RAPID DETECTION AND IDENTIFICATION OF DENGUE VIRUSES BY POLYMERASE CHAIN REACTION (PCR)

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Abstract. A polymerase chain reaction (PCR) method using sets of newly designed primers for rapid detection and simultaneous identification of dengue virus serotypes was developed and tested. The test is based on two sets of primers specific within the envelope (E) and non-structural (NS1) regions of the dengue-virus genome. Two sets of universal primers that bind to two target sequences which are shared by all the four serotypes of the virus within the E and NS1 regions are used. The resulting products are further amplified by another pair of inner or nested universal primers, which also bind to another set of shared sequences within the E and NS1 regions, respectively. The nested PCR of both the E and NS1 regions can detect dengue virus of all the four serotypes at a sensitivity of 1 plaque forming unit (pfu) or less. For the identification of serotypes, a mixture of four pairs of serotype-specific primers, specific to the E region, was used. The primers have been designed to bind to serotype specific sequences within the regions flanked by the outer universal primers, and giving the amplified products of different sizes, each corresponds to one particular serotype (405 bp for Den1, 346 bp for Den2, 196 bp for Den3, and 143 bp for Den4). A protocol has been developed and successfully applied to detect dengue virus in cell-culture supernatants and patients sera. The technique is simple and rapid, capable of not only detecting the dengue virus but also identifying the serotypes of the virus in clinical specimens.

INTRODUCTION

Dengue virus, the causative agent of dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), is a member of the family *Flaviviridae* (Westaway *et al,* 1985). It is one of the major cause of pediatric morbidity and mortality throughout the subtropical and tropical regions (Halstead, 1988). In general dengue infection in children usually results in mild febrile illness. However, in endemic regions, such as in Southeast Asia, where all four serologically related subtypes of dengue viruses are present, the infection often causes severer syndromes of DHF and DSS.

Laboratory diagnosis of dengue infection is normally based on detection of antibody and isolation of the dengue virus from patient's blood sample (Shope, 1990). Current technologies for the isolation and identification of the virus in biological samples are based on complicated systems of suckling mouse brain or mosquito inoculations or cell culture techniques, followed by identification of the virus by specific immunological methods. Thus, only in well equipped and highly experienced laboratories could a study of the types of dengue virus associated with clinical infections be carried out. The availability of the polymerase chain reaction (PCR) technique (Saiki *et al,* 1985, 1986; Mullis *et al*, 1986) has made it possible to perform rapid detection of the viral RNA. The development of the PCR technique for detection of dengue virus has previously been reported (Deubel *et al*, 1990; Eldadah *et al*, 1991; Henchal *et al*, 1991; Laille *et al*, 1991; Morita *et al*, 1991, 1994; Huo-sheng *et al*, 1992; Lanciotti *et al*, 1992; Maneekarn *et al*, 1993; Puri *et al*, 1994; Chang *et al*, 1994; Pierre *et al*, 1994). In this communication, we present an alternative design of the PCR technique that can detect and simultaneously identify the serotypes of the dengue virus. The method is sensitive, specific and can serve as an alternative technology for the identification of dengue virus in biological specimens.

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MATERIALS AND METHODS

Primer design and PCR strategy

The strategy of the primer design is to achieve two aims: first, to have a highly sensitive system of PCR that can amplify sequences unique only for dengue virus, and second, to be able to simultaneously identify the serotypes of the virus. The high sensitivity was achieved by using the nested PCR technique. Two tests, each specific for either the E or NS1 region within the dengue virus genome were designed. A total four pairs of primers, two specific for each corresponding region were synthesized. The two pairs of primers specific for each region consisted of one pair of outer universal primers and one pair of inner or nested universal primers. The sequences used for designing all pairs of primers were unique for dengue virus and were shared by all the four serotypes.

For the identification of dengue serotypes, four pairs of primers specific to sequences within the E region, each unique for one particular serotype, were designed so that they gave four different sizes of amplified products, each corresponding to its serotype. The PCR was carried out with all the eight primers present. The regions encompassed by all these eight primers were deliberately chosen to be within the region flanked by a pair of outer dengue specific primers used in the above nested PCR, so that nested amplification can be carried out.

