Optimization of the Dengue-2 Antigen ELISA Titer
for the Positive Case Detection of Dengue Virus Infection
by IgM ELISA

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Abstract: We have determined the dengue-2 antigen ELISA titer for the positive case detection of dengue virus infection by IgM ELISA using 33 paired serum samples of dengue patients admitted to the North Okkalapa General Hospital, Yangon, Union of Myanmar, in June and July, 1994. The antigen ELISA titer was found to be over 32 units in order to find out sufficient number of positive cases. This finding can be used as a reference for further experiments and the development of tetravalent dengue antigen for routine serodiagnosis of dengue virus infection.

Key words: Dengue virus infection, Antigen ELISA titer, Dengue-2 IgM ELISA

INTRODUCTION

Dengue virus infection draws attention to many researchers as being an enigmatic and growing public health problem in many tropical countries especially those of Southeast Asia (Halstead, 1980 & 1988). Confirmed laboratory diagnoses obtained either by virus isolation or serological diagnoses, are invaluable not only for the clinicians but also for the epidemiologists and the health administrators in treating and controlling this disease (Igarashi, 1994). Three conventional methods have been utilized in viral serology for long time; namely complement-fixation (CF), hemagglutination inhibition (HI) and neutralization (N) tests. Among these three methods, HI test has most widely been used as routine test because of its relative sensitivity and easiness to perform. All three classical serological methods require paired sera which were collected at acute and convalescent phase with appropriate interval in order to demonstrate significant rise in the antibodies. Since 19S or IgM-class antibodies are produced earlier and more specific than the 7S or IgG antibodies (Westaway, 1968) and persists transiently, the assay on IgM-antibodies was introduced to the viral serology with a potentiality to provide results even with a single serum specimen. The application of radioimmunoassay (Burke and

Nisalak, 1982), followed by enzyme−linked immunosorbent assay (ELISA) on IgM−antibodies (Bundo and Igarashi, 1985; Lam *et al.,* 1987; Innis *et al.,* 1989; Cardosa and Zuraini, 1991; Cardosa *et al.,* 1992) has gradually replaced or supplemented classical HI test.

The availability and the applicability of the high titered cell culture derived dengue anti-gens, as alternatives to the hemagglutinins derived from suckling mouse brain, have been studied by several workers (Soe Thein *et al.,* 1979; Cardosa and Zuraini, 1991; Mohamed *et al.,* 1995). In connection with these studies, we have performed the present study to determine the antigen ELISA titer of dengue type 2 virus (D2) for the positive case detection of dengue virus infection by D2 antigen.

MATERIALS AND METHODS

Samples

Thirty three paired serum samples of dengue patients admitted to the North Okkalapa General Hospital, Yangon, Union of Myanmar, in the period of June and July, 1994, were col-lected. These samples were primarily screened out to be dengue infection by hemag-glutination−inhibition test (HI) test (Clark and Casals, 1958).

Assay antigen production

Aedes albopictus, clone C6/36 cells (Igarashi, 1978) 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℃. When 80% monolayer was obtained, growth medium was removed from the cell culture and 1.5ml of seed virus (dengue type 2 virus, prototype New Guinea B strain) was inoculated. Adsorption was carried out for 2 hours at 28℃ spreading the inoculum over cell sheet every 30 minutes. After adsorption, 40ml of maintenance media (the same growth medium in which serum concentration was reduced to 2%) was poured into the culture bottle and incubated at 37℃. Infected culture fluid was harvested 5 days after infection, and the antigen detection ELISA was performed by micro sandwich method of Voller *et al.* (1976). The antigen ELISA titer of the harvested culture fluid was estimated by comparing their ELISA−OD with those of serially diluted standard positive specimens with predetermined en-dpoint titer (Igarashi *et al.,* 1981; Morita *et al.,* 1982). The harvested culture fluid of 64 ELISA unit was chosen as standard assay antigen for the further experiments, and stored at 4℃.

IgM−ELISA

IgM capture ELISA was performed as described previously (Bundo and Igarashi, 1985). Incubation at room temperature for 1hour and reagent volume of 100μl/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% NaN₃, and incubated at 4℃ overnight. The plates were inactivated by Block Ace (Yukijirushi, Japan), followed by washing with PBS−Tween (0.05% Tween 20 and 0.01% NaN₃ 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 ac-

cording to the set up protocol. The plates were washed as above. Dengue$-$2 virus antigen (64 ELISA unit) was 2 fold serially diluted with mock$-$infected culture fluid as a diluent, to prepare viral antigens with 32, 16, 8 and 4 ELISA units, and were added to the duplicate wells. After washing as above, the plates were reacted with horseradish peroxidase (HRPO)$-$conjugated antiflavivirus IgG at 1:100 dilution in PBS$-$Tween. This reagent was prepared by conjugating the antiflavivirus 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 0.05 M citrate phosphate buffer, pH5. After 30 minutes$-$1hour incubation in the dark at room temperature, the reaction was stopped by adding 100μl of 1N sulphuric acid to each well. The OD_{492} on each well was measured by an ELISA reader using 620nm as a reference wavelength. The samples were determined as positive when their ELISA$-$OD is twice or more than that of the cut$-$off value calculated from the average reading of the negative control sera (P/N ratio$\geq$2.0).

