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Original Research Article

Diagnosis of Dengue by NS1 Antigen Detection and Reverse Transcriptase PCR Assay

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Conflict of interest: Nil

Abstract:

Introduction: Dengue virus (DENV) belongs to the genus *Flavivirus* (family *Flaviviridae)* and has 4antigenically distinct serotypes; DEN1, DEN2, DEN3 and DEN 4. About, 40% of the world's population is at risk of acquiring dengue and around 50–100 million cases occur every year. An estimated 5,00,000 hospitalizations occur per year due to severe dengue disease out of which 2.5% are fatal.

Aim and Objectives: To assess the role of NS1 antigen determination in diagnosis of dengue within first week of fever, its comparison with rapid immunochromatographic test, ELISA and RT-PCR in diagnosis of dengue.

Material and Methods: Blood samples were received in Microbiology department of SGT Hospital Budhera, Gurugram from the 150 patients clinically suspected of dengue fever within 1 to 7 days of fever. All the samples were tested by rapid immunochromatographic test (ICT), ELISA and only 82 samples out of total 150 samples were tested by RT-PCR for NS1 antigen detection due to non- availability of kit. Samples found positive on RT-PCR were further processed by nested PCR for genotyping.

Results: Out of 150 patients, 85 (56.67%) patients were male and 65 (43.33%) were female. 47(55.3%) out of 85 males, 30 (53.8%) out of 65 females and 82 (54.7%) out of total 150 patients were found positive for NS1 antigen by rapid ICT. Maximum positivity of NS1 antigen by ICT and ELISA was 76.4% on day 4 of illness and 66.7% on day 1 respectively. NS1 antigen detection by rapid ICT showed a sensitivity & specificity of 95.8% & 75.6% as compared to ELISA.RT-PCR for Dengue was carried out in 82 samples, of which 33 were positive for DENV RNA. On performing the nested PCR all 33 samples were genotyped as DENV-3.The sensitivity of NS1 antigen by ICT was 84.8% and specificity 73.5% with 68.3% positive predictive value and 87.8% negative predictive value. Of 82 samples, 33 tested positive for dengue by PCR and only 31 samples were positive by ELISA. The sensitivity of ELISA for NS1antigen detection was 93.9% and specificity 100% with a positive predictive value of 100% and 96% negative predictive value.

Conclusion: NS1 antigen detection by ELISA and dengue group-specific reverse transcriptase RT-PCR are valuable techniques for the rapid and early diagnosis of dengue.

Keywords: Dengue, NS1 antigen Dengue Rapid, Dengue ELISA, PCR assay.

Introduction

Dengue is the most important emerging tropical viral disease of humans in the current world today. [1] The disease is endemic in more than 100 countries in WHO's Africa, Americas, Eastern Mediterranean, Southeast Asia and Western Pacific regions; the Americas, Southeast Asia and Western Pacific regions are the most seriously affected. Dengue fever has become a leading cause of hospitalization and death among children in the Southeast Asia region, following diarrheal diseases and ARDS infections. [2] Dengue is a mosquito-borne disease and is primarily transmitted by Aedes aegypti & Aedes albopictus is also known to be a vector. [3] The term dengue originated from two different words 'break-bone fever" and 'walk of a dandie'. Benjamin Rush had coined the term 'break bone fever' about the symptoms produced by the disease. [4] There is no specific treatment for dengue illness as yet, and vector control is the only preventive strategy. Dengue fever was reported in India in 1946, but a major effect was seen in Kolkata in 1964. [5] Epidemics are becoming more

frequent with the disease rapidly progressing to various parts of India specially the Northern and the Southern region. [6] During 1996, one of the most severe outbreaks of DF/DHF occurred in our country with a total no. of cases being 16,517 causing 545 deaths out of which 10,252 cases and 423 deaths were reported from Delhi only. In 2006, the country witnessed another outbreak of DF/DHF with 12,317 cases and 184 deaths, in this outbreak all four DENV serotypes were seen in circulation for the first time, but the outbreak was dominated by DENV- 3 serotype. [7] There is increase trend in Dengue cases every year in India e.g. 28292, 50222 and 75808 cases reported in year 2010, 2012 and 2013 respectively. [8]

Dengue virus poses a considerable global public health problem. Thus, rapid and reliable diagnostic methods are required for proper patient management and disease control. Regular scrutiny of the virus becomes a necessity in our country to establish molecular epidemiology to understand the evolving pattern of the viral pathogen. Dengue virus (DENV) infection is currently being diagnosed utilizing viral RNA detection by reverse transcriptase polymerase chain reaction (RT - PCR) or immunoglobulin M (IgM) capture enzyme–linked immunosorbent assay (ELISA), immunoglobulin G (IgG) ELISA and detection of NS1(nonstructural antigen 1) antigen.

