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Assessing the Diagnostic Accuracy of Three RT-PCR Kits for Dengue Virus Serotyping and Co-infection Identification in Clinical Samples

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Background & Objective: *Accurate identification of dengue virus (DENV) serotypes is essential for clinical management and epidemiological surveillance, especially in hyper endemic regions where co-infections are increasingly reported. While serological assays are limited in serotype differentiation, RT-PCR-based diagnostics offer both sensitivity and specificity. This study evaluated the diagnostic performance of three commercially available RT-PCR-based dengue serotyping kits in detecting and differentiating among the four DENV serotypes in clinical serum samples.*

Method: *A total of 80 serum samples (40 NS1-positives and 40 negatives by CLIA) were tested using each kit. Assay performance was assessed in terms of sensitivity, specificity and overall agreement using MedCalc software. Ct values were analyzed to compare relative analytical sensitivity.*

Results: *All three kits showed high specificity and comparable sensitivity, with TRUST gen-DS demonstrating 100% agreement with NS1-positive samples. Variability in DENV-1 and DENV-2 detection and co-infection patterns was observed across kits, likely due to differences in assay design and analytical stringency. Ct value distributions were not significantly different among kits.*

Conclusion: *All three kits show promise for routine clinical and surveillance use. However, standardized molecular diagnostics with high analytical sensitivity are crucial for reliably detecting co-infections and ensuring accurate serotype surveillance. Further validation against sequencing-based methods in larger datasets is recommended.*

Keywords: Dengue virus serotype, Real Time RT-PCR, Sensitivity, Specificity, Overall Agreement, Co-infection

Introduction

Dengue fever is a mosquito-borne viral illness caused by the dengue virus (DENV), a member of the *Flaviviridae* family. It is primarily transmitted to humans through the bite of Aedes aegypti mosquitoes. Despite continued control efforts, dengue remains a significant public health challenge, especially in tropical and subtropical regions [1]. Clinically, dengue is classified as dengue with or without warning signs, and severe dengue, which encompasses the conditions formerly known as dengue haemorrhagic fever (DHF) and dengue

shock syndrome (DSS) [2]. According to the World Health Organization (WHO), more than 14.4 million cases of dengue were reported globally in 2024, including 52,711 severe cases and over 11,000 deaths [3]. In India, 2,33,519 cases and 297 deaths were reported in the same year, highlighting the ongoing threat posed by the disease [4].

DENV comprises four closely related but antigenically distinct serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. These serotypes frequently co-circulate in hyperendemic regions. Infections may occur as mono-infections involving a single serotype or as co-infections with two or more serotypes [5]. Co-infections typically arise in regions where multiple serotypes co-circulate [6]. Co-infections can result from a single mosquito harboring multiple serotypes or from sequential bites by mosquitoes carrying different serotypes [7]. Additionally, asymptomatic human carriers may act as reservoirs, enabling the transmission of multiple serotypes to mosquito vectors.

While serological tests are commonly used for dengue diagnosis, they are limited in their ability to differentiate between serotypes. In contrast, molecular methods such as reverse transcription-polymerase chain reaction (RT-PCR) offer high sensitivity and specificity, along with the ability to identify specific serotypes [8]. These advantages make molecular diagnostics essential for both clinical case management and epidemiological surveillance.

Accurate identification of the infecting serotype plays an important role in clinical and epidemiological settings. Understanding which serotypes are circulating helps in predicting disease severity, optimizing treatment, and guiding public health interventions. Some studies suggest that infections with DENV-1 and DENV-3 are more frequently associated with severe disease manifestations, although the precise mechanisms of pathogenesis remain unclear. In regions with low levels of immunity, the introduction of a new serotype can also lead to widespread outbreaks [9,10]. Furthermore, serotyping allows distinction between mono and co-infections.

This distinction is becoming increasingly relevant, as recent years have seen a noticeable rise in reported dengue co-infections across India. For example, co-infection rates were 19% during an outbreak in Delhi in 2006 rising to 56.8% in Ernakulam, Kerala in 2009 [11,12]. Few studies have reported that co-infection rates reached as high as 100% during outbreaks in Kerala between 2013 and 2015 [13].

