

## Dengue virus type 2 unresponsive to the current PCR primer; construction of a new PCR primer to detect all strains of Dengue virus type 2.

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**Abstract:** We found that one strain of dengue virus (Trinidad 1751; TR) did not respond to the PCR primer for Jamaica/83. We investigated such property with other 10 strains of dengue virus type 2 and found 2 more unresponsive strains. All 3 strains were isolated from the central America. To detect the envelope gene of those 3 strains by PCR, we synthesized primers based on TR strain as the reference sequence. Using these primers, we could detect the 3 strains by PCR at the usual annealing temperature. We recommended the new primer for diagnosis of DEN 2.

*Key words:* PCR unresponsiveness, Dengue virus 2, Primer construction, RNA sequencing,

### INTRODUCTION

Dengue fever is the most important vector-borne viral disease of man, occurring in tropical latitudes where large epidemics occur involving up to several million people (Institute of Medicine, 1986). At present, there are no prophylactic and therapeutic strategies with which to combat dengue virus infection and the frequency of epidemics has not decreased. Rather, the number of patients has increased over the last 10 or so years in South-East Asia (Halstead, 1992), Africa (Halstead, 1993) and central America (Gubler and Trent 1994).

The disease is not native to Japan, but it is occasionally imported by travellers who had visited tropical countries and became infected with the virus. Patient sera are occasionally brought to our institute for diagnosis. The hemagglutination-inhibition (HI) test has been used for this purpose (Clarke and Casals, 1958) but PCR has recently been applied as a speedy diagnostic method (Deubel *et al.*, 1990; Eldadah *et al.*, 1991; Henchal *et al.*, 1991; Lancioti *et al.*, 1992; Morita *et al.*, 1991). Recently we found that a strain of dengue virus type 2 (DEN 2) was non-reactive to the current PCR primer based on the sequence of Jamaica/83. In this study, we reevaluated this primer using several strains of DEN 2, and then constructed a new primer with which we could detect this viral gene in question.

## MATERIALS AND METHODS

Virus: Eleven strains of DEN 2 were studied. Trinidad/1751 (TR), Thai (Th-CMP682) and Thai (Th-CMP982) were passaged in our laboratory through mouse brain. These viruses were passaged once in mosquito cell, C6/36 before use. New Guinea-C (NG-C), Thai (Th-NHP1193), Thai (Th-NH5293) and Vietnam (VNHH30591) were kindly supplied by Drs. A. Igarashi and K. Morita at the Institute of Tropical Medicine of Nagasaki University. These viruses had been passaged in C6/36. Jamaica/1983, Mexico/1983, Puerto Rico/1986 and Honduras/1984 strains grown in C6/36 were kindly supplied by Drs. D. Gubler and N. Karabatsos of the CDC. Dengue virus, DEN1 (Hawaii), DEN 3 (H87) and DEN 4 (H241) passaged in mouse brain were further passaged in C6/36 before use.

Synthesis of the PCR primer: Primers were synthesized with reference to the nucleotide sequence of the Jamaica/83 envelope gene (Morita *et al.*, 1991). As shown in Fig. 3, positions (267–286) and (477–496) were used as sense and reverse primers, respectively. A potential primer was identified in the flanking region of these primers based upon the nucleotide sequence of TR strain. The selected positions are shown in Fig. 3.

Oligonucleotide primers for both positions were constructed using betacyanoethylamide and an automated DNA synthesizer, model A391, (Applied Biosystems, California, USA) according to the manufacturer's instructions. Synthesized oligomers were cleaved with ammonium hydroxide and deprotected at 55°C for 14 hr. The ammonium hydroxide was evaporated using a vacuum-centrifuge. The oligomers were dissolved in Tris-EDTA buffer (pH 7.0) to an appropriate concentration for PCR reaction.

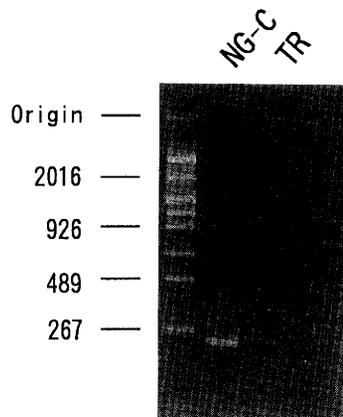
Nucleotide sequencing: Nucleotides were sequenced by a fully automated sequencer, model 370A (Applied Biosystems, California, USA). Primers for cyclesequencing were set at the region of 179–198 and 422–441. RT-PCR products of the region were used as templates, then the dye-terminator cycle sequence carried out using a kit (Applied Biosystems, California, USA) according to the supplied manual. The nucleotide sequences of 11 strains were submitted to the DSDB, DDBJ, EMBL and NCBI nucleotide sequence data bases, and accession numbers D45387 to D45396 and D45851.

