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Positive Case Detection of Anti-Dengue IgM Antibodies by IgM-Capture ELISA Using Monovalent and Tetravalent Dengue Antigens Prepared as Infected C6/36 Cell Culture Fluid

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Abstract: In order to improve the procedures in the IgM-capture ELISA which has recently been introduced to the dengue serodiagnosis, positive case detection rate in the assay on dengue patients' sera was compared using monovalent and tetravalent antigens prepared as infected C6/36 cell culture fluid. The results showed that the serotype of antigen which gave the highest positive case detection rate depended on the geographical area where the test sera were collected, and also that there were some sera which were scored as positive by monovalent antigen but were negative by the tetravalent antigen, and *vice versa*. Therefore, it is recommended that the test sera should first be examined by the tetravalent antigen, and the negative specimens should be re-examined with monovalent antigens in order to obtain highest positive case detection.

Key words: Dengue, IgM-ELISA, antigens, positive case detection

INTRODUCTION

Dengue virus infection could cause a spectrum of clinical diseases, from mild undifferentiated fever, dengue fever (DF), to dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) which could be fatal if not properly treated. Dengue virus infection has become a serious health problem in worldwide tropical areas, because of (1) increased number of patients, (2) expansion of epidemic areas, and (3) appearance of DHF/DSS (Halstead, 1993; Gubler, 1996). Laboratory diagnosis on dengue has most frequently been carried out by the serology particularly by the hemagglutination-inhibition (HI) test (Clarke and Casals, 1958; World Health Organization, 1986). The test has gradually been replaced or supplemented by the IgM-capture ELISA (Burke, 1983; Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989). The assay antigens in these tests have been prepared from infected suckling mouse brains by extraction using large quantities of organic solvent (Clarke and Casals, 1958). In order to simplify these procedures, the first author has been trying to prepare dengue antigens as infected C6/36 cell culture fluid. Previous studies using prototype dengue virus

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strains have shown that type 1 dengue (D1) antigen could be produced to high titer (Soe Thein *et al.*, 1979), and that type 2 dengue (D2) antigen production was increased by elevating incubation temperature of the infected C6/36 cell cultures (Mohamed *et al.*, 1995). Later studies showed that by selecting appropriate strains, type 3 dengue (D3) and type 4 dengue (D4) as well as D2 antigens could be produced to high titer in the infected C6/36 cell cultures at 28℃ (Igarashi *et al.*, unpublished).

The present paper describes the positive case detection of anti-dengue IgM antibodies in dengue patients' sera using monovalent and tetravalent dengue antigens in order to find out conditions for better performance of dengue IgM-capture ELISA

MATERIALS AND METHODS

Dengue virus strains: Following strains were used: D1: Hawaiian (prototype); D2: ThNH29/93 (isolated in Nakhon Phanom, Thailand, 1993, from a DHF case); D3: InJI-6/82 (isolated in Jakarta, Indonesia, 1982, from a DHF case); D4: CT93−158 (isolated in Bangkok, Thailand, 1993, from a DHF case). Each strain was inoculated to C6/36 cell culture and infected culture fluid was harvested after 7 days incubation at 28℃ to prepare seed viruses.

Cell culture and dengue antigen production: *Aedes albopictus* clone C6/36 cell line (Igarashi, 1978) was grown at 28℃ in Eagle's medium in Earle's saline supplemented with 10% heat-inactivated fetal calf serum (FSC) and 0.2 mM each nonessential amino acids, using 50ml for each Roux bottle. Growth medium was removed from C6/36 cell culture and seed virus was inoculated, followed by 2 hours virus adsorption at 28℃ spreading the inoculum over the cell sheet every 30 minutes. The cells were covered by the maintenance medium (the growth medium from which FCS concentration was reduced to 2%) using 45ml/Roux bottle, and incubated at 28℃ for 7 days. The infected culture fluid was harvested and kept at 4℃ as dengue antigen without further purification.