Sequences used in primer design

For the NS1 region (Table 1), a pair of primers that hybridized to regions unique for dengue viruses was designed from the published sequence data of the following viruses: Den1 (Nauru, M23027; Mason *et al*, 1987), Den2 (PR-159/S1, M19197; Hahn *et al*, 1988), Den2 (New Guinea C, M29095; Putnak *et al*, 1988), Den 2 (Jamaica, M20558; Deubel *et al*, 1988), Den2 (M1, X17338, M2, X17339, and M3, X17340; Fong *et al*, 1990), Den3 (H87, M93130; Osatomi and Sumiyoshi, 1990), and Den4 (Caribbean, M14931; Mackow *et al*, 1987). For the E region, the sequences from the following viruses as published in the GenBank database were used: Den1 (M23027), Den1CCV (D00501), Den1P83 (D00503), Den1TAH (D00502), Den2CGA (M29095), Den2JAMCG (M20558), Den2RCG (M19197), Den3 H87 (M93130), Den3SP5AA (M25277), and DenSTRA (M14931). Sequence comparison was performed using the MacVector computer program (International Biotechnologies, Inc, USA).

Dengue specific primers for nested PCR

Two tests, each specific for the E and NS1, were designed. For each region, two pairs of primers, one outer universal and one inner or nested universal primers (DEUL/DEUR and DENUL/DENUR for the E region, and DNS1UL/DNS1UR and DNS1NUL/DNS1NUR for the NS1 region) were synthesized (Table 1). Sizes of PCR products, when amplified with the outer and inner universal primers, were expected to be 641 basepairs (bp) and 434 bp for the E region, and 427 bp and 228 bp for the NS1 region, respectively.

Serotype-specific primers

Four pairs of serotype-specific primers (D1L/D1R, D2L/D2R, D3L/D3R, and D4L/D4R), all located within the E region, were synthesized. The regions covered by these serotype-specific primers were within the sequence flanked by the outer universal primers (DEUL/DEUR, see above). Sizes of DNA products after amplifications with the type-specific primers were expected to be 405 bp for Den1, 346 bp for Den2, 196 bp for Den3, and 143 for Den4, respectively. All the primers were synthesized by Research Genetics (USA) and Bioservice Unit of the Thai National Center for Genetic Engineering and Biotechnology (NCGEB, Bangkok, Thailand).

RNA extraction, reverse transcription (RT), PCR, and nested PCR

RNA extraction from culture medium and serum

Dengue-virus RNA was extracted from 100 µl of culture medium containing the viruses [6×10^5 to 8×10^7 plaque forming units (pfu) per milliliter] or from 100 µl of patient's serum by a single-step method using acid guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). RNA was finally dissolved in 5 µl of sterile distilled-water treated with diethyl pyrocarbornate

Table 1

PCR primers for amplifications of DNA fragments in the E- and NS1-regions of dengue-virus sequences.

Primer	Sequence	Position	Product size (bp)
E region			
DEUL	5'-TGGCTGGTGCACAGACAATGGTT-3'	1552-1574*	
			641
DEUR	5'-GCTGTGTCACCCAGAATGGCCAT-3'	2169-2192*	
DENUL	5'-GATCTCAAGAAGGAGCCATGCA-3'	1696-1718*	
			434
DENUR	5'-ATGGAACTTCCCTTCTTGAACCA-3'	2107-2129*	
NS1 region			
DNS1UL	5'-CATGCTGATATGGGTTATTGGATAGA-3'	3004-3029*	
			427
DNS1UR	5'-GTCTGATTTCCATCCCGTACCAGCA-3'	3406-3430*	
DNS1NUL	5'-AAAGTCACACACTCTATGGAGCAATG-3'	3099-3124*	
			228
DNS1NUR	5'-GTGGTTGTTCTTAAAGAGGGTCCTC-3'	3302-3326*	
Type-specific primers (in E region)			
D1L	5'-GGGGCTTCAACATCCCAAGAG-3'	1673-1694	
			405
D1R	5'-GCTTAGTTTCAAAGCTTTTTCAC-3'	2055-2077	
D2L	5'-ATCCAGATGTCATCAGGAAAC-3'	1744-1764	
			346
D2R	5'-CCGGCTCTACTCCTATGATG-3'	2070-2089	
D3L	5'-CAATGTGCTTGAATACCTTTGT-3'	1829-1848	
			196
D3R	5'-GGACAGGCTCCTCCTTCTTG-3'	2005-2024	
D4L	5'-GGACAACAGTGGTGAAAGTCA-3'	1892-1911	
			143
D4R	5'-GGTTACACTGTTGGTATTCTCA-3'	2012-2034	

* Position in relation to that of the Den2 Jamaica strain.

