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Original Article

Utility of Battery of Tests For Accurate Serodiagnosis of Dengue.

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ABSTRACT

Introduction: Inview of high morbidity and mortality associated with dengue, its imperative to have rapid and sensitive laboratory tools for early detection of the disease. Serological diagnosis of dengue by antibody testing though performed routinely is considered confirmatory if paired serum samples are tested. This is the practical problem in health care settings where patients after receiving adequate health care are discharged making followup difficult and only single serasample is available for serodiagnosis. Dengue diagnosis can also be arrived at by PCR and NS1 antigen detection. However, these tests also have their own set of limitations. This study is undertaken to access the utility of battery of these tests when carried out on a single specimen for accurate serodiagnosis of dengue. Materials and Methods: One forty four samples received from clinically suspected dengue cases with fever of 1-9 days were subjected to panel of tests viz. Dengue NS1 antigen capture ELISA, Dengue IgM antibody capture ELISA and Dengue PCR simultaneously. Results: Out of 144 samples tested, 28.4%, 34.02%, and 40.9% were positive for dengue NS1 antigen ELISA, PCR and IgM antibody ELISA respectively. Based on the dual combination, NS1 antigen ELISA+PCR, NS1 antigen ELISA+IgM antibody ELISA and PCR+IgM antibody ELISA, 37.5%, 48.6%, and 51.38% of patients were positive respectively. Combination of all the 3 tests yielded 63.19% of specimens positive for DENV infection. Conclusion: No solitary laboratory test is enough to diagnose all acute cases of dengue infection. Hence when NS1antigen detection or PCR is combined with antibody detection on same specimen, the possibility of missing out dengue infection be minimized and an accurate serodiagnosis of dengue infection can be achieved.

1. Introduction

Dengue fever is an acute arbo-viral disease affecting the tropical and subtropical regions of the world. The incidence of this disease has increased manifold over the last five decades with 100 million people affected each year and over 2.5 billion are at risk for infection. It is also associated with high mortality claiming around 30,000 deaths, mostly amongst children.[1] Dengue virus (DENV) which belongs to the genus flavivirus is transmitted by a mosquito vector, Aedes aegypti. It has been classified into four distinct serotypes (DENV 1-4) which are antigenicaly related. [2]

Laboratory confirmation of dengue relies on demonstration of the presence of DENV by (a) isolation of DENV from patient serum, (b) detection of viral RNA by reverse transcriptase polymerase chain reaction (RT-PCR) and (c) detection of dengue specific antibodies by using enzyme –linked immunosorbent assay (ELISA). [1]

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The first two assays have restricted scope as a routine diagnostic procedure. Viral isolation by cell culture and subsequent detection by immunofluorescence, though a gold standard,[3] cannot be used as a routine diagnostic procedure due to its low sensitivity, laborious procedure and time consumption. In case of molecular methods, the requirement of a highly trained staff, the need of sophisticated equipment as well the cost factor has limited its application as a routine diagnostic assay. [1]

The usage of dengue specific antibody detection assays, though cost-effective, is limited in the early phase of the disease as antibodies become detectable only around the fifth day upon the onset of the disease.[4] Moreover, a single serological detection of IgM is merely indicative of a recent dengue virus infection, and should not be interpreted as a confirmatory diagnosis of acute infection without a paired second serum sample. This is the practical problem in health care settings where patients after receiving adequate health care are discharged making follow-up difficult and only single serasample is available for serodiagnosis.

DENV comprises 7 non structural proteins NS1, NS2A, NS3, NS4A, NS4B and NS5.[5]Amongst them, NS1 – a highly conserved glycoprotein which is essential for virus replication is produced in both membrane associated and secretion forms. It is found in abundance in the serum of patients during the early stages of DENV infection. [6] Several studies have shown that testing for dengue NS1 which is detectable in blood from the first day after the onset of fever, before the formation of antibodies could be helpful in supplementing the early diagnosis. [7]

Hence considering the limitation of each of these tests, this study is carried out to assess the role of NS1 antigen ELISA, IgM antibody ELISA and PCR as an individual test, as dual combination and as a panel when carried out on the single specimen for accurate serodiagnosis of dengue.

2. Materials And Methods

This study was carried out in a tertiary care teaching hospital in Mumbai over a period of 6 months during January 2012 to June 2012. One forty four samples from the clinically suspected dengue cases were analyzed. These samples were received from the patients of all age groups. Serum was separated and subjected to a panel of tests viz. Dengue NS1 antigen capture ELISA, Dengue IgM antibody capture (MAC) ELISA and Dengue PCR simultaneously. Duration of fever in days and other relevant clinical information was recorded from the requisition form.

