Dengue Fever: Its Laboratory Diagnosis, with Special Emphasis on IgM Detection

by

N Sathish, T S Vijayakumar, P Abraham and G Sridharan[#]

Department of Clinical Virology, Christian Medical College, Vellore-632 004, India

Abstract

Rapid diagnosis of dengue is crucial for proper patient care. As IgM antibody appears early during the disease course, its detection is a valuable tool for rapid diagnosis.

We evaluated and compared two commercial tests, the PanBio Rapid Immunochromatographic Card Test (Brisbane, Australia) and the PanBio Microwell IgM ELISA with an IgM Capture ELISA (National Institute of Virology, Pune, India). A total of 154 samples from individuals with febrile illness having dengue fever (DF)-like symptoms were tested.

The NIV IgM Capture ELISA (MAC-ELISA) showed a high positivity rate (38.9%) as compared to the PanBio Rapid (22.7%) and the PanBio IgM ELISA (20.7%). The NIV MAC-ELISA showed a high sensitivity (96%) as compared to PanBio Rapid (73%) and PanBio IgM ELISA (72%). But the specificity was low (81%) when compared to PanBio Rapid (95%) and PanBio IgM ELISA (97%) using the latent class analysis model.

The MAC-ELISA, though a three-day procedure, should be a valuable screening test because of its high sensitivity rates. But rapidity gets compromised, as batch testing is required along with technical difficulty in performance. The "Rapid" test is an easier option in small peripheral laboratories in India because of its obvious advantages.

Keywords: Dengue, IgM detection, NIV IgM capture ELISA, PanBio Rapid Immunochromatographic Card Test, PanBio Microwell IgM ELISA.

Introduction

Early symptoms of dengue fever (DF) mimic other diseases often prevalent in areas where it is endemic, such as malaria and leptospirosis⁽¹⁾. Thus, a rapid differential diagnosis is crucial for proper patient care. The most straightforward diagnosis of a recent infection is achieved by the detection of the virus in the patient's blood, either by virus isolation in susceptible cell cultures⁽²⁾ or by identifying the viral RNA with PCR techniques^(3,4). But these methods are laborious and also require specialized

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[#] For correspondence: g_sridharan@yahoo.com

laboratory facilities. In addition, it has been shown that the level of the circulating virus wanes as the antibodies become detectable and so these procedures are successful only when done within a few days of the onset of illness⁽⁵⁾.

General methodological issues

The isolation of dengue viruses is usually carried out by inoculation into either 1-3-day-old mice, mammalian cell cultures, mosquito cell cultures or by intrathoracic inoculation of adult mosquitoes⁽⁶⁾. Inoculation into suckling mice, previously carried out in our laboratory, was seen to require numerous passages to adapt the viruses to replicate in mice. Mammalian cell cultures have many of the demerits associated with suckling mice and are also not recommended⁽⁶⁾.

Mosquito inoculation, though the most sensitive method for dengue virus isolation, has its associated demerits that include labour-intensiveness and requirement of an insectary to produce large numbers of mosquitoes for inoculation⁽⁶⁾.

For the serological diagnosis of dengue, traditionally in the past, the HI assay has been the most widely used method⁽²⁾. However, the variable potency of different haemagglutinins made in laboratories⁽⁵⁾, the extensive cross-reaction encountered and the non-availability of results within a short period of time due to the requirement of both acute and convalescent sera collected at least seven days apart, have compromised the general applicability of this assay in the diagnosis of dengue fever. Extensive cross-reactivity among the viruses posing a problem in HI

was encountered in our laboratory also⁽⁷⁾. Compliment Fixation Test (CFT), The another serological method of diagnosis, is not widely used currently for routine diagnostic testing. The major demerits of this test include difficulty in the performance of the test and the requirement of highly trained personnel⁽⁶⁾. As for HI, crossreactivity among the viruses was a problem encountered in our laboratory⁽⁸⁾. It was observed that CFT titers to other arboviruses was equal to and sometimes even higher than the agent involved and more often these heterologous titers were noted with Japanese encephalitis (JE) and (West Nile) WN antigens.

The neutralization test, though the most specific and sensitive serological test for dengue infections, also has major demerits. The turnaround time of the test, coupled with technical difficulty, have led to this test being less routinely employed⁽⁶⁾.

The most challenging problem associated with patient management in dengue infection is rapid diagnosis. Due to the aforesaid disadvantages of the various tests, they cannot be made use of for a rapid diagnosis of dengue fever. In this respect, commercially available ELISAs offer improvements over the HI assay for the serological diagnosis of dengue infections. Unlike HI assays, pre-treatment of sera is not required, and a diagnosis can be made from a single sample. Differentiation between primary and secondary infections may also be made with a single dilution of serum rather than with a series of dilutions.

