Clinical Applicability of Various Dengue Diagnostic Tests in Resource-Limited Endemic Settings

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ABSTRACT

Introduction: Dengue is one of the most important arboviral infections caused by one of the four dengue serotypes, 1-4. **Objective:** To study the applicability of different diagnostic methods in diagnosis of dengue viral infection. **Materials and Methods:** A total of 2101 blood samples were collected for confirmation of dengue viral infection. All the samples were tested by dengue-specific IgM ELISA, of which 111 were also tested for NS1 antigen detection and 27 acute samples (≤5 days) were further subjected for viral RNA detection by RT-PCR and isolation in C6/36 cell line. To detect the sensitivity of NS1 antigen for different dengue virus serotypes, four dengue serotype 1 and 12 dengue 3 were subjected for the NS1 antigen assay. **Results:** Most common age group affected was 16-45 years, with male to female ratio of 2.8:1. During first 3 days of illness virus isolation and RT-PCR were the most sensitive (83%) followed by NS1 antigen detection (75%) and IgM detection (37.5%). The positivity of IgM detection was found to be significantly higher as compared to NS1 detection during 4 to 5 days and also after 5 days of illness ($P < 0.05$). Dengue serotypes 1 and 3 were found to be co-circulated, dengue 1 being the predominant serotype. **Conclusion:** Virus isolation and RT-PCR were the most sensitive tests during the early period of illness whereas beyond third day, IgM antibody detection was found to be the most sensitive method of dengue diagnosis.

Key words: Dengue, Diagnosis, IgM antibody, NS1 Ag, RT-PCR

INTRODUCTION

Dengue viral infection is caused by one of the dengue virus types (DENV 1-4) belonging to the family Flaviviridae. A majority of patients suffer from self-limiting febrile illness (dengue fever; DF), whereas some may progress to severe life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[1,2]

Several diagnostic tests are presently available for diagnosis of dengue viral infection. Isolation of virus is confirmative but less sensitive and time consuming. The hemagglutination inhibition (HI) and complement fixation test (CFT) were previously the recommended tests by World Health Organisation (WHO). As these tests are time consuming, requires paired samples and tedious to perform presently replaced with dengue IgM antibody detection by MAC ELISA and dengue NS1 antigen by ELISA tests. Molecular techniques like RT-PCR, multiplex RT-PCR and real-time PCR system have also been reported for early detection of dengue RNA to warrant as acute dengue infection.[2] Thus, different diagnostic tests available for dengue viral infection testing are applicable at different phases of illness. Considering the above-mentioned kinetics, the present study compared the positivity of different tests during different phases of dengue viral illness in order to guide the microbiologists and clinicians to follow the appropriate diagnostic strategy.

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MATERIALS AND METHODS

The study was carried out in Dept. of Virology, Postgraduate Institute of Medical Education and Research, Chandigarh, which is one of the apex centers for advanced diagnosis of dengue, Japanese encephalitis, chikungunya and other arboviral infections under National Vector Borne Disease Control Programme, Ministry of Health and Family Welfare.

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A total of 2101 blood sample were received from clinically suspected dengue cases from Chandigarh and neighboring states during 2010. The inclusion criteria for dengue cases were considered as per the WHO criteria.[2] All these samples were tested for dengue IgM antibody by MAC ELISA as a routine diagnostic service of the department. One hundred and eleven samples were subjected to NS1 antigen ELISA whose information on duration of illness was available. Samples collected within 5 days of illness were further subjected for viral RNA detection and virus isolation in C6/36 cell lines. Dengue IgM was performed by MAC ELISA (National Institute of Virology, Pune, India) and dengue NS1 antigen detected by NS1 by ELISA (Panbio, Australia) as per manufacturer's instructions. The samples tested positive by any of the four mentioned tests were considered as positive for dengue viral infection and were included for analysis.

To detect the sensitivity of NS1 antigen for different dengue virus serotypes four dengue serotype 1 and 12 dengue serotype 3 (2 from 2010 and 10 from 2008) were subjected for NS1 antigen detection.

Virus isolation

Isolation of dengue virus was done as described before.[3]

Detection of dengue viral RNA

Briefly, viral RNA was extracted from 140 μL of serum sample and P2 supernatant fluid using QIAamp Viral RNA Mini Kit (Qiagen, USA). RNA was eluted in 50 μL of elution buffer and used as a template for cDNA synthesis. The extracted RNA was reverse transcribed on the same day with RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, USA) as per the manufacturer instructions.

