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Comparison of NS1 antigen detection ELISA, real time RT-PCR and virus isolation for rapid diagnosis of dengue infection in acute phase

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ABSTRACT

Background & objectives: Diagnosis of dengue infection in acute phase is important for clinical care, implementing control measures, surveillance and research. Currently, dengue fever is diagnosed by means of virus isolation, reverse transcriptase PCR or IgM and IgG based ELISA. Given the limitations of all the existing diagnostic methods, there is a need for rapid, sensitive and high throughput methods for detection of dengue virus in early stages of the disease. The study was conducted with the objectives to evaluate a dengue virus NS1 antigen detection ELISA and a TaqMan based real time RT-PCR for detection of all four serotypes of dengue virus, as diagnostic tools for acute dengue virus infection.

Methods: The acute phase serum samples of patients (n=153) presenting with dengue fever were subjected to NS1 antigen detection and real time RT-PCR. The results were compared to those of virus isolation in the C6/36 cell lines (n=55).

Results: The efficiency, sensitivity, specificity, positive and negative predictive values of NS1 Ag detection ELISA were 83.6, 73.5, 100, 100 and 70% respectively while for real time RT-PCR these were 87.3, 79.4, 100, 100 and 75% respectively. Maximum sensitivity of NS1 antigen detection ELISA was seen in two days of fever and that of real time RT-PCR in three days of fever.

Interpretation & conclusion: NS1 antigen detection ELISA and real time RT-PCR were found to be rapid, convenient and efficient tests for diagnosing of dengue fever in acute phase and the diagnosis could be made as early as within three days of onset of fever.

Key words Dengue; NS1 antigen detection; real time RT-PCR; virus isolation

INTRODUCTION

Dengue is a common mosquito-borne viral disease of humans that in recent years has become a major international public health concern. Dengue infection causes a wide range of symptoms, ranging from unapparent disease or mild non-specific fever to more severe and potentially lethal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)[1]. The mortality rate of DHF in most countries is about 5%, primarily among children and young adults. In several Asian countries, it is the leading cause of hospitalization and death in children[2]. Diagnosis of dengue infection in acute phase is imperative for clinical care, implementing control measures, surveillance and pathogenesis studies. Dengue virus (DENV) infection is currently being diagnosed by means of virus isolation, viral RNA detection by reverse transcription-polymerase chain reaction (RT-PCR) or immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (ELISA) and immunoglobulin G (IgG) ELISA.

Dengue IgM and IgG ELISA kits are widely used for diagnosis of dengue infection in routine laboratories. However, there are variations in detection limit during acute phase of the disease. After the onset of symptoms, it usually takes 4–5 and 1–14 days respectively for anti-DENV IgM and IgG antibodies to become detectable, depending on whether the patient has primary or secondary infection[3]. Isolation of virus in cell culture or in infant mouse brain remains the gold standard for diagnosis of acute cases. However, it requires specialized laboratories and takes more than a week for the test to be completed, making it impractical in most situations. Detection of viral RNA by RT-PCR also allows early diagnosis during febrile phase. However, the procedure is cumbersome and the interpretation is difficult; moreover, the results are not immediate, making its routine use in clinical diagnostic laboratories difficult.

In such a state of affairs, there is need for rapid, sensitive and high throughput methods for detection of dengue virus in the early stages of the disease. In the present study, we evaluated two new diagnostic tools for acute dengue virus infection. An enzyme immunoassay for detecting dengue virus NS1 antigen in human serum; and a dengue virus specific TaqMan based real time RT-PCR for detection of all four serotypes using a single probe primer set targeted against the 3′UTR.

MATERIAL & METHODS

Ethical approval

Approval of Institutional Ethics Committee was taken prior to beginning of the study. Before proceeding with any kind of test, informed consent was obtained from the subjects. In case of paediatric patients, consent from the parents was obtained.

Clinical samples

Aseptically collected and properly transported blood samples (n=153) were received in Virology Laboratory, All India Institute of Medical Sciences, New Delhi, India, from patients who were clinically suspected to be suffering from dengue fever due to presence of any or all of-fever/headache/myalgia/retro orbital pain/rash/hemorrhagic manifestations in the acute phase of their illness (1–6 days). Serum was separated aseptically from the clotted blood and was stored at –70°C until further processing.

