NS1 ANTIGEN DETECTION BY ELISA IN EARLY LABORATORY DIAGNOSIS OF DENGUE INFECTION

Deshkar Smita T[1], Tore Rajani P[2], Raut Sharmila S[3], Khadse Ravindra K[4]

[1]Assistant Professor, [2]Associate Professor, [3]Professor and Head, [4]Associate Professor, Department of Microbiology, Indira Gandhi Government Medical College and Mayo Hospital, Nagpur. Maharashtra.

ABSTRACT

Introduction: Dengue is a major public health problem in tropical and sub-tropical regions of the world and it is known for serious life threatening complications. Detection of IgM antibodies forms the mainstay for diagnosis of dengue infection. However, IgM antibodies develop after 4-5 days of infection and there is an urgent need for an alternative diagnostic tools that can detect dengue infection earlier. **Aim and Objectives:** To evaluate the efficacy of NS1 antigen ELISA for early diagnosis of dengue virus infection in a tertiary care hospital **Methods-** A total of 2106 serum samples from patients with suspected dengue infection were tested for dengue NS1 antigen and IgM antibody detection by ELISA. **Results:** 765 (36.32%) were positive for dengue NS1 antigen and 857 (40.69%) were positive for dengue IgM antibody. NS1 antigen was detectable in patient sera from day 1 onwards however; dengue IgM antibody was detected from day 3 onwards. Out of 765 NS1 antigen positive samples, 562 (73.46%) were positive in acute phase of illness and 203 (26.54%) were positive in convalescent phase of illness. Out of 857 MAC ELISA positive samples, 312 (36.41%) were from acute phase of illness and 545 (63.59%) were from early convalescent phase of illness. Combination of two tests resulted in increase in the positivity rate to 52.66% as against to independent positivity rate of 36.32% of NS1 ELISA and 40.69% of MAC ELISA. **Conclusion:** Combined use of NS1 antigen assay with MAC ELISA test could significantly improve diagnostic sensitivity of dengue infection.

Keywords: NS1 antigen; ELISA; Dengue; Early Diagnosis.

INTRODUCTION

Dengue is the most rapidly spreading mosquito-borne viral disease in the world. In the last 50 years, incidence has increased 30-fold with increasing geographic expansion to new countries and in the present decade, from urban to rural settings.[1] It has been recently estimated that 390 millions dengue infections occur every year (95% credible interval 284- 528 million), of which 96 millions (67-136 millions) manifest clinically with any severity of disease.[2,3]

In view of the high mortality rate and to reduce the disease burden, it is imperative to have a rapid and sensitive laboratory assay for early detection of the disease. Though, there are several laboratory methods available to diagnose dengue infection such as viral isolation, detection of RNA, antigen and antibody assays, haemagglutination inhibition (HI), plaque reduction neutralization test (PRNT), most of these are time-consuming costly methods and need a specialized laboratory with well-trained personnel which may not be widely available in many hospital settings.[1]

During last few years, non-structural 1 (NS1) antigen has emerged as a useful biomarker for early diagnosis of dengue virus (DENV) infection. Seven, non overlapping, virus NS proteins have been identified and mapped to the viral RNA by limited amino and carboxy terminal amino acid sequencing. Encoded 3' to the structural protein-coding region and following the E protein are NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5.[4,5]

NS1 is a highly conserved glycoprotein with molecular weight of ~48,000 kDa for all the serotypes and produced in both cell membrane-associated and secreted forms. It is essential for virus viability or replication, but has no biological activity and precise function has not yet been assigned to it. It stimulates a strong humoral response.NS1 antigen is detectable in blood from first day after the onset of fever up to day 9 and is also detectable in the presence of IgM antibodies and when viral RNA is negative by RT-PCR.[4-6]

Currently, NS1 antigen capture enzyme linked immunosorbent assay (ELISA) and rapid NS1 antigen commercial kits for detection of NS1 antigen are available. Its use has been suggested for early diagnosis of dengue

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Correspondence: Dr. Rajani P. Tore, Associate Professor, Department of Microbiology, Indira Gandhi Government Medical College and Mayo Hospital, Nagpur. Maharashtra440022. Email: smitadeshkar@ymail.com

infection after the onset of fever.[1] Considering this, the present study was conducted to evaluate the potential use of the dengue NS1 antigen ELISA test to improve dengue laboratory diagnosis and to compare it with the current antibody techniques available in our laboratory.

MATERIAL AND METHODS

Study design: An observational study

Ethics approval: Approval was obtained from the intuitional ethical committee.

