IgM CAPTURE ELISA FOR DETECTION OF IgM ANTIBODIES TO DENGUE VIRUS: COMPARISON OF 2 FORMATS USING HEMAGGLUTININS AND CELL CULTURE DERIVED ANTIGENS

Mary Jane Cardosa[1], Phaik Hooi Tio[1], Suchitra Nimmannitya[2], Ananda Nisalak[3] and Bruce Innis[3]

[1]School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia, [2]Children's Hospital, Mahidol University, Rajvithi Road, Bangkok 10400, Thailand, [3]AFRIMS, Rajvithi Road, Bangkok 10400, Thailand.

Abstract. The highly sensitive AFRIMS format IgM capture ELISA for the diagnosis of dengue virus infections requires the use of mouse brain derived hemagglutinins and consequently also the use of 20% acetone extracted normal human serum to eliminate high background. These reagents are not always easily available and we have thus compared the AFRIMS format with another published format which uses cell culture derived antigens (culture fluid, CF, format) in order to determine if it is reasonable to use cell culture derived antigens in situations where hemagglutinins and normal human serum are difficult to obtain. The study shows that using AFRIMS results as the reference point, the CF format described here has a sensitivity of 90% and a specificity of 96%.

INTRODUCTION

The laboratory diagnosis of dengue fever and dengue hemorrhagic fever has been historically determined by the use of the hemagglutination inhibition (HI) test described by Clarke and Casals (1958). Although the HI test has been extremely reliable, it demands the titration of paired sera taken 10 to 14 days apart, using 4 hemagglutinins in an assay which is very pH sensitive. Thus the advent of enzyme immunoassays has offered the possibility of designing tests which can determine the presence of dengue specific IgM in patient sera.

Many variations on the general theme of an IgM capture ELISA have been formulated as in-house tests in several research and diagnostic laboratories (*eg* Bundo and Igarashi, 1985; Lam *et al,* 1987; Innis *et al,* 1989; Cardosa and Zuraini, 1991). It is generally considered that hemagglutinins derived from mouse brain are the antigens of choice in this assay, and cell culture fluids are not considered useful. The format described by Innis *et al* (1989) is highly sensitive and is the best characterized. However, as a strict requirement, the AFRIMS format uses acetone extracted human serum as a blocking agent. If it is possible to produce high titered cell culture derived antigens to use in these ELISAs instead of hemagglutinins, the problem of high backgrounds necessitating the use of acetone extracted normal human serum may be circumvented.

This study compared the AFRIMS format (Innis *et al,* 1989) and the culture fluid (CF) format described by Cardosa and Zuraini (1991) in a collaborative study between the two groups in order to determine if culture fluid antigens may be reliably used in the IgM capture ELISAs for dengue diagnosis.

MATERIALS AND METHODS

The two formats compared in this study are summarized in Table 1, with details in the following sections.

IgM capture ELISA: AFRIMS format

This version of the IgM Capture ELISA was carried out on all specimens exactly as described by Innis *et al* (1989) using JE virus hemagglutinin and a tetravalent cocktail of dengue virus hemagglutinins as described. Essentially, 96 well microtiter plates were sensitized with a rabbit anti-human μ chain antibody (Behring, Germany). Patients' sera were diluted 1 : 100 in phosphate buffered saline (PBS), added to the wells and incubated at 4°C overnight. Plates were then washed with PBS

Table 1

Summary of formats

Step	AFRIMS	USM
Coating	Goat anti human mu	Rabbit anti human mu
Blocking	none	10% FCS
Patient serum:		
dilution	1 : 100	1 : 100
diluent	PBS	PBS + 1% FCS
time	overnight	2 hours
temperature	4°C	room temp
Antigen:		
type	Den 1 to 4 HA	Den 1 to 4 CF
diluent	PBS 20% NHS	PBS 1% FCS
time	overnight or 4 hours	overnight or 2 hours
temperature	4°C or room temperature	4°C or room temperature
Conjugate:		
type	pooled convalescent human antiflavivirus IgG conjugated with horseradish peroxidase	
diluent	PBS 20% NHS	PBS 1% FCS
Washing	PBS + 0.05% T20	PBS + 0.05% T20
Substrate	OPD	OPD

containing 0.05% Tween 20, and 50 µl of viral hemagglutinin (JE or tetravalent dengue cocktail) diluted in PBS containing 20% acetone extracted normal human serum was then added to the wells. After two hours at room temperature, antigen bound was detected using 25 µl per well of an optimal dilution of horseradish peroxidase conjugated human anti-flavivirus IgG prepared as described by Burke *et al* (1982). After one hour at room temperature, color development was achieved using o-phenylenediamine/hydrogen peroxide as described. Absorbance was read at 492 nm and results were calculated by reference to a weak positive control standard and units assigned as described by Innis *et al* (1989). All reagents were produced in the AFRIMS laboratory.

IgM capture ELISA: culture fluid (CF) format

Goat anti human IgM (Behringwerke, Germany) diluted 1 : 500 in 0.05M carbonate-bicarbonate buffer, pH 9.6, was used to coat flat bottomed microtiter plates at 100 µl per well. These were left overnight at 4°C, after which they were washed with PBS containing 0.05% Tween 20 (PBS-T), and blocked using 200 µl blocking buffer containing 10% fetal bovine serum in PBS. After a 2 hour incubation at room temperature, the plates were washed with PBS-T and stored dry at 4°C until use.