DENV isolation

As virus isolation could be done only on the samples for which proper cold chain was maintained throughout the transport, it was done in 55 out of 153 serum samples. It was carried out under biosafety level-3 setting in the C6/36 clone of *Aedes albopictus* (Diptera: Culicidae) cell lines as described earlier[4]. Briefly, one in ten dilution of each serum sample was inoculated in duplicate on a confluent monolayer of C6/36 cell line and was incubated at 25°C for 10 days. On Day 10, one tube was frozen at – 70°C and cells from the other tube were harvested and cell spots were made on Teflon coated slides from each sample. Uninfected clone of *Ae. albopictus* cell line was used as negative control and cell lines infected with dengue virus 1 to 4 (obtained from the National Institute of Virology, Pune, India) were included as positive controls in each run. Indirect fluorescent antibody assay (IFA) was performed on these spots using specific monoclonal antibodies to dengue virus types 1–4 (provided by Dr D.J. Gubler, then at CDC, Atlanta, USA during the 1996 outbreak). If the IFA was negative for dengue viruses on first passage, a blinded second passage was made and cells were again harvested on Day 10 for IFA. If the IFA was still negative for DENV, the sample was declared negative for virus isolation[5].

Dengue NS1 antigen detection

In all, 153 acute phase serum samples were tested for NS1 antigen using Platelia™ Dengue NS1 antigen-ELISA (Biorad Laboratories, Marnnes-la-Coquette, France). The manufacturer's instructions were followed in the procedure and results were interpreted as reactive, equivocal and nonreactive. The samples interpreted as equivocal (n=3) were excluded from further study.

RNA extraction

Real time RT-PCR was done in 150 samples. RNA was extracted using RNeasy Mini Kit, QIAGEN, Hilden, Germany as per manufacturer's instructions.

Real time RT-PCR

The extracted RNA samples (n=150) were subjected to real time RT-PCR developed by Gurukumar *et al*[6] targeting the 3′ UTR gene sequence. The primers and probe used targeted a stretch of nucleotides conserved in the four dengue virus serotypes. The probe used was TaqMan MGB probe labeled with FAM at 5′ end and non-fluorescent quencher at 3′ end. Cloned and amplified target sequence RNA as a positive control, and a no template control were included in each run. The real time RT-PCR primers, probe, enzyme mix, reaction buffer and positive control were provided by National Institute of Virology, Pune, India. The reaction mixture containing 20 µl of master mix and 5 µl of sample (or control) RNA was subjected to the following reaction conditions in ABI 7500 real time PCR system: reverse transcription at 45°C for 10 min, initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The test was considered valid if positive control showed a CT value <35. Samples that had a CT <35 were considered positive and value >35 were considered as noise.

Dengue IgM ELISA

Dengue IgM ELISA was done only on the serum samples from patients with duration of fever ≥4 days (n=41). The kit used was the µ-capture dengue IgM ELISA kit supplied by the National Institute of Virology, Pune; under the National Vector Borne Disease Control Programme. Manufacturer's instructions were strictly followed for performing the test and interpreting the results. The O.D. was measured at 450 nm using ELISA reader (BioTekR, Winooski, United States).

Dengue IgG antibody detection

Samples which were positive with any of the above tests or/and were from patients showing manifestations of hemorrhage or/and shock (n=67) were subjected to dengue IgG antibody detection for diagnosing dengue secondary infections. The kit used was rapid immunochromatographic test device–Dengue Duo Cassette-Panbio Diagnostics Inc., Australia.

Statistical analysis

Assessment of efficiency, sensitivity, specificity, positive and negative predictive values of the two tests were calculated by Fisher's exact test using GraphPad Prism v 5.0 software taking virus isolated from cell culture as gold standard. Concordance between two tests was calculated using the following formula:

$$\% \text{ Concordance (Agreement)} = \frac{\text{(No. of samples positive by both tests + No. of samples negative by both tests)}}{\text{Total No. of samples}} \times 100$$

RESULTS

Median age of the study population was 30 yr and the sex ratio (male/female) was 1.5. Out of 55 samples subjected to virus isolation, 34 (61.8%) were positive for dengue virus; among them 33 were dengue virus type-1 and one was dengue virus type-2. Real time RT-PCR was positive in 27 DENV-1 culture positive samples and negative for 7 (6 DENV-1 and the one DENV-2 sample). NS1 antigen was detected in 25 culture positive samples (24 DENV-1 and one DENV-2).

Overall performance of NS1 antigen detection and real time RT-PCR

Out of 153 samples subjected to NS1 antigen detection, 50 (32.7%) were positive, three were equivocal and 100 were negative (Table 1). The three equivocal samples were excluded from further investigation. Real time RT-PCR was done on 150 samples of which 51(34%) were positive, and 99 were negative. Overall efficiency of NS1 antigen detection was found to be 83.6%. The sensitivity and specificity were 73.5 and 100% respectively; and the positive and negative predictive values were 100 and 70% respectively. Overall efficiency of real time RT-PCR was found to be 87.3%. The sensitivity and specificity were 79.4 and 100% respectively; and the positive and negative predictive values were 100 and 75% respectively.

