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ROLE OF RTPCR IN THE DIAGNOSIS OF DENGUE

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ABSTRACT **Introduction:** Dengue is commonly found in tropical and subtropical zones. The gold standard for diagnosing and serotyping the virus is through virus isolation. However, a helpful biomarker for diagnosis is NS1, which is released by infected cells 5 to 6 days after the onset of the disease. In settings with a high sample load, the Dengue NS1 ELISA test is a cost-effective and more efficient option. NS1 antigen is considered a surrogate marker for viremia. Additionally, the Dengue RT-PCR test can detect viral RNA not only in primary and secondary dengue infections but also in cases of infection with DENV-4 within 24-48 hours of the acute stage of the disease. Therefore, the aim of this study was to evaluate the role of RT-PCR in patients with acute febrile illness caused by dengue. **Methods:** We analyzed a total of 150 samples from patients with acute febrile illnesses. Specifically, we included 50 samples from patients with NS1 ELISA-positive dengue fever, 50 samples from patients suspected of having dengue fever but with NS1 ELISA-negative results, and an additional 50 samples from patients with other types of acute febrile illnesses. We extracted viral RNA from whole blood samples collected from the 150 patients, and then performed a dengue Real Time PCR analysis. **Results:** Our study revealed that relying solely on NS1 ELISA testing may result in a missed diagnosis of dengue fever in 30% of acute febrile cases. **Conclusion:** For accurate diagnosis of dengue in the acute stage of febrile illness, we recommend using a combination of NS1 ELISA and dengue RT-PCR, especially when NS1 ELISA results are negative.

KEYWORDS : NS1 ELISA, Dengue RTPCR, DENV-4 infection, Dengue shock syndrome.

INTRODUCTION

Dengue virus infection is common in tropical and subtropical zones [1]. Clinical entities such as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome affect more than 50 million people annually in Southeast Asia, the Western Pacific, and Central and South America [2]. Virus isolation is the gold standard for the diagnosis and serotyping of dengue. But it is laborious and needs a well-equipped laboratory [3]. IgG antibodies are detected within 1 to 14 days, which is determined by primary or secondary dengue infection [4]. NS1 is a helpful biomarker for diagnosis.

It is released from infected cells after 5 to 6 days of disease onset in dengue. However, in patients with secondary dengue infection, there is an anamnestic rise in NS1 cross-reacting antibodies in the early phase of the disease.

These antibodies cause the sequestration of NS1 in immune complexes, which makes NS1 detection difficult [5-7]. False-negative results by NS1 ELISA are reported in infection with DENV-4 [5, 8, 9]. Therefore, with this background, the present study is aimed to evaluate the role of RT-PCR in patients with acute febrile illness due to dengue.

MATERIAL AND METHODS

The study was conducted in the Department of Microbiology, B.J. Govt. Medical College, Pune, after getting a due permission from Institutional Ethical Committee. The study was conducted over a period of 1 year from January to December 2018. Total 150 blood samples were collected from adult patients as follows-

A) Fifty samples from patients of acute febrile illness which were NS1 ELISA positive.
B) Fifty samples from patients of acute febrile illness which were clinically suspected as dengue but NS1 ELISA negative.
C) Fifty samples from patients of other acute febrile illnesses.

Patients with dengue haemorrhagic fever admitted in intensive care unit were excluded from the study. Detailed history of the patients was noted. Informed consent was obtained from patients prior to venepuncture.

Approximately five millilitres of blood was collected from each patient and transferred to EDTA and plain vacutainers in equal volume. Thus, a total of 150 serum samples in plain vacutainer were tested for dengue NS1 ELISA (J. Mitra & Co. Pvt Ltd.). Whole blood samples collected in EDTA vacutainer of these 150 patients were subjected to viral RNA extraction by QIAGEN kit followed by dengue Real Time PCR by Fast Track Diagnostics PCR kit.

RESULTS

In present study, out of a total 150 samples, 50 blood samples were collected from patients of acute febrile illness with positive NS1 ELISA. Another 50 blood samples were collected from patients of acute febrile illness with negative NS1 ELISA having clinical suspicion of dengue. Remaining 50 blood samples were collected from patients of other acute febrile illnesses. These 50 samples were found to be IgM ELISA positive for chikungunya.