Single-tube dengue multiplex RT-PCR

The synthesized cDNA was then subjected to single tube dengue multiplex PCR as described previously.[4] Briefly, viral cDNA was amplified using dengue-specific D1 upstream primer and four serotype-specific downstream primers TS1, TS2, TS3 and TS4. The thermal profile of PCR amplification cycle followed was: 35 cycles of denaturation at 95°C for 30 sec; annealing at 55°C for 45 sec and extension at 72°C for 2 min. The PCR products were visualized by 2% agarose gel electrophoresis under the digital gel documentation system (Alpha Innotech, San Leandro, CA).

Gel elution

The PCR positive samples were subjected to gel purification using the QIAquick Gel Extraction Kit (Qiagen, Germany) and the purified product was eluted in 30 μl of elution buffer which was used as a template for sequencing reaction.

Nucleotide sequencing

The purified PCR products were cycle sequenced in an ABI PRISM 310 genetic analyzer (PE Applied Biosystems Inc., Foster City, CA, USA) using an ABI PRISM Big Dye Terminator cycle sequencing kit (PE Applied Biosystems Inc., Foster City, CA). For confirmation of the sequencing product, BLAST programme was implemented.

Nucleotide sequence analysis

The obtained sequences were edited followed by BLAST search to confirm the identity of the sequences. The phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4. The phylogenetic tree was drawn by using the Neighbor-Joining method with bootstrap analysis of 1000 replicates. The sequences of dengue serotype 1 from different geographical regions were retrieved from GenBank and their accession numbers for genotyping and sub-typing.

Statistical analysis

All the data was analyzed using SPSS 17.0 software. The statistical analysis was performed at a significance level of *P* value <0.05 using the chi-square test.

RESULTS

Of the 2101 dengue suspected serum samples tested for IgM antibody, 745 (35.5%) were found to be positive. A majority of them were in the age group of 16-45 years (61%), with a male to female ratio of 2.8:1. The cases of dengue occurred from August through December, with a peak in October. Of the 111 tested samples 79 were positive by one of the four diagnostic tests applied and thus were included for analysis.

Result of samples collected within 1 to ≤3 days of illness

A total of eight samples were collected from patients with ≤3 days of illness of which six samples were tested by all the four tests where as two samples could not be subjected for virus isolation and RT-PCR due to less sample volume. Virus isolation and RT-PCR could detect maximum number of samples during this period with a positivity

of 83.3% (5/6) followed by NS1 antigen detection (75%: 6/8), and IgM antibody detection (37.5%:3/8 ($P = 0.180$) [Table 1].

The RT-PCR product revealed dengue type1 in majority (4/6) and type 3 in two samples. All the type 1 samples were confirmed by nucleotide sequencing.

Result of samples collected during 4-5 days of illness

Thirty-two samples collected from patients with 4-5 days of illness, of which 21 samples were tested by all four tests and the remaining 11 samples were tested only for IgM antibody and NS1 antigen detection.

IgM antibody could be detected in 30 of 32 samples tested during this period of illness (98%) whereas NS1 antigen could be detected in 20 of these samples with a sensitivity of 62.5%. The overall positivity of IgM antibody and NS1 antigen detection was found to be 90%, 54% respectively. The positivity of IgM antibody was found to be significantly higher than NS1 antigen detection ($P = 0.005$). The detail of dengue IgM and NS1 antigen positivity is depicted in Table 2.

Result of samples collected after 5 days of illness

A total of 39 samples collected after 5 days of illness were tested for IgM antibody and NS1 antigen detection. The positivity of dengue IgM antibody detection was found to be significantly higher as compared to that of NS1 antigen detection (97% Vs 44%; $P < 0.05$).

Table 1: Day wise positivity of different diagnostic tests for dengue viral infection

Day of illness	IgM +ve a/b (%)	NS1 +ve a/b (%)	RT-PCR +ve a/b (%)	Isolation +ve a/b (%)
1	0/2 (0)	1/2 (50)	2/2 (100)	2/2 (100)
2	1/2 (50)	1/2 (50)	1/1 (100)	1/1 (100)
3	2/4 (50)	4/4 (100)	2/3 (67)	2/3 (67)
4	17/19 (89.5)	12/19 (63)	1/12 (8.3)	1/12 (8.3)
5	13/13 (100)	8/13 (61.5)	0/9 (0)	0/9 (0)
6	16/17 (94.11)	8/17 (47)	ND	ND
>6	22/22 (100)	9/22 (41)	ND	ND

a: No. of positive samples, b: No. of tested samples, ND: Not done

Result of NS1 antigen in different dengue serotypes

NS1 antigen could be detected in all the 4 serum samples of dengue type 1 and 4 of 12 dengue type 3 giving a sensitivity of 100% and 33.3% for type1 and type 3, respectively.