Table 1. Overall performance of NS1 antigen detection and real time RT-PCR

Characteristic	NS1 antigen detection (%)	Real time RT-PCR (%)
Sensitivity	73.53	79.41
Specificity	100	100
Efficiency	83.64	87.27
Positive-predicted value	100	100
Negative-predicted value	70	75

Sensitivity of dengue NS1 antigen detection ELISA and real time RT-PCR on different days of illness

The sensitivity of NS1 antigen detection increased from 50% on Day 1 of illness, to a maximum of 100% on Day 2. In case of real time RT-PCR, the sensitivity increased gradually from 75% on D1, through 83.3% on D2, to a maximum of 92.9% on D3. On D4, the sensitivities of both NS1 antigen detection and real time RT-PCR dropped to 75% and then to 50% on D5 (Table 2).

Comparison of NS1 antigen detection and real time RT-PCR

Real time RT-PCR and NS1 antigen detection were done in a total of 150 samples. The results obtained showed 88.7% concordance, and 11.3% disconcordance (Table 3).

Concordance between dengue NS1 antigen detection ELISA and real time RT-PCR on different days of illness

The concordance between the performance of dengue NS1 antigen detection ELISA and real time RT-PCR in first three days of illness was 92.9, 94.6 and 91.4% on samples received on Day 1, 2 and 3 respectively. Concordance was maximum on D2 of illness. On D4 of illness, the concordance decreased to 89.5%. Minimum concordance was seen on D5 of illness, i.e. 58.3%; which again increased to 80% on D6 of illness (Table 4).

Table 2. Sensitivity of dengue NS1 antigen detection ELISA and real time RT-PCR on different days of illness

Investigation	Day of illness					Total
	Day 1	Day 2	Day 3	Day 4	Day 5	
No. of samples for which culture was done	7	11	25	5	5	55
Culture positive samples	4	6	14	4	4	34
NS1 antigen positive samples	2	6	10	3	2	25
Sensitivity of NS-1 antigen	50%	100%	71.4%	75%	50%	
Real time RT-PCR positive samples	3	5	13	3	2	27
Sensitivity of real time RT-PCR	75%	83.3%	92.9%	75%	50%	

Table 3. Comparison of NS1 antigen detection and real time RT-PCR

NS1 antigen detection	Real time RT-PCR		Total	Concordance	Disconcordance
	Positive	Negative		%	%
Positive	42	8	50		
Negative	9	91	100	88.67	11.33
Total	51	99	150		

Table 4. Concordance between dengue NS1 antigen detection ELISA and real time RT-PCR on different days of illness

Day of illness	NS1 antigen	Real time RT-PCR		Total	Concordance
		Positive	Negative		%
1	Positive	2	0	2	92.9
	Negative	1	11	12	
	Total	3	11	14	
2	Positive	12	2	14	94.6
	Negative	0	23	23	
	Total	12	25	37	
3	Positive	19	1	20	91.4
	Negative	4	34	38	
	Total	23	35	58	
4	Positive	6	1	7	89.5
	Negative	1	11	12	
	Total	7	12	19	
5	Positive	2	2	4	58.3
	Negative	3	5	8	
	Total	5	7	12	
6	Positive	1	2	3	80
	Negative	0	7	7	
	Total	1	9	10	

Comparison of NS1 antigen detection and real time RT-PCR with dengue IgM ELISA

Dengue IgM ELISA was done in 41 samples (on samples from patients with duration of fever ≥4 days); the results showed 73.2% concordance with real time RT-PCR and 65.8% with NS1 antigen detection ELISA (Table 5).

Dengue secondary infections

Out of 67 samples tested for dengue IgG antibodies, 12 showed positive results. Two of them were positive for both NS1 antigen and viral nucleic acid by real time RT-PCR as well. Two samples were positive by real time RT-PCR but negative for NS1 antigen, while one was positive for NS1 antigen and negative by real time RT-PCR.

DISCUSSION

In the present study, we looked into the worth of dengue virus NS1 antigen detection and dengue group specific real time RT-PCR for diagnosing dengue cases in acute phase of illness. Overall efficiency of the tests was found to be 83.64 and 87.27% respectively. Specificity of both the tests was 100%, whereas sensitivity was 73.53 and 79.41% for NS1 antigen detection and real time RT-PCR respectively. The maximum sensitivity of NS1 antigen detection was on D2 of illness and that of real time RT-PCR was on D3 of illness. The level of concordance between NS1 Ag ELISA and real time RT-PCR was 88.67%; maximum concordance was seen on D2 of illness (94.6%).

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. 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.