Despite the clinical and epidemiological importance of dengue serotyping, such data remain limited in India due to the absence of routine serotype-specific diagnostic testing. Although reports are available from states such as Andhra Pradesh, Delhi, Karnataka, Kerala, Odisha, Rajasthan, Uttar Pradesh and West Bengal, these are largely restricted to individual outbreaks and specific time periods [10,13-23]. Moreover, differences in serotyping methodologies particularly the widespread use of non-commercial, in-house RT-PCR assays have led to variability in diagnostic accuracy and hindered the comparability of results across studies.

Given these challenges, particularly the rising incidence of co-infections there is a clear need for consistent, year-round serotype surveillance using standardized and reliable diagnostic tools. In this context, the present study was undertaken to evaluate the diagnostic performance of three commercially available dengue serotyping kits in detecting and differentiating among the four DENV serotypes. Additionally, the study assessed the ability of these kits to identify both mono-infections and co-infections in clinical samples, with the goal of supporting more accurate clinical management and strengthening dengue surveillance systems in hyperendemic settings.

Materials and Methods

Serum Samples

Eighty anonymized serum samples collected from Hitech Diagnostic Centre, Chennai-84, were included in this study. These samples, stored at −80 °C, had previously been tested for the dengue NS1 antigen using the Autobio chemiluminescence immunoassay (CLIA), a qualitative method for NS1 detection. An overview of the study design is presented in Figure 1.

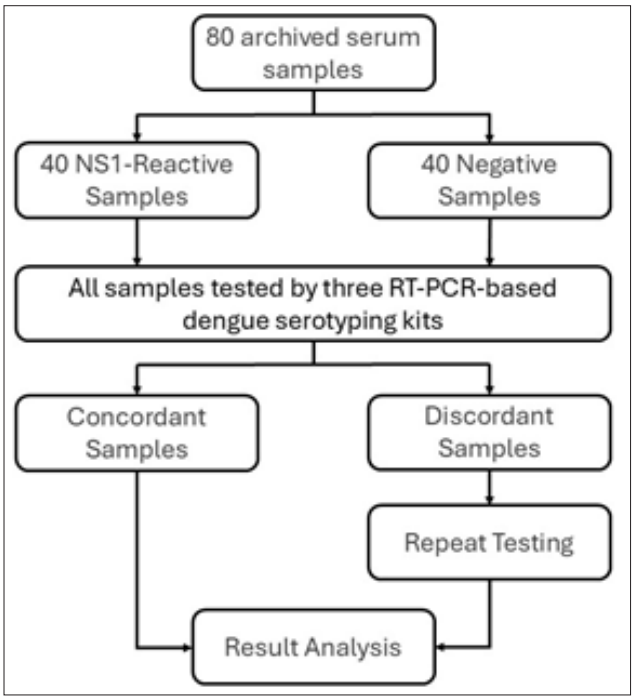

Figure 1: overview of the study design workflow

RNA Extraction

Viral RNA was extracted from the serum samples using the TRUPCR Magbead Viral RNA Extraction Kit (3B BlackBio Dx Ltd., India) on theZybioEXM3000 nucleic acid isolation system (Zybio, China), following the manufacturer's instructions. In brief, 200 µL of serum was used for each extraction. The RNA was eluted in 100 µL of elution buffer and stored at −80°C for downstream applications.

Dengue Serotyping RT-PCR Kits

Three different RT-PCR kits were tested in this study, namely TRUSTgen Dengue Serotyping Real Time PCR Kit (Athenese-Dx Pvt. Ltd., India), RealStar Dengue Type RT-PCR Kit 1.0

(Altona Diagnostics; Hamburg, Germany) and Hi-PCR Dengue Serotyping Probe PCR Kit 2.0 (HiMedia Laboratories Pvt. Ltd., India). For simplicity, these are referred to as TRUSTgen-DS, RealStar-DS, and Hi-PCR-DS, respectively. Among them, TRUSTgen-DS is a licensed in-vitro diagnostic (IVD) kit, RealStar-DS is specified for research use only (RUO) and Hi-PCR-DS is designated for in-vitro use only.

Each of these kits utilizes a two-tube, one-step real-time RT-PCR format, enabling simultaneous reverse transcription and amplification of dengue serotype-specific RNA targets. While the general workflow is consistent across kits, the design of primers, probes, and fluorophores used to detect each serotype varies.

In TRUSTgen-DS kit, tube 1 detects DENV-1(ROX) and DENV-4 (Cy5), while tube 2 targets DENV-2 (ROX) and DENV-3 (Cy5). An endogenous internal control is included in each tube, labelled with HEX.

