DETECTION OF DENGUE INFECTION BY COMBINING THE USE OF AN NS1 ANTIGEN BASED ASSAY WITH ANTIBODY DETECTION

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Abstract. We analyzed the utility of a commercial NS1 antigen based ELISA (Panbio Dengue Early ELISA) for detection of dengue infection during the early acute phase and anti-Dengue IgM capture ELISA for detecting dengue infection in patients in dengue endemic settings. A total of 145 serum samples collected from febrile suspected dengue patients were tested for the presence of anti-dengue IgM antibody using IgM antibody Capture ELISA (MAC ELISA) and the presence of dengue virus antigen using PanBio Dengue NS1 Antigen Capture ELISA. Of the 145 patient samples tested, 88 (60.7%) were positive for either NS1 antigen or IgM antibody by MAC ELISA. Dengue NS1 antigen-capture ELISA gave an overall positivity rate of 65.9% (58/88), and IgM ELISA gave an overall positivity rate of 60.2% (53/88). Only NS1 antigen can be used to test during the first two days of fever. MAC ELISA begins to show positive by the third day of illness and gradually its positivity increases. From Day 3 to Day 7, no significant difference in detection rates was seen between the NS1 assay and MAC ELISA. The NS1 antigen assay may be a useful tool for detecting dengue infection during first few days of fever.

Keywords: dengue, NS1 antigen, ELISA, IgM

INTRODUCTION

Dengue is an infection of great concern in India. The first dengue epidemic in India occurred in Kolkata during 1963-1964. Since then the epidemiology of dengue virus has continued to change (Sarkar *et al*, 1964). Dengue infection is endemic in both urban and semiurban India. Dengue outbreaks have been reported from many states in India, including Delhi; recently these epidemics have become more frequent (Bhattacharya *et al*, 2004). During outbreaks, all four dengue virus serotypes have been found circulating in India and the numbers of severe infections and cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are increasing (Chakravarti *et al*, 2002; Bharaj *et al*, 2008).

Diagnosis of recent dengue infection may be achieved by detection of the virus in the patient's blood, either by virus isolation in a susceptible cell culture or by identifying the viral RNA by PCR. Virus isolation is a lengthy process requiring specialized laboratory equipment (Sathish *et al*, 2003). The development of nested

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reverse transcription-PCR (RT-PCR) and realtime RT-PCR have significantly reduced processing times; however, these procedures are expensive and technically exacting. Laboratory contamination can yield false-positive results (Bessof *et al*, 2008). As a result, dengue culture and PCR have limited utility in routine clinical use. Serological diagnosis of dengue infection via detection of immunoglobulin M (IgM) by capture ELISA has many advantages: ease of performance in testing, sensitivity in detecting acute phase antibodies and non-requirement of sophisticated equipment (De Paula *et al*, 2004). IgM detection by capture ELISA (MAC ELISA) is the most widely used test for detecting dengue infection. However, time to IgM production varies considerably among patients. Some patients have detectable IgM by the third day of symptoms, others do not develop detectable IgM until the eighth day of symptoms. The sensitivity of this test is inadequate before the fifth day of symptoms (Xu *et al*, 2006). Because of the complications associated with dengue infection, it would be beneficial to have a diagnosis early in the illness.

Commercial assays based on a non-structural dengue NS1 protein in serum have become available. NS1 is a highly conserved glycoprotein, which appears essential for virus viability. During infection, NS1 is found associated with intracellular organelles or is alternatively transported through a secretory pathway to the cell surface. A soluble hexameric form is released in a glycosylation-dependent fashion from infected mammalian cells (Alcon *et al*, 2002). NS1 protein is found circulating in high concentrations in human serum during the acute phase of the disease (Young *et al*, 2000). Detection of dengue NS1 antigen by ELISA allows detection of infection prior to seroconversion. NS1 antigen can be detected in patient serum 1-9 days after the onset of fever (Alcon *et al*, 2002). Early diagnosis of dengue allows earlier monitoring, possibly reducing the risk for DHF/DSS.

Data regarding NS1 antigen based assays in India is limited. We performed a commercial NS1 antigen based ELISA using Panbio Dengue Early ELISA (Panbio Diagnostics, Australia) on serum samples from suspected dengue cases along with IgM capture ELISA.