DengueNS1, IgM and IgG ELISA kits are widely used for diagnosis of dengue infection in routine laboratories. After the onset of symptoms, NS1 can be detected from day 1 to day 7. And IgM and IgG antibodies become detectable in 4 – 5 days and 1 – 14 days respectively for anti DENV, depending on whether the patient has primary or secondary infection.

Reverse transcriptase polymerase chain reaction (RT-PCR) offers the potential for the rapid, highly sensitive and specific detection of dengue viruses.9RT -PCR also offers the advantage of serotyping the viruses. However, in peripheral laboratories and Primary health care facilities where facilities for PCR are not available, NS1 antigen detection offers on advantage for early diagnosis of dengue. NS1 antigen can be detected by rapid immunochromatographic tests or by ELISA.

With the emergence of dengue in North India in such high proportion, it is a reason for serious concern for the authorities to take immediate steps for its prevention. As the patients' health deterioration is very rapid in the disease, immediate diagnosis of the virus is of utmost importance for better clinical prognosis. The present study was carried out to assess the role of NS1 antigen determination in the diagnosis of dengue, its comparison with Immunochromatographic test and ELISA for NS1 antigen detection, evaluation of RT-PCR in the diagnosis of dengue and comparison of sensitivity and specificity of NS1 antigen detection with RT-PCR. This study provided information on the usefulness of NS1 antigen detection and RT-PCR for early diagnosis of dengue.

Material and Methods

This cross-sectional study was done from 01 August, 2016 to 01 October, 2017 on 150 blood samples collected from patients clinically suspected of having dengue fever due to presence of any or all of symptoms -fever, headache, myalgia, retro-orbital pain, rash, hemorrhagic manifestations in the acute phase of their illness day 1 to day 7. The samples were collected aseptically and transported properly to the Department of Microbiology at SGT Medical College in Gurugram, India. Approval of Institutional Ethics Committee of SGT Medical College and University, Gurugram, India was taken before commencing the study.

Serum Separation: The blood samples were incubated at room temperature for 2-3 hours for serum separation. The samples were then centrifuged at 2500 rpm for 15 min and then the serum speared. Serum samples were then aliquoted in 3 cryovials and stored at -80°C for NS1 Immunochromatographic test (ICT), NS1 ELISA and RT-PCR.

Rapid Dengue NS1 antigen detection test: Dengue NS1Antigen assay is a rapid immunochromatographic test for the qualitative detection of Dengue NS1Antigen detection in human serum /plasma.

Procedure: All 150 samples were tested by rapid dengue NS1 antigen using Advantage Dengue NS1 Ag Card (make- J-Mitra& Co. Pvt. Ltd). The manufacturer's instructions were followed in the procedure and results were interpreted as reactive, equivocal and nonreactive. (Fig 1)

Dengue NS1 Ag ELISA: Dengue NS1 Ag Microlisa is a solid phase enzyme linked immunosorbent assay (ELISA) based on the "Direct Sandwich" principle.

Procedure: Dengue NS1 antigen ELISA was performed on all 150serum samples. Dengue NS1 Ag Microlisa ELISA kit (make- J-Mitra & Co. Pvt. Ltd) was used for detecting NS1 Antigen the sample. The manufacturer's instructions were followed in the procedure. The O.D. was measured at 450 nm using on ELISA reader. (Fig 2)

PCR for detection of Dengue serotype: Dengue Virus Detection and Typing by PCR: Out of total 150 collected serum samples, only 82 samples were randomly selected and processed for serotyping and genotyping due non-availability of required kits. DENV was detected in the clinical samples by a

nested PCR reaction using published primers and cycling conditions.(Fig 3)

RNA Extraction (Qiagen Viral RNA Extraction Kit- Catalogue no: 52904) was used for extraction of RNA from the serum sample. The manufacturer's instructions were duly followed in the procedure. The eluted RNA was aliquoted and stored at −80°C until PCR was done.