Study location: The present study was carried out in department of Microbiology at a tertiary care hospital, in central India which is a sentinel surveillance site under National Vector Born Disease Control Programme (NVBDCP).

Study period: It was carried out from July 2014 to June 2016.

Sample size: Total 2106 serum samples from suspected dengue cases were included in this study.

Inclusion criteria: Study group consisted of clinically suspected cases of dengue infection, in the age group of 0-80 years and of both the sexes, admitted in the hospital as well as those who attended outpatient department.

Methodology: Detailed history was obtained from each patient. 2ml of blood samples were collected aseptically and serum was separated as per the standard guidelines.[7,8] Specimens were stored at 2 – 8°C till processing. Repeated freezing and thawing was avoided.

The serum samples were subjected to following tests for the diagnosis of dengue infection -

Dengue NS1 antigen detection with ELISA

Dengue IgM antibody detection with ELISA

Dengue NS1 antigen MICROLISA kit was provided by J. Mitra and Co. Pvt. Ltd. India. NIV DEN IgM Capture ELISA (MAC ELISA) kit was provided by NIV, Pune, India. Tests were performed as per manufacturer's instructions only [9,10].

Dengue NS1 antigen MICROLISA is a solid phase ELISA based on the direct sandwich principle. The microwells are coated with anti- dengue NS1 antibodies with high reactivity for dengue NS1 antigen. The samples were added in the wells followed by addition of enzyme conjugate. A sandwich complex was formed in the well wherein dengue NS1 was "trapped" or "sandwiched" between the antibody and antibody HRPO conjugate. Unbound conjugate was then washed off with wash buffer. Addition of the substrate buffer and chromogen, was followed by addition of stop solution and was read at 450 nm spectrophotometrically within 10 minutes after termination of reaction. Cut off values and dengue NS1 antigen units were calculated.

If the dengue NS1 antigen units were < 9 then the sample was interpreted as negative. If the dengue NS1 antigen units were in between 9 - 11 then the sample was interpreted as equivocal and if the dengue NS1 antigen units were > 11 then the sample was interpreted as positive for dengue NS1 antigen.

Dengue MAC ELISA is based upon the principle that IgM antibodies in the patient's serum are captured by anti-human IgM coated on to the solid surface. Dengue antigen, which was added in next step, binded to captured human IgM in the sample. Unbound antigen was removed during the washing step. In the subsequent step biotinylated flavivirus anti dengue monoclonal antibodies were added followed by Avidin –HRP. Subsequently, chromogenic substrate (YMB/H2O2) was added, the reaction was stopped by 1N H2SO4. The intensity of color/ optical density (OD) was measured at 450 nm within 10 minutes after termination of reaction. If OD value of sample tested was less than OD value of negative control by factor 2.0, sample was considered as negative. If OD value of sample tested exceeded OD of negative control by factor 3.0, sample was considered as positive. If OD value for sample tested exceeded OD of negative control by a factor 2.0 but was less than OD of negative control by factor of 3.0, the sample was considered as equivocal.

Statistical analysis: Data was analysed using statistical package for social sciences version 20 (SPSS V 20.0). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and % of accordance (agreement) were calculated. Relationship between nonparametric discreet observations was analysed by non parametric Chi square test. Significance value of less than 0.05 ($p < 0.05$) was considered for statistical tests.

RESULTS

Total 2106 serum samples from suspected cases were tested for NS1 antigen and IgM antibody for dengue by ELISA. Out of 2106 dengue suspected cases, 765 (36.32%) were positive for NS1 antigen and 857 (40.69%) were positive for dengue IgM antibody (Table 1). Results of NS1 ELISA were compared with MAC ELISA. Sensitivity, specificity, PPV, NPV were found to be 67.06%, 74.35%, 59.86% and 79.82% respectively. Percentage of accordance (agreement) was found to be 71.7% (Table 2).

NS1 antigen was detectable in patient sera from day 1 onwards and dengue IgM antibody was detected from day 3 onwards and it gradually increased in positivity towards the end of the acute illness phase of the disease (Table 3). Out of 765 NS1 antigen positive samples, 562 (73.46%) were from acute phase (≤ 5 days) of illness and 203 (26.54%) were from convalescent phase

(≥ 6 days) of illness. Out of 857 MAC ELISA positive samples, 312 (36.41%) were from acute phase of illness and 545 (63.59%) were from early convalescent phase of illness (Table 4).