Table 1: Comparative analysis of Dengue NS1 ELISA and Dengue RT-PCR in patients of acute febrile illness (n=150)

Test	NS1 ELISA Positive (n=74) Number (%)	NS1 ELISA Negative (n=76) Number (%)	Total
RT-PCR Positive	62(83.8)	31(40.8)	93
RT-PCR Negative	12(16.2)	45(59.2)	57
Total	74	76	150

Out of 74 NS1 ELISA positive samples, 62 (83.8%) were positive by both NS1 ELISA and dengue RT-PCR; whereas 12 (16.2%) samples were positive by dengue NS1 ELISA and negative by dengue RT-PCR. Out of the remaining 76 samples, 45 (59.2%) were negative by both NS1 ELISA and dengue RT-PCR; whereas 31 (40.8%) samples that were negative by NS1 ELISA showed positive result by dengue RT-PCR. Considering dengue NS1 ELISA as a gold standard, sensitivity, specificity, positive predictive value and negative predictive value of RT-PCR was 83.8%, 59.2%, 66.7% and 78.9% respectively.

Out of total 150 patients of acute febrile illness, 32 patients had fever since 2 days. In these 32 patients, concordance (agreement) between dengue NS1 ELISA and dengue RT-PCR was 75%. In patients of fever since 2 days, sensitivity of RT-PCR was found to be 85.7%. Dengue NS1 ELISA was considered as a gold standard.

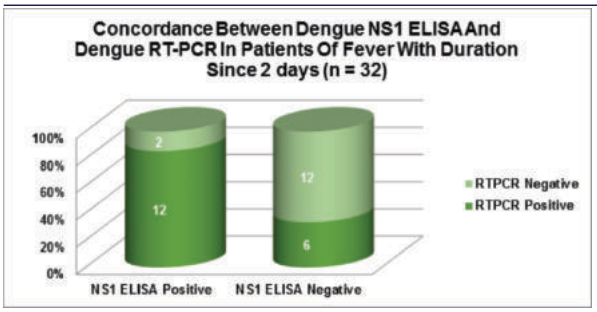

Figure 1: Concordance between Dengue NS1 ELISA and Dengue RTPCR in patients of fever since 2 days (n=32)

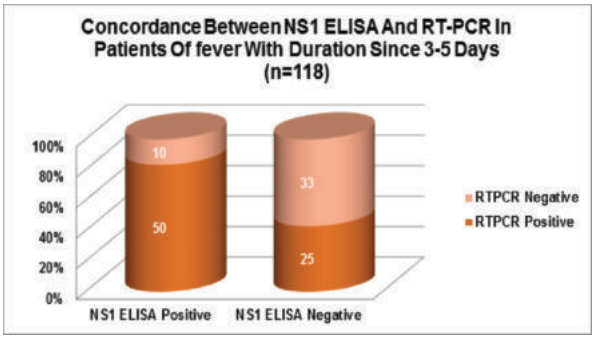

Figure 2: Concordance between Dengue NS1 ELISA and Dengue RTPCR in patients of fever since 3-5 days (n=118)

Out of total 150 patients of acute febrile illness, 118 patients were suffering from fever since 3 to 5 days. Considering this duration of fever since 3 to 5 days, concordance (agreement) between dengue NS1 ELISA and dengue RT-PCR was 70.3%. In patients having fever since 3 to 5 days, sensitivity of RT-PCR was 83.3%. Dengue NS1 ELISA was considered as a gold standard.

Table 2: Evaluation of Dengue RT-PCR in acute febrile Dengue NS1 ELISA negative patients (n=76)

Test	Clinically suspected Dengue cases negative by NS1 ELISA (n=50) Number (%)	Serologically confirmed cases of Chikungunya alone (n=26) Number (%)
RT-PCR Positive	15 (30.0)	16 (61.5)
RT-PCR Negative	35 (70.0)	10 (38.5)
Total	50	26

Out of 50 patients with clinical suspicion of dengue with negative NS1 ELISA, 35 (70.0%) patients were negative by both dengue NS1 ELISA and dengue RT-PCR. On the other hand, 15 (30.0%) patients were negative by dengue NS1 ELISA but found to be positive by dengue RT-PCR. In the present study, 26 patients of acute febrile illness were positive for IgM chikungunya alone by ELISA. Out of these 26 patients, 10 (38.5%) were negative by both dengue NS1 ELISA and dengue RT-PCR; whereas 16 (61.5%) were positive by dengue RT-PCR.

DISCUSSION

Dengue is a mosquito-borne viral infection. It is transmitted by *A. aegypti*, sometimes by *A. albopictus* [10]. Dengue infection shows wide range of clinical manifestations from asymptomatic stage to severe dengue. It may be associated with complications like dengue haemorrhagic fever and dengue shock syndrome [10, 11]. The global prevalence is also increasing making it a public health problem [12]. Early and accurate diagnosis of dengue is important in controlling epidemics as well as commencement of therapy [3, 10, 13].

