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Research Article

Molecular Identification of Dengue Virus Strains in Laboratory-Confirmed Dengue Cases

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A B S T R A C T

Background: Dengue fever is a rapidly spreading arboviral infection caused by the dengue virus, which has 4 antigenically distinct (DENV) serotypes—DENV1, DENV2, DENV3, and DENV4—transmitted by Aedes mosquitoes. Identification of circulating serotypes and their correlation with disease severity, outbreak prediction and clinical management may be necessary to initiate therapeutic, preventive and control measures. While NS1 antigen and IgM/IgG antibody tests are the widely used serological tests for diagnosis, RT-PCR provides definitive serotype identification.

Methodology: This study was performed at Chettinad Hospital & Research Institute between June 2023 and December 2023. A total of 100 laboratory-confirmed dengue-positive patients were included. Diagnosis was based on NS1 antigen and IgM/IgG antibody positivity using immunochromatography card tests and the ELISA method. RT-PCR (HELINI Dengue Genotyping Kit) was employed to detect and differentiate DENV1 to DENV4.

Results: Among 100 patients, 69% were males and 31% were females. The majority of the positive cases belonged to the 11–30 years of age group. NS1 antigen positivity was observed in 89%, and IgM in 30%; 19 patients showed dual positivity. RT-PCR was positive in 36 patients, and DENV3 was the predominant serotype. Three individuals were positive for the DENV-4 serotype. Five individuals were co-infected with the DENV-3 and DENV-4 serotypes.

Conclusion: DENV3 was the most prevalent serotype in this study. Young males were disproportionately affected. RT-PCR-based serotyping enhances diagnostic accuracy and supports informed clinical decisions during dengue outbreaks.

Keywords: Haemorrhagic fever, Molecular typing, Non-structural antigen, ICT, ELISA

Introduction

Globally, dengue fever is a viral infection caused by the Flavivirus group's serotypes (DENV1, DENV2, DENV3, DENV4), spread by Aedes aegypti and Aedes albopictus mosquitoes. It poses a serious public health concern in subtropical and tropical regions.[1] About 70% of the world's

illness load is found in Asia alone. Subsequent infections raise the likelihood of developing DSS and DHF, and one serotype provides lifelong immunity only to that particular type.[2]

Accurate identification of circulating DENV serotypes is critical for outbreak prediction, epidemiological surveillance, and clinical management. While traditional virus isolation methods are labour-intensive, molecular detection offers rapid and reliable serotyping.[3,4] Serological methods such as ELISA, which detect IgM and IgG antibodies or NS1 antigen, remain valuable for early diagnosis, though they show limitations in differentiating between primary and secondary infections. This study aims to identify the predominant DENV serotypes among laboratory-confirmed dengue patients. By exploring the validity and applicability of various diagnostic modalities, this investigation seeks to enhance understanding of dengue pathogenesis and support strategies for improved disease control and patient management.[5,6,7]

Methodology

Study Design: A prospective observational study conducted at Chettinad Hospital & Research Institute, from June to December 2023. Institutional Human Ethics Committee approval was obtained prior to study initiation.

Sample Size Calculation

Sample Size

n = Sample size

$Z_{\alpha/2}$ = (1.96 for 95% CI)

Z_{β} = (0.84 for 80% power)

r = expected correlation coefficient (effect size)

Correlation value (r = 0.3)

$n = (1.96+0.84)^2 / (0.3)^2$

$= (2.8)^2 / 0.09$

$= 7.84 / 0.09 = 87.11$

Approximately 88 samples. Hence, in this study the sample size was fixed as 100 (n=100).

Study Population

Individuals of all age groups, both male and female, presenting to the outpatient and inpatient departments with symptoms suggestive of dengue fever and positive for dengue by serology were included. Inclusion criteria were laboratory confirmation of NS1 antigen and IgG/IgM antibody of dengue positivity. Patients with other confirmed causes of pyrexia or negative dengue serology were excluded.