In RealStar-DS kit, tube 1 detects DENV-1 (FAM) and DENV-4 (Cy5), and tube 2 targets DENV-2 (Cy5) and DENV-3 (FAM). A heterologous internal control, labelled with JOE, monitors both RNA extraction efficiency and RT-PCR inhibition.

In Hi-PCR-DS kit, Tube 1 includes probes for DENV-2 (FAM), DENV-3 (Texas Red), and DENV-4 (Cy5), while Tube 2 detects DENV-1 (JOE) and contains an endogenous internal control (ROX).

Assays were performed according to the manufacturers' protocols. Each RT-PCR run included the extracted RNA, kit-provided positive controls, and no-template controls (nuclease-free water) to validate assay performance. Amplifications were carried out on Light Cycler® 96 real-time PCR instrument (Roche Diagnostics, Switzerland) and fluorescence was monitored using the appropriate detection channels. Due to instrument limitations, the ROX- and JOE-labelled targets were detected using the Texas Red and HEX channels, respectively. Samples with Ct values ≤ 40 were considered positive for the corresponding serotype. Any samples with inconclusive results or multiple serotype detections were retested to confirm findings.

Statistical Analysis

Sensitivity, specificity, and overall agreement with Dengue NS1 CLIA-positive and negative samples were evaluated using MedCalc (Version 23.0.9), with 95% confidence intervals [24]. Cycle threshold (Ct) values were summarized as medians with interquartile ranges, presented both at the kit level and for each serotype identified by the respective kits. To compare median Ct values across kits and across kit-serotype combinations, the Mann-Whitney U test, a non-parametric method, was applied using R (version 4.4.2) software [25].

Results

The performance of three commercially available dengue serotyping kits viz., TRUSTgen-DS, RealStar-DS, and Hi-PCR-DS was evaluated using 80 clinical samples comprising 40 Dengue NS1 CLIA-positive and 40 negative samples. Among these, 71 samples (88.75%) demonstrated fully concordant serotyping results across all three kits, while 9 samples (11.25%) showed discordant serotype results. All four dengue serotypes (DENV-1, DENV -2, DENV-3 and DENV-4) were identified in the 40 Dengue NS1 CLIA-positive samples.

The diagnostic performance characteristics, including sensitivity, specificity, and overall agreement (with 95% confidence intervals) for each kit, compared against NS1 CLIA test results, are presented in Table 1. TRUSTgen-DS achieved 100% sensitivity and specificity, correctly detecting serotypes in all 40 NS1-positive samples without reporting any false positives. Hi-PCR-DS and RealStar-DS each missed one positive case, resulting in 97.5% sensitivity, while still maintaining 100% specificity by correctly identifying all 40 negative samples. The overall agreement with the NS1 CLIA test was 100% for TRUSTgen-DS and 98.75% for both RealStar-DS and Hi-PCR-DS.

Result	Reference Dengue NS1 CLIA Test			%Sensitivity (95% CI)	%Specificity (95% CI)	%Agreement (95% CI)
	Positive	Negative	Total			
TRUSTgen-DS						
Positive	40	0	40	100% (91.19%-100%)	100% (91.19%-100%)	100% (95.49%- 100%)
Negative	0	40	40			
Total	40	40	80			
RealStar-DS						
Positive	39	0	39	97.5% (86.84% - 99.94%)	100% (91.19%-100%)	98.75% (93.23% - 99.97%)
Negative	1	40	41			
Total	40	40	80			
Hi-PCR-DS						
Positive	39	0	39	97.5% (86.84% - 99.94%)	100% (91.19%-100%)	98.75% (93.23%-99.97%)
Negative	1	40	41			
Total	40	40	80			

Table 1: Performance comparison of three Dengue Serotyping kits against the Dengue NS1 CLIA test for dengue detection and serotyping.

Serotypes Detected	No of Positive Samples Tested (N=40)		
	TRUSTgen-DS, N (%)	RealStar-DS, N (%)	Hi-PCR-DS, N (%)
Mono-infection			
DENV-1	17(42.5%)	14 (35.0%)	14 (35.0%)
DENV-2	3 (7.5%)	3 (7.5%)	4 (10.0%)
DENV-3	12 (30.0%)	12 (30.0%)	12 (30.0%)
DENV-4	1 (2.5%)	1 (2.5%)	1 (2.5%)
Total	33 (82.5%)	30 (75.0%)	31 (77.5%)
Co-infection			
DENV-1&DENV-2	0 (0.0%)	1 (2.5%)	1 (2.5%)
DENV-1&DENV-3	6 (15.0%)	3 (7.5%)	3 (7.5%)
DENV-2&DENV-3	0 (0.0%)	2 (5.0%)	0 (0.0%)
DENV-1,2 & 3	1 (2.5%)	3 (7.5%)	4 (10.0%)
Total	7 (17.5%)	9 (22.5%)	8 (20.0%)
No-infection			
Negative	0 (0.0%)	1 (2.5%)	1 (2.5%)

