Use of the Reverse Transcriptase Polymerase Chain Reaction for Diagnosis on Dengue Virus Infection Compared to IgM−ELISA

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Abstract: Applicability of the reverse transcriptase polymerase chain reaction (RT−PCR) was evaluated as a routine rapid diagnostic test for dengue virus infection. A total of 160 acute phase sera from patients with clinical diagnosis of dengue fever was examined both by the RT−PCR and IgM−ELISA. Of these, 9 (6%) were positive for both RT−PCR and IgM−ELISA, 61 were positive for IgM−ELISA only and 31 (19%) for RT−PCR only. Both techniques gave negative results in the remaining 59 (37%) specimens. The diagnostic efficiency of IgM−ELISA was statistically better than the RT−PCR even when the specimens were collected on the 3rd or 5th days of the disease. Considering the operational cost in the tests, the acute serum specimens should first be tested by the IgM−ELISA, followed by the RT−PCR on the negative specimens in order to increase the diagnostic efficiency with reasonable cost.

Key words: Dengue, RT−PCR, ELISA−IgM

INTRODUCTION

Since its first outbreak in Malaysia in 1902 (Skae, 1902), dengue virus infection remains to be a major problem in Malaysia and its prevalence has been increasing each decade. From 1956 to 1980, there were 5,163 cases as compared to 5,414 in 1981−1985, and 7,277 in 1986−1989 (data from Vector−Borne Disease Control (VBDC), Malaysia). In 1993 alone,

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5,589 dengue cases were reported with 23 deaths (VBDC, Malaysia, Annual Report, 1993). Hence, the availability of a rapid, sensitive, specific and economical laboratory diagnostic test is required to facilitate diagnosis, management, prevention and control of dengue.

Traditionally, the hemagglutination−inhibition (HI) test has been used in the laboratory diagnosis of dengue virus infection and still remains as the gold standard in the dengue serology. In this test, paired sera is needed which is not only inconvenient but also delays the diagnosis. Search for a serological test which can be carried out only with a single serum specimen for diagnosis led to the development of the IgM−capture ELISA for dengue (Burke 1983). Since then, several modifications of the technique have been reported (Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989).

Since 1991, the modified dengue ELISA−IgM (Lam *et al.*, 1987) has been introduced to the Arbovirus Laboratory, Virology Division of the Institute for Medical Research (IMR), Kuala Lumpur, to screen acute sera. In this test, a second sample is requested only when the first sample shows negative result. However, several studies have shown that IgM−ELISA is unable to pick up dengue IgM in acute phase sera from cases with primary infection (Lam *et al.*, 1987; Cardosa, 1989; Innis *et al.*, 1989). Hence, IgM−ELISA is not a satisfactory solution to the problem of rapid detection of dengue virus infection.

Around the early 1990s, several groups of investigators reported the application of RT−PCR to detect dengue viral genome (Deubel *et al.*, 1990; Morita *et al.*, 1991; Henchal *et al.*, 1991; Lanciotti *et al.*, 1992; Chungue *et al.*, 1993). Among them, the method by Morita *et al.* (1991) appeared to be simple enough to use for routine diagnostic method. This rapid RT−PCR method does not require any steps of RNA extraction because dengue virion can be solubilized by the nonionic detergent in order to expose genomic RNA. Secondly, all reagents reguired for the reverse transcription and PCR can be mixed together with the specimen in a single tube, in order to reduce the possibility of cross−contamination. Moreover, serotype of dengue viruses can be determined from the size of the PCR product which was amplified by the type−specific primer pairs to yield different size of the PCR product. This method was successfully applied to several recent dengue virus isolates from Thailand (Maneekarn *et al* ., 1993).

In this study, the usefulness of this rapid RT−PCR was evaluated as a routine and rapid diagnostic test for dengue virus infection.

Materials and Methods

Test sera: A total of 160 acute sera were collected in 1993 from patients admitted to the Kuala Lumpur General Hospital. These cases were referred to the Virology Division, IMR, by the clinicians as suspected of dengue virus infection. The diagnostic criteria was based on the patient's history and clinical findings suggestive of dengue virus infection, such as fever of more than 3 days duration, muscle and joint pains, skin rash, positive Tourniquet test and/or petechiae, thrombocytopenia. All sera were tested by the modified IgM−ELISA (Lam *et al.*, 1987) and the rapid RT−PCR (Morita *et al.*, 1991). A second (convalescent) sam-

ple was requested when the first sample was negative by the modified IgM−ELISA.

