

Research Article

# Unmasking Cross-Reactivity: A Comparative Evaluation of Serological and Molecular Diagnostics in Dengue and Chikungunya Co-infections

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## Abstract

**Background:** Dengue and Chikungunya are major arboviral diseases that frequently co-circulate in tropical regions like India, causing overlapping clinical symptoms and complicating diagnosis. This study aimed to evaluate the diagnostic performance of serological and molecular tests in differentiating these infections and to assess ELISA-based cross-reactivity, with a focus on Dengue Virus (DENV) serotype distribution.

**Methods:** A total of 566 dengue IgM-positive cases were screened, of which 132 samples collected within seven days of symptom onset were selected. These were tested for Chikungunya IgM, dengue NS1 antigen, and subjected to multiplex RT-PCR for dengue and Chikungunya viruses. Dengue-positive samples by Real-Time Reverse Transcription PCR (RT-PCR) were further serotyped. Concordance between assays was analyzed using Cohen's Kappa statistic.

**Results:** Among 566 cases, 132 were collected within seven days of illness onset and included for further analysis. Among these, 26 tested positives for Chikungunya IgM and were subsequently screened for dengue NS1 antigen using ELISA, of which 22 were found positive indicating concurrent positivity for CHIKV IgM and DENV IgM/NS1 by serological assays. Multiplex RT-PCR confirmed 12 dengue-only cases, 7 Chikungunya-only cases, and 2 co-infections. RT-PCR serotyping showed DENV-2 (41.67%) as the predominant serotype, followed by DENV-1, -3, and -4. Concordance between dengue NS1 and IgM ELISA was 84.6% ( $\kappa=0.69$ ), while RT-PCR and NS1 showed lower agreement (77.3%,  $\kappa=0.33$ ). Chikungunya IgM ELISA and RT-PCR showed poor concordance (31.8%,  $\kappa=-0.36$ ), suggesting false-positives and timing-related discrepancies.

**Conclusion:** The study highlighted the limitations of serological assays in differentiating dengue and Chikungunya infections due to cross-reactivity and timing of sample collection.

While combined IgM and NS1 testing is valuable for dengue diagnosis, reliance on Chikungunya IgM ELISA alone may be misleading and RT-PCR is essential.

**Keywords:** Dengue; Chikungunya; RT-PCR; ELISA; Cross-reactivity; Serotyping; Diagnosis

## Introduction

Dengue and Chikungunya are among the most prevalent mosquito-borne viral infections globally [1]. Both viruses are transmitted primarily by *Aedes aegypti* and *Aedes albopictus* mosquitoes and often co-circulate in the same geographic areas [2].

More than 5.6 billion people worldwide are at risk, and over 60 countries have reported the simultaneous presence of these arboviral infections [3].

In India, recurrent outbreaks of dengue and Chikungunya have led to substantial morbidity, mortality, and economic burden [4]. Dengue virus (DENV), a member of the Flaviviridae family, comprises four antigenically distinct serotypes (DENV-1 to DENV-4), all of which are known to circulate in India and have been implicated in major epidemics [5,6]. In contrast, Chikungunya Virus (CHIKV), an alphavirus, exists as a single serotype and has caused periodic outbreaks across Asia and Africa [7]. The clinical manifestations of both infections such as acute fever, arthralgia, myalgia, and rash are often indistinguishable, making clinical differentiation particularly difficult in resource-limited settings [8].

Laboratory confirmation is crucial for accurate diagnosis. ELISA is widely used for its affordability and speed [9] but is limited by cross-reactivity, especially among flaviviruses [10]. Cross-reactivity between DENV and the unrelated alphavirus CHIKV also occurs due to shared antigenic components in their envelope proteins, leading to false-positive IgM results of cases in endemic areas [11].

Although several studies in India and Southeast Asia have reported high serological coinfection rates, few have systematically examined the specificity of ELISA-based diagnoses using molecular confirmation [15,16]. This study was consequently initiated to evaluate and overcome the diagnostic challenges associated with serological testing in regions where both infections are prevalent. The objectives were to evaluate IgM-based ELISA cross-reactivity, validate ELISA findings using RT-PCR, and determine the circulating dengue virus serotypes.

## Materials and Methods

### *Study Design and Setting*

This study was conducted at the Viral Research and Diagnostic Laboratory (VRDL), a Biosafety Level II facility accredited by the Department of Health Research (DHR), located within the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi, India. The VRDL receives diagnostic referrals, including suspected dengue cases, from Sir Sunderlal Hospital, BHU. All cases referred to the VRDL between April 1, 2024, and April 5, 2025, were reviewed for inclusion in the study. Case specific data were extracted from Sample Request Forms (SRFs) completed by the referring physicians at the time of sample submission. Due to the retrospective nature of the study and the use of anonymized archival samples, individual patient consent was not required.