Comparison of detection rates of NS1 ELISA and MAC ELISA versus combined tests (NS1+ MAC ELISA) was also done. Combination of the two tests detected 1109 positive samples, which were 344 samples more than NS1 ELISA and 252 samples more than MAC ELISA (Table 5, 6).

Table 1. Positivity of dengue by NS1 ELISA and MAC ELISA

	NS1 ELISA (%)	MAC ELISA (%)
Positive	765 (36.32)	857 (40.69)
Negative	1341 (63.68)	1249 (59.31)
Total	2106	2106

Table 2. Comparison of NS1 ELISA and MAC ELISA

MAC ELISA	NS1 ELISA		Total
	Positive	Negative	
Positive	513	344	857
Negative	252	997	1249
Total	765	1341	2106

Table 3. Positive test results by day of symptoms in NS1 ELISA and MAC ELISA

Days	No. of sera tested	No. of NS1 ELISA Positive (%)	No. of MAC ELISA Positive (%)
Day 1	103	51 (49.51)	00
Day 2	127	62 (48.82)	00
Day 3	278	128 (46.04)	52 (18.71)
Day 4	332	148 (44.58)	93 (28.01)
Day 5	484	173 (35.74)	167 (34.50)
Day 6	341	114 (33.43)	197 (57.77)
Day 7	141	43 (30.49)	112 (79.43)
Day 8	106	30 (28.30)	89 (83.96)
Day 9	83	16 (19.27)	71 (85.54)
Day 10	53	00	39 (73.58)
Day 11	31	00	22 (70.97)
Day ≥ 12	27	00	15 (55.56)
Total	2106	765	857

Table 4. Positivity rate and time of detection in NS1 ELISA and MAC ELISA

Day post onset illness	No. of NS1 ELISA Positive (%)	No. of MAC ELISA Positive (%)
≤ 5 days	562 (73.46)	312 (36.41)
≥ 6 days	203 (26.54)	545 (63.59)
Total	765	857

Table 5. Comparative detection rates of NS1 ELISA versus combined tests (NS1+ IgM ELISA)

NS1 ELISA Test Results	Combined Test Results (NS1+ IgM ELISA)		
	Positive	Negative	Total
Positive	765	00	765
Negative	344	997	1341
Total	1109	997	2106

Table 6. Comparative detection rates of MAC ELISA versus combined tests (NS1+ IgM ELISA)

MAC ELISA Test Results	Combined Test Results (NS1+ IgM ELISA)		
	Positive	Negative	Total
Positive	857	00	857
Negative	252	997	1249
Total	1109	997	2106

DISCUSSION

Dengue fever is an acute systemic viral disease that has established itself globally in both endemic and epidemic transmission cycles in tropical and subtropical regions of the world and it represents a significant economic and disease burden.[1] Accurate and timely diagnosis of DENV infection remains a problem, especially in resource limited countries. It is acknowledged that the management of dengue fever is conservative; nevertheless, strict monitoring of clinical condition and hematological parameters is required to prevent complications, which makes early diagnosis pertinent.[1] Early diagnosis is also vital for exclusion, as dengue fever in most of the cases is clinically indistinguishable from other febrile illnesses prevailing in “dengue season”. Furthermore, early diagnosis plays a crucial role in forecasting a timely warning of an epidemic and in undertaking effective vector control measures.

In the present study, total 2106 serum samples from suspected dengue cases were tested for detection of NS1 antigen and IgM antibody by ELISA. Out of 2106 dengue suspected cases, 765 (36.32%) were positive for dengue NS1 antigen and 857 (40.69%) were positive for dengue IgM antibody. Our findings are in accord-

ance with Sahu SK et al who reported seropositivity of 36.03% by NS1 ELISA.[11] Ukey PM et al and Karoli R et al reported positivity of 31.3% and 39% respectively by MAC ELISA.[12,13] However, higher positive detection rates of 80.9% and 47.9% of NS1 ELISA and MAC ELISA respectively were observed by Anand AM et al.[6] The variability in detection rate might be because of variations in inclusion criteria of the study, method of estimation and prevalence of dengue virus in that particular region.

In the present study, we compared the results of NS1 ELISA with MAC ELISA. Sensitivity, specificity, PPV, NPV were found to be 67.06%, 74.35%, 59.86% and 79.82% respectively. Percentage of accordance (agreement) was found to be 71.7% (Table 2). There are several reports in literature assessing the usefulness of different NS1 ELISA for diagnosis of dengue infection and sensitivity of NS1 ELISA has been reported from 32.2%-95.9%.[6, 14-16]

We were able to detect NS1 antigen in patient's sera from day 1 onwards and upto day 9 of post onset of illness. Dengue IgM antibody was detected from day 3 onwards and gradual increase in positivity was observed towards the end of the acute illness phase of the disease (Table 3). Our findings are in accordance with Chakravarti A et al who reported positive results on patients during the first 2 days of symptoms by NS1 ELISA and dengue IgM antibodies were detected by the third day of symptoms.[17] Anand AM et al also observed NS1 antigen positivity as early as on day 1 of days post onset of illness (DPO) and it was detectable upto day twelve.[6] 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 clinicians.