Modified IgM−ELISA: The procedure described by Lam *et al.* (1987) was followed. The 96−well microplate was coated with rabbit anti−human IgM (mu−chain specific). After emptying and washing the plate, the test and control sera diluted 1:100 were added into each duplicate wells and incubated at 37℃ for 1 hour. The wells on the plate were washed and 50 hemagglutinating units of dengue 2 antigen (prepared in suckling mouse brains) was added and incubated at 37℃ for 1 hour. After washing, the wells were reacted with mouse monoclonal antibody to dengue type 2 virus (MCA 3H5−1−21−B71541) which was kindly provided from the Centres for Disease Control, Atlanta, GA, USA, at 1:500 dilution. The plate was incubated at 37℃ for 1 hour, washed, and then reacted with 1:1000 dilution of a goat anti−mouse IgG conjugated with horseradish peroxidase which is free from cross−reactions with human immunoglobulins. After 1 hour incubation at 37℃, the plate was washed, and added with substrate solution containing *o*−phenylenediamine dihyrochloride and hydrogen peroxide. After 30 minutes incubation at room temperature in the dark, the reaction was stopped by adding 4N sulphuric acid. The optical density (OD) at 492 nm was measured by an ELISA reader with 550 nm as a reference wavelength. A positive: negative (P:N) ratio was obtained by dividing the OD_{492} of the test specimen by OD_{492} of the standard negative specimen. The specimen showing P:N ratio greater than or equal to 2.0 was considered positive, provided the standard positive specimen definitely showed positive result.

Rapid RT−PCR: The procedure described by Morita *et al.* (1991) was followed with a modification using a different primers (Lanciotti *et al.*, 1992). Five microliter of serum was treated with an equal volume of 1% Nonidet P−40 and 30 units of RNase inhibitor (Takara, Kyoto, Japan) in PBS (−) in a 0.5 ml volume Eppendorf type tube for 1 minute at room temperature. This was followed by the addition of 90 μl of RT−PCR mixture (100 pmoles of each primers as shown in Table 1, 0.2 mM each of the 4 deoxynucleotide

Table 1. Nucleotide sequences of the dengue virus consensus primers (Lanciotti *et al* ., 1992)

Primers	Sequences	Genome position
D1	5'−TCAATATGCTGAAACGCGCGAGAAACCG−3'	134−161
D2	5'−TTGCACCAACAGTCAATGTCTTCAGGTTC−3'	616−644

triphosphate, 10 mM Tris−HCl, pH 8.09, 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg/ml bovine serum albumin, 0.1% sodium cholate, 0.1% Triton X−100, 5 units of reverse transcriptase [Life Sciences, FL, USA], and 4 units of Tth DNA polymerase [Toyobo, Osaka, Japan]). The reaction mixture was covered with 2 drops of mineral oil, and incubated at 53℃ for 10 minutes for reverse transcription. Then the mixture was subjected to 30 cycles of denaturation at 94℃ for 1 minutes, annealing at 53℃ for 1 minutes, and DNA chain elongation at 72℃ for 1 minutes. The final annealing step was followed by a prolonged elongation step for 5 minutes. In addition to the test sera, following controls were included in the test: negative

control, reagents control, and positive control. The PCR product was analyzed by agarose gel electrophoresis and amplified bands were visualized by ethidium bromide staining. Detection of the PCR products with estimated size of 511 bp, by comparing with the Phix 174 (Hae Ⅲ) marker, was considered as positive RT−PCR.

RESULTS

The results of the IgM−ELISA and rapid RT−PCR on 160 serum specimens were shown in Table 2. Sixty three percent (101/160) of the acute sera were found to be positive for dengue virus infection. Of these, 9 (6%) were positive for both RT−PCR and IgM−ELISA, 31 (19%) for RT−PCR only, and 61 (38%) for IgM−ELISA only. Both techniques were found to be negative in the remaining 59 specimens (37%). Therefore, the IgM−ELISA could detect more dengue cases than the RT−PCR on acute sera from dengue suspected cases, and the difference was statistically significant with 0.5% risk level by the chi−square test.

