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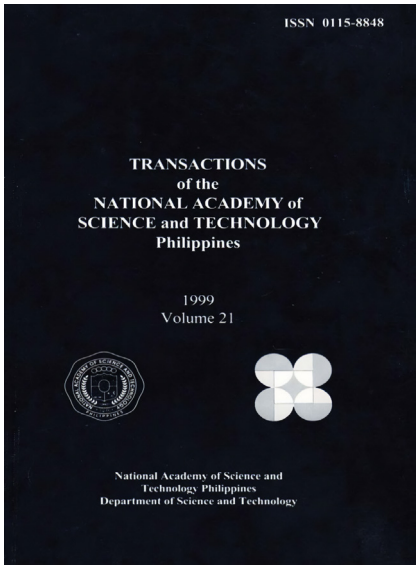

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DETECTION OF DENGUE VIRUS AND ANTIBODIES IN PATIENT SERA COLLECTED FROM 1995 TO 1999 IN THE PHILIPPINES

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ABSTRACT

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are persistent public health problems in the Philippines. In this study, two molecular techniques, reverse transcription-polymerase chain reaction (RT-PCR) and IgM-capture ELISA, are used to detect the presence of dengue virus and dengue antibodies in patient sera, respectively. Serum samples of clinically diagnosed DF and DHF patients from different hospitals in Metro Manila and from field collections of the Field Epidemiology Training Program of the Department of Health were used. From the samples collected from January 1995 to May 1999, a total of 1,429 sera were analyzed by RT-PCR and 149 (10.4%) samples were positive for dengue virus. Serotyping using serotype-specific primers showed that the predominant serotypes in the Philippines during this period are dengue serotypes 2 and 3. A total of 1,391 sera was tested for the presence of antibodies to dengue by IgM-capture ELISA using tetravalent dengue antigen. Seven hundred sixty two samples (57.8%) were found positive for anti-dengue IgM.

Key words: dengue virus, dengue fever, dengue hemorrhagic fever, molecular techniques, reverse transcription-polymerase chain reaction, IgM-capture ELISA, antibodies, serotype, tetravalent dengue antigen, anti-dengue IgM.

INTRODUCTION

Dengue virus causes millions of human infections each year. Illnesses resulting from dengue infections range from mild fever to severe hemorrhagic fever and sometimes fatal shock (Chan, 1987). The dengue virus is found in both tropical and subtropical regions of the world and its distribution overlaps with other pathogenic flaviviruses such as yellow fever and Japanese encephalitis virus. Hence, interpretation of both clinical and serological diagnosis can be complicated due to similar antigenic determinants that can elicit cross-reactive antibodies against these viruses (Bundo and Igarashi, 1985).

Hemagglutination-inhibition (HI) test and indirect immunofluorescent antibody tests (IFAT) have been used to detect and identify dengue viruses (Chan, 1987; Capeding *et al,* 1987; Hayes *et al* 1988; Henchal *et al*, 1982; Manoloto and Hayes, 1989). Virus serotype which infects is inferred by measuring a fourfold or greater increase in antibody titers to the particular infecting serotype. Specific diagnosis or identification may not be accurate due to the cross-reactivity of antibodies to different flaviviruses. In tests wherein paired serum samples are needed, this requirement causes delay in diagnosis. Virus isolation from patient serum has been achieved using cell culture methods as well as mosquito inoculation (Hayes *et al,* 1988). However, virus isolation takes from days to weeks and traditional virus identification is not always successful due to the small amount of viable virus in the sample. There is a need for an assay system that can be performed rapidly and is sufficiently sensitive and specific to be useful.

IgM antibodies have been reported to be more specific than total antibody titers in the detection of flaviviruses. Bundo and Igarashi (1985) developed an IgM capture ELISA that could differentiate Japanese Encephalitis infection from dengue infection. IgM has a distinct advantage of having multiple binding sites and is present during early stages of infection. Furthermore, polymerase chain reaction (PCR) development has greatly facilitated the development of several diagnostic protocols for detecting and identifying dengue viruses (Eldadah *et al,* 1991; Lanciotti *et al,* 1992; Morita, 1994; Seah *et al,* 1995; Thayan *et al,* 1995).

In this study, we have employed IgM-capture ELISA assay to detect anti-dengue antibodies in the serum samples collected by the different collaborating hospitals. RT-PCR was also used to detect and identify infecting serotype.