Sample Collection and Processing

After getting informed consent from all participants or guardians (in the case of the paediatric age group) before sample collection. Venous blood samples were collected in a plain vacutainer with a yellow top and an EDTA vacutainer. The plain vacutainer was processed for serum separation by centrifugation. Serum was used for serological tests like immunochromatography cards and ELISA, while EDTA blood plasma was used for RTPCR.

Samples were tested using immunochromatography (rapid card test) and ELISA methods. The SD Biosensor Ultra Dengue (Ag+Ab) Duo rapid test was used for testing of NS1 antigen, IgG, and IgM antibodies. The J Mitra kits were used for Dengue NS1, IgM and IgG ELISA

Procedure by Immunochromatographic Card Method for Dengue NS1 Antigen

10 µl of centrifuged serum sample was collected using the specimen transfer device until the marking was reached. The collected serum was then added to the specimen well of the cassette; the test result was read after 15 minutes up to 30 minutes.

Immunochromatographic card method for dengue IgM/IgG antibody

10 µl of centrifuged serum sample was collected using the specimen transfer device until the marking was reached. The collected serum was then added to the specimen well of the cassette, three drops of buffer were added into the well of the cassette. The test result was read after 15 minutes, up to 30 minutes.

Interpretation of test results for Dengue NS1 Ag & for Dengue IgM Ab Cassette: Positive result -The coloured bands in the control and test lines were observed.

ELISA Procedure for Dengue NS1 Antigen & IgG/IgM Antibody Detection - J. Mitra NS1 Ag and Dengue IgG & IgM Microlisa kits were employed. The NS1 antigen ELISA method works based on a direct sandwich format, while the IgM and IgG ELISA tests are based on the antibody capture ELISA technique.

ELISA Procedure for Dengue NS1 Antigen

The assay commenced with the addition of 50µL of diluent, controls, calibrators, and test samples into the designated wells of the microtiter plate. Conjugate of 100 µL was then dispensed to every well. After thorough mixing, incubation for 90 minutes at 37°C was performed. After which wells were washed six times with buffer wash to eliminate unreacted substances. 150µL of solution of substrate was dispensed into each and every well and

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was subjected to incubation; the reaction mixture was subjected to incubation at ambient temperature for thirty mins in the dark. Stop solution of 100 µL was introduced to terminate the reaction, and the absorbance was measured, and readings were obtained at wavelengths of 450 and 630 nm within 30 minutes.

ELISA Procedure for Dengue IgM/IgG Antibody Detection - For the antibody ELISA, 100µL of negative control (A1), calibrators (B1–D1), positive control (E1), and appropriately diluted patient samples (starting from F1) were dispensed to their specified microwells. The microtiter plate was incubated for 60 minutes at 37°C. Following this, the reaction chambers were washed five times to remove any unbound substances. Subsequently, 50µL of enzyme-labelled reagent was dispensed into all designated wells, and the plate was incubated again for 90 minutes at 37 °C. Post-incubation, the compartments were rewashed, and 100µL of chromogenic substrate solution was added. The plate was then kept in the dark at ambient temperature for 30 minutes. Finally, 50µL of stop reagent was added to each reaction site, and the optical density was read at 450nm with a reference wavelength of 630nm within 30 minutes.

Molecular typing

This was performed using the HELINI Dengue Genotyping Kit of RT-PCR for identification and differentiaion among all four DENV serotypes.

RNA Extraction

Viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen), which utilises a spin-column format. A volume of 5.6µL of carrier RNA was combined with 140µL of the sample in buffer AVL, vortexed thoroughly, and incubated at ambient temperature for 10 mins to ensure complete viral lysis. After adding 560µL ethanol and vortexing, the mixture was loaded onto the QIAamp column and centrifuged. The silica- based membrane tube was cleaned sequentially with 500µL of buffers 1 and 2, followed by high-speed centrifugation. Elution was performed using 60µL of room-temperature buffer, incubated for 1 minute and centrifuged. The extracted viral RNA (~90% yield) will be stable for a year at −30°C to −15°C or −90°C to −65°C.