Sandwich ELISA to assay dengue antigen titer: The principle of Voller *et al.* (1976) was followed with necessary modifications using 100μl/well reagents and 1 hour reaction at room temperature for each step, followed by 3 washings by PBS-Tween with 3 minutes intervals unless otherwise specified. The 96-well microplate was coated with anti-flavivirus IgG (20μg/ml), which had been purified from high titered DHF patient's sera by DEAE Sephacel column chromatography. After washing, test specimens and standard antigen diluted in serially 2-fold steps were distributed in duplicate wells for each sample. After incubation and washing, horseradish peroxidase (HRPO)-conjugated anti-flavivirus IgG at 1: 1000 dilution in PBS-Tween was distributed to all wells except blanks. The conjugation of HRPO to anti-flavivirus IgG was carried out according to Wilson and Nakane's method (1978). After the reaction and washing, substrate solution containing *o*-phenylenediamine and hydrogen peroxide was distributed to all wells for HRPO reaction to proceed in the dark. The reaction was stopped by adding 1N sulfuric acid and OD_{492} was recorded by a microplate ELISA reader. The ELISA titer of test specimen was estimated by comparing its ELISA-OD with those of serially diluted standard antigen (Igarashi *et al.*, 1981; Morita *et al.*, 1982).

Dengue IgM-capture ELISA: The method was essentially as described before (Bundo and Igarashi, 1985). The 96-well microplate was coated with anti-human IgM (μ-chain specific) goat IgG (Cappel, USA) at 4℃ overnight, followed by blocking with Blockace (Yukijirushi, Japan). After washing, the plate was distributed with test specimens as well as standard positive and negative sera at 1: 100 dilution in PBS-Tween. After the reaction and washing, the assay antigen diluted to 25 ELISA units was distributed, based on our previous studies showing that antigen titer over 16 ELISA units is required for reasonably efficient positive case detection (Khin Mar Aye *et al.,* 1995). The plate was washed and reacted successively with HRPO-conjugated anti-flavivirus IgG, substrate solution, stopping with sulfuric acid, and OD reading as described for the sandwich ELISA. The P/N ratio was calculated for each specimen by dividing its ELISA-OD by the ELISA-OD of the standard negative serum. The specimen with P/N ratio equal to or greater than 2.0 was considered as positive.
Test sera: The specimens were kindly supplied from the St. Luke's Medical Center, Metro Manila, the Philippines (21 sera), the North Okkalapa General Hospital, Yangon, Union of Myanmar (95 sera), and Udagaya University, Bali, Indonesia (115 sera).

RESULTS

Positive case detection of test sera using each of the 4 types of monovalent dengue antigen (D1, D2, D3, D4) each at 25 ELISA units, as well as tetravalent antigen (D1+D2+D3+D4) which contains all 4 types antigen at 25 ELISA units, was shown in Tables 1 (Philippine sera), 2 (Myanmar sera), and 3 (Indonesia sera), respectively. The results showed that many sera, which were scored as positive by any of the 4 types of monovalent antigen also showed positive results by the tetravalent antigen (18/20 or 90% of Philippine sera; 65/67 or 97% of Myanmar sera; and 68/85 or 80% of Indonesia sera, respectively). It was unexpected that some sera were scored negative by the tetravalent antigen although they

Table 1. Detection of anti-dengue IgM antibodies in the Philippine dengue patients' sera by the IgM-capture ELISA using monovalent and tetravalent antigens

Assay antigen					Number of specimens
D1	D2	D3	D4	Tetravalent	
+	+	+	+	+	6
-	+	-	+	+	5
-	+	-	-	+	4
-	+	+	+	+	2
-	+	-	-	-	2
-	-	-	-	-	1
+	+	-	+	+	1

Table 2. Detection of anti-dengue IgM antibodies in Myanmar dengue patients' sera by the IgM-capture ELISA using monovalent and tetravalent antigens