Genotyping and sub typing of dengue viruses

The dendogram showed the sequences of the present study isolates were clustered along with the genotype III and subtype 2 when compared among the reference sequences of dengue serotype 1 [Figure 1].

DISCUSSION

Dengue is a disease with wide spectrum of clinical manifestations mimicking several other illnesses. Early diagnosis of disease is of importance, as with timely intervention case fatality can be reduced to <1% in severe cases.[2]

In our study 35.5% of cases were serologically positive for dengue infection. The higher positivity among young adult males (61%) is consistent with previous dengue reports by the authors, as well as other Indian studies.[5-8] In several other studies pediatric population was most commonly affected.[9,10]

The knowledge of seasonal trends is important for timely implementation of effective control and preventive measures. Dengue cases are usually reported during post monsoon months as the climatic conditions during this period are considered favorable for breeding of *Aedes aegypti* mosquitoes.[5]

Confirmation of suspected dengue cases can be done by isolation of virus, viral RNA detection, viral antigen or IgM antibody detection in patients' blood sample.[2] Among these, serological test for IgM antibody detection is most widely employed because of its wide availability, high sensitivity and specificity and also as the immunoglobulin are stable at tropical room temperature, specimen transport does not pose any problem.[2]

Table 2: Dengue IgM antibody and NS1antigen positivity during different period of dengue viral infection

Days of illness	IgM+ve NS1 –ve a/b (%)	IgM+ve NS1 +ve a/b (%)	IgM-ve NS1 +ve a/b (%)	IgM-ve NS1 –ve a/b (%)	Total IgM positivity a/b (%)	Total NS1 positivity a/b (%)
1-3	1/8 (12.5)	2/8 (25)	4/8 (50)	1/8 (12.5)	3/8 (37.5)	6/8 (75)
4 to 5	12/32 (37.5)	18/32 (56.3)	2/32 (6.2)	0	30/32 (94)	20/32 (62.5)
>5	22/39 (56.4)	16/39 (41)	1/39 (2.6)	0	38/39 (98)	17/39 (44)

a: No. of positive samples, b: No. of tested samples

Because of transient viremia and time required for antibody development, it is widely considered that dengue infection of <5 days illness may be diagnosed by virus isolation, viral nucleic acid detection and recently by viral NS1 antigen detection either by ELISA or rapid tests.[2] The present study found virus isolation and RT-PCR as the most sensitive during first 3 days of illness, followed by NS1 antigen and IgM antibody detection. However, the difference was not found to be significant ($P = 0.180$) which could be due to less number of sample size. Whereas after day 3 of illness, positivity of IgM antibody detection was found to be significantly higher compared to NS1 antigen detection ($P < 0.05$). The early appearance of IgM antibody has been reported in secondary dengue infections as compared to primary dengue infection.[11] As Chandigarh is known for its endemo-epidemic status for dengue virus infection with sero-prevalence more than 70%, majority of dengue cases are considered to be secondary infections.[5] Secondly, the lower positivity of NS1 antigen after 3 days of illness in the present study could also be due to the secondary nature of dengue infection in this region due to endemicity. High titers of pre-existing IgG antibodies have been attributed to the lower positivity of NS1 antigen detection in acute secondary dengue infection as compared to that of acute primary infection.[12-16] Considering the observation of the present study and available literature, authors have tried to provide a practical approach to dengue diagnosis for endemic countries [Figure 2].

