

**GENOTYPING OF DENGUE VIRUS 2 USING RESTRICTION SITE-SPECIFIC
(RSS)-PCR**

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ABSTRACT

Dengue fever is considered the most important and widespread reemerging infectious disease in developing countries. Dengue virus belongs to the family *Flaviviridae*, a group of positive sense RNA viruses of 11 kb that are divided into four antigenically distinct serotypes (DENV-1 - DENV-4). Nowadays prevention is accomplished by using epidemiologic surveillance and vector control. Strain typing is a powerful tool for determining the geographic distribution of strains and understanding the epidemiology of infectious diseases. Strain typing can provide information on the relationship between disease manifestations and severity of disease. Harris et al. (*Virology* 253: 86-95, 1990), developed the RSS-PCR in order to genotype samples of *Dengue virus*, a rapid molecular strain-typing method that can be applied to analyze a large number of strains. The subtyping may identify viral factors that contribute to disease severity. This study was developed in order to genotype samples circulating in Minas Gerais state. Among the 34 clinical samples tested, 3 were DENV-1, 14 were DENV-2 and 17 were DENV-3. The samples were isolated using C6/36 cells. After RNA extraction using the silica method, RSS-PCR was executed. Different samples were used (1992-2005), including cases of DF (dengue fever) and DHF (dengue hemorrhagic fever). The results suggest genetic variability among the samples. The patterns seem to be related to the period of isolation, whether recent or from retrospective samples, as differences in patterns linked to severity of disease were not seen. The older sample (1992) seemed to have the same pattern as that ascribed by Harris et al, (1990) to the genotype "American". The other samples showed patterns not related to any other previously described genotype.

INTRODUCTION

Dengue is a viral disease transmitted by mosquitoes and caused by four viral serotypes (Dengue virus serotypes 1-4 [DENV 1-4]) belonging to the genus *Flavivirus* of the *Flaviviridae* family. Dengue viruses consist of a single-stranded positive-sense RNA genome of approximately 10,700 bases in length, surrounded by a nucleocapsid and a lipid envelope (Santos et al. 2002). In terms of illness and death, dengue is the most important viral disease transmitted to humans by mosquitoes (Diallo et al. 2003). At the beginning of the 21st century it is estimated that between 50 and 100 million cases of DF (dengue fever) and several hundred thousand cases of DHF (dengue hemorrhagic fever) occur each year, depending on epidemic activity (Gubler 2002). In Latin America, there has been an alarming increase in dengue transmission particularly in urban environments (Gubler & Trent 1994). Dengue has become one of the principal health problems of most Brazilian cities. It affects hundreds of people especially during the rainy season, when the density of the vector mosquito increases, and epidemics occur periodically affecting thousands of people. Nowadays there is co-circulation of DENV-1, 2 and 3 in Brazil. Dengue virus infections with a single subtype produce a classical febrile illness, whereas consecutive infections by different serotypes can lead to the more serious illness called DHF, of which there are about 1 million cases each year with a mortality rate of around 5% (Johansson et al. 2001).

Seroepidemiological surveillance of dengue is performed mainly by detection of DENV-specific IgM antibodies, while the detection of circulating serotypes is done by viral isolation and identification techniques. Reverse transcriptase-polymerase chain reaction (RT-PCR) assays have been used for the identification of DENV serotypes in supernatants

of infected cells and in clinical specimens (Balmaseda et al. 1999). When applied to clinical isolates from endemic areas, strain typing is a powerful tool for determining the geographic distribution of strains. Strain typing can provide information about disease manifestations and biological characteristics of an organism. In disease such as DF and DHF/DSS, where no appropriate animal model exists, this population-based epidemiological approach for subtyping may identify viral factors that contribute to disease severity (Harris et al. 1999).

The partial sequencing of various regions of Dengue virus has been used to determine the genetic variation of Dengue viruses and to characterize genotypes within each serotype (Rico-Hesse 1990, Blok et al. 1991, Deubel et al. 1993, Lewis et al. 1993, Lanciotti et al. 1994, Chungue et al. 1995, Harris et al. 1999). Sequencing is still needed in order to use phylogenetic classification based on sequences of different fragments.