MATERIALS AND METHODS

This study was conducted at the virology laboratory of Maulana Azad Medical College and Lok Nayak Hospital, Delhi. This center is part of 80 dengue sentinel surveillance sites in the country under the National Vector Borne Disease Control Program (NVBDCP) of India. The sentinel surveillance hospital duties include taking blood samples from patients with suspected dengue infection, maintaining records of positive dengue cases and capacity building of primary health centers within the district. Each sentinel surveillance site has been provided with an ELISA reader, ELISA washer and other necessary equipment for determining dengue serology. The IgM dengue ELISA capture test kits are supplied by National Institute of Virology (NIV), Pune under the NVBDCP.

As soon as a dengue case is confirmed by serological testing (IgM Capture ELISA), the district vector borne disease control officer/ district Chief Medical Officer or Municipal Health Officer are notified by telephone, e-mail or speed post so immediate vector control measures can be initiated in the affected area(s).

One hundred forty-five acute febrile

patients presenting to the outpatient or emergency departments of the hospital during August to November 2008, with clinical symptoms of dengue infection based on WHO criteria (WHO, 1997) were included in the study after obtaining informed consent. If routine lab tests or clinical features were observed during the 48 hours between admission/presentation and the time of patient blood collection for dengue serology suggested a different diagnosis, the case was excluded. Patients who refused to participate were also excluded from the study.

Acute phase clotted blood samples were collected from the 145 patients included in the study; the serum was separated aseptically and stored at -70ºC until further processing. All the samples were tested for the presence of anti-dengue IgM antibodies using dengue IgM ELISA developed and commercialized by NIV, Pune and provided by the NVBDCP. This assay is highly sensitive (96%) for detection of dengue infection (Sathish *et al*, 2002).

Samples were declared positive or negative based on the ratio of absorbance of the sample to that of the negative per the manufacturer's instructions.

All the samples were also tested for the presence of dengue virus antigen using PanBio Dengue NS1 Antigen Capture ELISA, per the manufacturer's instructions. The NS1 antigen ELISA was performed as follows: 100 l of diluted test mixture (prepared by mixing 1 part test serum with 9 parts diluent) and control was added to the well of a microtiter plate pre-coated with anti-NS1 monoclonal antibody and incubated at 37ºC for 1 hour. The plate was washed six times with diluted buffer to remove any residual serum prior to the addition of 100 l of anti-NS1 MAb-HRP conjugate. After an additional one hour of incubation at 37ºC, the plate was washed and a colorless substrate, tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2), was added. The plate was incubated for 10 minutes at room temperature and the colorimetric reaction was stopped with the addition of phosphoric acid. Within 30 minutes, the absorbance of each well was read at a wavelength of 450 nm with a reference filter of 600-650 nm. The results were interpreted per the manufacturer's instructions.

RESULTS

Of the 145 patient samples tested, 88 were positive for either NS1 antigen and/or IgM antibody by dengue IgM ELISA. Of these 88 patients, 26 were females and 52 males; the M:F ratio was 2.3:1. The most common age group affected was the 26-30 year old group, constituting about 30% of the total positive cases. The youngest patient was 2 years old and the oldest was 65 years old. The majority of patients (92/145, 63.6%) sought medical attention within 5 days of the onset of fever. The results of the two assays for dengue infection are shown in Table 1. Anti-dengue IgM antibodies only were detected in 30 out of 145 samples (34%). NS1 antigen only was detected in 35 of 145 samples (39.7%), while 23 samples (26%) were positive for both NS1 antigen and anti-dengue IgM antibody. NS1 antigen was detectable in patient sera from Day 1 onwards (Table 1) and dengue IgM ELISA was detected from Day 3 onward and gradually increased in positivity toward the end of the acute illness phase of the disease. The significance of the dengue detection methods by length of time from fever onset is shown in Table 2.

Table 1
Positive test results by day of symptoms.

Days after onset of symptoms	No. of sera tested (%)	Only NS1 ELISA positive	Only dengue IgM ELISA positive	Both positive
Day 1	3 (2)	2	0	0
Day 2	11 (7.5)	6	0	0
Day 3	16 (11)	6	4	3
Day 4	19 (13.1)	4	4	4
Day 5	43 (29.6)	8	9	9
Day 6	30 (20.6)	4	5	5
Day 7	22 (15.1)	5	7	2
Day 8	1 (0.6)	0	1	0
Total positive: 88	145	35	30	23

Table 2
Comparison of test results by day of symptoms between the 2 tested methods.