MATERIALS and METHODS

Serum Samples

Serum samples from patients suspected to have, or have been clinically diagnosed dengue fever or dengue hemorrhagic fever from the various collaborating hospitals were collected and sent to the Research and Biotechnology Division (RBD) of the St. Luke's Medical Center, Quezon City, Philippines. Serum samples were aliquoted in 1.5 ml cryovials and stored at -86°C until use.

Cell Culture and Virus Detection

Confluent cultures of *Aedes albopictus* C6/36 cells were inoculated with 20 µl of patient serum and the virus was allowed to adsorb for 2 hours at 28°C with agitation at 30 min. intervals. Cells were overlayed with 2 ml of Minimal Essential Medium containing 2% heat-inactivated fetal bovine serum and 0.2 mM each of nonessential amino acids and were kept at 28°C. Culture fluid and infected cells were harvested 7 to 10 days after inoculation. Dengue virus was detected from infected culture fluid and RNA extracts from infected cells by RT-PCR using dengue consensus primers. RT-PCR using dengue serotype specific primers was further performed on the culture fluid containing the virus.

RNA Extraction

The infected cell pellet was treated with 400 µl Trizol LS Reagent (Gibco, BRL). The cell lysate was passed several times through a pipette then transferred to a 1.5 ml eppendorf tube and kept for 5 min at room temp. Chloroform (150 µl) was added and the tubes shaken by hand vigorously for 15 sec. The tubes were then incubated at room temperature for 10 min and then centrifuged at 12,200 rpm for 15 min at 4°C. The aqueous phase was transferred to a sterile 1.5 ml tube and the RNA precipitated by adding 300 µl isopropyl alcohol. This was mixed by tube inversions and incubated overnight at –86°C. The samples were then centrifuged at 12,200 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet washed once with 500 µl 70% ethanol by vortexing. The pellet was then recovered by centrifuging for 5 min at 4°C. The supernatant was removed and the RNA pellet was vacuum-dried for 5 min. The RNA was then re-dissolved in 50 µl DEPC-treated water and incubated at 55°C for 10 min with little agitation. The RNA extract was stored at –86°C until use.

Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

For RT-PCR of infected culture fluid, 100 µl of ICF was heat-inactivated at 65°C for 10 min. 3 µl of detergent mix (1% NP-40 and RNAse inhibitor in 5:1 mixture) was mixed with 2.5 µl of the heat-inactivated ICF, and incubated at room temp. for 1 min. This was added to a master mix, previously prepared to give 50 µl final volume of each reaction, containing 200 nM each dNTPs, 25 pmol sense primer, 25 pmol antisense primer, 2.5 U Tth polymerase, and 5 U reverse tran-

scriptase. For RT-PCR of the RNA extract of infected cells, 2.5 μl of the RNA extract was added to the master mix to give 50 ul reaction mixture of the same composition as the above. The thermocyclers (Eppendorf Mastercycler 5330 and Perkin-Elmer Gene Amp PCR System 2400) were programmed to run the following reaction conditions in the order: reverse transcription at 53°C for 20 min, 35 cycles of denaturation at 94°C for 60 sec, annealing at 53°C for 60 sec, and extension at 72°C for 90 sec, then a final extension at 72°C for 10 min. RT-PCR products subjected to electrophoresis in 2% agarose gel were stained with ethidium bromide and visualized under UV illumination.

IgM-capture ELISA

The 96 well ELISA plate was coated with μ-chain specific anti-human IgM diluted in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6 containing 0.02% NaN3) and incubated at 4°C overnight. Nonspecific binding sites were blocked with blockace for 1 hour at room temperature followed by washing with PBS-Tween (0.05% Tween-20 in PBS-). The ELISA plates were reacted with the test and standard positive sera at appropriate dilutions for one hour at room temperature. The plates were washed as above. Assay antigen (monovalent and tetravalent antigen), 100 μl, containing 25 ELISA units of each dengue serotype was added into each well and incubated as above. After washing, the plates were reacted with antiflavivirus IgG for 1 hour, which had been previously conjugated to horseradish peroxidase (HRPO). The plates were washed as above and HRPO reaction was carried out using 0.05% o-phenylenediamine dihydrochloride and 0.01% H_2O_2 in 0.05 M citrate-phosphate buffer, pH 5.0 in the dark. The reaction was terminated by adding of 1 N H_2SO_4. The ELISA OD was measured at 492 nm. The ELISA titer of the test serum samples was estimated by comparing its OD with those of the serially diluted positive standard. The test specimen scored positive when its P/N ratio is equal to or greater than 2.