Non-sequencing methods to distinguish strains such as restriction analysis of labeled viral cDNA require radioactivity and large amounts of viral RNA (Vorndam et al. 1994). Typing by RFLP after a RT-PCR requires expensive restriction enzymes. The use of single-strand conformation polymorphism involves more elaborate electrophoresis and detection procedures (Farfan et al. 1997).

The restriction site-specific (RSS)-PCR technique is based on a single RT-PCR amplification using four primers that target regions spanning polymorphic restriction sites. The resulting products generate specific patterns of bands (Harris et al. 1999). Harris et al. (1999) selected a prototype strain (16681) and analyzed the restriction sites in the E recognition sequences.

The use of RSS-PCR offers a simple and rapid technique to identify the type of strain circulating in an epidemic area using materials normally used in a molecular biology laboratory.

MATERIAL AND METHODS

Samples and viral isolation.

The reference strains of dengue virus used in this study were DENV-1 (DEN1BRASIL/98), DENV-2 (DEN2/SPH) and DENV-3 (DEN-3 from the Evandro Chagas Institute, Belém, Brazil). Thirty-four samples were collected from patients suspected to be infected with dengue virus and stored at -70°C until use. Reference samples and clinical samples were propagated in C6/36 cells grown in L-15 medium containing 50mg/ml of fungizone, 200 mg/ml of gentamicin, 200U/ml of penicillin and 8% of fetal bovine serum (Invitrogen). After seven days at 28°C , the cells and supernatant were harvested and stored at -70°C until need.

RNA extraction.

RNA from serum was extracted using the “Trizol LS Reagent” (Invitrogen) method. RNA from isolates and the reference strains was extracted by a modified silica method according to Boom et al. (1990). Briefly, the material collected was treated with a lysis buffer containing guanidine isothiocyanate, Tris 0.1 M, EDTA 0.2 M, Triton X-100 mixed with sterilized silica. After centrifugation the pellet was washed with a buffer containing guanidine isothiocyanate followed by several washes with 70% ethanol and acetone. The material was re-suspended in TE (Tris 10mM, EDTA 1mM, pH 8.0) treated with RNAsin.

After centrifugation the RNA was collected in the upper phase and stored at -70°C until needed.

cDNA synthesis.

The cDNA synthesis was conducted in a 20 µl reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2,5 mM MgCl₂, 10 mM dithiothreitol, 1 mM each of the four dNTPs, 50 pmol of the anti-sense primer, 250 ng of viral RNA in 5µl volume and 200 U of reverse transcriptase enzyme (SUPERScript II RT, Invitrogen) at 42°C for 50 minutes. The enzyme was inactivated at 70°C for 15 minutes.

SEMI-NESTED PCR.

(i) Primers – Two pairs of primers were used in a semi-nested PCR assay according to Lanciotti et al. (1992) in order to detect the serotype of clinical samples. (ii) PCR assay – The PCR assay using 5µl of the cDNA was conducted in a reaction mixture of 50µl containing 10 mM Tris-HCl (pH 8.8), 80 mM KCl, 2mM MgCl₂, 0.01% gelatin, 80 mM of each of the four dNTPs, 50 pmol of each primers and 2 units of *Taq* DNA polymerase (Invitrogen). The thermal cycle used was that recommended by Lanciotti et al. 1992 with modifications. namely 94°C-5 min and then 94°C-1 min, 58°C-1 min, 72°C-1 min for 30 cycles followed by a final extension of 72°C-10 min. (iv) Semi-nested PCR – The semi-nested PCR was assayed using the same conditions as the first PCR assay. About 1 µl of the first PCR was used as a template for the semi-nested PCR. The thermal cycle used was 94°C-5 min and then 94°C-1 min, 60 °C-1 min, 72°C-1 min for 30 cycles followed by a final extension of 72°C-10 min. (v) Detection of amplified DNA – Amplified DNA was detected by electrophoresis of 10µl of the semi-nested PCR on a silver stained 8% polyacrylamide gel (PAGE).

RSS-PCR.

