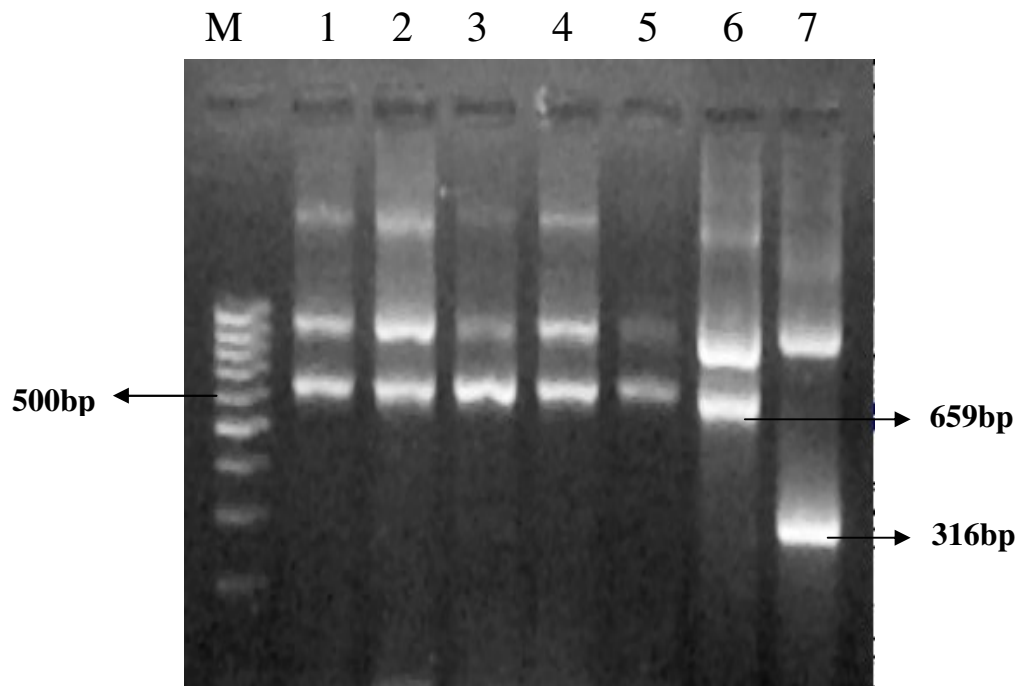
**Figure 1.** 2% agarose gel. M: Marker of a molecular weight of 100pb. Lines 1 to 5: fragments of 958bp of sera samples. Line 6: positive control, DENV-2 Ceara. Line 7: negative control, supernatant of cells C6/36 not infected.

**Molecular characterization of DENV by hemi-nested PCR.**

The amplicons of 78 positive samples were submitted to hemi-nested PCR, resulting in the characterization of DENV-3; the results of samples by municipality are shown in Table 3. Seventy eight samples generated fragments of 659bp characterizing DENV-3 (Figure 2).

**Table 3.** Results by municipal district of the characterization of samples through hemi-nested-PCR.

Municipalities	Characterization of DENV3
Ariquemes	01
Cacoal	02
Colorado do Oeste	08
Jaru	02
Ouro Preto do Oeste	16
Porto Velho	45
Vilhena	04
Total	78



**Figure 2.** Agarose gel of 2.5%. Line M: marker of a molecular weight of 100bp. Line 1 to 6: fragment of 659bp of serum samples characterized as DENV-3. Line 7: positive control DENV-2 Ceará, fragment of 316bp.

#### **Nucleotide sequences analysis.**

Comparison of the nucleotide sequence analysis of NS5 gene of DENV3 of isolates in Colorado do Oeste, Jaru, Ouro Preto do Oeste, Porto Velho and Vilhena with nucleotide sequences of DENV-3 deposited in Genbank using the BLAST (Basic Local Alignment Search Tool) software (NCBI, USA). In concordance with the datas shown by the software, the analyzed samples presented 99% of similarity with the DENV-3 when compared with the isolated samples in the state of Rondônia, see Figures 3 and 4.

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>gi|37545157|gb|AY099340.1|AY099340S1  Dengue virus type 3 isolate D3/H/INTSSA-MART/2001/2012 polyprotein
gene, partial cds
Length = 2880

Score = 664 bits (335), Expect = 0.0
Identities = 342/343 (99%), Gaps = 1/343 (0%)
Strand = Plus / Minus

Query: 1   tctttttggggagctctgcttgggaattgtattgctctgtccaggatggacctcgttggg 60
          |||
Sbjct: 2536 tctttttggggagctctgcttgggaattgtattgctctgtccaggatggacctcgttggg 2477

Query: 61   gacgaagattccacttccacatttgagttctttgcctttccagttataacacatcccat 120
          |||
Sbjct: 2476 gacgaagattccacttccacatttgagttctttgcctttccagttataacacatcccat 2417