Table 2: Distribution of Dengue Serotypes detected by the three Dengue Serotyping kits across 40-NS1 Positive samples

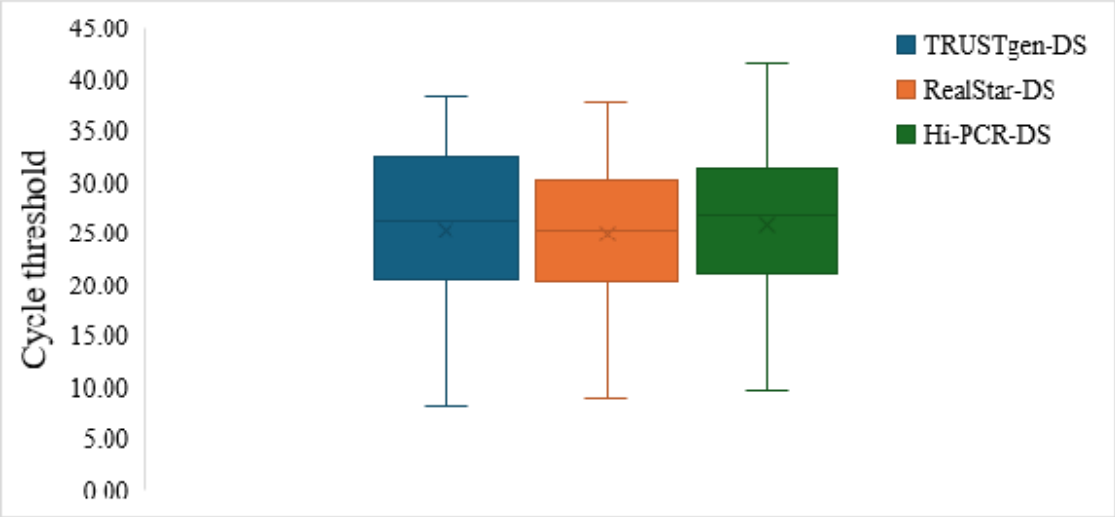

Figure 2: Comparative Ct value Distribution across kits for dengue serotype detection

Kits	Ct Values in Median (IQR)			
	DENV-1	DENV-2	DENV-3	DENV-4*
TRUSTgen-DS	23.79(18.49 - 29.21)	24.91(20.69 - 33.60)	27.14(22.13 - 32.70)	35.6
RealStar-DS	26.19(21.62 - 29.99)	25.25(21.61 - 30.53)	23.77(19.69 - 31.47)	29.73
Hi-PCR-DS	26.85(21.16 - 29.90)	25.48(21.20 - 31.18)	25.15(23.28 - 32.28)	27.53

Table 3: Median Ct Value comparison of three Dengue Serotyping kits. *: Interquartile range could not be calculated due to the availability of only one sample.

Discussion

Dengue fever has remained a persistent public health challenge in India since the first reported outbreak in Calcutta (West Bengal) in 1963 [26]. Over the decades, the country has witnessed repeated outbreaks, and all four dengue virus (DENV) serotypes have been detected, classifying India as a hyperendemic region. The co-circulation of these serotypes, along with periodic shifts in the dominant strain, has been well documented. Sirisena et al. in his review reported that 2.5–30% of all dengue infections in hyper endemic geographical settings are due to co-infections [5]. More recently, reports of co-infections involving multiple DENV serotypes have become increasingly common across various parts of the country [10,13,16,18,19]. While the exact clinical implications of DENV co-infections and their role in disease severity remain an area of ongoing investigation, identifying the infecting or co-infecting serotypes remains important, particularly for assessing the risk of severe disease in individuals. On a

broader scale, serotype surveillance is crucial for public health preparedness, aiding in the anticipation of outbreak severity and tracking viral transmission patterns.

