

Application of IgM-capture ELISA on Myanmar Dengue Patients' Serum Samples Collected in 1994

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Abstract: Seventy serum samples from clinically diagnosed dengue patients admitted to the North Okkalapa General Hospital, Yangon, Union of Myanmar, in 1994, were used to obtain the confirmed virological diagnosis by IgM-capture ELISA using all four types of dengue antigens raised by cell culture method. Sixty-two out of 70 specimens (88.6%) showed positive IgM ELISA titer against one or more assay antigen. Single application of dengue 2 antigen could detect 59 out of 62 positive sera (95.1% of total positives), whereas three more samples could be detected by using other serotypic antigens (either single antigen or double antigens in combination). Ten samples showing positive by single dengue antigen but negative results by the rest could possibly be due to the primary infection which showed serotype specific immune response.

Key words: Dengue virus infection, IgM-capture ELISA, Myanmar

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) has first been introduced to the field of medical science in the 1970's (Engvall and Perlmann, 1971). Since the ELISA methods can be used to assay both antibodies and antigens, the wide applicability of these techniques are evident nowadays especially in the fields of infectious diseases (Sever and Madden, 1977; Voller and Ceska, 1978). The first use of ELISA for the measurement of viral antibodies was done by Voller and Bidwell (1975) on rubella virus. IgM-capture ELISA was proved to be an efficient diagnostic tool for the rapid diagnosis of dengue infections, due to its rapidity, simplicity, reproducibility, acceptable running cost and diagnostic capability even with a single serum specimen. Because of these characteristic features, it has been successfully used as a supplement and/or substitute to the pre-existing classical serological tests, namely complement-fixation (CF), hemagglutination-inhibition (HI) and neutralization (N) tests. Many researchers have utilized this technique for the last two decades (Burke, 1983;

Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989; Cardosa and Zuraini, 1991; Cardosa *et al.*, 1992). We do also apply the IgM-capture ELISA technique in this study on Myanmar dengue patients' serum samples using all four dengue serotypic antigens derived from cell culture methods, in order to confirm its diagnostic capability.

MATERIALS AND METHODS

Samples

Seventy serum samples from the patients clinically diagnosed as dengue virus infection, and admitted to the North Okkalapa General Hospital, Yangon, Union of Myanmar, in the period of June and July, 1994, were used in this study. The clinical diagnosis was made according to the WHO criteria (WHO, 1986).

Assay antigens production

Three Roux bottles of *Aedes albopictus*, clone C6/36 cells (Igarashi, 1978), and one Roux bottle of vero cells were grown in 40 ml/Roux bottle of Eagle's medium in Earle's saline supplemented with 0.2 mM each nonessential amino acids and 9% heat-inactivated fetal calf serum, at 28°C for C6/36 cells and at 37°C for vero cells. When 80% monolayers were obtained, growth media were removed from the cell cultures and 1.5 ml each of seed viruses {prototypes: Hawaiian strain for dengue type 1 virus (D1), New Guinea B strain for dengue type 2 virus (D2), H-87 strain for dengue type 3 virus (D3) and H-241 strain for dengue type 4 virus (D4)} were inoculated. Adsorption was carried out for 2 hours at 28°C and 37°C for C6/36 cells and vero cells, respectively, and spreading the inoculum over cell sheet every 30 minutes. After adsorption, 40 ml of maintenance medium (the same growth medium in which serum concentration was reduced to 2%) was poured into each culture bottle. Then D1 culture bottle was incubated at 32°C for 5 days, D2 culture bottle at 37°C for 5 days, D3 culture bottle at 28°C for 7 days, and D4 culture bottle at 37°C for 7 days. After the infected culture fluid of D1, D2, D3 and D4 viruses were harvested accordingly, the antigen detection ELISA was performed by micro sandwich method of Voller *et al.* (1976). The antigen ELISA titers of the harvested culture fluid were estimated by comparing their ELISA-OD with those of serially diluted standard positive specimens with predetermined endpoint titer (Igarashi *et al.*, 1981; Morita *et al.*, 1982). The titers of harvested culture fluid were found to be 16, 64, 3 and 4 ELISA units for D1, D2, D3 and D4 viruses, respectively. These culture fluids were stored at 4°C until using as assay antigens for the further experiments.