PCR Procedure: Modified RT-PCR was performed according to Morita *et al.* (1991). Briefly, 10 $\mu$ l of virus lysate in phosphate-buffered saline with 0.5% NP-40 and 10U of RNase inhibitor (Takara Co. Kyoto, Japan) was added to 90 $\mu$ l of RT-PCR cocktail [100 pmole of each primer, 0.2mM deoxynucleoside triphosphates, 10mM Tris, 1.5 mM MgCl<sub>2</sub>, 80 mM KCl, 10U of AMV reverse transcriptase (Life Science Inc. Florida, USA) and 2U of DNA polymerase for PCR (Toyobo Co., Osaka, Japan)]. After incubating the reaction mixture for 10 min at 53°C, it was amplified by PCR (92°C for 60 s, 53°C for 60 s, and 72°C for 60 s) in a thermal sequencer (Iwaki glass Co., Tokyo, Japan). The PCR product resolved by agarose gelelectrophoresis was visualized by ethidium bromide staining.

## RESULT

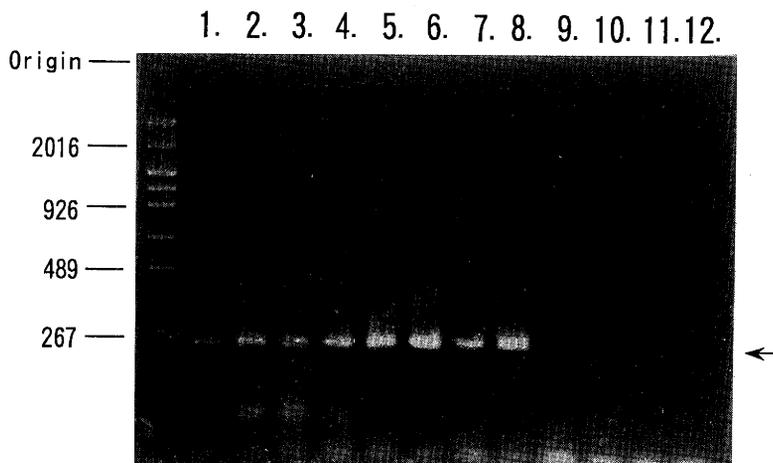
Figure 1 shows that the dengue virus type 2 (DEN 2) Trinidad 1751 (TR), did not react with the current primer. This was not due to the modified PCR method used in this study because it worked with the DEN 2 strain, prototype NG-C.

To clarify whether or not the failure of PCR was specific to the TR strain, 11 strains including TR were tested with this primer in PCR. As shown in Fig 2, Mexico/84 and Hon-



**Fig. 1.** TR strain is not recognized by Jamaica/83 primer.

PCR of NG-C and TR were performed as described in Materials and Methods and the products resolved by agarose gel-electrophoresis were stained by ethidium bromide.



**Fig. 2.** Screening of TR like strains after PCR with Jamaica/83 primer.

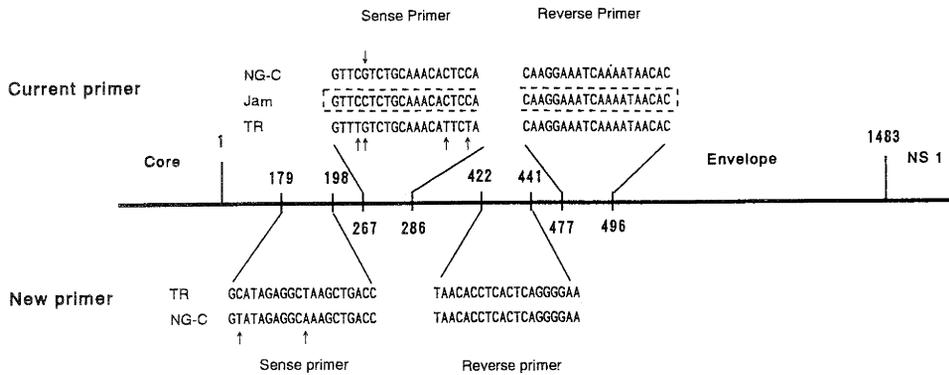
Eleven strains of DEN 2 were amplified by PCR using the Jamaica/83 primer. Arrows show the PCR products. Lanes were as follows:

- |                             |                          |                       |
|-----------------------------|--------------------------|-----------------------|
| 1. New Guinea-C (Prototype) | 2. Vietnam (VN HH 30591) | 3. Thai (Th NHP 1193) |
| 4. Thai (Th NH 5293)        | 5. Thai (Th CMP 982)     | 6. Thai (Th CMP 682)  |
| 7. Puerto Rico/86           | 8. Jamaica/83            | 9. Mexico/83          |
| 10. Honduras/84             | 11. Trinidad 1751        | 12. Control (Mock)    |

dulus/86 strains, were not recognized by the current primer.