Patients' sera were diluted 1 : 100 in diluent buffer (PBS containing 1% fetal bovine serum) and dispensed at 100 µl per well in duplicate. This was incubated at room temperature for 2 hours and after washing with PBS-T, 100 µl antigen or uninfected control was added to each well of a pair, thus each serum was reached with antigen as well as uninfected control. The plate was then incubated at 4°C overnight. After washing as before, antigen bound was detected using a rabbit anti-flavivirus antibody (gift of Dr James Porterfield) followed by swine anti rabbit IgG conjugated with horseradish peroxidase (Dako, Denmark). Each of these two layers was subjected to a 1 hour incubation at room temperature followed by a washing step.

Presence of dengue specific IgM was detected with a chromogenic substrate using o-phenyledia-

mine/hydrogen peroxide. Absorbance was measured at 492 nm wavelength in a microplate spectrophotometric reader, and results were calculated by dividing the absorbance of antigen containing well by the absorbance of the uninfected control well for each specimen (OD_{Ag}/OD_C).

Hemagglutinins were prepared by AFRIMS by sucrose acetone extraction of dengue virus infected suckling mouse brain (Clarke and Casals, 1958).

Cell culture antigens were prepared as described in Cardosa and Zuraini (1991). Briefly C6/36 *Aedes albopictus* cells were infected with various dengue virus serotypes in Leiboviz 15 media containing 1% heat inactivated fetal bovine serum and 10% tryptose phosphate broth. Culture supernatants were collected when syncytia formation was evident. Uninfected control antigens were harvested from mock infected cells at the same time.

Viruses used for both forms of antigen were the protypes Den1 Hawaii, Den2 New Guinea C, Den3 H87 and Den4 H241.

Specimens from patients with suspected dengue fever or dengue hemorrhagic fever were collected in Thailand and Malaysia during 1989. There were 67 patients from the state of Perak in Malaysia and 81 patients from Bangkok, Thailand. Of these, convalescent specimens were available from only 97 of the 148 patients. Normal serum specimens were obtained from blood donors in Penang, Malaysia.

Evaluation of results. The method of determination of positive and negative results in the MAC ELISA of the AFRIMS format has been described by Innis *et al* (1989). By reference to a weak positive control assigned a value of 100 units, specimens scoring 40 units or more were considered positive.

In the case of the CF format, it was necessary to first evaluate the performance of normal human sera in the test. 217 serum specimens from Malaysia blood donors were tested in the CF format to assess background levels. The mean OD_{Ag}/OD_C was 1.272 with a standard deviation of 0.225. We thus determined the scores of patient sera using various cutoff values starting with a cutoff determined by adding 3 standard deviations to the mean (where $OD_{Ag}/OD_C = 2$).

RESULTS AND DISCUSSION

Table 2 shows the results obtaining when comparing 97 cases with paired sera using the two formats described. **Using the AFRIMS format as the reference method,** we have found that using a cutoff OD_{Ag}/OD_c value of 2.0, 4 non-dengue cases were scored as positive by the CF format (specificity = 85.7%). When the cutoff OD_{Ag}/OD_C was increased to 2.5 or 3.0 only 1 false positive was observed (specificity = 96.4%) in either situation.

Again using the AFRIMS format as the reference here, the sensitivity of the CF format was 91.3% (cutoff $OD_{Ag}/OD_C = 2.0$), 89.9% ($OD_{Ag}/OD_C = 2.5$) and 87.0% ($OD_{Ag}/OD_C = 3.0$) respectively.

There is no doubt that the AFRIMS format is relatively more sensitive than the USM format. However, if a cutoff OD_{Ag}/OD_C of 2.5 is used in the CF format, reasonable sensitivity (89.9%) and specificity (96.4%) can be expected.

This study shows that it is possible to use cell culture derived antigens in an IgM capture ELISA for dengue if availability of hemagglutinins and acetone extracted normal human serum is difficult. The detecting antibody in the CF format described here was a polyclonal anti-flavivirus antibody raised in rabbits and this probably compromises the sensitivity of the test. We have recently replaced the rabbit antiserum with monoclonal antibodies which are dengue group reactive, and preliminary results show that cell fluid derived antigens can in fact be very reliably used without compromising sensitivity or specificity.

ACKNOWLEDGEMENTS

This work was supported by a grant from the International Development Research Centre and IRPA funds from University Sains Malaysia.

REFERENCES

Bundok, A. Antibody-capture ELISA for detection of immunoglobulin M antibodies in sera from Japanese encephalitis and dengue haemorrhagic fever patients. *J Virol Methods* 1985; 11 : 15-22.

Cardosa MJ, Zuraini I. Comparison of an IgM capture ELISA with a dot enzyme immunoassay for laboratory diagnosis of dengue virus infections. *Southeast*

Table 2

Comparison of AFRIMS and CF format: paired specimens (by case).

	Cutoff fluid format								
	Cutoff = 2.0			Cutoff = 2.5			Cutoff = 3.0		
	+	−	Tot	+	−	Tot	+	−	Tot
AFRIMS format:									
> 40 units	63	6	69	62	7	69	60	9	69
< 40 units	4	24	28	1	27	28	1	27	28
Total	67	30	97	63	34	97	61	36	97

Asian J Trop Med Public Health 1991; 22 : 337-40.

Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. *Am J Trop Med Hyg* 1958; 7 : 561-73.

Innis BL, Nasalak A, Nimmannitya S, *et al.* An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg* 1989; 40 : 418-27.

Lam SK, Devi S, Pang, T. Detection of specific IgM in dengue infection. *Southeast Asian J Trop Med Public Health* 1987; 18 : 532-38.