Dengue NS1 antigen early ELISA

The assay was performed in accordance with manufacturer's instructions (Panbio, Brisbane, Australia). The sensitivity and specificity of dengue NS1 antigen capture ELISA kit is 76% and 98.4% respectively as mentioned in the kit literature. Briefly, 100 microlitres of diluted (1:10 in serum diluent) patient serum, positive control, negative control, and calibrator was added to microwells precoated with a polyclonal capture anti-NS1 antibody and then incubated for 1 h at 37ºC. The plates were washed six times and incubated for an additional 1 h at 37ºC following the addition of HRP-conjugated anti-NS1 MAb. After an additional six washes, antibody complexes were detected by adding TMB and incubating samples for 10 min at room temperature. The reaction was stopped by adding stop solution (1M H3PO4), and the plates were read. The cut-off value was determined by multiplying the average OD of the calibrator (tested in triplicate) by the lot-specific calibration factor (provided in the kit insert). An index value was calculated by dividing the average OD of each sample by the cut-off value. Index values of <0.9, 0.9 to 1.1, and >1.1 were considered negative, equivocal, and positive, respectively.

Dengue IgM Antibody capture ELISA

These kits were supplied by National Institute of Virology (NIV), Pune. Test procedure and Interpretations were made as per the kit literature. The sensitivity and specificity of MAC ELISA kit is 98% and 94.22% respectively as mentioned in the kit literature. Briefly, Wells were washed thrice with wash buffer, 50 microlitres of diluted (1:100 in serum diluent) patient serum, positive control, negative control, was added to microwells precoated with anti-human IgM antibody and then incubated for 1 h at 37ºC and washed for five times. Then 50 microlitres of dengue antigen was added and incubated for an additional 1 h at 37ºC and then wells were washed five times. Again 50 microlitres of Anti dengue monoclonal antibody was added and incubated for 1 h at 37ºC and washed five times. 50 microlitres of Avidin HRP was added and incubated for 30 min at 37ºC, this was followed by washing the plate five times. 100 microlitres TMB/H2O2 (substrate) was added and incubated for 10 min at room temperature. The reaction was stopped by adding stop solution (1 N H2SO4), and the plates were read at 450 nm. If OD value of sample tested exceeds OD of Negative control by a factor 4, the sample was considered as 'Positive'.

Dengue genome (PCR) detection

Dengue genomic RNA was detected by real time reverse transcriptase – polymerase chain reaction (RT-PCR) method by the TaqMan® RNA Amplification Kit (Roche Molecular Systems, Inc.). This test is considered as gold standard for serological diagnosis and was carried out at the reference laboratory for PCR testing for MCGM Hospitals.

Ethics Consideration: There is no conflict of interest between the suppliers and the producers of the kits and the Public Health Laboratories. We have analyzed only information retrieved from laboratory databases. All samples included in the study were received by the laboratory for dengue diagnosis. The identities of the patients were kept confidential and biological samples were identified only by numbers.

3. RESULTS:

Table 1: Results of samples (n=144) tested by NS1 antigen ELISA, PCR and IgM antibody ELISA tests.

Test	NS1 antigen ELISA	PCR	IgM antibody ELISA
Positive	41 (28.4%)	49 (34.02%)	59 (40.9%)
Negative	103 (71.52%)	95 (65.97%)	85 (59.1%)

Samples included in the study were received from day 1 to 9 of fever. Viremia in DENV infection generally lasts for 4 to 5 days and antigens remain detectable in the blood up to 5 days after the onset of symptoms and rapidly disappear following the appearance of specific antibodies.[8] Here out of 41 samples tested positive by NS1 antigen ELISA, 70.73% (29/41) were positive on day 1 and 2 of fever and decreased up to 4.87% (2/41) on day 5 of fever. Similar findings were observed for PCR, out of 49 PCR positive samples 81.63% (40/49) were positive on day 1 and 2 of fever and decreased up to 2.04% (1/49) on day 8 of fever. From 59 dengue IgM antibody positive samples, 5.06% (3/59) were detected from day 5 onwards and gradually increased up till day 9 in 50.84% of samples (Figure 1).

Figure 1: Co-relation of Dengue test with days of fever.

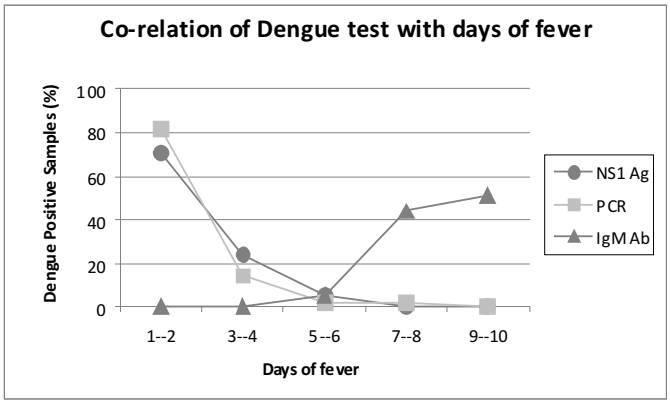

Table 2: Test results of various combinations.

