Rev. Inst. Med. trop. São Paulo
39 (2): 79-83, março/abril, 1997

DETECTION AND IDENTIFICATION OF DENGUE VIRUS ISOLATES FROM BRAZIL BY A SIMPLIFIED REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION (RT-PCR) METHOD

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SUMMARY

We show here a simplified RT-PCR for identification of dengue virus types 1 and 2. Five dengue virus strains, isolated from Brazilian patients, and yellow fever vaccine 17DD as a negative control, were used in this study. C6/36 cells were infected and supernatants were collected after 7 days. The RT-PCR, done in a single reaction vessel, was carried out following a 1/10 dilution of virus in distilled water or in a detergent mixture containing Nonidet P40. The 50 µl assay reaction mixture included 50 pmol of specific primers amplifying a 482 base pair sequence for dengue type 1 and 210 base pair sequence for dengue type 2. In other assays, we used dengue virus consensus primers having maximum sequence similarity to the four serotypes, amplifying a 511 base pair sequence. The reaction mixture also contained 0.1 mM of the four deoxynucleoside triphosphates, 7.5 U of reverse transcriptase, 1U of thermostable Taq DNA polymerase. The mixture was incubated for 5 minutes at 37°C for reverse transcription followed by 30 cycles of two-step PCR amplification (92°C for 60 seconds, 53°C for 60 seconds) with slow temperature increment. The PCR products were subjected to 1.7% agarose gel electrophoresis and visualized by UV light after staining with ethidium bromide solution. Low virus titer around $10^{3.6}$ $TCID_{50}$/ml was detected by RT-PCR for dengue type 1. Specific DNA amplification was observed with all the Brazilian dengue strains by using dengue virus consensus primers. As compared to other RT-PCRs, this assay is less laborious, done in a shorter time, and has reduced risk of contamination.

KEYWORDS: Dengue detection; Dengue diagnosis; RT-PCR.

INTRODUCTION

Since the mid-1980s, epidemics of dengue virus types 1 and 2 have occurred in all of Brazil's geographic area, causing millions of infections [6]. While the vast majority of the cases were not life-threatening, hundreds of cases of dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) occurred in the States Rio de Janeiro and Ceara and smaller numbers in other States, causing many deaths [6, 17, 21, 22, 25]. Presently, dengue types 1 and 2 are endemic and the Brazilian Ministry of Health reports 125000 dengue cases during 1995 and 107000 in the first semester of 1996.

The four dengue virus serotypes are single-stranded positive sense, RNA viruses belonging to the genus *Flavivirus*, family *Flaviviridae*[24], transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes[7].

The virologic diagnosis of dengue is classically done by virus isolation followed by identification with type-specific monoclonal antibodies in a slide immunofluorescence test using infected mosquito cells[9]. Dengue virus RNA transcription to a complementary DNA followed by PCR DNA amplification has been used for *flavivirus* and dengue detection and diagnosis [1, 10, 11, 12, 13, 14, 15, 20]. We show here the application of a simplified method of RT-PCR for detection and identification of dengue virus isolates from Brazil.

MATERIALS AND METHODS

Virus strains

Five dengue virus isolates from Brazilian patients were used in this study (Table 1). Dengue type 1 RioH 28973 was supplied by Dr. Hermann Schatzmayr (Oswaldo Cruz Institute, Rio de Janeiro), dengue type 2 TOC 213 and dengue type 2 CEA 2462

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were supplied by Dr. Amélia Travassos da Rosa (Evandro Chagas Institute, Belém), and dengue type 2 SPH 125367 was supplied by Dr. Luiza Teresinha Madia de Souza (Adolpho Lutz Institute, São Paulo). The yellow fever vaccine virus 17 DD (Fiocruz, Brazil), was used as a negative control.

The viruses were grown in C6/36 *Aedes albopictus* cells as previously described[4] and tissue culture fluids were collected for RT-PCR after 7 days of infection. Confirmation of cell infection was done by indirect immunofluorescent test [4]. The infected tissue culture fluids were stored at -70°C until use.

Serial ten-fold dilutions of culture supernatants of Dengue type 1 RibH830 virus strain were inoculated in ninety six-well microplates containing C6/36 cells. After 7 days the infected cells were processed as previously described[5]. Briefly, the cells were fixed using formalin and an indirect enzyme immunoassay was performed. The 50% tissue culture infecting dose, $TCID_{50}$ was calculated by the method of REED & MUENCH [18].