(DEPC) and kept at -70°C. This RNA sample was used within a few days.

Reverse transcription (RT)

Complementary DNA (cDNA) was synthesized from RNA by reverse transcriptase (RT) reaction in a 0.6-ml microtube. The reaction mixture contained 5 µl of RNA solution, 1 µl of 10 × PCR buffer I (containing 100 mM Tris-HCl, pH 8.3 and 500 mM KCl), 2 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs (Promega, USA), and 1 µl of 10 pmol/µl of righthand-side (R) primer. After incubation at 70°C for 10 minutes and cooling on ice, 1.5 µl of 8 units/µl avain myeloblastosis virus (AMV) reverse transcriptase and 0.5 µl of 40 units/µl of rRNasin (both from Promega, USA) were added into the mixture, and incubated at 37°C for 1 hour.

PCR

PCR was carried out in the same microtube by adding the following solutions into the RT mixture: 2 µl of 10 × PCR buffer I, 4 µl of 25 mM $MgCl_2$, 1

μl of 10 pmol/μl of lefthand-side (L) primer, 0.25 μl of 5 units/μl of Taq DNA polymerase (Pharmacia, USA), and 31 μl of DEPC-treated distilled-water. The total volume of PCR mixture was 50 μl. It was overlaid with light mineral oil and the reaction tube was placed in a Thermal Cycler (Perkin-Elmer-Cetus, USA). The machine was programmed to perform 40 step cycles comprising of 94°C for 5 minutes for denaturation in first and 1 minute in subsequent cycles, 45°C for 1 minute for annealing, and 72°C for 1 minute for extension (5 minutes for the last cycle).

Nested PCR

The reaction mixture for nested PCR (25 μl) was made up by adding 1 μl of the primary PCR product into a new 0.6-ml microtube containing premixed 2.5 μl of 10 × PCR buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 1% gelatin), 0.5 μl of 10 mM dNTPs, 0.5 μl each of 10 pmol/μl of L- and R-primers, 0.1 μl of 5 units/μl of Taq DNA polymerase, and 20 μl of DEPC-treated distilled-water. The mixture was covered with the mineral oil and the reaction tube was placed in the Thermal Cycler similarly programmed as above with the annealing temperature set at 62°C.

For negative and carry-over controls, 5 μl of RNA solution in the RT reaction mixture were replaced with 5 μl of the extraction from uninfected cell-culture medium, and with 5 μl of sterile distilled water, respectively.

Detection of PCR products

The PCR products were detected by agarose-gel electrophoresis. Five μl each of the PCR mixtures were applied into wells of 2% agarose mini-gel slab and electrophoresed at 100 volts and 80 mA for about 1 hour. The gel was stained in 2 μg/ml ethidium bromide solution.

Direct sequencing of PCR product

To prove that the PCR product contained nucleotide sequence of the dengue virus, DNA amplified from the E region of Den2 virus by using the universal DEUL/DEUR and type-specific D2L/D2R primers was sequenced by asymmetric PCR (McCabe, 1990) and cycle sequencing (Lee, 1991).

Dengue and other flaviviruses

Four different serotypes of dengue viruses, one Japanese encephalitis (JE) and West Nile (WN) viruses were kindly provided by Dr Bruce Innis of the Armed Forces Research Institute of Medical Science (AFRIMS), Bangkok. Strains of the four serotypes of dengue viruses were: Den1 (Hawaii), Den2 (16681 and New Guinea C), Den3 [CH 53489(H)], and Den4 (814669). These viruses were individually cultured in C6/36 *(Aedes albopictus)* cells at 28°C. Concentrations of the viruses in pooled culture media collected between two and seven days after inoculations were determined by plaque assay, using PS clone D cell-monolayers and staining with naphthalene black (Cardosa *et al*, 1986).

A serum sample containing hepatitis C (HC) virus was obtained from the Department of Immunology, Faculty of Medicine Siriraj Hospital. The presence of HC virus in this serum sample was determined by PCR using primers specific to several regions of HC virus-genomic sequence.