Disease mortality can be reduced by prompt laboratory intervention followed by proper management [3, 13]. Appropriate, time saving and affordable laboratory tests are the mainstay of early diagnosis of acute stage infection [1, 3]. Virus isolation in the cell culture or infant mouse brain is the gold standard for diagnosis of dengue virus infection. This technique is very costly and time consuming. It requires 1 to 2 weeks and hence cannot be employed in clinical settings [3, 4, 9]. Dengue NS1 antigen ELISA has the ability to detect dengue infection from day 0 to day 6 of the illness. Several studies have shown that dengue NS1 ELISA has maximum sensitivity during acute stage of disease [13]. NS1 antigen detection can be done at the same time as viral RNA and before the antibody response in primary infection. Hence, NS1 is labelled as a surrogate marker for viraemia [5].

RT-PCR can detect viral RNA within 24 to 48 hours in the samples collected within 5 days after onset of illness [9]. Molecular methods such as RT-PCR are highly sensitive and specific as compared to NS1 ELISA especially when the sample is collected within 3 days of onset of illness [14, 15].

In the current study, out of 150 patients, 74 (49.3%) were found to be positive by dengue NS1 ELISA. Out of these 74 NS1 ELISA positive samples, 62 (83.8%) were positive by both dengue NS1 ELISA and dengue RT-PCR; whereas 12 samples were positive by NS1 ELISA but negative by dengue RT-PCR. Out of 76 NS1 ELISA negative samples, 45 (59.2%) were negative by both NS1 ELISA and dengue RT-PCR; whereas 31 samples were negative by NS1 ELISA showed positive result by dengue RT-PCR.

Considering NS1 ELISA as a gold standard, sensitivity, specificity, positive predictive value and negative predictive value of RT-PCR was 83.8%, 59.2%, 66.7% and 78.9% respectively. Ahmed and Broor found that sensitivity and negative predictive value of RT-PCR as 79.41% and 75% respectively, which was comparable with the present study [4]. Specificity and positive predictive value of RT-PCR in the present study was found to be lower as compared to a study by Ahmed and Broor [4]. Gurukumar et al. revealed 100% specificity of RT-PCR in acute stage of illness, which was found to be higher than the present study [16].

Out of a total 150 patients of acute febrile illness, it was found that 32 patients had fever since 2 days. In these 32 patients, concordance (agreement) between NS1 ELISA and RT-PCR was 75%. In patients with fever since 2 days, the sensitivity of dengue RT-PCR was found to be 85.7%. Gaikwad S et al., in their study, compared NS1 antigen detection by rapid immunochromatography and ELISA with real time PCR as gold standard. They reported the average concordance between NS1 ELISA and RT-PCR within 2 days of illness as 70.65% that was similar to the present study [17]. In the study by Ahmed and Broor, the average concordance within 2 days of illness was found to be 93.75%, which was found to be higher than the present study [4]. Dissimilarity in the concordance could be due to difference in the number of samples collected within 2 days of illness. Ahmed and Broor, in their study reported the average sensitivity of RT-PCR within 2 days of illness as 79.15%, which was comparable to the present study [4].

In this study, among the total of 150 patients with acute febrile illness, 118 patients were suffering from fever since 3 to 5 days. Considering this duration of fever (i.e., 3 to 5 days), the concordance (agreement) between NS1 ELISA and RT-PCR was 70.3%. Ahmed and Broor reported the average concordance between NS1 ELISA and RT-PCR within 3 to 5 days of illness as 79.73%, which is similar to the present study [4]. The present study showed that the sensitivity of dengue RT-PCR was 83.3% in patients having fever since 3 to 5 days. Ahmed and Broor in their study reported the average sensitivity of RT-PCR between 3 to 5 days of illness as 72.63% which was comparable to the present study [4]. Ahmed and Broor also revealed that there was a drop in the sensitivity of RT-PCR as days of illness progressed [4]. As duration of illness increased, fall in the sensitivity of RT-PCR was also reported by Pok et al. [15].

In this study, 76 cases tested negative by NS1 ELISA were also evaluated by dengue RT-PCR. Out of these 76 cases, 50 were clinically suspected dengue and 26 were patients of acute febrile illness found to be positive for chikungunya IgM by ELISA. Out of 50 patients with clinical suspicion of dengue with negative NS1 ELISA, 35 (70.0%) patients were negative by both dengue NS1 ELISA and dengue RT-PCR. On the other hand, 15 (30.0%) patients were negative by dengue NS1 ELISA but were found to be positive by dengue RT-PCR. Colombo et al. evaluated DENV-4 false negative results by dengue NS1 ELISA in Brazil. They found that, among 100 samples negative by dengue NS1 ELISA, 15% samples were positive by multiplex-nested-PCR. They showed that false negative results of NS1 ELISA were reported when DENV-4 was circulating [8].