Serological IgM-ELISA and IgG-ELISA are currently widely used for dengue fever diagnosis in routine laboratories. The tests are user friendly and robust; however, the sensitivity of these tests does not become acceptable until five days after the onset of fever. Moreover, a single serological detection of IgM is merely indicative of a recent dengue virus infection and a paired second serum sample is required for confirmation. Additionally, IgM detection is not always conclusive because

Table 5. Comparison of NS1 antigen detection and RT-PCR with dengue IgM ELISA

Dengue IgM ELISA	RT-PCR		Total	NS1 antigen detection		Total
	Positive	Negative		Positive	Negative	
Positive	5	3	8	4	4	8
Negative	8	25	33	10	23	33
Total	13	28	41	14	27	41
Concordance %	73.2				65.8	

of cross reactivity with other flaviviruses[7]. Virus isolation is carried out only by reference laboratories and is a time-consuming and expensive technique. The use of dengue RT-PCR in most laboratories is currently difficult, due largely to the cumbersome procedure, difficult interpretation and the time taken[8–10].

NS1 protein is highly conserved for all dengue serotypes, circulating in high levels during the first few days of illness. There is no cross-reaction of dengue NS1 protein with those of other related flaviviruses[11]. Plasma viremia levels would be associated with the detection of plasma NS1; since NS1, like virions, is a product of infected cells. Srivastava *et al*[12] have reported that viremia levels were significantly higher in patients who were NS1-positive versus those who were NS1-negative. A previous study has demonstrated that the level of NS1 of dengue-2 virus in plasma was correlated with viremia levels and was significantly higher in patients with dengue hemorrhagic fever than in patients with dengue fever within 72 h of onset of illness[13]. The development of a quantitative test of NS1 could probably be of interest as a prognosis factor in the future. NS1 antigen detection ELISA can be implemented in routine diagnostic laboratories and can be easily automated. The test has great potential value for use in epidemic situations, as it could facilitate the early screening of patients and limit disease expansion.

The most widely used PCR based method for detecting DENV nucleic acid in the serum is the nested RT-PCR developed by Lanciotti *et al*[8], and it's modification by Harris *et al*[9]. The nested RT-PCR is considerably economical and has the advantage of sero-typing, which may be relevant for public health and for patient management as well. However, important drawbacks are cumbersome procedure, the need to repeat several tests due to inconclusive bands of low intensity, and the time taken to obtain the result. Many of these shortcomings can be taken care of in single-tube multiplex reverse transcriptase-PCR (M-RT-PCR); which has been developed for use in the detection, quantitation, and serotyping of dengue viruses from patient serum or plasma. There is evidence in recent literature that this test is having high sensitivity and specificity, and that reaction set-up to result time that is much shorter[14–16]. We have evaluated the utility of real time RT-PCR, which is more sensitive, can be automated, interpretation is easier and the hands on time is less. The real time RT-PCR can also be used to quantitate the viral load in blood samples, making it a useful tool to investigate the role of viremia in pathogenesis of dengue. The ability of the real time RT-PCR assay to detect DENV-2 in inoculated mosquitoes has been reported earlier[6], which makes it a potential tool for detecting DENV in field caught mosquitoes; thus, making it useful in surveillance of vector population and providing early warning of a possible outbreak of the disease.

In the present study, we found that the specificity of NS1 antigen detection and real time RT-PCR was 100% and overall sensitivities were 73.5 and 79.4% respectively. In published literature, the sensitivity of NS1 antigen detection ranges from 76 to 97% and specificity from 98 to 100%[17–19]. Gurukumar *et al*[6] have reported 100% sensitivity and specificity of real time RT-PCR in diagnosing dengue in acute phase. We found the overall performance of real time RT-PCR to be better than NS1 antigen detection, in terms of sensitivity, efficiency and negative predictive value. Moreover, a higher detection rate by both the tests was found during the first four days of illness. Gurukumar *et al*[6] have reported that the real time RT-PCR assay is able to detect DENV RNA up to 10 days post-onset of fever. Previous studies have reported that NS1 antigen was found circulating up to Day 9 of illness; however, the level of NS1 decreased significantly after the acute phase. The 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[17–18]. Efforts to dissociate immune complexes have shown to enhance the sensitivity of NS1 antigen detection ELISA[19].

With the expansion of the geographic range of dengue fever and the increasing number and severity of reported cases, the use of NS1 antigen detection and real time RT-PCR could allow clinical diagnostic laboratories to identify dengue virus infections early enough to adjust patient management, reducing the time between detection of the first cases, and the notification of public health authorities, including vector control teams. To conclude, NS1 antigen detection, and dengue group specific real time RT-PCR are valuable techniques for the rapid and early biological diagnosis of dengue.

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