TABLE 1
Dengue virus isolates

Isolates	Place of patient infection	Year
DEN 1 RibH830	Ribeirão Preto, SP (FIGUEIREDO et al., 1992)	1991
DEN 1 RioH28973	Rio de Janeiro, RJ (SCHATZMAYR et al., 1986)	1986
DEN 2 SPH 125367	Rio de Janeiro, RJ (NOGUEIRA et al., 1990)	1991
DEN 2 TOC 213	Araguaina, TO (VASCONCELOS et al., 1993)	1991
DEN 2 CEA 2462	Fortaleza, CE (VASCONCELOS et al., 1995)	1994

Primers

Dengue virus consensus primers DEN-S and DEN-C define a 511 base pair sequence located at the beginning of 5' - nontranslated region of the genome which also includes the capsid gene. DEN1-C is a dengue type 1 specific primer. DEN-S and DEN1-c define a 482 base pair sequence located in approximately the same region defined by DEN-S and DEN-C [14]. The dengue type 2 specific primers, DEN2-S and DEN2-C, define a 210 base pair sequence of the envelope protein gene [3,12]. The primers were synthesized by GIBCO BRL (USA). Primer sequences and position in the virus genome are showed in Table 2.

RT-PCR

The test was done in 1µl of tissue culture fluid serial dilutions in phosphate buffered saline containing 0.5% bovine albumin. Comparison was made between 5µl of a detergent mixture including 2% Nonidet P-40 and 5µl of distilled, deionized water, both containing 5 U of RNase inhibitor (Pharmacia - USA). The tissue culture fluids were incubated 1, 3, 5 and 10 minutes at room temperature with either water or the detergent mixture. A reaction mixture containig 50 pmol of each primer (dengue type 1 and type 2 specific primers were used simultaneously), 100 µM of the four deoxynucleoside triphosphates, 7.5 U of Moloney murine leukemia virus reverse transcriptase (Pharmacia, USA). 1U of thermostable Taq DNA polymerase (Pharmacia, USA), 5 µl of a buffer solution containing 5 mM Tris (pH 9.0), 750 µM $MgCl_2$, 25 mM KC1. The volume was brought up to 50 µl by adding distilled, deionized water. The reaction mixture covered by 2 drops of oil was incubated for 15 minutes at 37°C for reverse transcription followed by 30 cycles of a two step PCR amplification (92° C for 60 seconds, 53° C for 60 seconds) by using a thermal cycler (Techne, UK). The temperature increment was slow, taking 120 seconds from 53° C to 92° C, and 15 seconds between 70°C and 75°C. The total time for the test was 150 minutes.

TABLE 2
Nucleotide sequences and genome position of dengue primers.

Code *	Sequence	Position
DEN-S	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	106-134
DEN-C	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC	616-644
DEN1-C	CGT CTC AGT GAT CCG GGG G	569-588
DEN2-S	GTT CCT CTG CAA ACA CTC CA	1203-1222
DEN2-C	GTG TTA TTT TGA TTT CCT TG	1432-1413

* S-sense primer, C-complementary primer.

Eight microliters of each PCR product were subjected to 1.7% agarose gel electrophoresis and the amplified DNA was visualized by UV light after ethidium bromide staining. The size of the amplified DNA fragments was determined by comparison with a 100 base pair molecular weight marker (Pharmacia, USA).

All the virus samples were tested at least three times with the three primer pairs in order to evaluate the reproducibility of the RT-PCR.

RESULTS

The amplification of dengue virus isolates after water and 2% nonidet P-40 detergent incubation at different times was comparable as estimated by visual inspection of the agarose gel shown in Figure 1. Consequently, for practical reasons, 3 to 5 minutes water incubation was used thereafter.

The sensitivity of RT-PCR as estimated by visual inspection of agarose gels was evaluated with a $10^{4,6}$ $TCID_{50}$/ml dengue type 1 RibH830 seed. It was possible to detect the virus genome

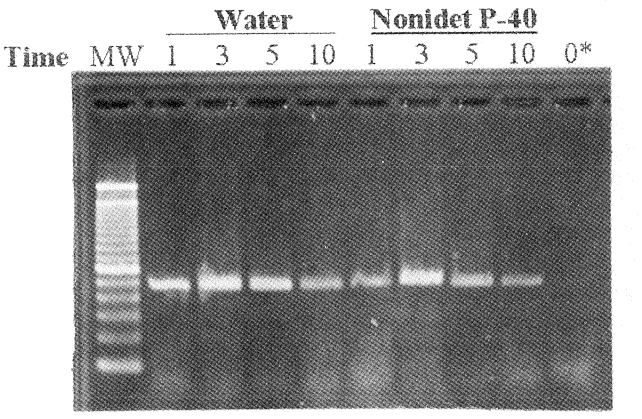

Fig. 1 - Agarose gel stained with ethidium bromide, showing 482 DNA base pair bands obtained from dengue type 1 RioH 28973 incubated at different times in water or a detergent mixture includind nonidet P40. Time in minutes * 0- Water dilution immediately followed by RT-PCR processing.

at 1/10 dilution with dengue virus consensus primers (511 base pair band) and dengue type 1 specific primers (482 base pair band) as shown in Figure 2. A smaller than 100 base pair band, probably related to a primer-dimer formation, was also seen in two gel tracks in Figure 2.