IgM-capture ELISA

IgM-capture ELISA was performed as described previously (Bundo and Igarashi, 1985). Incubation at room temperature for 1 hour and reagent volume of 100 µl/well for each reaction step were applied unless otherwise specified. The 96-well flat bottom microplates (Nunc, Denmark) were coated with anti-human IgM (µ-chain specific) goat IgG (Cappel,

PA, USA) diluted 1: 200 with 0.05M carbonate–bicarbonate buffer, pH 9.6, containing 0.01% NaN_3 , and incubated at 4°C overnight. The plates were inactivated by Block Ace (Yukijirushi, Japan), followed by washing with PBS–Tween (0.05% Tween 20 and 0.01% NaN_3 in PBS) four times without incubation, using a ICN Titertek microplate washer (Flow, USA). Test sera, known dengue IgM positive and negative control sera were diluted 1: 100 with PBS–Tween, and added to duplicate wells according to the set up protocol. The plates were washed as above. D1, D2, D3 and D4 antigens were added to the duplicate wells. After washing as above, the plates were reacted with horseradish peroxidase (HRPO)–conjugated anti-flavivirus IgG at 1:100 dilution in PBS–Tween. This reagent was prepared by conjugating the anti-flavivirus IgG with HRPO (Sigma, type VI, USA), using Wilson and Nakane's method (1978). After washing as above, color reaction was carried out by the addition of substrate solution of 0.5mg/ml *o*–phenylenediamine dihydrochloride (OPD) and 0.02% H_2O_2 in 0.05M citrate phosphate buffer, pH 5. After 1 hour incubation in the dark at room temperature, the reaction was stopped by adding 100 μ l of 1N sulphuric acid to each well. The OD_{492} on each well was recorded by an ELISA reader using 620nm as a reference wavelength. The samples were determined as positive when their ELISA–OD is twice or more than that of the cut–off value calculated from the average reading of the negative control sera (P/N ratio ≥ 2.0).

RESULTS AND DISCUSSION

Positive case detective capability of four different types of dengue antigens by IgM–capture ELISA on Myanmar dengue patients' serum samples was shown in Table 1. Out

Table 1. Dengue positive case detection by IgM–ELISA on Myanmar dengue patients' serum samples using different assay antigens

Assay antigens				Samples showing response	% to total test sera (70)
D1	D2	D3	D4		
+	+	+	+	29	29/70 (41.4%)
+	+	+	–	13	14/70 (20%)
+	+	–	+	1	
+	+	–	–	3	9/70 (12.9%)
+	–	+	–	1	
+	–	–	+	1	
–	+	+	–	3	
–	+	–	+	1	
–	+	–	–	9	10/70 (14.3%)
–	–	+	–	1	
–	–	–	–	8	8/70 (11.4%)

(Source of the samples: North Okkalapa General Hospital, Yangon, Union of Myanmar, in 1994)

of 70 serum samples tested, 62 samples (88.6%) showed positive IgM-ELISA titer against one or more assay antigen. Twenty-nine samples (41.4%) showed positive with all four assay antigens, whereas 14 samples (20%) and 9 samples (12.9%) were positive with triple and double antigens, respectively. There is no single specimen which was positive by either D1 or D4 antigen, but negative by other triple antigens. There are 9 specimens which were positive by D2 antigen but negative by other antigens, while single specimen showed positive by D3 antigen with negative results of the rest. Eight samples (11.4%) showed negative by all four types of assay antigens.

Even using only D2 antigen could detect 59 out of 62 positive sera (i. e. 95.1% of total positives) (Table 2). This is a reasonably high sensitivity rate which is essential for routine diagnostic assays. By using other serotypic antigens (either single antigen or double antigens in combination), three more serum samples could be detected. This additive role of serotypic antigens other than D2 antigen is an interesting outcome of this study. Nine samples showing positive by single D2 antigen, and one sample showing positive by single D3 antigen (Table 1) could possibly be due to the primary infection which showed serotype specific immune response.

Table 2 shows that 64 ELISA unit D2 antigen could detect 95.1% of total positive sera, whereas 16 unit D1 antigen, 3 unit D3 antigen and 4 unit D4 antigen could detect 77.4%, 75.8% and 51.6% of total positive sera, respectively. It might be due to the fact that high titered dengue antigen could detect the higher number of serum samples than the low titered antigens do. On the other hand, it might possibly reflect the distribution pattern or the prevalence of dengue serotypes in Yangon community in 1994.

Since IgM-capture ELISA method has been used to detect dengue virus infection in Myanmar patients' serum samples (May La Linn *et al.*, 1985; Khin-Mar-Aye *et al.*, 1995), and to detect chikungunya virus infection in Yangon, Myanmar (Soe Thein *et al.*, 1992), our recent study serves as an additional indicator for its application in Myanmar.

Table 2. Dengue positive case detection by IgM-ELISA using different serotypic antigens raised by cell culture methods

Assay antigen	ELISA unit	Positive samples	% to total positive sera (62)	% total test sera (70)
D1	16U	48	77.4%	68.5%
D2	64U	59	95.1%	84.2%
D3	3U	47	75.8%	67.1%
D4	4U	32	51.6%	45.7%

(Source of the samples: North Okkalapa General Hospital, Yangon, Union of Myanmar, in 1994)

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