(i) Samples – positive isolates amplified as DENV-2 and reference samples were used in this assay. (ii) Primers – Four primers were used in a RSS-PCR assay according to Harris et al. (1999) in order to detect the type of DENV-2 samples (Table 1). (iii) RT-PCR assay – “One-step kit” (Eppendorf) was used; 7µl of the RNA was used in a reaction of 20µl containing 25 pmol of each primer. The thermal cycle used was that recommended by Lanciotti et al. (1992) with modifications. The thermal cycle used was 95°C-5 min and then 94°C-1 min, 58 °C-1 min, 68°C-1 min, for 30 cycles and 68°C-10 min. (iv) Detection of amplified DNA – amplified DNA was detected by electrophoresis of a 10µl of the RSS-PCR reaction on a silver stained 8% polyacrylamide gel (PAGE).

The reproducibility of the RSS-PCR was tested amplifying each sample more than once and comparing the patterns produced. The reference samples DENV-1 and DENV-3 were used to assure that the primers were specific for DENV-2 RSS-PCR.

Table 1. Primers used in the RSS-PCR technique.

Primer	Sequence	Genome position- strain 16681 (Blok et al. 1989)	Strand
RSS1	5'-GGA TCC CAA GAA GGG GCC AT	1696-1715	+
RSS2	5'-GGC AGC TCC ATA GAT TGC T	2277-2259	-
RSS3	5'-GGT GTT GCT GAT GGA A	1524-1542	+
RSS4	5'-GTG TCA CAG ACA GTG AGG T	2371-2353	-

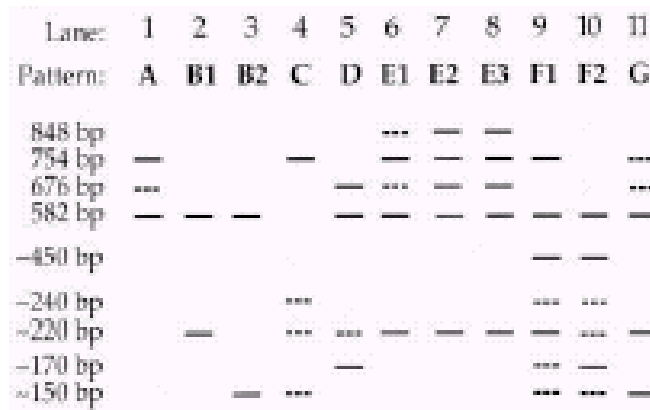
RESULTS

Among the 34 clinical samples tested, 3 were DENV-1, 14 were DENV-2 and 17 were DENV-3 (Table 2), results obtained using the nested-PCR procedure. Three samples characterized as DENV-2 could not be isolated using C6/36 cells and were not included in the RSS-PCR study.

Table 2. Information regarding the 14 DENV-2 clinical samples used in this study.

Sample No.	Year	Clinical case	Isolation
409	2004	FHD	Positive
427	2005	FHD	Positive
80	2003	FD	Positive
25	2003	FD	Positive
152	1999	FHD	Positive
86	2003	FD	Positive
15	2003	FD	Positive
318	2004	FD	Positive
154	2002	FD	Positive
167	2001	FD	Positive
144	2003	FD	Positive
12737	2002	FD	Negative
164	2003	FD	Negative
125	2001	FD	Negative

The pattern of bands generated for each strain was assigned an RSS-PCR designation (A-G). The patterns of RSS-PCR for DENV-2 can be consulted in the Figure 1.



Harris et al. 1999.

Figure 1. RSS-PCR patterns of DENV-2 strains established by Harris et al. (1999). The dotted line designates bands that display sample-to-sample variation. Lane (1) Jamaica genotype; Lanes (2) and (3) samples from Thailand; Lane (4) samples from Trinidad; Lane (5) samples from Philippines; Lanes (6), (7), and (8) samples from Indonesia, Sri Lanka and Burkina Faso; Lanes (9) and (10) American genotype; Lane (11) samples from Africa.

The genotype “American-Asian” of DENV-2 was observed only in the reference samples isolated in 1992. In this case, amplifications of 754bp, 582pb and 180bp were observed after electrophoresis in a polyacrylamide gel. The band of 180bp was described by Balmaseda et al. (1999) as an additional band in the pattern of this genotype, observed in samples studied in the epidemic period of Nicaragua in 1998.