As IgM antibody appears early during the disease course, and as a diagnosis can be made from a single serum sample, detection of IgM antibody seems to be a valuable tool

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for the rapid diagnosis of acute viral infection.

Previously in our laboratory, from the middle of 1996 to the end of 1999, the dengue IgM blot (Genelabs Diagnostics), a qualitative enzyme immunoblot assay, was used for the detection of dengue IgM antibodies. This test was not very rapid and involved a series of incubation steps with addition of various reagents after the incubation steps. Even the shortened version the original protocol (a two-day of procedure, with overnight incubation in between) was a day-long procedure. As it was a long procedure, only batch testing of samples could be performed. All these samples were later confirmed by the NIV MAC-ELISA. For detection of dengue IgG antibodies, a separate dengue IgG blot was available. Since 1999, the blot assays were replaced by the PanBio Rapid Immunochromatographic Card test which was guick to perform and both dengue IgM and IgG responses could be detected using a single card.

Specific methodology

In a more recent study carried out in our department of Clinical Virlology, three tests employed for the detection of dengue IgM antibody from serum samples, the PanBio Rapid Immunochromatographic Card Test (Brisbane, Australia), the PanBio INDX^RIVDTM Microwell ELISA for IgM only (Baltimore, USA), and the IgM antibody Capture ELISA (MAC-ELISA) (National Institute of Virology, Pune, India) were compared.

Serum samples were received at our department from 154 patients with DF-like febrile illnesses attending the Paediatric and

Medicine clinics of the Christian Medical College, Vellore, India, during June 2000-September 2000. The clinical basis for diagnosing the patients as having dengue fever was based on standard criteria like presentation of febrile illness of 2-7 days' duration, with features like headache, myalgia, arthralgia, rash, haemorrhagic manifestation and leucopenia. Clinical information about the stage of the disease at which sampling was done was unavailable for most samples, and hence not taken into account.

PanBio Rapid Immunochromatographic Card Test

This is a rapid (<10 minutes) immunochromatographic test for the detection of both dengue specific IgM and IgG in a testcard format. Anti-dengue virus IgM and IgG antibodies in the test sample (30µl of undiluted serum per test) were detected simultaneously on the same card by using an antibody capture format with a cocktail of all the four dengue virus serotypes. The test is considered positive for primary dengue virus infection if two purple/pink lines (IgM and Control) are seen in the viewing window after five minutes⁽⁹⁾. This test format has been earlier described by others⁽⁹⁾. In this study, we were primarily interested in the IgM antibody results for comparison with the other two tests.

PanBio INDX[®]IVD[™] Microwell ELISA (IgM only)

In the PanBio Microwell ELISA kit, wells were coated with purified dengue virus type 2 antigen cultured in Vero cells. The test was performed according to the manufacturer's instructions.

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Samples were considered negative if the optical density (OD) values were in the range 0.0-0.3 OD units, weak positive if in the range 0.5-1.0 OD, and positive if >1.0 OD. Samples were interpreted as indeterminate if the OD values were in the range 0.3-0.5 units.

IgM Antibody Capture ELISA (MAC-ELISA) (NIV, Pune, India)

The test is designed to detect IgM antibodies to any one or three of the flaviviruses prevalent in India (DEN-2, JE and West Nile). The procedure starting with the coating of anti-IgM was performed according to the protocol provided. For each test sample the number of antibody units was calculated based on the formula:

$$\left\{ \begin{array}{l} \underline{OD (Test) - OD (NC)^{\star}} \\ OD (Weak PC)^{\star \star} - OD (NC) \end{array} X 100 \right\}$$

*NC: Negative Control

**Weak PC: Weak Positive Control

The test sample was considered positive for IgM antibody against that particular antigen if the antibody units were =50; if <50 the sample was considered negative.

Statistical analysis

As there was no gold standard among the three tests done, the accuracy of the tests assessed through "Latent Class was Models" (10, 11, 12) The true prevalence, sensitivity and specificity of the three tests were estimated using 2LC latent class model using expectation-maximization algorithm. The analysis was done using Latent software version 3.0 (Courtesy Dr SD Walter, McMaster University, Canada). As the conservative, asymptotic Gausian confidence

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interval has the overshooting aberration when the parameter estimates are at or near the maximum and minimum values (1 or 0), the Wilson 'score' method⁽¹³⁾ with continuity correction was employed to obtain the 95% confidence intervals.

