Dengue IgM–capture ELISA Using Heat–inactivated and Unheated Control Antigens Prepared as Infected C6/36 Cell Culture Fluid

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Abstract: A panel of 91 dengue patient's sera were examined in the IgM–capture ELISA using each of the 4 types of dengue antigens prepared as infected C6/36 cell culture fluid, with or without brief heat inactivation at 56℃. The result did not show any significant difference between the results obtained by both antigens. Therefore, dengue antigens for serodiagnosis by the IgM–capture ELISA could be prepared as infected C6/36 cell culture fluid with brief heat–inactivation for biosafety requirement.

Key words: Dengue, IgM–ELISA, antigen, heat–inactivation

Introduction

Dengue is a serious public health problem in worldwide tropical areas, with epidemics involving hundred thousands of people in tropical Asia, Australia and the Americas where *Aedes aegypti* is present. Dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) have emerged as the most important arthropod-borne viral diseases of human (Monath & Heinz, 1996; World Health Organization, 1997). The continent of South America was reinvaded by *Aedes aegypti* resulting in large outbreaks and establishing endemic pattern of dengue infections (Pan American Health Organization, 1996).

As for other viral diseases, laboratory diagnosis on dengue can be achieved by virus isolation and serology (Igarashi, 1994). Although virus genome detection by the reverse transcriptase-polymerase chain reaction has significantly improved to detect causative agents, serology has still most frequently been used in the laboratory diagnosis on dengue. Classical serodiagnosis depends on the demonstration of four fold or greater antibody titer rise in paired sera, by the hemagglutination-inhibition (HI), complement fixation (CF), or neutralization (N) tests (Clarke & Casals, 1958; Russell & Nisalak, 1967). The method has gradually

been replaced or supplemented by the IgM ELISA, which could provide diagnosis even for a single serum specimen (Burke, 1983; Bundo and Igarashi, 1985; Lam et al., 1987; Innis et al., Vorndam & Kuno, 1997). Assay antigen for serodiagnosis on dengue and other arthropod-borne viruses was originally prepared from infected suckling mouse brains (Clarke & Casals, 1958). The method requires animal handling and extraction with a large quantity of flammable organic solvents.

Our previous studies showed that all 4 serotypes of dengue antigens could be produced as infected C6/36 cell culture fluid with sufficient titer for use in serological tests if appropriate strains were used (Kyaw-Zin-Thant, et al., 1995; Igarashi & Antonio, 1997). Our unpublished studies also showed that the virus infectivity in the infected culture fluid could be inactivated by brief heating at 56℃ without losing its antigenicity or reactivity in IgM-capture ELISA

The objective of this study was to evaluate the dengue antigens, which were prepared as infected C6/36 cell culture fluid and their infectivity was removed by brief heating, for use in the IgM-capture ELISA on a panel of dengue patient's sera.

MTEARIALS AND METHOD

Serum specimens: Ninety one single serum specimens, which had been obtained from patients clinically diagnosed as dengue, in Yangon, Union of Myanmar (Burma), 1994, were used in this study (Khin-Mar-Aye et al., 1995; Kyaw-Zin-Thant et al., 1996).

Cell culture and production of dengue antigens: Aedes albopictus clone C6/36 cell line (Igarashi, 1978) was grown at 28℃ with cell growth medium which is composed of Eagle's medium in Earle's saline supplement with 0.2 mM each nonessential amino acids and 9% heat inactivated fetal calf serum (FSC), in Roux bottles. When the cells grew up to 80% confluence, growth medium was removed and 1 ml of seed virus was inoculated in each bottle. Following dengue stains were used in this study: dengue type 1 (D1) Hawaiian, dengue type 2 (D2) ThNH29/93, dengue type 3 (D3) InJ-I6/82, dengue type 4 (D4) No. 17, respectively. Adsorption was carried out for 2 hours at 28℃, spreading the inoculum over the cell sheet every 30 minutes. Then, the cells were covered by the maintenance medium (cell growth medium from which FCS concentration was reduced to 2%), and were incubated at 28℃. Afer 7 days, the infected culture fluids were collected and kept at 4℃ as dengue antigens. BHK21 cells were grown at 37℃ using the same growth medium as C6/36 cells.