RT-PCR Procedure

All kit components were thawed, gently mixed by inversion, and briefly centrifuged prior to use. Each run included both positive and negative controls, and an internal control template was added during RNA extraction or directly to the purified RNA (2.5µL). The reaction volume was standardised to 25µL. Each reaction comprised 8 µL of 1-step Master Mix, 2µL of RT-Taq enzyme mix, and 5 µL of dengue virus primer-probe mix (Set 1-DENV1&2; Set 2 -DENV3&4). A total of 10µL of purified RNA was added per reaction. To minimise pipetting errors, an additional 0.5µL was incorporated.

Multiplex fluorescent signal detection was carried out using FAM (targeting DENV &3), HEX (for DENV2&4), and Cy5 (serving as the internal control). No passive reference dye was included in the assay. Fluorescence data were acquired under default ramping parameters using a PCR system. The thermal cycling protocol included reverse transcription at 50°C in 20 minutes. Then by initial denaturation at 95 °C for 15 minutes and 45 cycles of amplification consisting of denaturation (95 °C in 20 s), annealing with fluorescence detection (56 °C in 20 s), and extension (72 °C in 20 s).

Amplification curves were interpreted using fluorescence thresholds set just above the negative control baseline. The Cy5 signal was used to assess internal control validity (expected Ct: ~21 ±10). A Ct > 31 suggested suboptimal RNA purity or PCR inhibition. FAM and HEX channels were analysed for genotype-specific detection, with flat curves in NTC wells confirming the absence of contamination. Each genotype-specific signal was confirmed by expected amplification in the corresponding positive controls.

Results

During this study period, 100 samples of dengue NS1 or IgM or IgG positive samples were collected. This pie chart represents the distribution of gender among dengue-positive individuals. This data includes 100 dengue-positive cases, with the distribution split between males and females. Male patients (blue section) were 69 (69%). Female patients were 31 (31%). Males were more affected by dengue compared to females, as depicted in Figure 1.

This bar chart (figure 2) illustrates the dengue-positive cases distribution based on age and gender, with a sample size total of 100 cases. Male cases (blue bars) are significantly higher in all age groups compared to female cases (orange bars). Among males, the highest incidence of dengue positivity was recorded in the 11–20 years age group, accounting for 25 cases (25%). In contrast, females showed the highest number of positive individuals in the 21–30 years of age group, comprising 9 cases (9%). The 11-20-years-olds and 21-30-years-old were the most affected age groups. Male patients dominated across all age groups. The number of cases gradually decreased as the age increased, with age groups >51 years having the least cases. In the 41-50 years group, there were no female cases (0%). The age groups >51 years has the lowest number of cases (5 males and 1 female). Young adults (11-30 years) were the most affected by dengue infection. Cases decreased with increasing age, suggesting that younger populations may be more vulnerable to dengue infections.

This pie chart (figure 3) represents the diagnostic methods that were used for detecting dengue among 100 cases. The

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sample size consists of 100 dengue-positive cases diagnosed by using two different methods. The rapid card test was positive in 78 cases (78%). The ELISA test was positive in 22 cases (22%).

This bar chart (figure 4) represents the positivity distribution of NS1, IgM, and IgG among 100 dengue-positive cases, indicating how different diagnostic markers were detected. NS1 antigen was detected in 89 cases out of 100 cases. IgM Antibody (30 cases, 30%) was found in a smaller proportion. IgG antibody (0 cases, 0%) was not detected in any case. Therefore, The NS1 antigen was most frequently detected (89%) among the other two markers, indicating that the the majority of cases were in the early phase of dengue infection (first 5-7 days). IgM antibody positivity (30%) suggests that some patients may have progressed to the acute or recent past infection stage. IgG Antibody (0%) means that no cases were in the late or past infection stage, implying most of the infections are recent.