In the present study, out of 765 NS1 antigen positive samples, 562 (73.46%) were positive in acute phase of illness and 203 (26.54%) were positive in convalescent phase of illness. This dropped off of 46.92% in NS1 antigen detection in acute phase and convalescent phase sera is statistically highly significant ($p < 0.00001$). Out of 857 MAC ELISA positive samples, 312 (36.40%) were positive during acute phase of illness and 545 (63.60%) were positive during early convalescent phase illness. This increase of 27.2% in IgM antibody detection in acute phase and convalescent phase sera is statistically highly significant ($p < 0.00001$). Similar findings have been reported in previous studies where NS1 antigen was though found to be circulating up to day 9 of illness; the level of NS1 antigen decreased significantly after the acute phase.[18,19] The sensitivity of MAC ELISA was found to be inadequate before the fifth day of symptoms by Xu H et al.[20]

The reason for differences in positivity rates of different tests can be attributed to difference in physiological mechanisms guiding positivity of dengue virus. NS1 antigen circulates uniformly in all serotypes of dengue virus and it circulates at high level during the first few days of illness.[21] NS1 antigen levels varies from 0.04 - 2 µg/ml in acute-phase serum samples, to only 0.04 µg/ml or even less in convalescent phase serum. [22] Decreased levels of NS1 after 4 days of illness can be because of formation of immune complexes; due to which the target epitopes are not accessible to monoclonal antibodies.[23-24] However, efforts to dissociate immune complexes have shown to enhance the sensitivity of NS1 antigen detection ELISA.[25]

Dengue IgM antibodies are detectable in 50% of patients by 3-5 days after onset of illness, increasing to 80% by day 5 and 99% by day 10. Dengue IgM antibody levels peak about two weeks after the onset of symptoms and then decline generally to undetectable levels over 2–3 months.[1] This explains significant increase in IgM antibody detection in convalescent phase sera compared to acute phase sera.

In the present study, we compared detection rates of NS1 ELISA and MAC ELISA versus combined tests (NS1 + MAC ELISA). Combination of the two tests (NS1 + MAC ELISA) detected 1109 positive samples, which were 344 samples more than NS1 ELISA and 252 samples more than MAC ELISA. This resulted in raise up in the positivity rate to 52.66% as against to independent positivity rate of 36.32% of NS1 ELISA and 40.69% of MAC ELISA (Table 5, 6). Combination of two tests also increased the sensitivity to 77.28% as opposed to the sensitivity of 67.05% of NS1 ELISA only (Table 2, 6).

Similar findings have been reported in a study by Datta and Wattal, the independent positivity of NS1 assay and MAC-ELISA were 23.3% and 39.1% respectively which increased to 53.3% when both the tests were used in combination.[26] In another study, overall detection rate of cases increased to 65.38% by combination of two ELISAs as compared to 25.59% and 17.3% by NS1 antigen ELISA and MAC ELISA alone respectively.[27]

The IgM capture ELISA is most commonly used in India due to its low cost, ease of handling and non-requirement of sophisticated equipment.[28] 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. Here it is important to understand that, NS1 antigen detection assay has an advantage of detecting infection very early, however it disappears early also and is of little use in the early convalescence phase when IgM antibody is useful. Since it is also difficult to judge accurately which post infection day the sample is being tested, it is ideal to use both assays even though it would double the cost, it significantly adds to detection rate especially in

acute phase of illness.

CONCLUSION

With the widespread geographic expansion of dengue fever along with the increasing number and severity of reported cases, the use of NS1 antigen detection could allow clinical diagnostic laboratories in early identification of dengue virus infections, providing with enough time to optimise patient management, reducing the time between detection of the first cases and the notification of public health authorities, including vector control teams. It would be safe to say that ELISAs based on a single biomarker, NS1 antigen or IgM antibody have limitations when such tests are used individually. The findings in present study, in general, substantiating the findings as reported in earlier studies and re-affirm the use of NS1 antigen and IgM antibody detection as complementary diagnostic tests in suspected cases of dengue infection.

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