Days after onset of symptoms	Cumulative NS1 ELISA positive	Cumulative MAC ELISA positive	*p*-value
Day 1	2/3 (66.7%)	0/3 (0.0%)	<0.05; S
Day 2	6/13 (46.1%)	0/13 (0.0%)	<0.05; S
Day 3	9/22 (41.0%)	7/22 (32.0%)	>0.05; NS
Day 4	8/30 (27.0%)	8/30 (27.0%)	>0.05; NS
Day 5	17/35 (49.0%)	18/35 (51.4%)	>0.05; NS
Day 6	9/31 (29.0%)	10/31 (32.2%)	>0.05; NS
Day 7	7/11 (64.0%)	9/11 (81.8%)	>0.05; NS
Day 8	0/1 (0.0%)	1/1 (100%)	NE[a]
	58[b]	53[b]	

S, Significant; NS, Not significant
[a] NE, Not evaluated for significance since the sample size was too small (only 1 case).
[b] Includes 23 dengue positives (positive with both NS1 and dengue IgM ELISA).

DISCUSSION

Some dengue diagnostic methods are unable to determine emerging epidemics in a timely manner at a reasonable cost. Consequently, epidemics may be at peak transmission before they are recognized and confirmed as dengue infection. By that time there is significant morbidity and mortality attributable to the disease and preventive measures have less impact on transmission and the course of the epidemic. A rapid, reliable dengue diagnostic method, which is technically less demanding, is needed.

In this study, we carried out a commercial dengue NS1 protein based capture immunoassay (PanBio) and dengue IgM ELISA on clinical samples collected from suspected dengue patients.

The NS1 antigen ELISA gave positive results on patients during the first 2 days of symptoms.

Dengue IgM antibodies began to be detected by the third day of symptoms. On the third and fourth days of illness, we found no significant difference between dengue detection rates between the 2 methods used (Table 2). In 8 of 88 patients (9%) the dengue IgM ELISA results were positive and the dengue NS1 antigen assay results were negative (Table 1). These results cannot be explained on the basis of inhibition of NS1 detection due to the appearance of an early IgM since it has been observed the presence of IgM does not hinder NS1 detection (Alcon *et al*, 2002; Xu *et al*, 2006). One possible reason could be the lower sensitivity of the NS1 commercial kit in detecting dengue virus serotype 4 (Bessof *et al*, 2008). All 4 dengue serotypes have been shown to be circulating in Delhi.

During the fifth to seventh days of symptoms (Table 2), the overall detection rates of the 2 methods were not significantly different ($p > 0.05$), although 13 of 88 patients (14.7%) had a positive dengue NS1 antigen assay and a negative dengue IgM ELISA (Table 1). In these patients, IgM seroconversion might not have occurred yet or these patients may have had a secondary dengue infection, where IgM production is known to be reduced or absent (Innis *et al*, 1997). The NS1 antigen capture ELISA has been shown to be useful in determining dengue infection in acute phase sera during both primary and secondary dengue infections, although the sensitivity of detection is higher in primary infections (Kumarasamy *et al*, 2007; Sekaran *et al*, 2009).

In this study, NS1 antigen was detectable in patient serum from the first to the seventh days of illness. NS1 antigen has been demonstrated in patient sera during the first day to the ninth day of the onset of symptoms (Alcon *et al*, 2002). We did not test the upper limit of duration of NS1 positivity, since there was only one patient in the study with a duration of fever of more than seven days.

The sensitivity of the Panbio NS1 protein assay as reported in literature is 72-93% (Kumarasamy *et al*, 2007; Bessof *et al*, 2008); in RT-PCR the specificity is 97.8% - 100%.

An additional 35 cases (39.7%) had a positive result with the dengue NS1 antigen (Table 1). The NS1 assay has a distinct advantage of being able to detect dengue antigen during the first 2 days of symptoms. For patients with fever less than 2 days in duration, the addition of the dengue NS1 antigen assay may improve the diagnostic arsenal of the clinician. The simple ELISA format requires minimal infrastructure and may provide an early, sensitive and reliable test for dengue infection in areas where it has not been previously available. The NS1 antigen should be evaluated in diverse geographical Indian settings for sensitivity in detecting different dengue serotypes in primary and secondary dengue infections before widespread use.

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