Out of 100 dengue-positive cases, 89 patients tested positive for NS1, while 30 patients were positive for IgM. Notably, 19 patients showed concurrent positivity for both NS1 and IgM, indicating an overlap between early viral detection and the host's immune response. This dual positivity suggests a transitional phase in the infection, where active viral replication is still present alongside the body's initial immune activation. (Figure 5)

RT-PCR results

Out of 100 samples tested for dengue virus serotypes 1,2,3 and 4, 36 (36%) were positive for DENV by RTPCR. Of these, 28 (28%) of the RTPCR positives belonged to the DENV 3 type, and 3 were positive for DENV 4 (Figure 6 & Table 1). 5 samples were positive for both DENV3 and DENV4. None of the samples tested positive for DENV1&2.

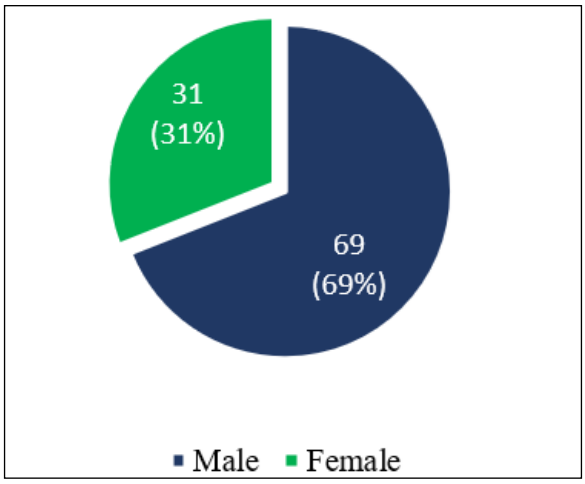

Figure 1.Gender wise distribution for total dengue positive samples

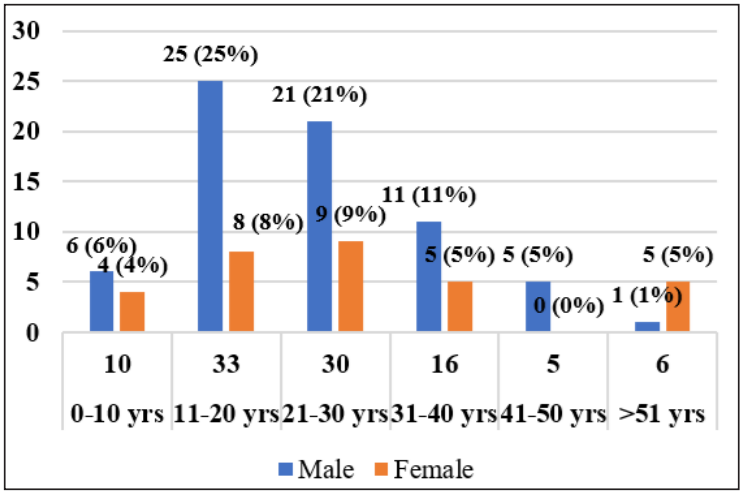

Figure 2.Age and gender-wise Distribution of dengue positive cases

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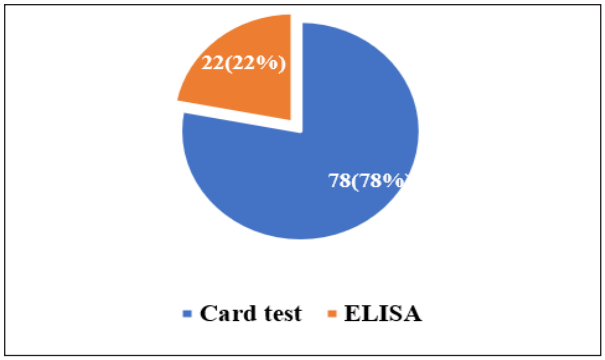