Sandwich ELISA to assay antigen titer: Micro sandwich method of Voller et al. (1976) was used with some modifications (Igarashi et al., 1981; Morita et al., 1982), by incubation at room temperature for 1 hour and reagent volume of 100 μl/well for each reaction step. A 96 well ELISA flat bottom plate (Nunc, Denmark) was coated with anti-flavivirus IgG (20 μl/ml) in coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.0) at 4℃ overnight. The plate was inactivated with Blockace (Yukijirushi, Japan). After washing with PBS-Tween 3 times

for 3 minutes each, test specimens, standard D2 antigen in serial 2-fold dilution, and negative control (maintenance medium alone) were distributed in duplicate wells. The plate was incubated and washed as above, and horseradish peroxide (HRPO)-conjugated antiflavivirus IgG (1: 1000 dilution in PBS-Tween) was distributed into all wells except the blanks. Unbound conjugate was washed off as above, and the plate was incubated with the substrate solution (0.5 mg/ml o-phenylendiamine dihydrochloride and 0.02% hydrogen peroxide in 0.05 M citrate-phosphate buffer, pH 5.0) for 1 hour in the dark. The reaction was stopped by adding 1 N sulfuric acid, and optical density (OD) at 492 nm was measured using ELISA Microplate reader. Antigen titer of the test specimen was determined by comparing its ELISA-OD with those of serially diluted standard D2 antigen with predetermined endpoint titer (Igarashi et $al.$, 1981; Morita et $al.$, 1982).

IgM-capture ELISA: The procedures were as described before (Bundo and Igarashi, 1985) with similar incubation time and reagent volume as sandwich ELISA unless otherwise specified. ELISA plates were sensitized with anti-human IgM (μ-chain specific) goat IgG (Cappel, USA) diluted 1: 200 in the ELISA coating buffer overnight at 4℃. After blocking and washing, test sera, along with the standard positive and negative sera (all diluted at 1:100 in PBS-Tween), were distributed. After incubation and washing, heat-inactivated and unheated dengue antigens diluted to 25 ELISA units were distributed (Khin-Mar-Aye et $al.$, 1995; Igarashi and Antonio, 1997). After incubation and washing, HRPO-conjugated anti-flavivirus IgG diluted to 1:1000 in PBS-Tween was distributed to all wells except blanks. The plate was incubated and washed as above, and HRPO reaction and plate reading were carried out as in the case of sandwich ELISA. The P/N ratio was calculated for each specimen by dividing its ELISA OD with that of the negative control. The specimens showing P/N ratio equal to or greater than 2.0 were considered as positive.

Infectivity assay of dengue viruses: Focus titration of Okuno et $al.$ (1985) was used with some modifications. Dengue virus specimens were serially diluted in 10-fold steps in virus diluent (cell growth medium from which FCS concentration was reduced to 5%). BHK21 cells were grown on 96 well microplate using 100 μl cell suspension/well and incubated in 5% CO_2-atmosphere. When the cells grew up to 80% confluence, growth medium was removed by suction and diluted virus specimens were inoculated using 50 μl/well volume. Adsorption was carried out at 37℃ for 2 hours in 5% CO_2-atmosphere, spreading the inoculum over cell sheet every 30 minutes. The cells were then covered by 100 μl/well overlay medium (0.5% methylcellulose 4000 in the maintenance medium), and incubated as above for 3 days for D2 and D4, while D1 and D3 infected cells were incubated for 4 days. Dengue virus antigens in the cytoplasm of infected BHK21 cells were visualized by immunoperoxidase staining. Overlay medium was removed from BHK21 cells on the plate and the plate was rinsed with PBS(+). The cells were fixed with 5% formaldehyde in PBS for 20 minutes at room temperature. After rinsing with PBS, the cells were permealized with 0.1% Nonidet P40 in PBS for 20 minutes at room temperature. After blocking with Blockace solution for 30 minutes,