Although we did not look for DENV serotypes in this study, these 15

cases could have been due to infection by DENV-4. Colombo et al. also stated that variations in secretion of NS1 antigen along with variations in viraemia in a person might contribute to the reduced NS1 ELISA sensitivity [8]. This could be the reason for 15 cases positive by dengue RT-PCR but negative by NS1 ELISA in present study. Felix et al. performed NS1 ELISA and IgG avidity test on 379 dengue RT-PCR positive samples. They detected 68.6% cases with secondary dengue. Out of these, they found only 20.7% cases positive by NS1 ELISA. The reason for NS1 negative but dengue RT-PCR positive was explained as NS1 could be sequestered into immune complexes with IgG [7]. Mardekian and Roberts also reported that lower sensitivity of NS1 might be due to existence of anti-NS1 antibodies in secondary dengue [6]. In the present study, 15 cases positive by dengue RT-PCR but negative by NS1 ELISA could be due to acute febrile illness due to secondary dengue.

A study based on evaluation of dengue NS1 by rapid kits and ELISA by Pal et al. demonstrated that NS1 was detected in early course of the disease in both primary and secondary dengue infections. They found that NS1 ELISA sensitivity was dependent on DENV serotypes. In their study, false-negative results of NS1 were attributed to low level of viraemia in DENV-4 infection and large antigenic distance within DENV-4 strains [13]. In the present study, these 15 cases of acute febrile illness detected additionally by dengue RT-PCR would have missed if we had performed dengue NS1 ELISA only. Thus, we suggest that diagnosis of acute febrile illness due to dengue should not be confirmed by dengue NS1 ELISA alone. In such cases, dengue RT-PCR should also be performed especially when patient is negative by NS1 ELISA.

In the current study, 26 patients of acute febrile illness were positive for IgM chikungunya alone. Out of these 26 patients, 10 (38.5%) were negative by both dengue NS1 ELISA and dengue RT-PCR, whereas 16 (61.5%) patients were detected to have dengue infection by dengue RT-PCR. These 16 cases would have been missed if only NS1 ELISA was performed. Chua found that IgM antibodies for chikungunya were present till 11 to 14 months after onset of disease. They described an association of persistent chikungunya IgM antibodies in patients suffering from chronic arthralgia. They discussed that in patients with chronic joint disease, persistence of chikungunya IgM antibodies was observed in response to occult viral persistence [18]. In the present study, 16 (61.5%) cases were found to be positive for chikungunya IgM antibodies. These chikungunya IgM antibodies could be persistent as a result of chronicity of illness due to chikungunya. Thus, these 16 cases could be suffering from acute febrile illness due to dengue with fever ≤ 5 days and showed positivity by dengue RT-PCR which were detected additionally.

Dengue NS1 ELISA helps in diagnosing dengue during acute stage in a setting where sample load is high; it is also inexpensive. It can be performed without much technical expertise in clinical laboratory for routine diagnosis as compared to RT-PCR. Presence of dengue NS1 antigen in the blood correlates well with viraemia. Hence, NS1 antigen is considered as surrogate marker for viraemia. In spite of all these advantages of NS1 ELISA, it has lower sensitivity in patients with secondary dengue infection and infection due to DENV-4 serotype. Both NS1 ELISA and dengue RT-PCR can diagnose acute febrile illness due to dengue from day 1 to day 5. However, higher sensitivity and specificity with RT-PCR is observed especially in situations like secondary dengue and DENV-4 infection.

In conclusion, acute febrile illness due to dengue has potential to cause life-threatening complications such as dengue haemorrhagic fever and dengue shock syndrome. Timely, accurate laboratory diagnosis with appropriate treatment plays the pivotal role in increasing chances of survival. NS1 ELISA is better and cost-effective diagnostic tool for high throughput screening for dengue during acute phase of the disease. NS1 antigen is considered as a surrogate marker for viraemia.

In spite of all the advantages of NS1 ELISA, it has lower sensitivity in patients with secondary dengue infection and infection due to DENV-4 serotype. Higher sensitivity and specificity with RT-PCR is observed in secondary dengue and DENV-4 infection. RT-PCR has better negative predictive value as compared to dengue NS1 ELISA. In the present study, we found that diagnosis of dengue would have been missed in 30% of the acute febrile cases if only NS1 ELISA was performed. Thus, we conclude that, dengue RT-PCR should be used in conjunction with NS1 ELISA for accurate diagnosis of dengue in acute stage of febrile illness especially when NS1 ELISA is negative. In well-equipped clinical laboratory, dengue RT-PCR can offer additional diagnostic opportunity where an expert microbiologist is available.

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Conflict of interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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