Complementary DNA (cDNA) Synthesis: The cDNA was synthesized from the extracted RNA using a high capacity cDNA Reverse Transcription Kit. An aliquot of 3 µl of cDNA was used for further amplification and the rest was stored at -20°C for further use.

External PCR reaction: The cDNA amplification reaction was carried out in 25 µl reaction with cDNA, Go Taq Green Master Mix (2x) (Promega USA), forward primer (D1) and reverse primer (D2), nuclease free water and cDNA. The PCR reaction was carried out in a Veriti thermal cycler (Applied Biosystems).

The amplified product was diluted in 1:5 ratio before proceeding to the next step.

Nested PCR Reaction: In the second round of semi-nested PCR, the external amplicon was amplified using published primers. The 1µl of the diluted amplicon was amplified with serotype-specific primers TS1, TS2, TS3 and TS4 for DENV-1, DENV-2, DENV-3 and DENV-4 respectively.

The nested PCR reaction was set up with primers (TS1, TS2, TS3, TS4 and D1), Go Taq Green MM (2x) (Promega, USA), Nuclease free water and DNA, in a 25 µl reaction.

Agarose Gel Electrophoresis: 2% agarose gel was prepared by mixing 3gm of agarose (Sigma Aldrich Corp., USA) and 150 ml of TBE buffer (1X). An aliquot of 10 µl of PCR product was loaded in the wells with 3µl of bromophenol blue dye. An aliquot of 1µl of 100bp DNA ladder was also loaded in the agarose gel. 3µl of the ethidium bromide (10mg/ml) was added to the running buffer (1XTBE) in the gel electrophoresis tank (Bio-Rad, USA). The voltage was set at 100V. After one and a half hours, the gel was visualized under the UV light using gel documentation system (Wealtec, Sparks, NV, USA).

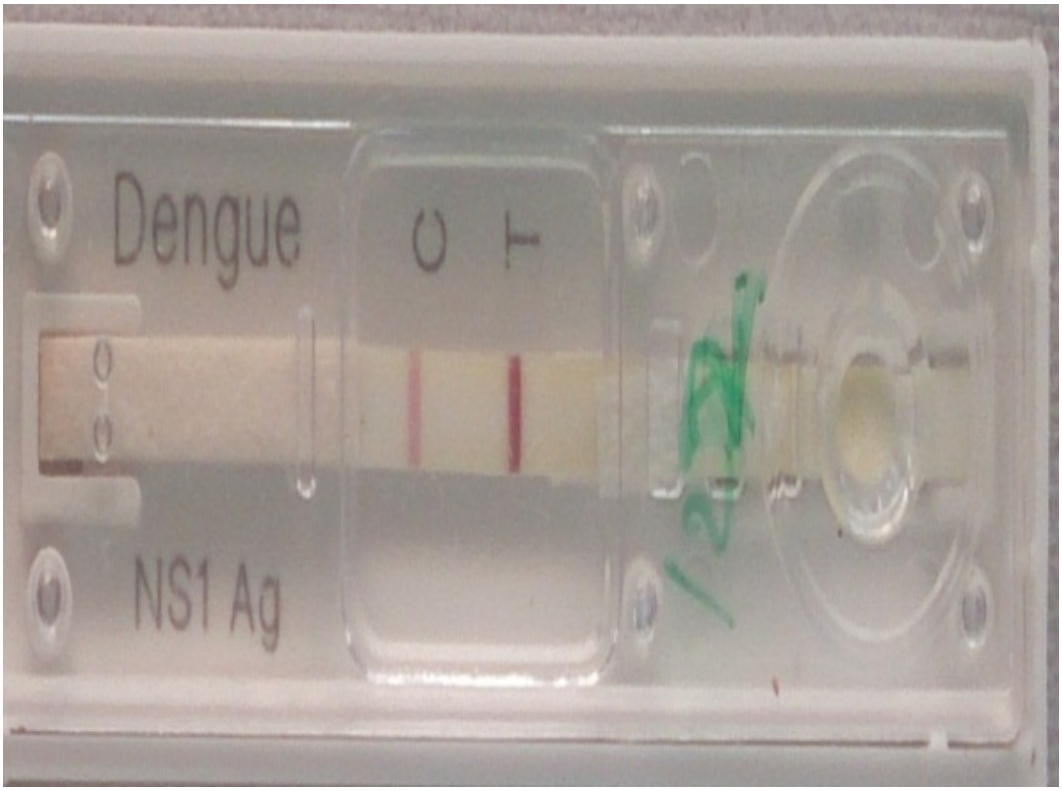


Figure 1: ICT for NS1 antigen: A pink line is observed in T line in positive sample and C line (quality control)