Sixty cell-culture supernatants containing different types of dengue viruses (11 Den1, 19 Den2, 11 Den3, and 18 Den4) isolated from patients with dengue virus infection by C6/36 cell culture, collected from a period spanning 10 years, were kindly provided by Dr Ananda Nisalak of AFRIMS, Bangkok. The viruses were used for testing the accuracy of serotype detection and the procedures were carried out blind.

Serum samples

Three serum samples from two sibs who both died of suspected dengue shock syndrome (DSS) were tested by the PCR method developed. These samples were negative for anti-dengue-virus antibody by HI test, but positive by dot enzyme immunoassay (DEIA) (Cardosa *et al*, 1988), and enzyme-linked immunosorbent assay (ELISA) (Innis *et al*, 1989; Cardosa *et al*, 1992).

RESULTS

Amplifications with universal primers

Results of the amplification of the cDNA reverse-transcribed from RNA of four serotypes of

dengue viruses using the universal primers specific either to the E or NS1 regions are shown in Fig 1. Amplifications of dengue-virus cDNAs with the DEUL/DEUR primers and subsequently with DENUL/DENUR, specific to the sequences within the E region, resulted in PCR products with the sizes of approximately 641 and 434 bps respectively, for all dengue serotypes (Fig 1A). Amplifications of dengue cDNAs with DNS1UL/DNS1UR and then with DNS1NUL/DNS1NUR, specific to the NS1 region, gave rise to PCR products with sizes of about 427 and 228 bp, respectively (Fig 1B). Only Den1 showed an extra smaller PCR product when amplified with the DNS1NUL/DNS1NUR primers, possibly a result of non-specific amplification.

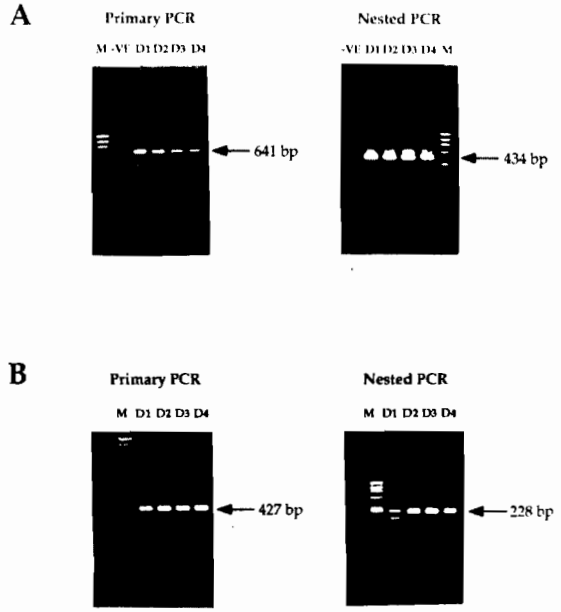

Fig 1–Amplifications of cDNAs from 4 serotypes of dengue viruses (D1, D2, D3, and D4) from cell-culture supernatants with the universal primers specific to : (A) the E region, and (B) the NS1 region. In primary PCR, the cDNAs were amplified with outer universal primers. In nested PCR, the primary PCR products were amplified with nested universal primers. For the E region, primary PCR using the outer universal (DEUL/DEUR) and nested PCR using the nested universal (DENUL/DENUR) primers resulted in PCR products of 641 and 434 bps, respectively. For the NS1 region, primary PCR using the outer universal (DNS1UL/DNS1UR) and nested PCR using the inner universal (DNS1NUL/DNS1NUR) primers gave rise to PCR products of 427 and 228 bps, respectively. Lanes M are *Hae*III-PhiX174 DNA markers and lanes "-ve" are negative controls.

Sensitivity of the nested PCR method was tested with cultured Den2 (16681) virus, using the DEUL/DEUR and DENUL/DENUR primers. Fig 2A shows the results of the nested PCR performed on a series of ten fold dilutions (in culture media) of Den2 (16681) virus. 0.1 pfu of the virus can be detected. Similar results were obtained when the viruses were diluted in human sera (results not shown). No difference in the sensitivity was observed when sera with different HI-titer were used.