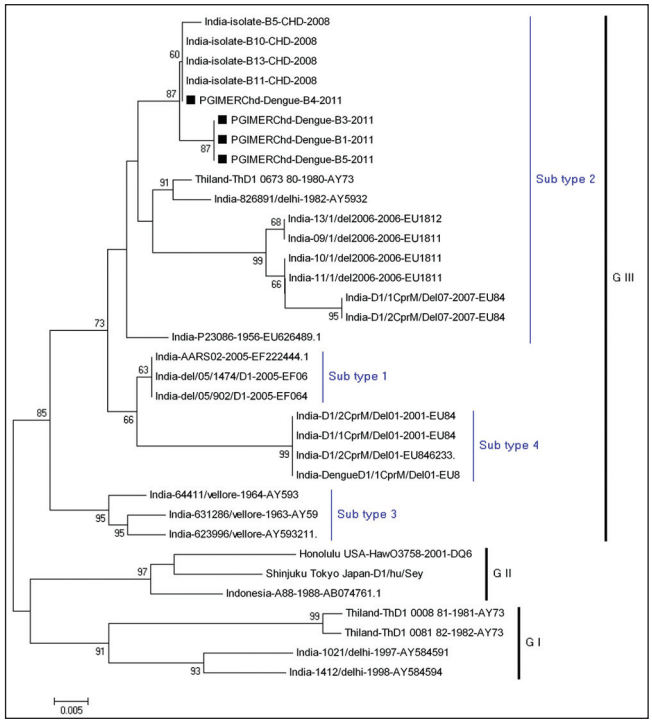

Figure 1: Phylogenetic analysis of dengue virus

Diagnosis of dengue based on IgM antibody detection by MAC ELISA from single serum specimen in absence of positive RT-PCR in acute phase sample has been classified as recent probable dengue infection.[17] This could be either due to the persistence of dengue IgM antibody for 2-3 months after acute illness or due to the cross reactivity with other Flaviviruses. However, a late acute phase sample (around day 5 of illness) from a suspected dengue patient if found negative for IgM antibody as well as by RT-PCR is labeled as a laboratory-indeterminate case and in such cases a either a convalescent sample should be asked to confirm the diagnosis[17] or sample may be subjected for NS1 antigen detection.

The sensitivity of NS1 antigen testing in different dengue virus serotypes infection has been reported variedly in different studies.[18,19] Studies from Venezuela and Vietnam have shown lower NS1 sensitivity for dengue virus-2 infections.[16,20] The present study found lower sensitivity of NS1 for dengue virus-3 serotypes which needs to be confirmed with large number of samples from both primary and secondary dengue infection.

The role of viral RNA detection by RT-PCR though limited only to the early part of dengue diagnosis, the method has the potency of dengue virus typing which is important to assess the severity of outbreak and to take timely appropriate control measures.

Circulation of various dengue types have been reported in the past decade. Dengue types 1, 2 and 3 have been found to be circulated in Chandigarh over the last few years.[3-5,21]

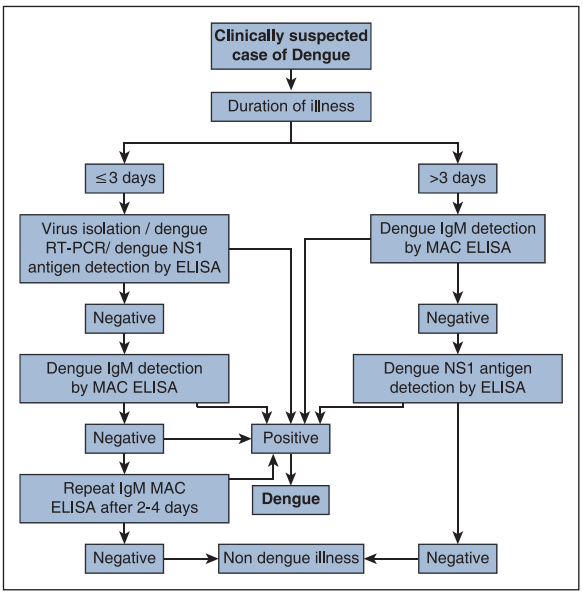

Figure 2: Algorithm for laboratory diagnosis of clinically suspected dengue cases in endemic setting

The present outbreak revealed the co-circulation of dengue types 1 and 3 with the predominance of dengue type 1, upon phylogenetic analysis revealed they were belong to subtype 2 under genotype III [Figure 1].

The present study thus concludes that wherever facilities for isolation and molecular techniques are available, diagnosis of acute dengue infection should be done by RT-PCR or virus isolation. Though NS1 antigen is a good early marker, the samples negative for NS1 antigen during acute phase should be tested for IgM antibody detection where secondary dengue infections are common, before reporting as dengue negative.

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