All the 5 dengue virus isolates were detected by RT-PCR using the dengue virus consensus primer pair (Figure 3). RT-PCR of dengue type 1 strains RibH830, RioH28973, and dengue type 2 strains SPH125367, TOC213, and CEA2462 by using the specific dengue type 1 and type 2 primer pairs produced amplicons, as shown in Figure 4. DNA amplification was not observed with the negative control (Figures 3 and 4).

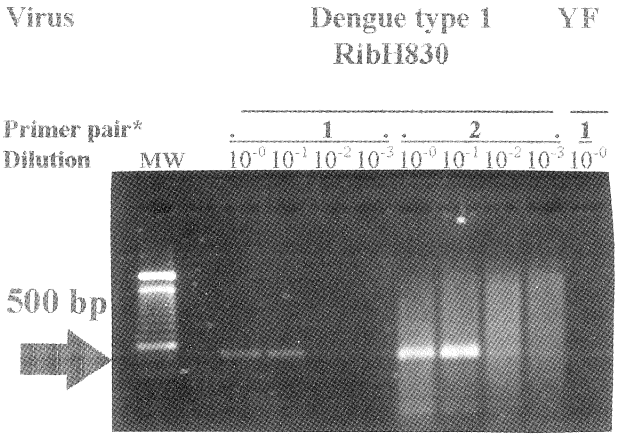

Fig. 2 - Agarose gel stained with ethidium bromide, showing 511 DNA base pair bands obtained from a tenfold dilution titration of dengue type 1 RIBH830 amplified by RT-PCR using dengue consensus primers and 482 DNA base pair bands obtained with dengue type 1 specific primers. The RT-PCR of yellow fever (YF) virus using dengue consensus primers was used as a negative control.
* Primer pairs: 1 = DEN-S / DEN-C; 2 = DEN-S / DEN1-C. MW - Molecular weight marker.

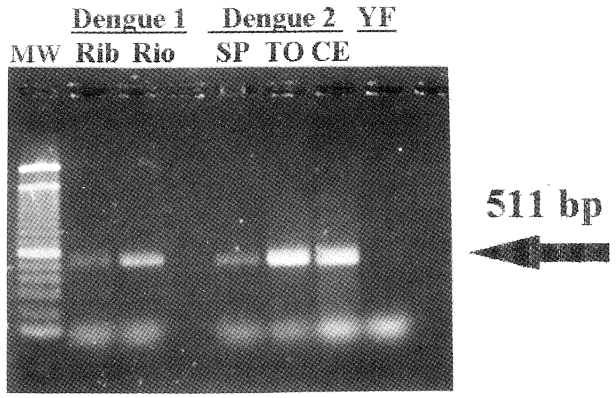

Fig. 3: Agarose gel stained with ethidium bromide, showing 511 DNA base pair bands obtained from the 5 Brazilian dengue isolates amplified by RT-PCR using dengue consensus primers. The RT-PCR of yellow fever (YF) virus using dengue consensus primers was used as a negative control.
MW - Molecular weight marker; Dengue type 1 **Rib**H 830; **Rio**H 28973; Dengue type 2 **SP**H 125367; **TOC** 213, and **CEA** 2462; **YF** = Yellow fever.

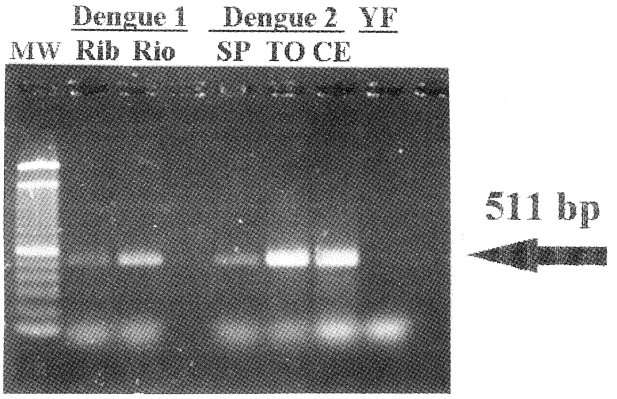

Fig. 4: Agarose gel stained with ethidium bromide, showing 511 DNA base pair bands obtained from the 5 Brazilian dengue isolates amplified by RT-PCR using dengue type 1 and dengue type 2 specific primers. The RT-PCR of yellow fever virus using the 2 primer pairs was used as a negative control.
MW - Molecular weight marker; Dengue type 1 **Rib**H 830; **Rio**H 28973; Dengue type 2 **SP**H 125367; **TOC** 213, and **CEA** 2462; **YF** = Yellow fever.