The samples collected after this point have a polymorphism characterized by a differentiate pattern of amplification. The amplifications were 180bp, 220bp, 510bp and 850bp (Figure 2). This type of amplification was observed in the majority of the samples in

this study. The material isolated in 2005 showed the most different pattern, not presenting the amplifications of 180bp and 220bp.

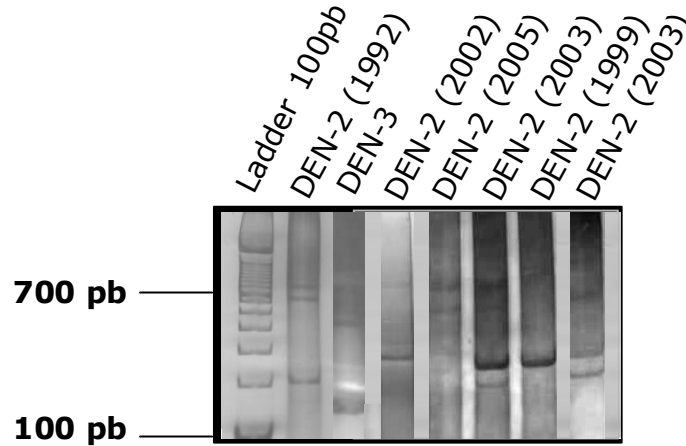


Figure 2. RSS-PCR results. Lane (1) 100 bp ladder (Invitrogen); Lane (2) reference sample of DENV-2; Lane (3) reference of DENV-3; Lane (4), (5), (6), (7) and (8) material amplified from clinical samples 154 (2002), 427 (2005), 25 (2003), 152 (1999) and 80 (2003), respectively

The RSS-PCR patterns of sample showed no correlation whether the samples caused FD or FHD, not showing a significant virulence marker reflected in any alteration regarding the pattern. Besides, there was amplification of the reference sample of DENV-3 using the primers designed for DENV-2 RSS-PCR, although the reference sample of DENV-1 was not amplified using the same primers mentioned above (data not shown). The DENV-3 reference sample presented amplification of 120bp and 420bp.

DISCUSSION

Genetic variability occurs with DENV and could be demonstrated with the amplification of the reference pattern of RSS-PCR for DENV-2 using the primers described by Harris et al. (1999). Only one sample after amplification showed the reference pattern of DENV-2 RSS-PCR, being a sample isolated in 1992. The samples used by Harris et al. (1999) were from the American region isolated between 1969 and 1986. The samples isolated in the decade of the 90's seem to show the pattern described by Harris et al. (1999).

In fact, DENV have a RNA genome more susceptible to mutation than DNA viruses. Holmes & Burch (2000) affirmed that DENV is highly mutable, since the RNA-dependent RNA polymerase produces approximately one error in each genome replication. Moreover these viruses present a very high replication rate.

Apart from mutation, recombination between different samples could occur, being another cause of genetic diversity among DENV. During replication, if the genomes of two viruses of the same serotype infected the same cell, at the end of the process they could form a hybrid molecule (Holmes & Burch 2000).

The region of the genome amplified was the protein E. The E gene has the highest mutation rate among the *Flaviviruses*, mainly because it interacts directly with the immune system of the host, being under high selective pressure (Chen et al. 2003).

The genetic modifications of DENV could contribute in part to the fact that primers designed for DENV-2 amplify the reference sample of DENV-3. According to the phylogenetic tree, DENV-2 is located nearer DENV-3, than DENV-1 (Holmes & Twiddy 2003). Thus, DENV-1 could not be amplified using the primers designed to amplify

DENV-2 in RSS-PCR. In order to confirm the results obtained using the RSS-PCR procedure, sequencing of samples should be done.

We can conclude that the RSS-PCR technique using the primers designed by Harris et al. (1999) is useful to genetically monitor DENV-2 samples isolated in the 90's or before. Furthermore, the results shown here indicate a higher genetic variation occurring among DENV-2 subtypes along time as these samples showed a different pattern of amplification.

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