Query: 121  gtcagcttgaccacagctcccagatagagtgaatgattcctatcgcaatgcatgaaaa 180
          |||
Sbjct: 2416 gtcagcttgaccacagctcccagatagagtgaatgattcctatcgcaatgcatgaaaa 2357

Query: 181  tgacatggatgtgttttttgaattcaaccctatccaagtcagagaactcctattccgat 240
          |||
Sbjct: 2356 tgacatggatgtgttttttgaattcaaccctatccaagtcagagaactcctattccgat 2297
    
```

**Figure 3.** Result of BLAST of nucleotide sequence of DENV-3 isolated from Porto Velho with the “Genbank”.

DENVRO1

GGGGGTGTGTGACTACCATGGCTAAGAACAAGCCAACGTTGGATATAGAGCTT  
CAGAAGA 180

DENVRO2

GGGGGTGTGTGACTACCATGGCTAAGAACAAGCCAACGTTGGATATAGAGCTT  
CAGAAGA 180

DENVRO3

GGGGGTGTGTGACTACCATGGCTAAGAACAAGCCAACGTTGGATATAGAGCTT  
CAGAAGA 180

DENVRO4

GGGGGTGTGTGACTACCATGGCTAAGAACAAGCCAACGTTGGATATAGAGCTT  
CAGAAGA 180

DENVRO5

GGGGGTGTGTGACTACCATGGCTAAGAACAAGCCAACGTTGGATATAGAGCTT  
CAGAAGA 180

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DENVRO1

CCGAGGCCACCCAATTGGCGACCCTAAGGAAGCTATGCATTGAGGGGAAAATT  
ACCAACA 240

DENVRO2

CCGAGGCCACCCAATTGGCGACCCTAAGGAAGCTATGCATTGAGGGGAAAATT  
ACCAACA 240

DENVRO3

CCGAGGCCACCCAATTGGCGACCCTAAGGAAGCTATGCATTGAGGGGAAAATT  
ACCAACA 240

DENVRO4

CCGAGGCCACCCAATTGGCGACCCTAAGGAAGCTATGCATTGAGGGGAAAATT  
ACCAACA 240

DENVRO5

CCGAGGCCACCCAATTGGCGACCTTAAGGAAGCTATGCATTGAGGGGAAAATT  
ACCAACA 240

\*\*\*\*\*

**Figure 4.** Result of alignment through “BLAST” of (nucleotides) of DENV-3 isolates from Porto Velho with the “Genbank”.

## DISCUSSION

Arboviruses present a major problem to public health in Brazil. Among arboviruses transmitted by insects, the flaviviruses are the most important in terms of outbreaks in Brazil and countries with a tropical or subtropical climate. The DENV is the most important flavivirus today and has been the cause of significant outbreaks over the last few years. During the past few decades there has been a significant rise in virus transmission

throughout the world resulting in an expansion of geographical distribution. Transmission continues of viral serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) and is related to the process of urbanization together with a lack of effective control of vector (PAHO 2003, Chambers et al. 1997, Pinheiro 1997).

Through virus isolation it is possible to identify and characterize circulating molecular serotypes, and to have epidemiological surveillance in order to detect new serotypes. One of the risk factors for developing DHF is the concurrence of more than one circulating serotype. This study analysed 160 samples of serum from patients with suspected cases of dengue. The first stage of this work referred to viral isolation using C6/36 cells. All samples were submitted to viral RNA extraction followed by RT-PCR for viral identification. Of one hundred sixty samples analyzed, 78 were identified as DENV, using the primers FG1 and FG2 and characterized by Hemi-nested-PCR as serotype 3. The sensitivity and efficiency of this method is related to the high viremia presented by these patients. The amplicons of samples characterized as DENV-3 were sequenced and submitted to a search for similarity using the BLAST program. This software finds regions of local similarity between sequences. The program compares nucleotide sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. The result of concordance of similarity showed that sequences of characterized samples in the state of Rondonia showed a similarity of 98% when compared to the same region of genome of samples of DENV-3 from other regions of Brazil (Osatomi et al. 1990, Peyrefitte et al. 2003).

In accordance with data obtained in this study the results were reproduced using the RT-PCR and hemi-nested-PCR techniques in order to identify and characterize the serotype of DENV-3 circulating between 2004 and 2006 in the state of Rondonia. These techniques show the importance of this method when it is used in the quick detection of new viral strains. The state of Rondônia is a favorable region for the propagation of DENV, as well as other arboviruses, due to its geographical location, being served by road to the state of Acre and other regions of Brazil as well as forming a frontier with Bolivia for an overall distance 1,342 km, together with rivers making possible the entrance of new serotypes via the frontier, as was seen in 1982 with the entrance of DENV-1 and DENV-4 serotypes over the Venezuela/Roraima state frontier (Osanai 1986). The use of these techniques contributes to the monitoring of circulating dengue virus serotypes, and to adapting preventative measures in the dissemination of these new serotypes in the state of Rondonia, as well as the development of future control strategies.

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