Figure 3.Test Methodology use for Diagnosis

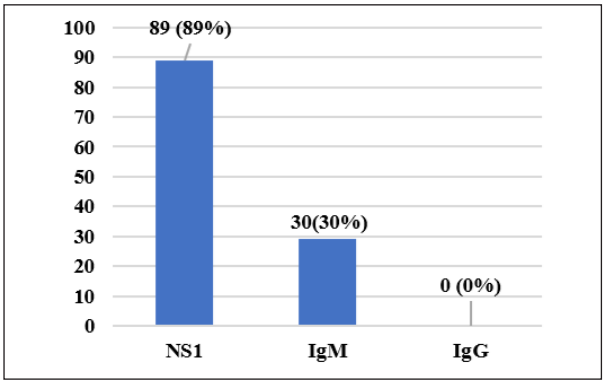

Figure 4.Positivity distribution of NS1, IgM and IgG

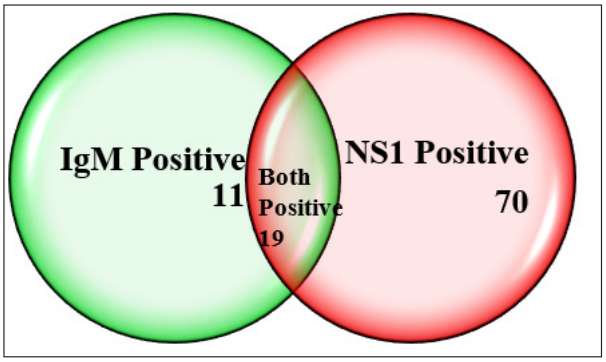

Figure 5.NS1 & IgM positivity prevalence cases

N = 100
Total NS1 Ag Positive= 89
Total IgM Positive = 30
Both NS1 Ag & IgM Ab Positive =19

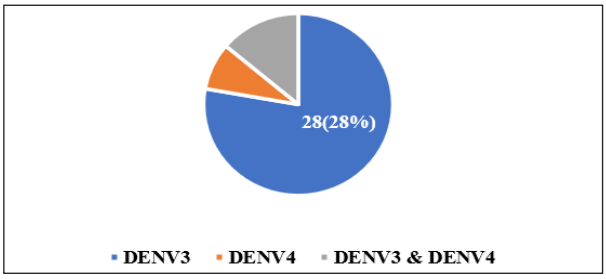

Figure 6.Distribution of Dengue virus serotypes

28 (28%)DENV 3
3 (3%)DENV 4
5 (5%)DENV3 & DENV4
Both NS1 Ag & IgM Ab Positive =19

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Table 1.Age & Gender distribution of RTPCR Positive patients in Dengue (n = 36)

Age & Gender distribution of only DENV3 Positive patients	Total (n=28)	Male	Female
Less than 10 years	1	1	0
11 to 20	12	8	4
21 to 30	7	3	4
31 to 40	4	2	2
41 to 50	2	2	0
Above 51	2	0	2
Age & Gender distribution of only DENV4 Positive patients	**Total (n=3)**	**Male**	**Female**
Less than 10 years	1	0	1
11 to 20	1	1	0
21 to 30	1	0	1
31 to 40	0	0	0
41 to 50	0	0	0
Above 51			
Age & Gender distribution of both DENV3&4 Positive patients	**Total (n=5)**	**Male**	**Female**
Less than 10 years	1	1	0
11 - 20	1	1	0
21 - 30	1	0	1
31 - 40	1	1	0
41 - 50	1	1	0
Above 51	0	0	0

Discussion

This study assessed the laboratory-confirmed dengue cases over a six-month period with a focus on evaluating the diagnostic tools employed and the distribution of dengue virus serotypes. The diagnostic approaches included rapid immunochromatographic card tests, enzyme-linked immunosorbent assays (ELISA), and RT-PCR, with the latter serving as the definitive tool for serotyping (Shamala DS et al. 2015 Jun).[8]