and rinsed with PBS, the cells were successively reacted with DHF patient's serum at 1:500 dilution in PBS followed by HRPO-conjugated antihuman IgG (Cappel, USA) at 1:500 dilution in PBS, for 1 hour each. Afer washing, the HRPO reaction was carried out for 5 minutes using the substrate solution containing 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.02% hydrogen peroxide. The plate was rinsed with tap water and foci of brown colored cells were counted using a magnifying lens. The infectivity titer was expressed as focus forming unit (FFU)/ml.

<div align="center">RESULTS</div>

Heat inactivation of dengue virus infectivity and antigenicity in the infected C6/36 cell culture fluid

Aliquots of dengue virus-infected C6/36 cell culture fluid were heated at 56℃ and specimens were cooled on ice after every minutes up to 5 minutes heating. Residual virus infectivity and antigenicity were determined by focus assay and sandwich ELISA, respectively, as described in the Materials and Methods. Table 1 shows dengue virus infectivity and antigenicity remaining after heating at 56℃ in the culture fluid of C6/36 cells infected with each of the 4 types of dengue viruses. All 4 types of dengue virus infectivity was reduced to
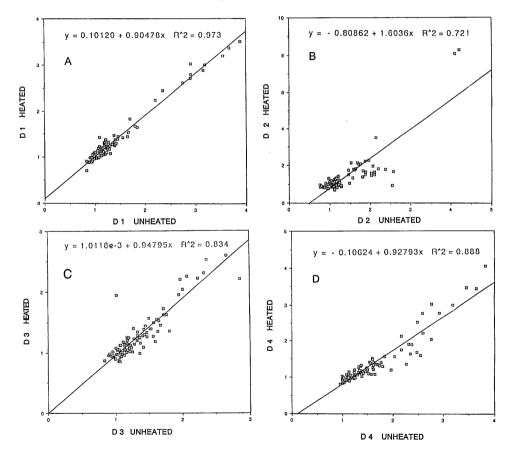
Table 1. Residual infectivity and antigenicity (percentage) after heating at 56℃ in the culture fluid of C6/36 cell line infected with each of the 4 types of dengue viruses*

Heating at 56℃ in min	D1		D2		D3		D4	
	FFU	Ag	FFU	Ag	FFU	Ag	FFU	Ag
0	2.0×10^4	6	2.8×10^5	28	4.0×10^3	10	1.4×10^5	9
	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
1	8.0×10^3	5	2.1×10^5	30	1.1×10^3	8.5	1.3×10^5	5.5
	(40)	(83)	(75)	(100)	(27.5)	(85)	(92.8)	(61)
2	9.0×10^3	5.2	1.4×10^5	29	1.1×10^3	6.5	5.0×10^4	7
	(45)	(86)	(49.3)	(100)	(28.5)	(65)	(35.7)	(77)
3	8.0×10^2	2.3	9.0×10^4	24	2.8×10^2	4	3.6×10^3	6
	(5)	(38)	(32.1)	(85.7)	(9)	(40)	(2.5)	(60.6)
4	70	2.8	2.0×10^3	20	<20	4	90	4
	(0.35)	(46)	(0.7)	(71)	(0)	(40)	(0.06)	(44.4)
5	<20	1.6	60	8	<20	1.5	<20	2
	(0)	(36)	(0.02)	(28.5)	(0)	(15)	(0)	(22.2)

* Infectivity was shown by FFU/ml, while antigenicity by ELISA units. Figures in parentheses indicate percentage remaining compared with the titer of the specimen before heating (0 minutes).

almost undetectable level after 5 minutes heating at 56℃, while antigenicity remained around 15−36%. The results confirmed our preliminary study and indicated that brief heating would be an appropriate method to remove virus infectivity from infected C6/36 cell culture fluids to prepare biologically safe assay anitigen.