RESULTS AND DISCUSSION

Table 1. and Fig. 1 show the correlation between antigen ELISA units used for the D2 IgM ELISA and the number and percentage of positive samples detected. When the titer of D2 ELISA antigen was reduced from 64 units to 32 U, 16U, 8U and 4U, the percentage of positive samples (both acute and convalescent sera) detected was also reduced from 86 to 79, 62, 52 and 26, respectively. There was a dose dependent relationship between these two parameters, that is, highest titer of DEN$-$2 ELISA antigen could detect largest number of positive samples. Lowering the titer of ELISA antigen especially below 32 units leads to significant reduction in the number of positive samples detected. Therefore it is important to determine the best antigen ELISA titer for the most efficient positive case detection in dengue virus infection. Best antigen ELISA titer determination based upon two factors: (1) acceptably high positive case detection rate and (2) producibility of such high titered antigen by cell culture methods. According to our results, we would like to suggest that antigen ELISA titer of 32 units or more should be used. Preliminary results of the similar experiments using other dengue serotype antigens also indicated the dose dependent fashion as mentioned above (data not shown).

Table 1 Dengue positive case detection by dengue$-$2 IgM ELISA using 33 paired serum samples from Myanmar in 1994.

Antigen ELISA units used for detection	Acute sera positive	Convalescent sera positive	Total sera positive
64	28/33 (85%)	29/33 (88%)	57/66 (86%)
32	26/33 (79%)	26/33 (79%)	52/66 (79%)
16	20/33 (61%)	21/33 (64%)	41/66 (62%)
8	17/33 (52%)	17/33 (52%)	34/66 (52%)
4	6/33 (18%)	11/33 (33%)	17/66 (26%)

Cut off value: P/N ratio$\geq$2.0
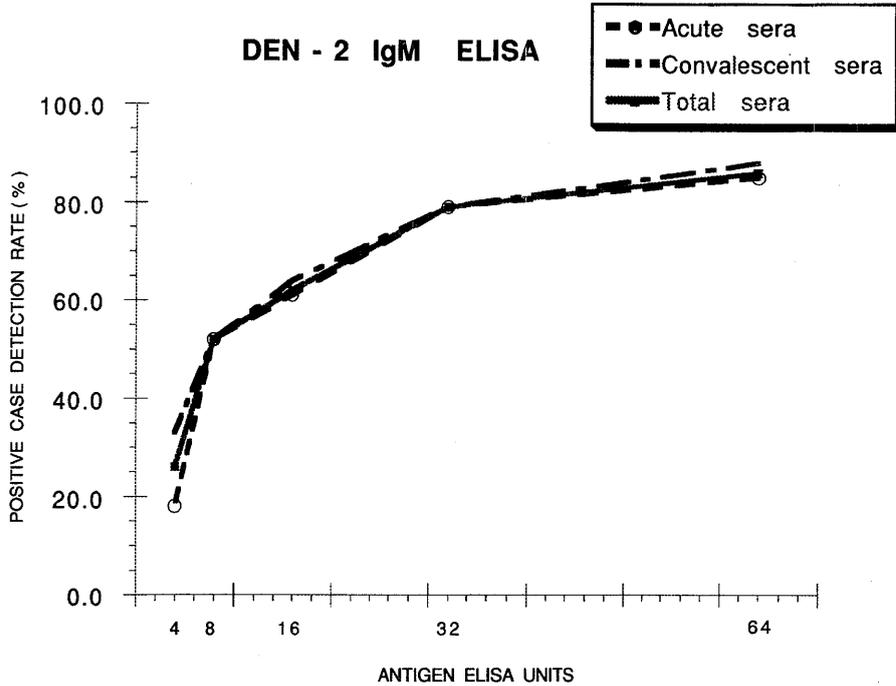
Fig. 1. Correlation between antigen ELISA units and dengue positive case detection rate by dengue−2 IgM ELISA using 33 paired serum samples from Myanmar in 1994.

In our study, only acute serum showed positive for IgM ELISA in two pairs of sera, whereas only convalescent serum showed positive in three pairs. In general, there was no marked difference in positive case detection rate between acute sera and convalescent sera, as well as combination of both (acute and convalescent sera), especially with high titers of ELISA antigen. It indicates that only a single serum specimen (either acute or convalescent serum) can provide the sufficient evidence for the positive dengue infection detected by IgM−capture ELISA.

May La Linn *et al.* (1985) reported that IgM capture ELISA has advantages over the HI in terms of its diagnostic efficiency, rapidity, simplicity, capacity of getting results by single serum specimen and differential diagnosis with other related flavivirus infections like Japanese encephalitis. They proposed to perform IgM capture ELISA on a single serum using only D4 antigen without losing so many positive cases.

In this study, we used single assay antigen (D2 antigen) for IgM capture ELISA, and achieved the positive case detection rate of over 80% by using 64 units of ELISA antigen. The higher positive case detection rate might possibly be achieved by using all four types of dengue antigens. However, our recent finding can be used as a reference for further experiments and formulating the tetravalent dengue antigen for routine clinical diagnosis using cell culture derived dengue antigens.

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