RESULTS

A total of 1,429 serum samples was processed for virus detection by RT-PCR using flavivirus specific primers. Of these serum samples, 149 (10.4%) were RT-PCR positive (Table 1). Ninety (90) samples were serotyped by RT-PCR using serotype specific primers. There were 46 dengue serotype 2 (den 2) viruses detected followed by 31 den 3, 12 den 1 and 1 den 4 (Table 2). Figure 1 shows the RT-PCR products of 3 dengue positive isolates using serotype-specific primers.

A total of 1,391 serum samples were tested for the presence of IgM. Of these serum samples, 762 (57.8%) were IgM positive (Table 3). A comparative experiment using the individual 4 monovalent dengue antigens or combined (tetravalent) as assay antigen was done. Table 4 shows that out of 179 serum samples, 73 (41%) serum samples reacted with den 1, while 68 (38%) samples

Table 1. Detection of dengue virus by RT-PCR using dengue consensus primers.

	1995	1996	1997	1998	1999	Total
Number of samples tested	469	166	74	523	197	1,429
RT-PCR +	59	16	17	51	6	149
Percentage	12.6	9.6	23	9.8	3	10.4

Table 2. Serotyping of dengue virus by RT-PCR using type specific primers.

	1995	1996	1997	1998	1999	Total
Dengue 1	1	1	0	5	5	12
Dengue 2	21	8	8	8	1	46
Dengue 3	19	2	4	6	0	31
Dengue 4	0	0	1	0	0	1
Total	41	11	13	19	6	90

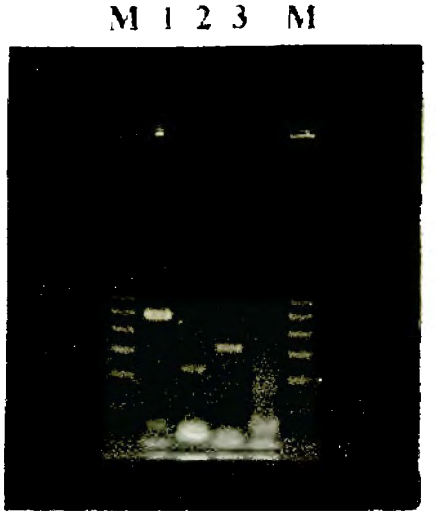

Figure 1. RT-PCR products of 3 dengue isolates using dengue serotype-specific primers. *Lane 1,* 99 St-12 is Den 1 (490 bp); *lane 2,* SLMC 70 is Den 2 (230 bp); *lane 3,* DOH 109 is Den 3 (320 bp). *M,* 100-bp marker.

Table 3. Detection of dengue virus by IgM Capture ELISA using tetravalent dengue antigen.

	1995	1996	1997	1998	1999	Total
Number of Samples Tested	164	67	23	821	244	1,391
IgM ELISA +	91	27	10	529	105	762
Percentage	55.5	40.3	43.5	64.4	43	57.8

Table 4. Comparison of the sensitivities of different monovalent antigens and tetravalent antigen used as an assay antigen in IgM capture ELISA.

n = 179	Monovalent Antigen				Tetravalent Antigent
	Den 1	Den 2	Den 3	Den 4	
1995	14	17	12	9	32
1996	2	2	2	2	2
1997	3	2	3	0	5
1998	54	47	45	26	78
Total	73	68	62	37	117

reacted with den 2 and 62 (35%) samples reacted with den 3. Only 37 (21%) serum samples were positive using den 4 as an assay antigen. On the other hand 117 (65%) serum samples gave a positive result when all 4 dengue serotypes were used as an assay antigen.

IgM and RT-PCR results were correlated with the collection day of the serum samples relative to the onset of fever (Table 5). There was an increase in the number of IgM positive cases if and when the serum was obtained several days after the onset of fever. On the other hand, the highest number of RT-PCR positive results was obtained 2-3 days after onset of fever. The number of positives declined thereafter.

