

COMPARISON OF AN IgM CAPTURE ELISA WITH A DOT ENZYME IMMUNOASSAY FOR LABORATORY DIAGNOSIS OF DENGUE VIRUS INFECTIONS

MJ Cardoso and Ismail Zuraini

School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.

Abstract. This study describes the use of an IgM capture ELISA using cell culture derived antigens and a polyclonal rabbit anti-flavivirus antisera for the detection of dengue positive cases. The IgM capture ELISA is compared with the dot enzyme immunoassay and the results are discussed in the context of dengue endemicity.

INTRODUCTION

The increasing importance of dengue fever and dengue hemorrhagic fever in Asia, South America and the Caribbean has underlined the importance of early detection as an aid to control of the spread of the disease. The hemagglutination inhibition test has served an important role in the detection and determination of these disease entities, but a need has arisen for a single dilution test accessible to less endowed laboratories with few highly trained personnel, in order to decentralize and democratize the means to perform confirmatory laboratory diagnosis.

There are already several versions of IgM capture ELISA for dengue virus antigen in print eg, Bundo and Igarashi, 1985; Lam *et al.*, 1987; Innis *et al.*, 1989) and this paper offers another modified protocol for IgM capture ELISA using cell culture derived antigens and polyclonal rabbit anti-flavivirus antisera. This test is compared with the dot enzyme immunoassay method of detecting antibodies to dengue virus antigens.

MATERIALS AND METHODS

Specimens

Serum specimens from suspected dengue patients presenting at several hospitals in the northern Malaysian states of Penang and Perak were received by our laboratory for confirmation

of dengue infection. This study includes specimens from 308 cases during the period 1989 and 1990. 160 cases had only acute phase sera available while 148 cases had paired sera collected 5 to 20 days apart. All sera were stored at -30°C until use.

Virus antigens

Dengue viruses 1 to 4 were grown in C6/36 *Aedes albopictus* cells maintained in Leibovitz 15 medium containing 10% tryptose phosphate broth, 1% heat inactivated bovine serum albumin and antibiotics. Dengue virus antigens used were a cocktail of equal volumes of all 4 serotypes. Cell culture supernatants were harvested when syncytia formation was evident and clarified by centrifugation. Uninfected control antigens were prepared from cell culture supernatants of uninfected C6/36 cells.

Dot enzyme immunoassay (DEIA)

This was performed essentially as described previously (Cardoso *et al.*, 1988a,b). Briefly, nitrocellulose filters (pore size 0.45 µm) were coated with dengue virus antigens and uninfected control antigens. Unbound sites were blocked with 5% skimmed milk in phosphate buffered saline (SMB), washed and dried. Filters were incubated for 1 hour at room temperature with serum diluted in SMB and washed with phosphate buffered saline. Antibody bound was detected with Protein A conjugated with horseradish peroxidase and color development was achieved using a 4-chloro-1-

naphthol/hydrogen peroxide substrate system.

A color reaction over the dengue antigen spot indicated that antibodies to dengue virus were present in the serum. All serum specimens were tested at 1:1000 dilution initially, and results were scored as positive (+), weak (+/-) or negative (-). Only those specimens with a (+) score were recorded as dengue positive cases. All other scores required a second specimen for confirmation of the diagnosis. On receipt of a second specimen, each pair of sera was retested at 1:500 and 1:1000 dilutions to determine if seroconversion had occurred. Thus if the first specimen was (-) converting to (+/-) or (+), a seroconversion was considered to have occurred. Similarly if the first specimen was (+/-) converting to (+), a seroconversion was recorded.

IgM capture ELISA

Goat anti human IgM (Behringwerke, Germany) diluted 1:500 in 0.05 M carbonate-bicarbonate buffer, pH 9.6, was used to coat flat bottomed microtiter plates (Falcon, Becton Dickinson, USA) 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 incubated 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 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-phenylenediamine (OPD) and hydrogen peroxide. Absorbance

was measured at 492 nm wavelength in a micro-plate 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. A ratio of 3 or more was considered positive.

RESULTS

An analysis of 160 single specimens showed that 60 of these contained IgM to dengue virus antigens, and of these IgM positive cases, 39 or 65% were DEIA positive (+) as seen in Table 1, and all IgM negative cases were DEIA negative [(-) or (+/-)].

148 cases with paired sera were tested for the presence of dengue specific IgM, and it was shown that 93 cases were IgM positive in one or both of the specimens in each pair (Table 2A). Of these IgM positive cases, 83 were also positive by DEIA as determined by a positive (+) DEIA score on both specimens of a pair, or by DEIA seroconver-

Table 1

Comparison of IgM and DEIA results for 160 single specimens.