Combinations	Positive	Negative
NS1 antigen ELISA + PCR	54 (37.5%)	90 (62.5%)
NS1 antigen ELISA + IgM antibody ELISA	70 (48.6%)	74 (51.4%)
PCR + IgM antibody ELISA	74 (51.38%)	70 (48.62%)
NS1 antigen ELISA + PCR + IgM antibody ELISA	91 (63.19%)	53 (36.81%)

4. DISCUSSION

In this study, 144 serum samples were tested for the presence of DENV infection. It was found that when tested by individual test, 28.4% (41/144) were positive by NS1 antigen ELISA, 34.02% (49/144) were positive by PCR and 40.9% (59/144) were positive by IgM antibody ELISA. (Table 1)

To increase the chances of detection of DENV, dual and triple test combination were tried. The various combinations tested were NS1 antigen ELISA + PCR, NS1 antigen ELISA + IgM antibody ELISA, PCR + IgM antibody ELISA and NS1 antigen ELISA+ PCR + IgM antibody ELISA

Combination of NS1 antigen ELISA + PCR

Based on this combination, 37.5% (54/144) of patients were found positive (Table 2). Out of these 54 positive samples, 36 were positive by both tests. PCR detected 13 NS1 antigen negative samples, while NS1 antigen test was positive for 5 PCR negative samples. Rest (90/144) samples were tested negative by both methods. Similar results were also obtained in the study by Kassim FM et al, where out of 208 tested samples, 50% (104/208) of patients were found positive by the combination of NS1 antigen ELISA + PCR. Out of these 104 positive samples, 43 were positive by both tests. PCR detected 37 NS1 antigen negative samples, while NS1 antigen test was positive for 24 PCR negative samples. Rest (104/208) samples were tested negative by both methods.[9]

Combination of NS1 antigen ELISA + IgM antibody ELISA

Based on this combination, a total of 48.6% (70/144) patients were positive for DENV infection. Out of 70 positive samples, 30 were positive by both tests. NS1 antigen was detected in 11 IgM antibody negative samples while IgM antibody was detected in 29 NS1 negative samples. Rest (74/144) samples were tested negative by both methods. The possible explanation for reduced NS1 sensitivity in the presence of a measurable anti-DENV antibody response is that the plasma NS1 is sequestered in immune complexes and the target epitopes are not accessible to either the plate-bound anti-NS1 antibody or HRP-conjugated anti-NS1 MAb in the NS1 ELISA. They may already have established an anamnestic humoral immune response characteristic of a secondary infection and are therefore more likely to be NS1-negative. [10] This positivity was about 11.1% higher than the combination of NS1 antigen ELISA + PCR tests.

Combination of PCR + IgM antibody ELISA

When PCR and IgM antibody tests were combined, 51.38% (74/144) of patients were positive for DENV infection. Out of 74 positive samples, 34 were positive by both tests. IgM antibody was detected in 25 PCR negative samples while 15 PCR positive samples

were IgM antibody negative. Rest (70/144) samples were negative by both the method. This positivity was 13.88% and 2.78% higher than the NS1 antigen ELISA + PCR and NS1antigen ELISA + IgM antibody ELISA combinations, respectively.

The study by V. Doung et al also showed when RT-PCR was used alone the sensitivity was 77.3%, which increased up to 95.4% when PCR and MAC-ELISA combined together. This combination of RT-PCR with IgM antibody detection showed slightly higher sensitivity than the combination of NS1-antigen capture assay and IgM antibody detection for dengue diagnosis. [11]

Combination of NS1 antigen ELISA+ PCR + IgM antibody ELISA

When this three test panel was used, 63.19% of patients (91/144) were found positive for DENV infection, this positivity was 25.6%, 14.59% and 11.81% higher than the dual combination of NS1 antigen ELISA + PCR, NS1 antigen ELISA + IgM antibody ELISA, and PCR + IgM antibody ELISA respectively.

The results obtained from this study allow the laboratory to consider and choose a suitable combination of tests that increases test sensitivity. The detection rate of DENV infection was similar between the combinations of PCR + IgM Antibody ELISA (51.38%) and NS1 antigen ELISA + IgM Antibody ELISA (48.6%) (z =0.716; 95% CI).

PCR and NS1 antigen ELISA are both useful in detecting dengue in early stages of fever. PCR test can not be used as the routine diagnostic test due to the requirement of sophisticated equipments and technical expertise, when PCR test is combined with any other test the total cost increases imposing the financial burden on the patient or the hospital, also the funds required to establish PCR labs may be limited in smaller set-ups and in rural areas. NS1 antigen ELISA can be used as an effective alternative to PCR and when combined with IgM antibody ELISA, this combination become simple, easy to perform and economical. Studies by Sekaran et al and Blacksell et al also showed that the NS1 antigen ELISA when used in conjunction with IgM capture ELISA is sufficiently sensitive and specific to be clinically informative in diagnosing the dengue in endemic settings. [8, 12]

5. CONCLUSION

Solitary laboratory assay in isolation is not adequate enough to diagnose all acute cases of dengue. NS1 antigen and PCR test are potentially useful during early febrile stage, while IgM antibody test is useful after 5th day of fever and requires paired sera sample. Hence when combination of NS1 antigen ELISA or PCR is performed with IgM Antibody ELISA simultaneously on the single specimen the possibility of missing out the dengue infection will be minimized. Considering the limitations of PCR as a routine diagnostic tool, NS1 antigen detection may act an effective alternative and prove to be an economical, simple and easy to perform aiding accurate serodiagnosis of dengue infection.

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