### *Sample Selection, Screening, and Testing*

All suspected dengue cases referred to the VRDL between 01 April 2024 and 05 April 2025 that tested positive for dengue IgM by ELISA were included in the study. From these, samples were selected for further analysis based on the following inclusion criteria:

- Collection within 7 days of symptom onset
- Adequate serum volume without visible signs of hemolysis

The screening and selection workflow is illustrated in Fig. 1.

All selected samples were first screened for Chikungunya Virus (CHIKV) IgM antibodies using a commercial ELISA kit. Samples that tested positive for CHIKV IgM were further tested for dengue NS1 antigen by ELISA. NS1-positive samples then underwent RNA extraction using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). RNA concentration and purity were evaluated using a NanoDrop spectrophotometer (Fig. 2). Real-Time Reverse Transcription PCR (RT-PCR) was performed on a Bio-Rad CFX96 system using the TRUPCR Dengue, Chikungunya and Malaria Detection Kit Version 1.0, with appropriate positive and negative controls included in each run (Fig. 3). Samples confirmed positive for Dengue Virus (DENV) by RT-PCR were subsequently serotyped for DENV-1, DENV-2, DENV-3, and DENV-4 using a multiplex RT-PCR assay with the TRUPCR DENV Serotyping Kit Version 1.0 (Fig. 3).

### *Statistical Analysis*

The statistical analysis in this study included descriptive statistics to report frequencies and percentages of RT-PCR results, dengue serotype distribution, and test concordance. Concordance rates were calculated between RT-PCR, NS1, and IgM ELISA

assays. To assess inter-assay agreement beyond chance, Cohen's Kappa statistic was applied between Diagnostic Tests for Dengue and Chikungunya.

## Results

### *Sample Selection and Testing Overview*

Out of 566 dengue IgM-positive samples, 132 were collected within 7 days of illness onset and included for further analysis. These 132 samples were screened for Chikungunya IgM antibodies by ELISA, with 26 testing positive. The 26 positive samples were then subjected to NS1 ELISA to determine the antigenic component, and 22 tested positive. These samples were subsequently analyzed by multiplex RT-PCR targeting dengue and Chikungunya genes. The results are illustrated in Table 1. Twelve samples that tested positive for dengue by RT-PCR were subjected to RT-PCR serotyping, and the results are shown in Table 2.

| RT-PCR Result                       | Number of Samples | Percentage (%) |
|-------------------------------------|-------------------|----------------|
| Dengue Positive Only                | 12                | 54.5%          |
| Chikungunya Positive Only           | 7                 | 31.8%          |
| Co-infection (Dengue + Chikungunya) | 2                 | 9.1%           |
| RT-PCR Negative for Both            | 5                 | 22.7%          |
| Total                               | 22                | 100%           |

Note: co-positive cases (2) are included in the individual counts

**Table 1:** Multiplex RT-PCR Results for Detection of Dengue and Chikungunya Viral Genes.

| Dengue Serotype | Number of Samples (out of 12) | Percentage (%) |
|-----------------|-------------------------------|----------------|
| Serotype 1      | 4                             | 33.33%         |
| Serotype 2      | 5                             | 41.67%         |
| Serotype 3      | 2                             | 16.67%         |
| Serotype 4      | 1                             | 8.33%          |