	IgM + ve (%)	IgM - ve (%)	Total
DEIA + ve	39 (65.0)	0 (0.0)	39
DEIA - ve	21 (35.0)	100 (100.0)	121
Total	60	100	160

Table 2

A. Comparison of IgM and DEIA results for 148 paired specimens: analysis case by case.

	IgM + ve (%)	IgM - ve (%)	Total
DEIA + ve	83 (89.2)	4 (7.3)	87
DEIA - ve	10 (10.8)	51 (92.7)	61
Total	93	55	148

B. Comparison of IgM and DEIA results for 148 paired specimens: analysis of seroconverting pairs.

	IgM + ve	IgM SC*	IgM - ve	Total
DEIA + ve	50	0	2	52
DEIA SC*	14	19	2	35
DEIA - ve	7	3	51	61
Total	71	22	55	148

*Seroconverting

sion as described above. Thus 89.2% of the IgM positive cases were also positive using the DEIA method.

Of the 55 IgM negative cases shown in Table 2A, 51 were also negative using DEIA. Thus 4 cases appeared to be positive cases by DEIA, but did not have IgM. Table 2B shows that of these 4 apparently false positives by DEIA, 2 showed clear seroconversions by DEIA, although no seroconversion was apparent by IgM. The other 2 cases were probably false positives by DEIA. Thus taking this into consideration, 51 of 53 negative dengue cases were also negative by DEIA, making a specificity of 96.2% for paired sera.

Table 2B also shows that 22 of the 93 IgM positive cases seroconverted for IgM from negative to positive IgM, thus showing that 23.7% of dengue positive cases seroconvert from no detectable IgM in the acute serum to positive IgM in the second serum, indicating that IgM may not always be present in the acute serum specimen in dengue cases.

Thirty-five of the total 87 DEIA cases positive by DEIA were seroconversions, thus showing that 40.2% of cases seroconverted by DEIA.

DISCUSSION

It is clear that for both the IgM capture ELISA and the DEIA described in this paper, paired serum specimens are required for optimum sensitivity and specificity. With only a single specimen available the DEIA only picks up 65% of the positives detected by IgM capture ELISA.

Analyzing paired sera, the IgM capture ELISA is able to detect 76.3% (71/93) of cases when only the acute phase specimen is tested, requiring a second specimen for the other 23.7%. The DEIA is able to detect 89.2% of all these cases positive for IgM, but only 64.5% (60/93) when only the first specimen of the pair is tested. This percentage is similar to that obtained in the analysis of single specimen in which the DEIA detects 65% of the sera positive by IgM capture ELISA.

Whether single or paired specimens are available, specificity of the DEIA and the IgM capture ELISA is not a problem.

These data are cumulative results obtained in a country where dengue is endemic but where the level of endemicity has not yet reached such proportions that most cases presenting with dengue fever or dengue hemorrhagic fever are experiencing a secondary immune response to dengue. The pool of cases from which this study draws tends to be about 60% secondary dengue, and we believe that these are the cases which are detected most readily by the DEIA.

Thus in situations where the majority of cases are second dengue infections, there is reason to use the DEIA when the IgM capture ELISA is not readily available or when the facilities for the optimum performance of ELISA are lacking.

When dengue fever and dengue hemorrhagic fever is a recent phenomenon, and most cases are experiencing primary dengue, then the IgM capture ELISA is clearly a more desirable test to use.

The IgM capture ELISA described in this study makes use of cell culture derived antigens rather than mouse brain extracts, and the detecting anti-flavivirus antibody is a polyclonal rabbit antiserum, both reagents being readily prepared as in-house reagents. This IgM capture ELISA protocol is not nearly as sensitive as the protocol used by Innis and coworkers (1989) but the reagents are more readily prepared and no acetone extracted normal human serum is required, thus making this a more accessible test for less endowed laboratories.

ACKNOWLEDGEMENTS

This study was carried out when the authors were at the School of Medical Sciences, Universiti Sains Malaysia, and we thank the Dean for all assistance rendered.

REFERENCES

- Bundo K, Igarashi 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, Tio PH, Noor Sham S. Development of a dot enzyme immunoassay for dengue 3: a sensitive method for the detection of antidengue antibodies. *J Virol Methods* 1988; 22 : 81-8.
- Cardosa MJ, Noor Sham S, Tio PH, Lim SS. A dot enzyme immunoassay for dengue 3 virus: comparison with the haemagglutination inhibition test. *Southeast Asian J Trop Med Public Health* 1988; 19 : 591-4.
- Innis BL, Nisalak 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.
-