MOLECULAR DETECTION AND SEROTYPING OF DENGUE VIRUS IN METRO MANILA, PHILIPPINES

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ABSTRACT

Dengue virus infection is a persistent health problem in the Philippines. Surveillance and diagnosis have been limited to recognizing the clinical symptoms. In this study, a reverse transcription-polymerase chain reaction (RT-PCR) assay complemented with virus isolation was used to detect and isolate dengue virus from sera of patients admitted to different medical institutions during the period 1995-1998. RT-PCR was done directly on serum samples. Additionally, serum samples were inoculated on *Aedes albopictus* cells and RT-PCR was done on infected culture fluid or on RNA extracted from infected cells. Dengue virus was detected and isolated in 95 out of 738 serum samples. Serotyping of the virus indicated the prevalence of Den 2 and Den 3.

INTRODUCTION

Dengue fever (DF) and dengue haemorrhagic fever (DHF) are major health problems in many countries of Southeast Asia and the Latin Americas. It has recently been identified as one of the emerging or re-emerging infectious diseases in industrialized countries. DF and DHF are caused by different serotypes of dengue viruses (Henchal and Putnak, 1990). Hence, viral isolation and confirmation as well as serotyping are important for epidemiological studies and eventual understanding and control of the disease. In this study, we applied reverse transcription-polymerase

chain reaction (RT-PCR) in detecting dengue viruses from serum samples obtained from different hospitals in Metro Manila, Philippines from January 1995 to April 1998. Dengue virus serotypes were further determined using type specific primers.

MATERIALS AND METHODS

Serum samples

Blood samples from clinically diagnosed dengue patients were collected from several collaborating medical institutions namely, Bureau of Research and Laboratories (BRL), Cardinal Santos Medical Center (CSMC), National Children's Hospital (NCH), Philippine General Hospital (PGH), Makati Medical Center (MMC), Philippine Children's Medical Center (PCMC), San Lazaro Hospital (SLH), St. Luke's Medical Center (SLMC), and University of Santo Tomas(UST) Hospital. Sampling was done by venipuncture using vacutainers. Sera were separated by centrifugation and brought to the Research and Biotechnology Division (RBD) of the St. Luke's Medical Center, Quezon City, Philippines and to the Dept. of Molecular Epidemiology, Institute for Tropical Medicine, Nagasaki University in Japan. Serum samples were aliquoted in 1.5 mL cryovials and were stored at –86°C until use.

Virus detection and mosquito cell culture

Confluent cultures of *Aedes albopictus* C6/36 cells (Igarashi, 1978) were inoculated with 10 μL of patient serum and the virus was allowed to adsorb for 2 hours at 28°C with agitation at 30 min intervals. Thereafter, cells were overlayed with Minimal Essential Medium containing 2% heat-inactivated fetal bovine serum and 0.2 mM each of the non-essential amino acids and were incubated at 28°C for 7 days. An aliquot of the infected culture fluid was harvested 7 or 10 days after inoculation and subjected to RT-PCR. RNA was further extracted from infected cells. Cells were briefly lysed in the culture dish by adding 750 μL of Trizol LS reagent. The lysate was transferred into 1.5 mL precipitated from the upper aqueous layer by adding 500 μL of isopropyl alcohol. The RNA pellet was washed once with ethanol, dried, and resuspended in sterile, RNAse-free distilled water and stored at –80°C until use. Flavivirus on serum samples was detected using yellow fever virus primer pair (YFI:5'-GGTCTCCTCTAACCTCTAG-3' and YF3: 5'-GAGTGGATGACCACGGAAGACATGC-3'; Rice et al., 1998) Dengue virus was detected using a dengue consensus primer pair (DC1: 5'-TCAATATGCTGAAACG-CGCGAAACCG-3'and DC2: 5'TTGCACCAACAGTCAATGTCTTGAGGTTC-3'; Lanciotti et al., 1992). Dengue serotypes were determined using serotype specific primers (for D1S: 5'-GGACTGCGTATGGAGTTTTG-3' and D1C: 5'ATGGGTTGTGGCCTAATCAT-3', Mason et al., 1987; for D2S; 5'GTTCCTCTGCAAACACTCCA-3' and D2C: GTGTTATTTTGATTTCCTTG-3', Hahn et al., 1998, Deubel et al., 1988; for D3S: 5'-GTGCTTACACAGCCCTATTT-3' and D3C: 5'-TCCATTCTCCCAA-GCGCCTG-3', Osatomi and Sumiyoshi, 1990; for D4S: 5'-CCATTATGGCTGTGTTGTTT-3' and D4C: 5'-CTTCATCCTG-

CTTCACTTCT-3', Mackow et al., 1987, Zhao et al., 1986). RT-PCR reaction using either serum samples or, culture fluid from *Aedes albopictus* C6/36 cells inoculated with sera or RNA extracted from infected cells as template for cDNA amplification was carried out for 35 cycles using a thermocycler (Eppendorf Mastercycler) with 94°C denaturation for 1 min, followed by 53°C annealing for 1 min, and 72°C primer extension for 1.5 min. A final extension at 72°C for 10 min was included after the last cycle. RT-PCR products subjected to electrophoresis in 3% agarose gel were stained with ethidium bromide and visualized under UV illumination.