Assay antigen					Number of specimens
D1	D2	D3	D4	Tetravalent	
+	+	+	+	+	21
-	-	-	-	-	28
+	+	+	-	+	17
-	+	-	-	+	8
-	+	+	-	+	7
-	-	+	-	+	5
-	+	+	+	+	2
+	+	-	-	+	2
+	+	+	+	-	1
+	-	-	-	+	1
+	+	-	-	+	1
-	+	+	+	+	1
+	-	-	-	-	1

Table 3. Detection of anti-dengue IgM antibodies in Indonesia dengue patients' sera by the IgM-capture ELISA using monovalent and tetravalent antigens

Assay antigen					Number of specimens
D1	D2	D3	D4	Tetravalent	
+	+	+	+	+	23
-	-	-	-	-	28
-	-	+	-	+	16
+	+	+	-	+	8
-	-	+	-	+	7
-	+	-	-	-	6
-	-	+	-	-	4
-	+	+	+	+	4
-	+	+	-	+	3
+	-	+	-	-	3
+	+	+	-	-	2
+	-	+	-	+	2
-	-	-	-	+	2
+	-	+	-	-	1
-	-	+	+	+	1
-	-	+	+	-	1
-	+	-	-	+	1
+	-	-	-	+	1
+	-	+	+	+	1
-	-	+	+	+	1

were positive by the monovalent antigen(s). The number and percentage of such sera were: 2 (9.5%) for Philippine sera, 2 (2.1%) for Myanmar sera, and 17 (14.7%) for Indonesia sera, respectively. On the other hand, 2 (1.7%) Indonesia sera, which showed negative results by any of the 4 types of monovalent antigen, were scored positive by tetravalent antigen. Therefore, it is recommended to examine test specimens first by the tetravalent antigen, and re-examine negative specimens by monovalent antigens in order to obtain better positive case detection rate.

The positive case detection of anti-dengue IgM antibodies by monovalent and tetravalent antigens was summarized in Table 4. The antigen which gave the highest detection rate depends on the location where the test sera were collected: D2 for Philippine, tetravalent for Myanmar, and D3 for Indonesia, respectively. The results could very probably reflect predominant serotypes of dengue virus circulating in each area.

Table 4. Positive case detection of anti-dengue IgM antibodies by IgM-capture ELISA using monovalent and tetravalent dengue antigens

	Location where the sera were collected		
Antigen	Philippines	Myanmar	Indonesia
D1	7 (37.3%)	44 (46.3%)	41 (35.7%)
D2	20 (95.2%)	60 (63.2%)	47 (40.9%)
D3	8 (38.1%)	54 (56.8%)	77 (67.0%)
D4	14 (66.7%)	25 (26.3%)	31 (27.0%)
Tetravalent	18 (85.7%)	65 (65.4%)	70 (60.9%)
No. of specimens	21	95	115

DISCUSSION

Faced with worldwide expansion of dengue, its laboratory diagnosis is required to provide accurate information on causative agents, to the clinicians for proper case management, to the epidemiologists for better understanding the epidemic situation, and to health authorities for planning and implementing effective control measures. Because of its relative simplicity, rapidity, and reasonable cost, IgM-ELISA is the method of the 1st choice for the laboratory diagnosis on dengue. However, it requires 2 key reagents, assay antigen and HRPO-conjugated anti-flavivirus IgG. The results in the present paper showed that dengue antigens which were prepared as infected C6/36 cell culture fluid could successfully be used in the test. The test specimens should first be examined by the tetravalent antigens followed by testing negative specimens with monovalent antigens in order to obtain better positive case detection rate. From the biosafety standpoint, it is recommended to inactivate infective virus in the antigen without losing its antigenicity to detect IgM antibodies. Our recent study

to disrupt dengue virion by nonionic detergents, such as Nonidet P-40 or Triton X-100, resulted in significant reduction in the antigenicity to detect IgM antibodies, although the antigen titer measured by the sandwich ELISA was not so much affected (Maha and Igarashi, 1997). Further studies are required to overcome this problem.

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