Comparative IgM-capture ELISA using heat-inactivated and unheated control dengue antigens

A panel of 91 serum specimens from patients with clinical diagnosis of dengue was tested by the IgM-capture ELISA using heat-inactivated and unheated control antigens. The P/N ratio on each individual serum specimens was summarized in Figure 1, while the

Fifgure 1. Correlation between the P/N ratio obtained by heat-inactivated and unheated control dengue anitigens in the IgM-capture ELISA

Each dot represents each test serum with its P/N ratio obtained by heat-inactivated antigen on the ordinate, and that by unheated control antigen on the abscissa, respectively. Results obtained by D1 antigen in panel A, by D2 antigen in panel B, by D3 antigen in panel C, by D4 antigen in panel D, respectively.
Correlation coefficiency and equation of regression line are shown on each panel.

averaged P/N ratio with standard deviation on positive and negative serum specimens was shown in Table 2.

These results indicated IgM-capture ELISA gave almost comparable results both by the heat-inactivated and unheated control antigens.

Table 2. Average and standard deviation of the P/N ratio calculated for the positive and negative serum specimens examined by heat-inactivated and unheated control antigens of each of the 4 serotypes of dengue antigens in the IgM capture ELISA

Positive specimens examined by

Antigens	D1	D2	D3	D4
Heat-inactivated	2.89±0.38	2.63±2.08	2.33±0.23	2.36±0.76
Unheated control	3.04±0.58	2.27±0.74	2.34±0.30	2.68±0.52

Negative specimens examined by

Antigens	D1	D2	D3	D4
Heat-inactivated	1.14±0.17	1.19±0.90	1.11±0.18	1.32±0.24
Unheated control	1.15±0.19	1.17±0.14	1.19±0.19	1.12±0.17

DISCUSSION

Our previous studies showed that all 4 types of dengue virus antigens could be produced sufficient titer in the infected C6/36 cell culture fluid when appropriate virus strains were used. According to the biosafety recommendation by WHO, it was necessary to inactivate infective virus in the infected culture fluid without losing it antigenicity to detect IgM antibodies in order to be used as assay antigens for serodiagnosis.

Initial attempts to destroy virus infectivity by nonionic detergent treatment, however, resulted in markedly reduced reactivity of the antigen in the IgM-capture ELISA, although antigen titer measured by the sandwich ELISA was retained (Maha & Igarashi, 1997). Therefore, another preliminary experiment was designed to inactivate virus infectivity by brief heating without losing antigenicity and reactivity in the IgM-capture ELISA using a particular positive patient's serum specimen. This experiment gave promising result, however, it remained to examine whether the heat-inactivated antigen could provide similar results as unheated antigen to assay a number of sera in the IgM-capture ELISA. The results in this study indicated that dengue antigens which were prepared as infected C6/36 cell culture fluid and their infectivities were inactivated by brief heating could be used as diagnostic antigen with biosafety requirement for use in the IgM-capture ELISA.

REFERENCES

1) Bundo, K. & Igarashi, A. (1985): Antibody-capture ELISA for detection of immunoglobulin M antibodies in sera from Japanese encephalitis and dengue hemorrhagic fever patients. J. Virol. Methods., 11, 15−22.

2) Burke, D. S. (1983): Rapid methods in the laboratory diagnosis of dengue virus infections. pp72−84 *In* T. Pang and R. Pathmanathan (eds). Proceedings on the International Conference on Dengue/Dengue Haemorrhagic Fever. University of Malaya, Kuala Lumpur, Malaysia

3) Clarke, D. H., & Casals, J. (1958): Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg., 7, 561−573.

4) Igarashi, A. (1978): Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J. Gen. Virol., 40, 531−544.