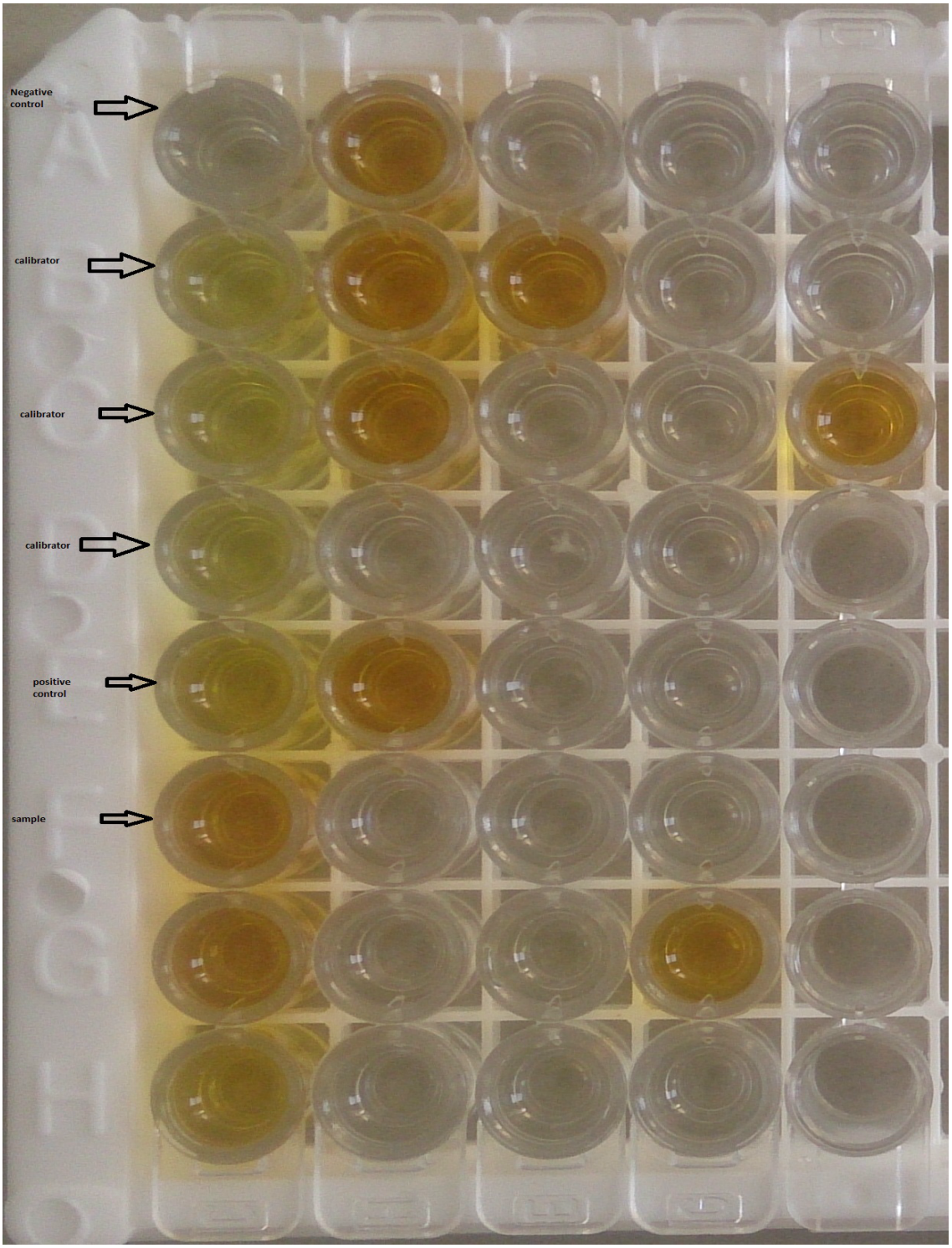


Figure 2: Microtitre for NS1 antigen

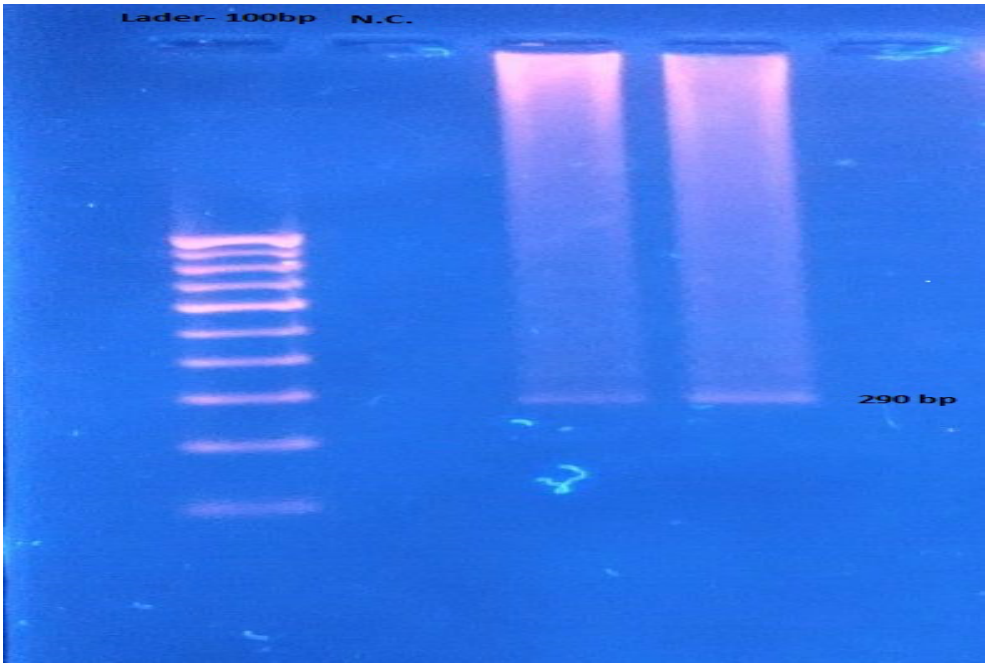


Figure 3: Agarose gel photograph of nPCR

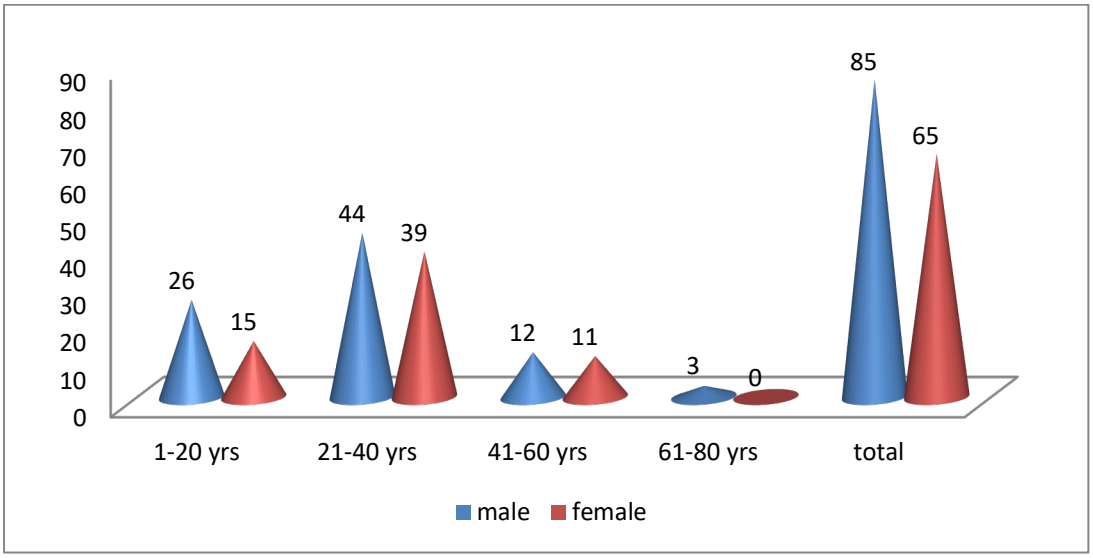
Results: Patients aged 1-80 years were selected randomly for the study. Out of 150 patients, 85 (56.67%) patients were male and 65 (43.33%) were female (Table 1). The male to female ratio was 1.3:1.