Specificity of the nested PCR using the sets of universal primers (DEUL/DEUR and DENUL/

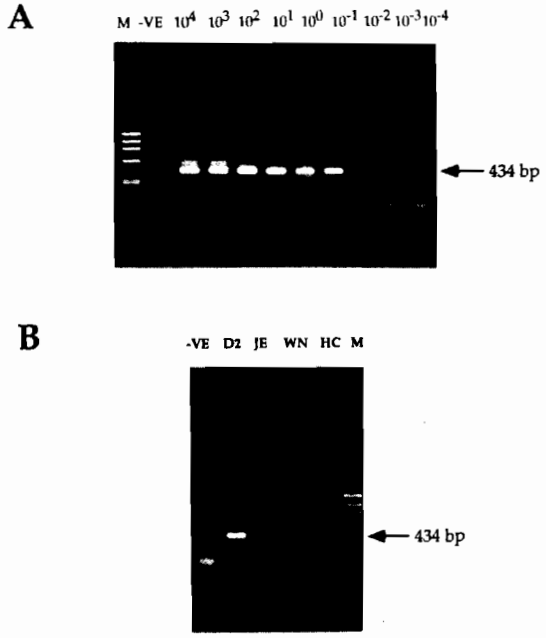

Fig 2–A) Sensitivity of the PCR method. Den2 virus was serially diluted with culture medium and its cDNA was amplified with the universal DEUL/DEUR and nested universal DENUL/DENUR primers. The PCR product was detected by agarose-gel electrophoresis and ethidium bromide staining. As low as 10^{-1} pfu per reaction of Den2 virus could be detected. B) Specificity of the PCR method using the same sets of primers. Den2, JE, WN, and HC viruses were tested but only Den2 virus were positive. The band that moved faster than the 434-bp PCR product was primers. Lanes M are *Hae*III-PhiX174 DNA markers and lanes -ve are negative controls.

DENUR) was evaluated by amplifications of cDNAs synthesized from RNA samples extracted from JE, WN, and HC viruses. No PCR product was detected from all the other flaviviruses tested (Fig 2B).

Amplifications with serotype-specific primers

RNAs were extracted from cell-culture supernatants containing each serotypes of dengue viruses (Den1, Den2, Den3, or Den4). PCRs were primarily performed with the outer universal primers (DEUL/DEUR) specific to the sequences in the E region. The amplified products were diluted 10,000 fold and 1 µl of the diluted products was used in the secondary amplifications with four separate sets of serotype-specific primers (D1L/D1R, D2L/D2R, D3L/D3R and D4L/D4R). The results (Fig 3) showed that each set of serotype-specific primer specifically amplified the corresponding dengue serotype without cross-amplification. As expected, sizes of PCR products obtained by using the serotype-specific primers for Den1, Den2, Den3, and Den4 were found to be about 405, 346, 196 and 143 bp, respectively.

The mixture of the four sets of serotype-specific primers was tested in amplifications of the diluted primary PCR products from the individual dengue serotype. The mixture gave the right sizes of PCR products of the respective serotypes as shown in Fig 4.

Sequencing of PCR product

To confirm that the amplified PCR products contain sequences not different from the original dengue viruses, nucleotide sequence of the PCR product obtained from amplifications of Den2 virus (New Guinea C) with DEUL/DEUR and D2L/D2R primers was determined by direct sequencing