5) Igarashi, A., (1994): Principle of laboratory diagnosis and epidemiological surveillance on dengue and Japanese encephalitis. Trop. Med., 36, 220−227.

6) Igarashi, A., Bundo, K., Matsuo, S., Makino, Y., & Lin, W.-J. (1981): Enzyme-liked immunosorbent assay (ELISA) on Japanese encephalitis virus. I. Basic conditions of the assay on human immunoglobulin. Trop. Med., 23, 49−59.

7) Igarashi, A. & Antonio, M. T. A. (1997): 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. Trop. Med., 39, 81−87.

8) Innis, B. L., Nisalak, A., Nimmannitya, S., Kusalerdchariya, S., Chongswasdi, V., Sunthyakorn, S., Puttisri, P., & Hoke, C. H. (1989): An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis cocirculate. Am. J. Trop. Med. Hyg., 40, 418−427.

9) Khin-Mar-Aye, Kyaw-Zin-Thant, Tan-Swe & Igarashi, A. (1995): Optimization of the dengue-2 antigen ELISA titer for the positive case detection of dengue virus infection. Trop. Med., 37, 123−128.

10) Kyaw-Zin-Thant, Khin-Mar-Aye & Igarashi A. (1995): Anitigen production of dengue type 4 virus strains in *Aedes albopictus* clone C6/36 and Vero cell cultures. Trop. Med., 37, 115−121.

11) Kyaw-Zin-Thant, Khin-Mar-Aye, Soe-Thein, Tan-Swe & Igarashi, A. (1996): Application of IgM-capture ELISA on Myanmar dengue patients serum samples collected in 1994. Trop. Med., 38, 1−6.

12) Lam, S. K., Devi, S. & Pang, T. (1987): Detection of specific IgM in dengue infections. Southeast Asian J. Trop. Med. Publ. Hlth., 18, 532−538.

13) Maha, M. S., & Igarashi, A. (1997): The effect of nonionic detergent on dengue and Japanese encephalitis virus antigens in antigen detection ELISA and IgM-capture ELISA. Southeast Asian J. Trop. Med. Publ. Hlth., 28, 718−722.

14) Monath, T. P. & Heinz, F. X. (1996): Chapter 31. Flaviviruses. pp961−1034. *In* B. N. Fields, D. M. Knipe, P. M. Howley, *et al.* (eds), Fields Virology, 3rd Ed., Lippincott-Raven Publishers, Philadelphia.

15) Morita, K., Bundo, K. & Igarashi, A. (1982): Enzyme-liked immunosorbent assay (ELISA) on Japanese encephalitis virus. IV. A computer system to calculate ELISA endpoint from ELISA OD at a single dilution of test sera. Trop. Med., 24, 131−137.

16) Okuno, Y., Fukunaga, T., Tadano, M., Okamoto, T., Onishi, T., & Takagi, M. (1985): Rapid focus reduction neutralization test of Japanese encephalitis virus in microtiter system. Arch. Virol., 86, 129−132.

17) Pan American Health Organization (1996): Guidelines, 1996.

18) Russell, P. K. & Nisalak, A. (1967): Dengue virus identification by the plaque reduction neutralization test. J. Immunol., 99, 291−296.

19) Voller, A. Bidwell, O & Bartlett, A. (1976): Microplate enzyme immunoassays for the immunodiagnosis of viral infections. pp506−512. *In* N. R. Rose & N. Friedman (eds), Manual of Clinical Immunology, American Society for Microbiology, Washington, DC.

20) Vorndam, V. & Kuno, G. (1997): Laboratory diagnosis of dengue virus infections. pp313−333. *In* D. J. Gubler & G. Kuno (eds), Dengue and Dengue Hemorrhagic Fever, CAB International, New York.

21) World Health Organization (1997): Dengue haemorrhagic fever. Diagnosis, treatment, prevention, and control, 2nd Ed., WHO, Geneva.