Demographics of patients enrolled in the study: Patients were divided into four groups based on their age into >1-20, 21-40, 41-60 and 61-80 years (Table 1, Graph 1).

Maximum numbers of patients were in 21-40 years of age group i.e. 83(55.3%). Only three cases were in the age group of 61-80 years.

Table 1: Age and Gender wise distribution of the patients enrolled in the study

Age group	Male n (% of total in age groups)	Female n (% of total in age groups)	Total
1 -20yrs	26(63.4%)	15 (36.6%)	**41**
21-40yrs	44 (53%)	39 (47%)	**83**
41-60yrs	12 (52.2%)	11 (47.2%)	**23**
61-80yrs	3 (100%)	0	**3**
Total	**85 (56.7%)**	**65 (43.3%)**	**150**



Graph 1: Age and gender wise distribution of patients enrolled in the study

NS1 antigen positivity (by ICT card) by gender-wise and day of illness:

Table 2: Gender-wise ICT result

Gender	Number	Positive on ICT	Negative on ICT	% positivity
Male	85	47	38	55.3%
Female	65	35	30	53.8%
Total	150	82	68	54.7%

No significant variation seen in positivity for NS1 antigen in male and female patients on ICT (Table 2).

Table 3: NS1 antigen positivity (by ICT card) by day of illness

Day of illness	No. of NS1 antigen Positive (%) RDT	Total Tested
1	3 (100%)	3
2	23(45.0%)	51
3	25(54.3%)	46
4	13(76.4%)	17
5	13(65%)	20
6	3(37.5%)	8
7	2(40%)	5

The NS1 antigen was detected from day 1 to day 7 of the illness. 82(54.7%) samples were found positive by rapid ICT card with maximum positivity of 13(76.4%) on day 4 of the illness.

With day 1 fever, only 3 patients were enrolled and all were found positive for NS1antigen by ICT (Table 3).

Detection of NS1 antigen by ELISA:

All150 serum samples were tested for anNS1 antigen using NS1 antigen ELISA kit and 64 (42.67%) was found to be positive for NS1 antigen. NS1 antigen was detected by ELISA from day 1 to day 7 of illness with maximum positivity on day 1 and 4 (66.7%), (52.9 %) respectively (Table 4).

Table 4: Detection of NS1 antigen by ELISA

Day of illness	No. of NS1 antigen positive by ELISA	Total Tested
1	2 (66.7%)	3
2	19 (37.2%)	51
3	20 (41.3%)	46
4	9 (52.9%)	17
5	9 (50.0%)	20
6	3 (37.5%)	8
7	2 (40.0%)	5

Comparison of NS1 antigen by ICT and ELISA:: In comparison, NS1 antigen by ICT has a sensitivity of 95.8% as compared to ELISA but a low specificity of 75.6% as compared to ELISA (Table 5).

Table 5: Comparison of NS1 antigen rapid immunochromatographic test and NS1 antigen ELISA

NS1 antigen ELISA (gold standard)	NS1 antigen ICT Positive	NS1 antigen ICT Negative	Total	Sensi-tivity	Speci-ficity
Positive	61	3	64	95.8%	75.6%
Negative	21	65	86		
Total	82	68	150		

Reverse transcriptase polymerase chain reaction (RT-PCR) for Dengue: RT-PCR for Dengue was carried out on randomly selected 82 samples out of total collected 150 samples using primer from the pre-M region. Of 82 samples, 33 were found positive by RT-PCR. On performing the nested PCR all 33 samples were genotyped as DENV-3

Comparison of RT-PCR with NS1 antigen detection by ICT and ELISA (among 82 samples processed by RT-PCR): Table 6 The maximum number of samples (12) which were found positive for NS1 antigen by all three assays used was from day 2. On day four, 6 samples were positive by PCR but only 5 of them were positive for NS1 antigen by ELISA and 4 by ICT. On day five, 4 samples were positive out of 12 by PCR and NS1 antigen by ELISA. By ICT 3 additional samples were positive for NS1 antigen. On day 7, none of the samples (2) were positive in any of the tests.