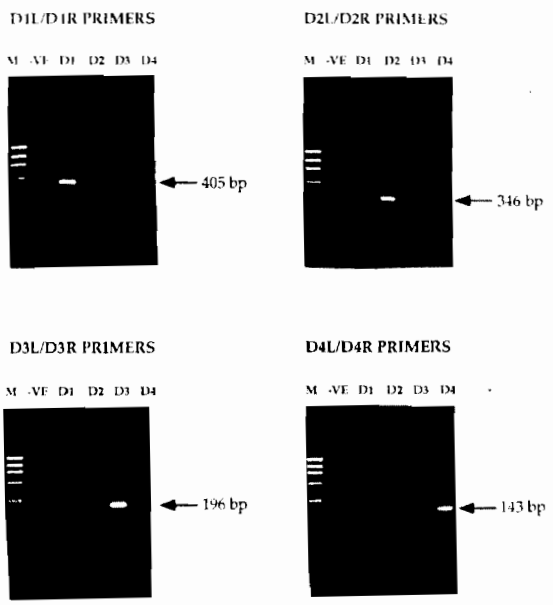

Fig 3–Amplifications with serotype-specific primers. cDNAs from the four serotypes of dengue viruses were initially amplified with the outer universal (DEUL/DEUR) primers specific to the E region, and the amplified products were diluted 10,000 folds before amplifying with four separate sets of the serotype-specific (D1L/D1R, D2L/D2R, D3L/D3R, and D4L/D4R) primers. Sizes of PCR products were 405, 346, 196, and 143 bps for Den1, Den2, Den3 and Den4, respectively. Lanes M are *Hae*III-PhiX174 DNA markers and lanes -ve are negative controls.

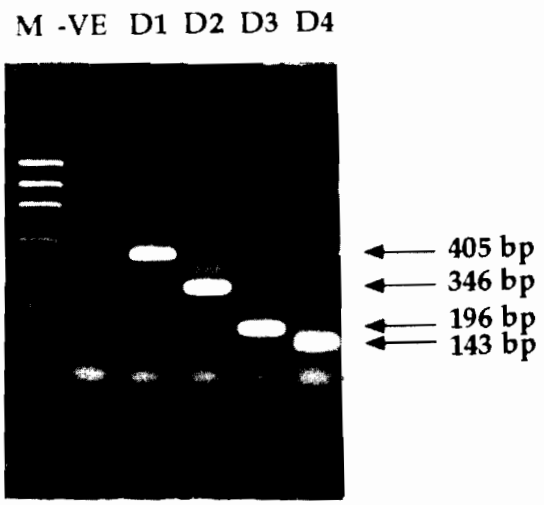

Fig 4–Amplifications with mixture of serotype-specific primers. cDNA from each serotype of dengue viruses was initially amplified with the outer universal (DEUL/DEUR) primers specific to the E region, and the amplified products was diluted 10,000 folds before amplifying with mixture of the four sets of serotype-specific (D1L/D1R, D2L/D2R, D3L/D3R, and D4L/D4R) primers. A single PCR product with the expected size for each serotype of dengue viruses was observed. Lane M is *Hae*III-PhiX174 DNA markers and lane -ve is negative control.

method, using a combination of asymmetric PCR and cycle sequencing procedures. The sequence of the 157 nucleotides completely matches those of Den2CGA (M29095) as published in the GenBank database.

Accuracy of serotype detection and application in serum samples

To test the accuracy of serotype detection, the method was applied blind to test 60 samples of dengue virus culture supernatant of known serotypes. The results of 59 samples were in total agreement. Only in one sample (Den1 serotype), the inner serotype-specific primers failed to amplify the product from the outer universal primers (DEUL/DEUR).

The PCR method was applied to three serum samples (Two from venous-blood and one from heart-blood samples) from two children in the same family who died of dengue shock syndrome. All three samples were positive for Den3 virus (Fig 5).

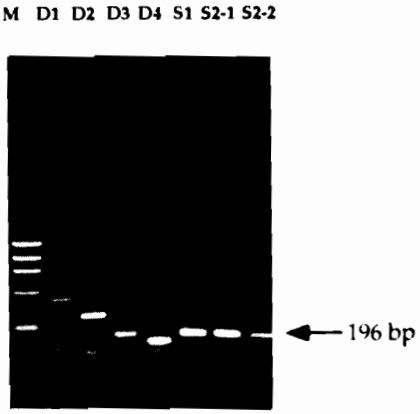

Fig 5–Detection and identification of dengue viruses in serum samples by the PCR method, using the outer universal and the mixture of serotype- specific primers. Three serum samples, two from venous-blood (lanes S1 and S2-1) and one from heart-blood (lane S2-2) from two sibs who probably died of DSS. All three samples were positive for Den3 virus. Lane M is *Hae*III-PhiX174 DNA markers, and lanes D1, D2, D3, and D4 are positive control for the four serotypes of dengue viruses.

DISCUSSION

We have reported a sensitive nested PCR technique for detection of dengue virus in biological samples. The method not only is capable of detecting the presence of dengue virus at a dilution of 1 pfu (or 10-100 viral particles/100 µl sample) or less but can also simultaneously identify the serotype of the virus by the unique sizes of the PCR products. The technique could correctly identify the serotypes of all except one of 60 dengue viruses isolated from patients with dengue virus infection, collected in Bangkok spanning a period of 10 years.