The immunochromatographic test, which detects NS1 antigen, IgM, and IgG antibodies, serves as a rapid and accessible diagnostic method. NS1 antigen is detectable from the first day of onset of fever and persists for up to nine days. It is a key marker for early infection. Identification of IgG and IgM antibodies provides insights into the phase of infection, with IgM indicating recent infection and IgG pointing to past or secondary infections (Hedge SS et al. 2022 May).[9]

A notable finding in this study was the higher prevalence of dengue among males (69%) compared to females (31%). (figure 1) This observation was consistent with epidemiological data from several Asian countries, where greater exposure to mosquito vectors due to outdoor occupational and recreational activities increases infection risk among males (Prattay KM et al; Apr 2022).[10] Biological factors, such as hormonal and immune response variations, may also contribute to the observed gender disparity (Anwar SL et al; July 2016).[11] This pattern mirrors the findings by Priyadarshini et al. (2016), who reported that 77.6% of adult dengue-positive patients were male.[12]

Age-wise distribution analysis revealed that the 11–30 years age group was the most affected population by dengue. (figure 2) Young adults and adolescents are often more active outdoors, increasing their exposure to mosquito bites, which likely contributes to this trend. Kumar M, et al. highlighted similar age-related vulnerability in their study, attributing it to behavioural and environmental factors. The declining number of cases in older age groups may reflect a combination of reduced exposure and acquired immunity. However, this study observed no female cases in the 41–50 age group and very few in the age above-51-years category, suggesting potential age- and gender-linked differences in vulnerability and immune response.[13]

The study demonstrated that the rapid card test was the most frequently used diagnostic tool, accounting for 78% of cases. This high usage is likely due to its affordability,

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quick turnaround time, and suitability for use in point-of-care settings. Although ELISA offers higher sensitivity and specificity, particularly for differentiating between primary and secondary infections, it was limited to 22% of cases in this study. (figure 3) This finding emphasised the importance of balancing accessibility and accuracy in diagnostic testing. Kabir MA et al. and Raafat N et al. emphasised that using a combination of diagnostic methods could significantly enhance case detection and improve patient management during outbreaks.[14, 15]

Among the 100 laboratory-confirmed cases, NS1 antigen was detected in 89 patients, highlighting its utility as a reliable early marker for dengue infection. IgM antibodies were detected in 30 patients, which is consistent with their appearance 3–5 days after the onset of symptom indicating recent or acute infections. No patients tested positive for IgG, suggesting an absence of past infections or secondary infection of dengue (figure 4). This high NS1 positivity correlated with the findings from Sharada T et al., who demonstrated the diagnostic value of combining NS1 and IgM tests to enhance sensitivity and early detection. [(16)] In Mohanam L, Shanmugam P., et al.' study showed the concurrent detection of NS1 antigen and IgM antibody proves valuable in identifying the early phase of infection, indicating that immunochromatographic testing (ICT) can serve as an effective primary screening tool.[17]

Our study results depicted in (Figure 5) demonstrated the utility of NS1 and IgM testing in identifying different phases of dengue infection, with 89 out of 100 (89%) NS1 positivity indicating early viral replication and 30 out of 100 (30%) IgM positivity reflecting immune activation. The overlap of 19 cases with dual positivity suggests a transitional phase. Similar to our study, Anand AM et al. concluded that research in Southern India showed NS1 detection rates of 80.9% during the acute phase, highlighting its sensitivity for early diagnosis.[18]

RT-PCR identified dengue virus infection in 36% of the total samples analysed. Among the positive cases, DENV-3 was identified as the most prevalent serotype, detected in 28 patients. The highest number of DENV-3 cases (12) occurred within the 11–20 years age group. DENV-4 was detected in three patients across various age groups, with a predominance among females. Five patients exhibited co-infection with both DENV-3 and DENV-4. Notably, no samples tested positive for DENV-1 or DENV-2. (Figure 6 and Table 1depict the serotype distribution, highlighting DENV-3 as the predominant strain among RT-PCR-positive cases.