Results

Of the 154 serum samples tested, 60(38.9%) were positive by the MAC-ELISA (NIV, Pune, India) while 35(22.7%) were positive by the PanBio Rapid Immunochromatographic card test, and 32 (20.7%, excluding 15 indeterminate results) were found positive by the PanBio Microwell IgM ELISA. The number of samples positive in a single or in a combination of tests is shown in Table 1.

Table 1.	Reactivity patterns of 154 samples in
	the three dengue IgM assays

Positive status category	No. of samples positive	Percentage positivity
NIV IgM Capture only	23	14.9
PanBio rapid only	5	3.2
PanBio ELISA only	3	1.9
Three methods (NIV, PanBio Rapid & ELISA)	20	12.9
NIV IgM Capture & PanBio Rapid	9	5.8
NIV IgM Capture & PanBio ELISA	8	5.2
PanBio Rapid & PanBio ELISA	1	0.6

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The estimated prevalence of dengue in the study population was 25%. The sensitivity and specificity of the three tests computed using 2LC Latent class model are presented in Table 2. The estimated sensitivity was 96% in the NIV MAC-ELISA, while it was 73% and 72% in PanBio Rapid and PanBio ELISA respectively. However, the PanBio Rapid and the PanBio ELISA tests had a high specificity as compared to the NIV MAC-ELISA. The difference in sensitivity and specificity amongst the three tests was found to be significant.

 Table 2. Prevalence, sensitivity, specificity

 and their 95% confidence intervals (CI) of

 three tests using 2LC Latent Class model

Tests	Prevalence	Sensitivity	Specificity
	(95%CI)*	(95%CI)*	(95%CI)*
NIV MAC-		0.96	0.81
ELISA		(0.91, 0.98)	(0.74, 0.87)
PanBio	0.25	0.73	0.95
Rapid	(0.19, 0.33)	(0.65, 0.80)	(0.90, 0.98)
PanBio		0.72	0.97
ELISA		(0.64, 0.78)	(0.92, 0.99)

*95% CI Wilson 'score' method with continuity correction was used.

Difference in sensitivity of NIV MAC-ELISA in comparison to PanBio Rapid and PanBio ELISA was statistically significant (p < 0.001).

Difference in specificity of NIV MAC-ELISA in comparison to PanBio Rapid and PanBio ELISA was statistically significant (p < 0.001).

A total of 68 samples (of the original 154) were analysed by the NIV MAC-ELISA format for IgM antibodies additionally to two other flaviviruses (JE and WN). The total number of samples which were positive for IgM antibodies to one, two or all three flaviviruses is shown in Table 3 along with the findings in the PanBio Rapid and PanBio

ELISA on those samples. (The first 68 samples were chosen based on the NIV MAC-ELISA kit availability). This was based on the known cross-reactivity among the flaviviruses, expecting 30-70% of the sera to show heterologous cross-reactivity. Investigation of this phenomenon needs a sample size of 30 positives (confidence level 99.9%). In the 68 samples tested for the three agents, there were 31 positive samples, thus this panel was found to be sufficient to examine for heterologous reactivity.

Table 3. Positivity in the PanBio Rapid and
PanBio ELISA tests on serum samplescategorized on the basis of flavivirus reactivity
in the IgM Capture ELISA (NIV MAC-ELISA)

Positive category by NIV IgM MAC- ELISA (n)	No. positive by PanBio Rapid Dengue IgM n (%)*	No. positive by PanBio Dengue IgM ELISA n (%)*
Dengue only (8)	2 (25.0)	2 (25.0)
Dengue and JE IgM (10)	1 (10.0)	Nil
Dengue and WN IgM (4)	2 (50.0)	2 (50.0)
Dengue, JE and WN IgM (7)	5 (71.4)	5 (71.4)
JE and WN IgM (2)	1 (50.0)	Nil

* row percentages

JE: Japanese B encephalitis, WN: West Nile

Discussion

A rapid and accurate method for the diagnosis of dengue fever is important for proper patient management, keeping in view the complications and fatality rate associated with it. Several studies have compared and

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evaluated the different serological tests available for the diagnosis of dengue viral infection.

The PanBio Rapid Immunochromatographic card test, following its introduction, has been evaluated in two separate studies^(9,14). In the first study⁽⁹⁾, it was seen that the test demonstrated 100% sensitivity in the diagnosis of dengue viral infection and was able to distinguish between primary and secondary dengue virus infections through separate determinations of IgM and IgG. It was seen that cross-reactivity with JE was only a concern for a small proportion of the patients with secondary dengue infections. In the other study⁽¹⁴⁾, it was seen that, of the total number of patients with primary infection, 88% were IgM positive and IgG negative while, of the total number with secondary infection, 97% were IgG positive with or without IgM. Both the studies, alluded to the finding that the rapid test could be a useful aid in rapid diagnosis of dengue viral infection.