In this context, the present study assessed the ability of three commercially available dengue serotyping kits viz., TRUSTgen-DS, RealStar-DS, and Hi-PCR-DS, to detect and differentiate among the four DENV serotypes and to identify both mono-infections and co-infections in NS1 CLIA-positive and negative samples. A high level of concordance was observed among the kits, though minor discrepancies suggest some variability in serotype detection between assays. Although all three kits demonstrated comparable diagnostic accuracy, only the TRUSTgen-DS kit successfully identified serotypes in all 40 NS1 CLIA-positive samples without any false negatives.

Most of the discrepancies observed were in the detection of DENV-1 and DENV-2 mono-infections, whereas identification of DENV-3 and DENV-4 showed consistent results across the kits, indicating better agreement for these two serotypes. In addition, multiple co-infection patterns were observed, including DENV-1 and DENV-2, DENV-1 and DENV-3, DENV-2 and DENV-3, as well as a triple co-infection involving DENV-1, DENV-2, and DENV-3. These findings are consistent with previous reports that documented similar co-infection combinations [13,17,20]. Likewise, the predominance of the DENV-1 and DENV-3 co-infection observed in the present study was also reported in Kerala [18]. Vaman et al. described that the higher frequency of certain co-infection combinations may be attributed to selection processes occurring during viral replication and transmission, wherein specific serotype pairings interact more stably or compatibly within the mosquito midgut, thereby increasing their chances of successful transmission [18].

The variation in the number and type of co-infection cases detected by each kit highlights differences in their ability to reliably detect mixed serotype infections. These discrepancies likely stem from differences in several technical parameters. One key factor is the specificity of primers and probes. Kits with highly specific oligonucleotide sequences are better equipped to avoid cross-reactivity and false-positive results, which is particularly important when detecting multiple serotypes in co-infected samples [27]. Additionally, assay optimization and the degree of analytical stringency can influence performance. Kits that are finely tuned to reduce non-specific amplification and to interpret weak or borderline signals more precisely are more likely to produce accurate results. Sensitivity differences, especially in detecting low-copy targets, may also contribute to missed minor serotypes in co-infected samples. However, directly comparing these aspects remains challenging due to the limited publicly available data on each kit's manufacturing and validation processes.

Although TRUSTgen-DS exhibited lower median Ct values for DENV-1 and DENV-2, RealStar-DS for DENV-3, and Hi-PCR-DS for the single DENV-4 positive sample, the differences in Ct value distributions across the kits were not statistically significant. This suggests that, despite subtle variations, all three kits offer comparable sensitivity for dengue serotype detection.

This study has certain limitations that should be acknowledged. Although the sample size was adequate for preliminary evaluation, the limited representation of some serotypes, particularly DENV-4, restricted the ability to generalize findings or conduct robust statistical comparisons. The reference method used was the Dengue NS1 CLIA test, which, while reliable for determining infection status, does not provide serotype-specific information. As a result, serotype confirmation relied solely on cross-comparison among the evaluated kits rather than on sequencing-based methods. Lastly, the proprietary nature of the kits limited access to information regarding their primer/probe design and assay optimization, making it difficult to dissect the technical reasons behind observed discrepancies.

In summary, the comparative evaluation of the TRUSTgen-DS, RealStar-DS, and Hi-PCR-DS dengue serotyping kits demonstrated that all three exhibited comparable sensitivity and specificity in detecting DENV serotypes. The observed variability in DENV-1 and DENV-2 detection, along with differences in reported co-infection patterns, likely reflects variations in assay design and analytical sensitivity. Kits with higher analytical sensitivity are better positioned to detect all circulating serotypes while minimizing false amplification or misinterpretation of weak signals. Given the increasing prevalence of co-infections and the limitations of serological tests, standardized molecular diagnostics are essential for accurate clinical management and effective dengue surveillance. Although these kits show promise for routine dengue surveillance and clinical use, further validation with larger datasets and comparison against sequencing-based methods is recommended.

Credit Authorship Contribution Statement

Fairoz Banu: Conceptualization, Project Administration, Formal analysis, Writing – original draft. Co-Author 1: Methodology, Data curation, Validation. Co-Author-2: Resources, Investigation. Co-Author-3: Funding acquisition, Supervision, Writing – review and editing

Ethical Considerations

This study adhered to the principles outlined in the Declaration of Helsinki. Leftover clinical samples, previously used for Dengue NS1 testing, were fully anonymized and used without subjecting them to any additional procedures. To ensure patient confidentiality and privacy, all samples were coded and handled.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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