PCR and NS1 antigen by ICT and ELISA positivity by day of illness:

Table 6: Positive samples by ICT, ELISA and RT-PCR on different days of illness

Day of illness	Total	Positive by ICT	Positive by ELISA	Positive by PCR
1	2	2	2	2
2	27	16	12	12
3	23	9	7	8
4	9	4	5	6
5	12	7	4	4
6	7	3	1	1
7	2	0	0	0
Total	82	41	31	33

Detection of NS1 Antigen on different days of illness by RT-PCR, ELISA and RAPID ICT: A total of 82 samples were tested by RT-PCR. 33 samples were found positive by RT-PCR. 31 and 41 samples were positive for NS1 antigen by ELISA and rapid ICT assays respectively among these 82 samples.

Comparison of NS1 antigen rapid ICT and reverse transcriptase polymerase chain reaction (RT-PCR) (gold standard): In the present study out of 82 samples 33 samples were positive by RT-PCR. Of these 41 were positive for NS1 antigen by rapid ICT. The sensitivity of NS1 antigen by rapid ICT is 84.8% and specificity 73.5% with 68.3% positive predictive value and 87.8% negative predictive value.

Comparison of NS1 antigen ELISA and reverse transcriptase polymerase chain reaction (gold standard): Out of 82 samples processed by RTPCR, 33 samples tested positive for dengue PCR. ELISA was positive in 31 samples out of these 82 samples. The sensitivity of ELISA is 93.9% and specificity 100% with a positive predictive value of 100% and 96% negative predictive value.

Discussion

Dengue fever is a mosquito borne tropical disease caused by the dengue virus. Numerous Aedes species of mosquitoes, most notably A. aegypti, are responsible for the transmission of dengue. There are five distinct kinds of the virus; an infection with one type typically results in lifetime immunity to that type and only temporary immunity to the others. Severe consequences are more likely when a different sort of illness occurs later on. Each year between 50 to 528 million people is infected with dengue virus and approximately 10,000 to 20,000 die. [10] Apart from eliminating the mosquitoes, work is ongoing for medication targeted directly at the virus. It is classified as a neglected tropical disease. [11,12]

In 2016, a partially effective vaccine for dengue fever became commercially available in the Philippines and Indonesia. [13,14] The vaccine is produced by Sanofi and goes by the brand name Dengvaxia. [15,16] It is based on a weakened combination of the yellow fever virus and each of the four dengue serotypes. Two studies of a vaccine found that it was 60% effective and prevented more than 80 to 90% of severe cases. [17]

In the present study, the sensitivity and specificity of NS1 antigen by ICT were found to be 90.3% and 74.5%, respectively. Gaikwad et al. in 2017 reported sensitivity and specificity of NS1 antigen by ICT were 88.5% and 66.7%. [18]

The sensitivity and specificity of NS1 antigen by ELISA were 93.9% and 100%, respectively. Ahmed and Broor in 2014 reported the sensitivity, specificity, PPV, and NPV of NS1 antigen ELISA to be 73.51%, 100%, 100%, and 70%, respectively. [19] In 2014, Hunsperger et al. reported sensitivity and specificity of NS1 antigen by ELISA to be 60-75% and 71-80% respectively. [20] Furthermore, a comparative study of NS1 antigen by ICT and ELISA was done by Reddy et al (2022). [21].

In our study out of 150 patients, 85 (56.7%) patients were male and 65 (43.3%) were female. Of 85 males and of 65 females, 47 (55.3%) and 35 (53.8%) were positive for NS1 antigen respectively by ICT. Thus, the study shows a male preponderance which is concordant with a study by Gaikwad et al.2017. [18] This may be since males have more exposure to outdoors than females.