DISCUSSION

The dengue virus of which there are 4 serotypes (den 1, den 2, den 3 and den 4) causes dengue fever and dengue hemorrhagic fever. In the Philippines, epidemic proportions of DHF have been occurring every 3 to 5 years (Chan, 1987). Serological diagnosis by HI have been performed to confirm initial clinical diagnosis and virus isolation using AP-61 cells and identification by

Table 5. Effect of the time of serum collection on the sensitivity of IgM capture ELISA and RT-PCR.

Age of Serum (Date of blood extraction-Date of onset of fever)	No. of IgM positive cases n = 762	No. of RT-PCR positive cases n = 149
0 - 1	18	11
2 -3	69	54
4 -5	170	36
6 -7	151	13
> 7	194	7
no data	160	28

immunofluorescent antibody tests using specific monoclonal antibodies was done at NAMRU-2 (Chan, 1987; Hayes *et al*, 1988). The predominant serotype from 1983 to 1984 as determined by Hayes *et al* (1988) was den 2 followed by den 1 and den 3. Den 4 was the least common. Den 3 was the most common serotype isolated from 1983 to 1986 (Manaloto and Hayes, 1989). Capeding *et al* (1997) isolated 20 dengue viruses from 143 serum samples submitted from 1992 to 1993. Of the 20 isolates, 16 belong to serotype 1 and 4 belong to serotype 2 complex. There was no reported isolation of den 3 or den 4. Capeding *et al* (1997) used the immunofluorescent test using type-specific monoclonal antibodies. In this study, we have used RT-PCR to detect and identify as well as specifically serotype the dengue virus. Similar to the NAMRU-2 study, we isolated and identified more den 2 viruses. On the other hand, we detected more den 3 than den 1 serotypes. Again, den 4 was the least detected.

The specificity of the RT-PCR assay depends on the ability of the type specific primers to recognize unique and specific nucleotide sequences in the virus genome (Lanciotti et al, 1992). Although false positive PCR results have been described in other PCR-based assays, this can be avoided by routinely performing several precautionary measures such as physical separation of pre- and post PCR procedures, use of positive displacement pipettes, careful handling and UV irradiation of reaction mixtures (Lanciotti *et al*, 1992; Morita, 1994). The use of appropriate positive and negative control also helps differentiate tube to tube contamination. The accuracy, sensitivity and speed of the RT-PCR assay makes it an effective method both for diagnosis and epidemiological surveillance (Eldadah *et al*, 1991; Seah *et al*, 1995; Thayan *et al* 1995). This assay method can be used to complement existing techniques such as the IgM capture ELISA method.

Furthermore, it can be used to amplify other regions of the virus genome for faster sequence analysis, which is very much useful for genetic and evolutionary studies.

Acute dengue cases have been diagnosed using IgM capture ELISA (Bundo and Igarashi, 1985). IgM antibodies are specific and indicative of early infection. Furthermore, IgM antibodies directed towards dengue antigen usually persist for only 2 to 3 months after an acute infection. Using IgM capture ELISA, it is possible to make a presumptive diagnosis using a single serum sample. In this study, 762 out of the 1,391 serum samples analyzed by IgM capture ELISA were positive suggesting early infection. More serum samples containing the anti-den IgM antibody were detected when all four dengue serotypes were used as the assay antigen. Using individual serotypes as assay antigen, den 1 seems to detect the highest number of sera containing IgM anti-den antibodies, followed by den 2 and den 3. Den 4 was the least reactive. While RT-PCR showed that den 2 and 3 are the predominating co-circulating serotypes, den 1 assay antigen is cross reactive to the other 4 serotypes. This probably explains why previous authors identified more den 1 serotypes using immunofluorescent antibody tests even with the use of monoclonal antibodies. Furthermore cross-reactivity to other flaviviruses always exists. It is therefore suggested that antigens from other flaviviruses endemic in that particular area be included in the future assay to make definitive diagnosis.

While it is tempting to compare the 2 diagnostic protocols (IgM-capture ELISA and RT-PCR), caution must be made in doing so. The former detects and measures antibodies whereas the latter detects virus genome. There are few cases which simultaneously have both anti-dengue IgM and the dengue virus or just the virus without anti-dengue IgM antibodies (Table 6). Most of the serum samples tested contained only anti-dengue IgM antibodies (Table 6). This is probably because most of the DHF cases would be secondary infection which shows shorter viremic period than the primary infection or most of the sera were collected at a

Table 6. Correlation between the presence of anti-dengue IgM antibodies and dengue virus.