The unresponsiveness of these strains to the primer was confirmed by the nucleotide sequence in GenBank. When the nucleotide sequence of TR was compared with that of Jamaica/83, of which the nucleotide sequence of E protein was used to synthesize the current PCR primer, the primer sequence region of TR differed by 4 of 20 bases as shown by arrow (Fig. 3). Twenty bases of the reverse primer completely coincided with the primer sequence from the reference strain (Fig. 3).

To expand this analysis, we determined the nucleotide sequences of 11 strains at the same 20 base-position for the sense primer. The results are summarized in Table I. It was



**Fig. 3.** Physical map of DEN 2 and the strategy for constructing the new primer.

Base sequence for the current primer was that of Jamaica/83 and that of the new primer was based TR. Arrows are different positions from the primer sequence. The sequence numbers of envelope show the range used as primers. Sequences were derived from the Gene Bank.

**Table 1.** Nucleotide sequences of the corresponding to the current primer in 11 strains of DEN 2.

Virus strain	Nucleotide sequence of E protein genome from 267 to 286
Jamaica/83*	GTTCC <u>T</u> CTGCAAACACTCCA
NewGuinea-C	GTT <u>C</u> GTCTGCAAACACTCCA
Vietnam(VN HH 30591)	GTT <u>C</u> GTCTGCAAACACTCCA
Thai(Th NH 5293)	GTT <u>C</u> GTCTGCAAACACTCCA
Thai(Th NHP 1193)	GTT <u>C</u> GTCTGCAAACACTCCA
Thai(Th CMP 982)	GTT <u>C</u> GTCTGCAAACACTCCA
Thai(Th CMP 682)	GTT <u>C</u> GTCTGCAAACACTCCA
Puerto Rico/86	GTTCC <u>T</u> CTGCAAACACTCCA
Mexico/83	GTTT <u>G</u> TCTGCAAAC <u>A</u> TTCCA
Hondulas/84	GTTT <u>G</u> T <u>T</u> TGCAAAC <u>A</u> TTCCA
Trinidad/1751	GTTT <u>G</u> TCTGCAAAC <u>A</u> TT <u>T</u> CTA

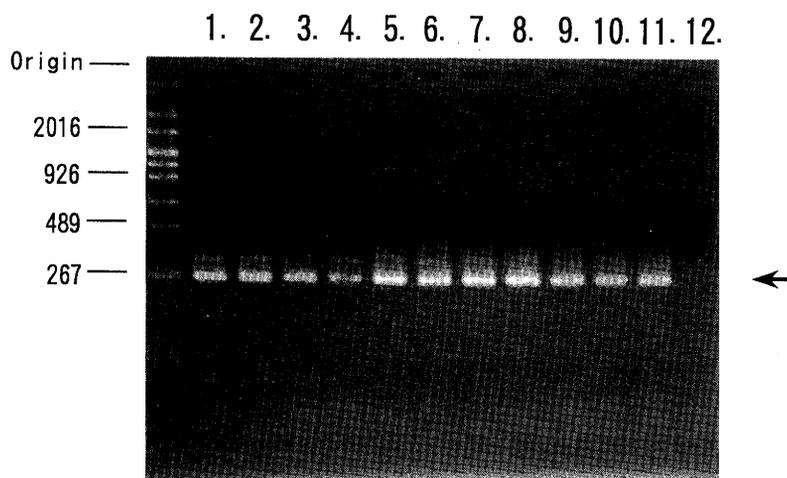
Nucleotides different from those of Jamaica/83 are underlined.

\* This sequence was used as the current primer.

confirmed again that TR differed at 4 positions from Jamaica/83. Honduras and Mexico differed by 4 and 3 bases from Jamaica/83 respectively. NG-C, Vietnam and four strains from Thai differed by only one base from those of Jamaica. Puerto Rico possessed completely the same sequence as Jamaica.