To achieve the highest sensitivity of detection of the virus, the nested PCR technique was used, with two pairs of primers annealing to the regions within the E or NS1 shared amongst the four serotypes. For the detection of dengue serotypes, four pairs of serotype-specific primers, each hybridized to the unique (to each serotype) regions within the area flanked by the pair of outer E-region universal primers, were applied. The design of serotype-specific primers and the PCR protocol in this report differed from those reported by Lanciotti and co-workers (Lanciotti *et al*, 1992). In our work, all the four pairs of serotype-specific primers were independent from the outer universal primers, whereas four single-sided serotype-specific primers had been used in conjunction with one universal primer in Lanciotti's method. Generally, pairs of serotype-specific primers will be more specific than single-sided serotype-specific primers.

The development of PCR technique for detection and typing of dengue viruses has previously been reported by several groups (Deubel *et al*, 1990; Eldadah *et al*, 1991; Henchal *et al*, 1991; Laille *et al*, 1991; Morita *et al*, 1991, 1994; Huosheng *et al*, 1992; Lanciotti *et al*, 1992; Maneekarn *et al*, 1993; Puri *et al*, 1994; Chang *et al*, 1994; Pierre *et al*, 1994). In principle, the techniques differed in two ways, first whether nested PCR had been used in the amplification of dengue-virus cDNA, and second, what techniques had been used for the detection of the PCR products. Hybridization with specific DNA probes (Deubel *et al*, 1990; Laille *et al*, 1991) or synthetic oligonucleotides (Henchal *et al*, 1991) has been the common method used for the identification of the PCR product. Although the hybridization with specific DNA probes could enhance both sensitivity and specificity, it is tedious and complicated, requiring a

well trained operator. Agarose-gel electrophoresis of the PCR product is another simpler method used, including ours (Eldadah *et al*, 1991; Morita *et al*, 1991; Huo-sheng *et al*, 1992; Lanciotti *et al*, 1992).

To enhance sensitivity and/or specificity, additional steps were added to the procedures, including: initial amplification of the virus by prior culturing the virus in cells (Morita *et al*, 1991), cleavage of the PCR products with restriction endonuclease (Eldadah *et al*, 1991), or nested PCR (Lanciotti *et al*, 1992). Two groups (Morita *et al*, 1991; Lanciotti *et al*, 1992) demonstrated the use of serotype-specific primers for identification of all four dengue serotypes and detection of the PCR products by the simple method of agarose-gel electrophoresis. We preferred the method of detection of the PCR products by agarose-gel electrophoresis since it is generally a simpler technique and according to our design, it allowed the identification of the serotypes by the unique sizes of the PCR products. The sizes of the PCR products could also be used to check whether the amplification was specific, since sizes other than those designated for each serotype would indicate non-specific amplification.

Our PCR method is sensitive and can detect the virus as low as 0.1 pfu (1-10 viral particles) (Fig 2A) in culture and 0.1-1 pfu in serum samples, comparable to those reported by Deubel *et al* (1990), Morita *et al* (1991) and Lanciotti *et al* (1992), but with higher sensitivity than the method described by Henchal *et al* (1991). It should be noted that in the reconstitution test the presence of serum with anti-dengue antibodies did not affect the sensitivity of detection (data not shown). The same finding has also been observed by Henchal *et al* (1991). When the method was used to detect samples of virus in cell-culture supernatants, it was necessary to dilute the primary PCR products 10,000 folds before applying the nested amplification. This process is not necessary when serum samples (which contain fewer virus particles) were used. Specificity of our PCR method using the same set of primers was demonstrated by negative amplification with a number of flaviviruses including JE, WN, and HC viruses (Fig 2B).

At present, we are carrying out a collaborative study to compare and evaluate our PCR technique with those methods reported by others (Henchal *et al* 1991; Morita *et al*, 1991; Lanciotti *et al*, 1992). Result of this study will be reported elsewhere.

In conclusion, the new sets of primers and PCR protocol reported in this communication have been shown to be efficient for the detection and identification of dengue viruses in clinical specimens. The method is sensitive and specific. The procedure is also simple and rapid. The whole process of detection and identification can be finished within one day. The method has added another alternative technique for the rapid detection of dengue virus.

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