	IgM+/RT-PCR+	IgM-/RT-PCR+	IgM+/RT-PCR-	IgM-/RT-PCR-
1995	4	11	84	57
1996	2	6	24	31
1997	3	10	5	3
1998	17	27	254	120
1999	2	4	82	106
Total	28	58	449	317

later part of the disease. Table 5 shows that if the serum samples are collected at a later time, the chances of detecting the virus by RT-PCR decreases whereas the chances of detecting anti-dengue IgM antibodies increases. It has been shown by previous authors that the highest detection rate of dengue virus infection by IgM capture ELISA was in the convalescent phase, while RT-PCR detection was more successful in the acute phase (Saat et al, 1994).

In conclusion, since one of the drawbacks of RT-PCR is that it is expensive, we recommend that serum specimens obtained from acute dengue cases first be tested by the IgM capture ELISA. Samples that are found to be negative should then be examined by RT-PCR to increase the diagnostic efficiency.

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REFERENCES

Bundo, K. and A. Igarashi. 1985. Antibody-capture ELISA for detection of immunoglobulin Mantibodies in sera from Japanese encephalitis and dengue hemorrhagic fever patients. *J. Virol. Methods* 11:15-22.

Capeding, M. R. Z., F. J. E. Paladin, E. G. Miranda, and X. R. Navarro. 1997. Dengue surveillance in Metro Manila. *Southeast Asian J Trop Med Pub Hlth* 28(3):530-534.

Chan, V. 1987. Virological and epidemiological studies of dengue haemorrhagic fever in the Philippines. *Southeast Asian J Trop Med Pub Hlth* 18(3): 275-277.

Eldadah, Z. A., D. M. Asher, M. S. Godec, K. L. Pomeroy, L. G. Goldfarb, S. M. Feinstone, H. Levitan, C. J. Gibbs, and D. C. Gajdusek. 1991. Detection of flaviviruses by reverse-transcriptase polymerase chain reaction. *J Med Virol* 33:260-267.

Hayes, C. G., C. R. Manaloto, A. Gonzales, and C. P. Ranoa. 1988. Dengue infections in the Philippines: clinical and virological findings in 517 hospitalized patients. *Am J Trop Med Hyg* 39(1):110-116.

Henchal, E. A., M.K. Gentry, J. M. McCown, and W. E. Brandt. 1982. Dengue virus specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *Am. J. Trop. Med. Hyg* 31(4):830-836.

Henchal, E. A., S. L. Polo, V. Vorndam, C. Yaemsiri, B. L. Innis, and C. H. Hoke. 1991. Sensitivity and specificity of a universal primer set for the rapid diagnosis of dengue virus infections by polymerase chain reaction and nucleic acid hybridization. Am J Trop Med Hyg 45(4):418-428.

Igarashi, A., H. Mohamed, A. Yusof, M. Sinniah, and H. Tanaka. 1995. Production of type 2 dengue (D2) monoclonal antibody and cell culture derived D2 antigen for use in dengue IgM capture ELISA. *Trop Med* 37(4):165-173.

Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G. Chang, and A. V. Vorndam. 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase polymerase chain reaction. *J Clin Microbiol* 30(3):545-551.

Manaloto, C. R. and C. G. Hayes. 1989. Isolation of dengue viruses from hospitalized patients in the Philippines, 1983-1986. *Southeast Asian J Trop Med Pub Hlth* 20(4):541-547.

Morita, K. 1994. Principle of PCR and its application for the diagnosis of dengue and Japanese encephalitis. *Trop Med* 36(4):228-234.

Saat, Z., R. Thayan, V. Balasubramaniam, K. Morita, H. Tsuchie, M. Sinniah, A. Igarashi, and H. Tanaka. 1994. Use of the reverse transcriptase polymerase chain reaction for diagnosis of dengue virus infection compared to IgM-ELISA. *Trop Med* 36(3):75-81.

Seah, C. L. K., V. T. K. Chow, and Y. C. Chan. 1995. Semi-nested PCR using NS3 primers for the detection and typing of dengue viruses in clinical serum specimens. *Clin & DiagnosticVirol* 4:113-120.

Thayan, R., B. Vijayamalar, S. Zainah, T. K. Chew, K. Morita, M. Sinniah, and A. Igarashi.1995. The use of polymerase chain reaction (PCR) as a diagnostic tool for dengue virus. *Southeast Asian J Trop Med Pub Hlth* 26(4):669-671.