NS1 antigen by ICT was maximally positive i.e. 13(76.4%) on day 4 of illness. Only 3 patients were enrolled and all were positive for NS1 antigen on day 1 of illness. Similarly on day 6 of illness only 3 (37.5%) were positive by NS1 antigen ICT. Out of total of 46 patients tested for NS1 antigen 25(54.3%) were positive, but in a study by Gaikwad et al.2017 maximum positivity by NS1 antigen ICT was on day 3 of illness. [18] Out of a total of 150 serum samples tested for NS1 antigen ELISA, 64(42.7%) were positive for NS1 antigen which was detected from day1 to 7 of illness. Maximum positivity was detected on day 4 and 5 as 52.9% and 50.0% respectively. Two (66.7%) out of 3 enrolled patients were positive on day 1 for NS1 antigen by ELISA. Only 2(40.0%) patients were positive for NS1 antigen out of 5 enrolled patients on day 7 of illness, but in a study by Ahmed and Broor, 2014, NS1 antigen ELISA was maximally positive (100%) on day 2 of illness

whereas in our study NS1 antigen ELISA was positive in 19 (37.2%) on day 2 of illness. Thus, it can be concluded that positivity by NS1 antigen ELISA may vary among different serotypes. [19]

In contrast to ELISA, the current study's observations indicate that the NS1 antigen via ICT has a low specificity of 75.6% and a sensitivity of 95.8%. Out of a total of 64 patients positive by NS1 antigen ELISA, 61 (95.3%) were positive by NS1 antigen ICT and 3 (4.7%) were negative by NS1 antigen ICT, whereas out of 86 patients negative by NS1 antigen ELISA, 65 (75.6%) were negative by NS1 antigen by ICT and 21 (24.4%) were positive by NS1 antigen ICT. Out of 150 serum samples collected, RT-PCR was carried out on randomly selected 82 samples. Of the 82 samples 33(40.24%) were positive by RT-PCR and all the 33 samples were genotype as DENV- 3 by performing nested PCR.

In the study on comparing RT-PCR with antigen detection by ICT and ELISA, a maximum number of samples (12) that were positive by all three assays belonged to day 2 of illness. On day 4, six samples were positive by PCR but only 5 of them were positive for NS1 antigen by ELISA and 4 were positive by ICT. On day 7, none of the 2 samples were positive in any of the 3 tests.

Out of the total of 82 samples tested by RT-PCR 33 were positive by RT-PCR, 31 were positive for NS1 by ELISA and 41were positive for NS1 antigen by ICT. On comparison of NS1 antigen ICT and RT-PCR for 82 samples, the sensitivity of NS1 antigen by ICT is 84.8% and specificity is 73.5% with positive predictive value 68.3% and 87.8% negative predictive value.

Similarly, in the study, out of 82 samples, comparing NS1 antigen ELISA and RT-PCR, 33 samples tested positive for dengue by PCR whereas 31 were positive by ELISA out of 33 samples subjected to PCR. The sensitivity, specificity, positive predictive value, and negative predictive value of ELISA are 93.9%, 100%, 100%, and 96% respectively. According to our study, a higher detection rate by rapid and NS1 ELISA test was found from day 4 of illness. To further analyze the sensitivity of these tests numerous cases should be tested. Multiple studies have demonstrated a diagnostic strategy combining NS1 antigen (ICT) detection and NS1 antigen ELISA in acute phase sera providing a sensitivity of about 92.1% for dengue diagnosis. Raafat et al (2019) [22] reviewed the advantages, uncertainties, and variability of the above-described diagnostic methods used for dengue and discussed their implications and applications for dengue surveillance and control.

Conclusion

NS1 antigen detection and dengue group specific reverse transcriptase RT-PCR are valuable techniques for the rapid and early microbiological diagnosis of dengue. NS1 antigen ELISA can be implemented in diagnostic laboratories for diagnosis of dengue in the acute phase of illness and the diagnosis can be made as early as within three days of onset of fever.

The test also has great potential value for use in epidemic situations, as it facilitates the early screening of patients. However, NS1 antigen detection by ICT may give false positive and false negative results as compared to ELISA and thus Microbiology laboratories should confirm all NS1antigen positive results by ICT with ELISA.

RT-PCR is more sensitive and specific but requires well equipped laboratory with trained staff. In comparison with other laboratory diagnostic tests such as MAC ELISA for anti-DENV IgM antibody detection. NS1 antigen becomes positive earlier and thus helps in diagnosis of early infection, whereas IgM antibodies appear by 6-7 days post fever and last for about 3 months. This study thus showed the usefulness of NS1antigen detection in the early diagnosis of dengue.

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