Table 2. Number of specimens diagnosed as dengue virus infection by IgM−ELISA and RT−PCR on acute serum specimens

		IgM−ELISA (+)	IgM−ELISA (−)	Total
PCR	(+)	9	31	40
	(−)	61	59	120
total		70	90	160

$\chi^2=8.67$,
difference is statistically significant at 0.5% risk level

The second serum specimens were requested from the 90 patients whose acute sera were negative by the IgM−ELISA. However, the convalescent sera were obtained only from 41 cases (46%) for repeated IgM−ELISA. The results showed that 28 (68.3%) of them were positive for IgM−ELISA (Table 3). As shown in Table 3, RT−PCR had detected dengue

Table 3. Number of convalescent sera tested for dengue IgM−ELISA and the comparison of their results with the test results on acute sera

Acute sera			Convalescent sera		
Test results		No.	IgM−ELISA results		not received
IgM−ELISA	PCR		(+)	(−)	
(−)	(+)	31	17	1	13
(−)	(−)	59	11	12	36
Total		90	28	13	49

viral genome in 17 (41.5%) of these 41 cases which were re−examined by the IgM−ELISA on the convalescent serum. Excepting a single specimen which was still IgM−ELISA negative in the convalescent specimen, all 17 specimens which were negative IgM−ELISA but positive RT−PCR in the acute serum showed seroconversion in their convalescent serum specimen. This single case was confirmed as dengue virus infection by virus isolation (data not shown).

The data were rearranged to show the relationship between RT−PCR positive rate and the day of the disease when the serum specimens were collected, and the result was shown in Table 4. From the data, the diagnostic efficiency was considered to be higher for IgM−ELISA than the RT−PCR even on those specimens which were collected on the 3rd or 5th day of the disease.

Table 4. Diagnosis on dengue virus infection by RT−PCR and IgM−ELISA on acute serum specimens collected on different days of the disease

Day of the disease	RT−PCR*	IgM−ELISA*	χ^2	difference significant	risk level
1	1/ 2	0/ 2	1.33	no	
2	2/ 7	2/ 7	0	no	
3	10/50	19/50	3.93	yes	5%
4	18/51	19/51	0.04	no	
5	9/41	23/42	9.89	yes	0.5%
6	0/ 2	1/ 2	1.33	no	

*Figures represent the number of positive/number of tested

DISCUSSION

The rapid RT−PCR (Morita *et al.*, 1991) is a relatively simple test to perform compared to the conventional RT−PCR as described in the Text. The method was modified to use consensus primers homologous to conserved dengue virus RNA sequences in order to detect all serotype genome sequences with reduced cost in the reaction. Re−examination of the positive specimen using type−specific primer pairs can determine serotype of the infecting dengue virus. However, our result showed that detection rate of dengue virus infection was higher by the IgM ELISA than RT−PCR in the acute serum specimens. The reason for the apparent lack of sensitivity of the present RT−PCR could be due to the following factors: (1) cases were primarily from adult medical wards and would predominantly be the secondary infection which shows shorter viremic period than the primary infection (Lam *et al.*, 1987; Innis *et al.*, 1989; Cardosa, 1989), and (2) most of the sera were collected late during the course of the disease. Table 4 showed that 89% (143/160) of the specimens were collected from day 3 to day 5 of the disease. Chen *et al.* (1992) and Chan *et al.* (1993) showed that the highest detection rate of dengue virus infection by IgM−ELISA was in the convalescent phase, while the PCR was in the acute phase. Our result showed similar tendency, the

highest detection rate for PCR was day 1 (50%), although the number of specimen is small, The detection rate then generally decreased along with the day of the disease, reaching to 0% on day 6. While, detection rate by IgM−ELISA showed increasing trend starting from day 2 (28.5%) reaching to 54.7% on day 5.

Compared with IgM−ELISA which was estimated to cost 9 Malaysian dollars (RM) for a specimen, RT−PCR is manifold expensive (RM 70 for a specimen). While the time taken to perform rapid RT−PCR followed by agarose gel electrophoresis is 3 hours as compared with 5 hours for IgM−ELISA.

Followings are our conclusions: (1) RT−PCR is a rapid but expensive test, (2) acute serum specimens suspected of dengue should first be examined by the IgM−ELISA, (3) the negative IgM specimens should then be examined by the RT−PCR in order to increase the diagnostic efficiency with relatively reasonable cost.

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