This rapid test seemed to have definitive advantages over the previously available Blot tests in the market, in that no pre-treatment of sera to remove competing IgG or rheumatoid factor was required. Preparation and dilution of reagents along with multiple incubation steps as in Blots could be avoided.

PanBio has also marketed the PanBio Dengue Duo ELISA, which is a capture ELISA format for the detection of IgM and IgG in two separate assays. A study which evaluated this assay⁽¹⁵⁾ revealed that the ELISA was able to diagnose significantly more cases of dengue than the HI assay (55% versus 14%)

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through the use of the first serum sample alone. The results of the IgG Capture ELISA gave a significant correlation with those of the HI assay and it could be used to distinguish primary from secondary dengue infection. One demerit of the PanBio Dengue Duo ELISA was the need to run two separate assays (for IgM and IgG), thereby preventing laboratories from using this due to financial constraints.

To overcome this disadvantage, screening ELISAs were introduced by PanBio, wherein both anti-human IgG and anti-human IgM were applied as a coating to the same assay wells. The level of the anti-human IgG applied as a coating to the well was set to detect high levels of IgG characteristic of secondary dengue infections alone. A study⁽¹⁶⁾, which evaluated this kit, found a sensitivity of 95% and a specificity of 94% in primary and secondary dengue.

In a study⁽¹⁷⁾, comparing two commercially available IgM capture ELISAs (MRL Den Fever Virus IgM Capture ELISA and PanBio Duo ELISA), it was seen that both these performed well, taking into consideration both sensitivity and specificity.

In another study⁽¹⁸⁾, comparing six immunoassays for the detection of dengue IgM and IgG antibodies, the authors arrived at the conclusion that the PanBio Rapid Immunochromatographic test (RIT) was the simplest and the fastest assay to perform. It was seen that the best complete dengue IgM and IgG detection system was the PanBio ELISA. If separate assays were to be performed, a combination of PanBio RIT for IgM detection and PanBio IgG ELISA was found to be the most sensitive and specific combination.

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In the light of all these studies, we chose to compare three tests useful in the diagnosis of dengue viral infection. In our study, the PanBio Rapid Immunochromatographic Card test did not show a good correlation with the NIV IgM Capture ELISA format. Though the sensitivity of this test was found to be low in comparison to the NIV IgM Capture ELISA, it had other advantages. It is a simple and a rapid test that can be performed in five minutes, does not require highly trained personnel or sophisticated equipment such as in field situations thus, enabling its performance even in areas lacking extensive laboratory facilities. Due to the use of standardized reagents for the test, inter-laboratory variation stands reduced. The "Rapid" test is also capable of detecting antibodies to all the four dengue virus types, as the antigen employed for the test is a cocktail of all the four serotypes, enabling its use in any geographical location. The test gives information about both the dengue IgM and IgG responses.

The IgG response detected with this test is set to detect high levels of IgG (HI Titer-1:2560), thus indicative of a secondary infection and just not residual antibodies from a previous infection. Thus, the IgG response detectable by this assay may be helpful for clinicians in diagnosing a susceptibility to DHF, and hence can be used as an initial screening test. This kit also has an extended shelf-life of approximately a year when stored at 4°C.

In comparison to IgM-detecting ELISAs,

laboratory. This might specially prove useful in epidemic situations and in post-monsoon months where many samples are expected to be received in the laboratory, thereby ensuring rapidity in giving the results.

The PanBio Rapid Immunochromatographic Card test has now been by replaced а Rapid Immunochromatographic Strip test, which is essentially based on the same principle as above, except that the card is replaced by a strip. This is presently being used in our laboratory.

The PanBio Microwell IgM ELISA can be modified according to the manufacturer's instructions for the detection of total Ig (IgM and IgG, by avoiding treatment with antihuman IgG) against dengue, but this might not provide useful information, as it merely gives an indication about the antibody response with no differentiation. As the current study focused only on IgM detection, the samples were treated with anti-human IgG supplied with the kit, and carried through the assay. The test, being an ELISA, requires batch testing; thereby rapidity stands compromised. As the microtiter wells are coated with DEN-2 antigen, it cannot be guaranteed that the serotypes of dengue virus other than DEN-2 will be detected with equal efficiency, though cross-reaction exists among the four dengue serotypes. Also, for some samples, the kit gives indeterminate results from which a definitive diagnosis cannot be reached. The protocol requires an ELISA reader for the measurement of OD values, thereby compromising the utility of And And the annual of a second se

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