DISCUSSION

The data shown herein confirm previously published results obtained in different countries with other dengue virus isolates utilizing the same primers[12, 14, 15, 20]. The fact that the amplicons obtained were of the expected sizes, and the absence of DNA amplification of yellow fever virus with all primers strongly suggest that they are dengue-specific. However, a definite proof of this specificity could only be obtained by sequencing the PCR products or analysing the amplicons by digestion with restriction enzymes or hybridization with dengue virus specific probes.

The primer combinations used in this study are suitable because they permit the identification of dengue types presently circulating in Brazil. Moreover, the use of dengue consensus primers offers a possibility of detection of new dengue types eventually introduced in the area.

The 5 dengue viruses whose cell infection was confirmed by immunofluorescence were amplified by RT-PCR showing a clear equivalence of both techniques.

The RT-PCR showed high sensitivity, since dengue type 1 virus, at titer as low as $10^{3.6}TCID_{50}$/ml, was detected with both dengue type 1 specific and dengue consensus primers in tissue culture fluid. Virus titers in the blood during the first 6 days of dengue infection are commonly higher than this, indicating the potential clinical usefulness of this assay [7].

Water dilutions of dengue virus produced amplicons with adequate reproducibility and generated bands equal to those obtained with a detergent mixture including Nonidet P-40. We suppose that disruption of cells probably caused by freeze-thaw cycle, changes in pH or tonicity, liberated viral RNA to the medium, which constituted target for amplification in this RT-PCR [24]. Therefore, water used as diluent could have reduced RNAse, reverse transcriptase, DNA polymerase inhibitors from the C6/36 cells, or reduce RNA excess present in straight samples inhibiting reverse transcriptase.

Two-step PCR cycles have been reported to generate as much DNA as three-step cycles, specially for short fragments [2]. A suitable extension of DNA in two-step PCR is done by the high processivity of taqDNA polymerase, which occurs during the temperature transition from 53°C to 92°C. The optimal taq DNA polymerase action range temperature of 70 to 75°C took 15 seconds in the extension time of our RT-PCR [2].

Compared to other reported assays [10, 11, 14, 15, 20] our RT-PCR is less laborious, has a shorter turn around time, utilizes a single reaction vessel, thus reducing the risk of contamination and utilizes lower amounts of reagents, which represents an economy that may be significant for Third World countries.

In summary this simplified method for dengue virus RT-PCR is suitable for confirming and typing isolates from patients or mosquitoes, in tissue culture fluids. We intend to extend the use of this technique to the detection of dengue virus genome in clinical specimens.

ACKNOWLEDGEMENTS

We thank Dr. Eurico Arruda for reviewing the manuscript. This research was supported by Japan International Cooperation Agency, and Brazilian Government Research Agencies (CNPq and CAPES).

RESUMO

Detecção e identificação de vírus do dengue brasileiros utilizando uma reação em cadeia da polimerase simplificada, precedida de transcrição reversa (RT-PCR)

Apresentamos neste trabalho uma técnica simplificada de RT-PCR para identificação de vírus de dengue. Cinco estirpes brasileiras de vírus de dengue dos tipos 1 e 2, isoladas de pacientes e o vírus da vacina de febre amarela como controle negativo, foram utilizadas nos testes. Células C6/36 foram infectadas e os fluidos de cultura coletados após 7 dias. A RT-PCR, era efetuada em um único tubo contendo 1µl de fluidos infectados e diluídos 1/10 em água destilada ou em mistura detergente contendo Nonidet P40. A mistura de reação, num volume de 50 µl, continha 50 pmol de primers especificos, que amplificavam seqüência com 482 pares de bases para vírus do dengue tipo 1 e 210 pares de bases para dengue tipo 2, ou, ainda, primers para grupo dengue que amplificavam seqüência com 511 pares de bases. A mistura de reação, também, continha 0.1 mM dos 4 desoxinucleotídeos, 7.5 U de transcriptase reversa e 1 U de Taq DNA polimerase termoestável. A mistura era incubada por 5 minutos a 37° C para a transcrição reversa e processada em 30 ciclos de um PCR com 2 passos (92° C por 60 segundos, 53° C por 60 segundos) e incremento lento da temperatura. Produtos do PCR foram visualizados à luz ultra-violeta após eletroforese em gel de agarose e coloração em brometo de etídio. Baixos títulos virais como $10^{3.6}TCID_{50}$/ml foram detectados com o RT-PCR para dengue tipo 1. Amplificação genômica tipo-específica e grupo-específica para dengue foi obtida com todos os vírus do dengue estudados. Este RT-PCR, comparado a testes similares, é menos trabalhoso, mais rápido e possui reduzido risco de contaminação.

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Recebido para publicação em 05/12/1996
Aceito para publicação em 28/02/1997