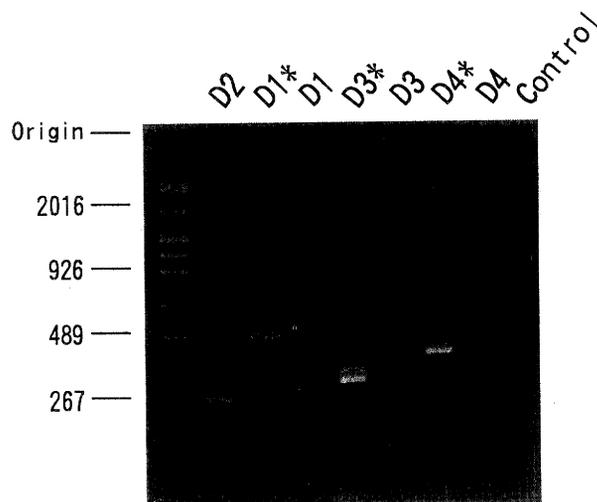
In order to construct new primer, we compared the TR sequence directly with that of NG-C which was the prototype strain. Regions that were sufficiently conserved and matched the criteria for PCR primers were selected. These are shown in Fig. 3 as sense and reverse primers. In the selected region for the sense primer (179–198), when the sequence of TR was compared with that of another strain (for example NG-C), 2 bases (180 and 189) differed. To verify the new primer as a tool to identify DEN 2, the reactivity of the new primer for TR was tested against 11 strains. The results are shown in Fig. 4. All strains reacted with the new primer to generate a band of 263 bases.

The specificity of the new primer was confirmed using other types of dengue virus. The primer did not anneal with the DEN-1, 3, and 4 strains (Fig. 5). Type specific PCR products in the figure were easily recognized by their different molecular weight.



**Fig. 4.** Identification of 11 strains of DEN 2 by PCR with the new primer. PCR and electrophoresis proceeded as described in Materials and Methods. Arrow means the PCR product of the new primer. Lanes were as follows:

1. New Guinea-C (Prototype)	2. Vietnam (VN HH 30591)	3. Thai (Th NHP 1193)
4. Thai (Th NH 5293)	5. Thai (Th CMP 982)	6. Thai (Th CMP 682)
7. Puerto Rico/86	8. Jamaica/83	9. Mexico/83
10. Honduras/84	11. Trinidad 1751	12. Control (Mock)



**Fig. 5.** Type specificity of the new primer. Lanes were as follows:  
 No mark: PCR with new primer  
 \*: PCR with the primer to each specific type

#### DISCUSSION

The TR strain has been passaged through the mouse brain in our laboratory for a long period. We initially considered that the failure of TR to anneal in RT-PCR with the current primer was due to a mutation during brain passage. This was indicated by sequencing the PCR product. Our TR virus varied less in the sequence of PCR product, having 97% homology against the reported TR (data not shown). In the region of the sense primer, the sequence was completely coincident with that reported (Fig. 3 and Table I). The sequence of the TR sense primer differed by 4 bases from that of Jamaica/83. This difference was estimated 10°C below at melting temperature in PCR. This fact suggested that the viral gene would not anneal to the current primer under the usual PCR conditions. Mexico and Honduras strains differed by 3 and 4 bases respectively in the sense primer from Jamaica. These differences affected the usual annealing temperature of the PCR so that TR was not amplified by PCR. On the other hand, only one base difference in NG-C, Vietnam and four Thai strains were not effective on the performance of PCR.

The characteristics that caused the PCR failure were not restricted to TR, since it was found in other 2 strains. Therefore, we considered that the region used for the sense primer is not always conserved in DEN 2 but is relatively variable. Hence, analysis of this region might support further classification of the 5 subtypes of DEN2 (Lewis *et al.*, 1993). These 3 strains of the TR-type were focused in the same geographic area of the central America, and might have the same evolutionary origin.

Dengue virus type 2 is the most well studied among the four types of DEN viruses in terms of the pathology of dengue hemorrhagic fever. The disease has not been clarified because of the complicated pathology involved in the pathogenicity of the virus strain and

the host defence mechanisms including specific and nonspecific immunity. The type 2 virus caused the highest clinical incidence of dengue shock syndrome in Thailand (Sangkhawibha *et al.*, 1984). We do not have any animal models of dengue virus infection. Analysis on the isolated virus and serum specimens collected from dengue patients is the only readily available approach to explore the pathogenesis of dengue haemorrhagic fever. Rapid and precise diagnosis is required to resolve this problem. The primer constructed here that detected all viruses tested may be useful for studies of dengue fever. We propose this primer instead of the current primer.

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