RESULTS AND DISCUSSION

A total of 738 serum samples was analyzed for the presence of dengue virus by means of RT-PCR using at least one of the following sources of templates for cDNA amplification: (1) serum samples, (2) infected culture fluid of C6/36 *Aedes albopictus* cells, and (3) RNA extracted from serum-inoculated C6/36 cells. Yellow fever primers, used in detecting flaviviruses, were used for serum samples while Dengue Consensus primers, used in detecting dengue virus, were used in infected culture fluid and RNA extracts. Results showed 95 out of the 738 samples (13%) were positive for the presence of dengue virus (see Figure 1 for some samples positive for dengue virus). The breakdown of positive samples according to year of collection is as follows: for the year 1995, 59 out of 470 samples (13%); for 1996, 16/164 (10%); for 1997, 17/73 (23%), and for January to April 1998; 3/31 (10%). A possible reason for the low percentage of positive samples is that blood collection from patients may have been done after the onset of fever. The viremic period is within 3-5 days from the onset of fever (Seah et al., 1995).

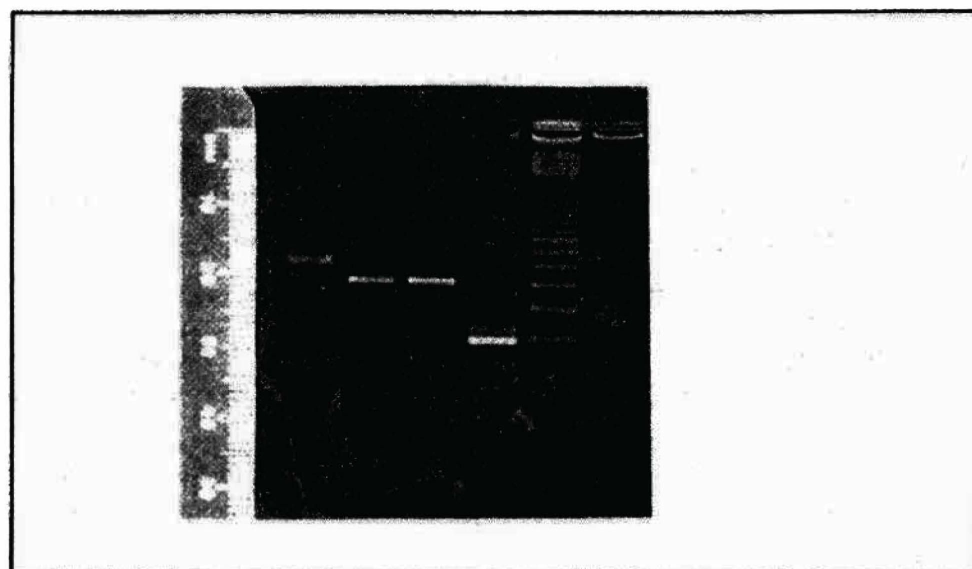

Figure 1. PCR products of two Dengue isolates using different sets of primers. SLMC 50 (*lanes* 1-4) and DOH 143 (*lanes* 6-8). *Lane* 1, RT-PCR of serum using Yellow fever primers; *lanes* 2, 3, 6, and 7, RT-PCR on infected culture fluid using Dengue consensus primers; *lanes* 4 and 8, RT-PCR on infected culture fluid using Dengue serotype 3 primers.

RT-PCR protocol using YF primers on serum samples detected only 40 out of 565 samples (7%). On the other hand, 62 out of 307 infected culture fluid samples (20%) were positive using dengue consensus primers. Furthermore, 65 out of 274 serum samples (24%) were positive when RNA extracts were used as templates for RT-PCR. These results would indicate the efficiency of using RNA extracts for viral detection. The low percentage of detection by direct RT-PCR of serum may be due to the presence of inhibitors of Taq polymerase which decreases the sensitivity of the technique. It is also possible that RNAses are present which destroy the RNA template (Rolf et al., 1992).

Fifty-nine out of 95 dengue viruses detected were serotyped using dengue serotype specific primers. Thirty-five samples were identified as Dengue 2 while 21 were Dengue 3. To date, only one sample was identified as Dengue 1 and two samples as Dengue 4. For this batch of samples, Dengue 2 and 3 are the more prevalent serotypes.

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