These findings correlated with the findings of Gupta A et al., who also described DENV-3 as the predominant serotype. In contrast, other regions such as Indonesia have shown variable serotype distributions. For instance, DENV-1 and DENV-2 were more common overall, except in Bali, where DENV-3 accounted for 48% of cases. These regional variations highlight the dynamic nature of serotype prevalence and the importance of localised surveillance. Co-infections, as observed in this study, have been associated with increased disease severity and underscore the clinical implications of identifying multiple circulating serotypes.[19]

In our study, DENV3 was the most prevalent serotype in both NS- positive and NS1-negative patients. Dinkar A, et al., concluded that the decline in occurrences of dengue virus serotypes DENV-1 and DENV-2 in recent times can be attributed to several interrelated factors where the predominant circulating serotypes have shifted, with DENV-3 and DENV-4 becoming more prevalent in certain regions. This shift may lead to a decrease in the incidence of DENV-1 and DENV-2 as populations become more susceptible to these other serotypes. Vector control measures have successfully reduced populations of Aedes mosquitoes in various regions. This has led to a temporary decrease in dengue cases, including those caused by DENV-1 and DENV-2.[20]

The RT-PCR positivity rate was relatively low, with only 36% of NS1 and IgM positive samples testing positive. Several factors may account for this, including the timing of sample collection. RT-PCR is most sensitive during the initial stages of infection, typically within the first five days of onset of fever, when viremia is at its peak. Singh K et al. also concluded and pointed out that the sensitivity of RT-PCR declines significantly after the five days of onset of fever due to a decrease in the viral load or the short-lived nature of dengue viremia.[21]

Furthermore, Santiago GA et al. highlighted that low levels of viral RNA load may result in false-negative RT-PCR outcomes, especially when the viral RNA concentration falls below the detection threshold.[22] Technical issues such as sample degradation, improper storage or handling, and the presence of PCR inhibitors can also affect results; these findings were concluded by Barkham TM et al.[23] In addition, Gusti et al. reported that negative RT-PCR results may stem from viral mutations or primer mismatch, both of which can reduce assay sensitivity and produce false negatives.[24]

Despite these limitations, RT-PCR remains the gold standard for serotyping due to its ability to precisely identify viral genotypes, which is critical for tracking epidemiological patterns and guiding public health interventions. The predominance of DENV-3, as shown in this study, reinforces the need for integrated vector control planning and public health preparedness strategies tailored to prevailing serotypes.

In conclusion, this study highlights key epidemiological features of dengue in the study population, with DENV-3

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found to be the most prevalent serotype. These findings emphasise the importance of early diagnosis using NS1 antigen testing, the value of combining diagnostic modalities for improved detection accuracy, and the necessity of continuous serotype surveillance.

Conclusion

This study provides a wide understanding of the prevalent dengue virus (DENV) serotypes. Among the 100 laboratory-confirmed dengue-positive patients, RT-PCR identified DENV3 as the most dominant serotype, with five cases of co-infection involving DENV3 and DENV4. The predominance of DENV3 suggests circulating serotypes, which may have implications for disease severity, outbreak patterns, and future epidemiological surveillance.

The majority of positive cases were male, with a higher prevalence in the 11–30 years age group. Serological testing indicated that the NS1 antigen was positive in 89% of cases, while 30% tested positive for IgM, highlighting the utility of early diagnostic markers. However, RT-PCR remained the most definitive tool for serotyping, and it played a role in accurate diagnosis and outbreak monitoring.

Overall, this study enhanced the importance of continuous dengue surveillance, particularly in identifying circulating serotypes and monitoring their impact on clinical outcomes. The predominance of DENV3 highlights the need for region-specific dengue management strategies. Additionally, early detection through RT-PCR may help in timely intervention, reducing complications and improving patient outcomes. Strengthening diagnostic capabilities and public health measures will be crucial in mitigating future dengue outbreaks and enhancing disease control efforts.

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