**Table 2:** Dengue Virus Serotyping Results by RT-PCR.

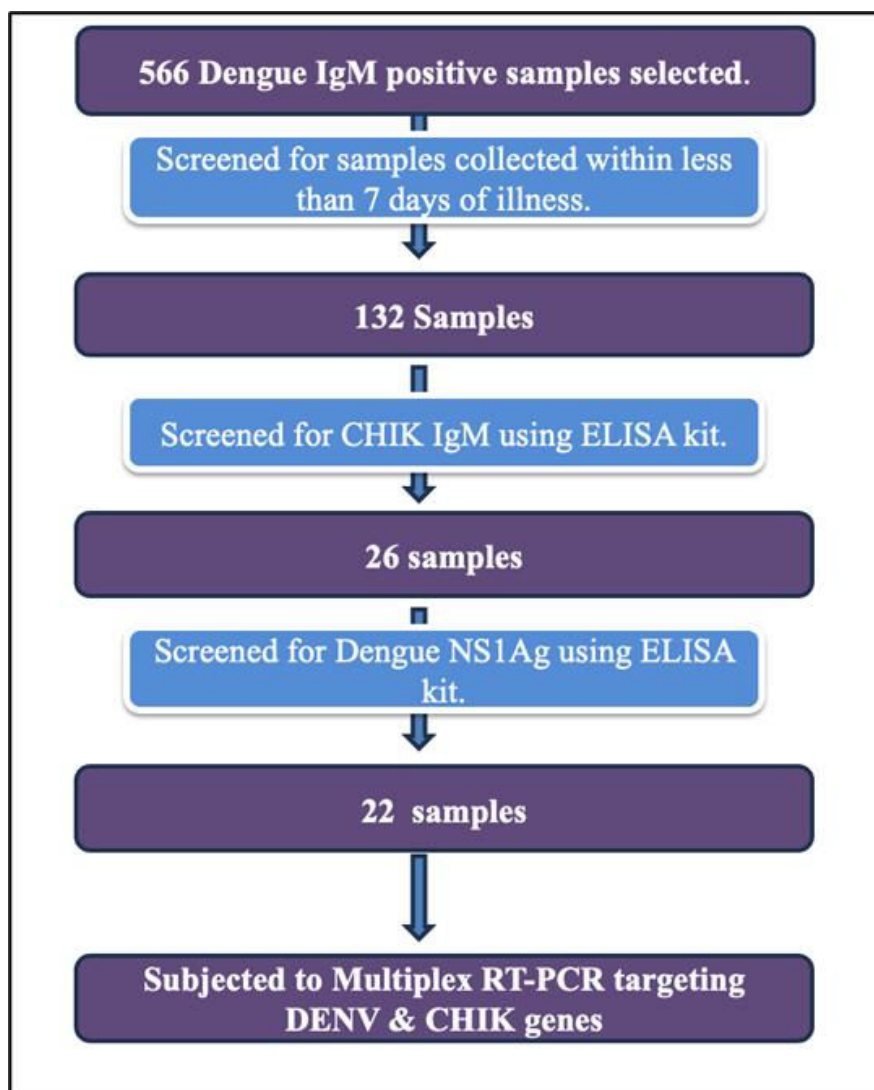
| Assays                                                   | Value                  |
|----------------------------------------------------------|------------------------|
| NS1 Dengue Concordance with IgM Dengue ELISA             | 22 / 26 = <b>84.6%</b> |
| Dengue RTPCR Concordance with Dengue NS1 ELISA           | 12/22 = <b>77.3%</b>   |
| Chikungunya RTPCR Concordance with Chikungunya IgM ELISA | 7/22 = <b>31.81%</b>   |

**Table 3:** Concordance rates among RT-PCR, IgM ELISA, and NS1 ELISA test results.

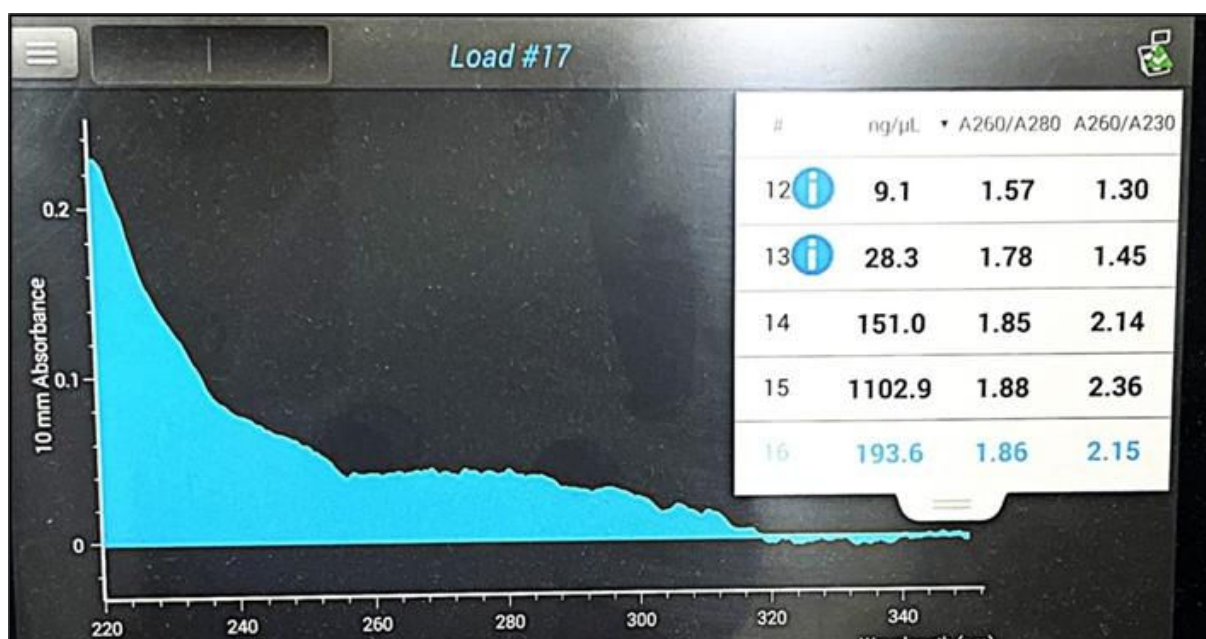
| Comparison                        | Cohen's Kappa ( $\kappa$ ) | Level of Agreement            |
|-----------------------------------|----------------------------|-------------------------------|
| NS1 ELISA vs IgM ELISA (Dengue)   | 0.69                       | Substantial agreement         |
| RT-PCR vs NS1 ELISA (Dengue)      | 0.33                       | Fair agreement                |
| RT-PCR vs IgM ELISA (Chikungunya) | -0.36                      | Poor agreement (below chance) |

NOTE: Kappa values range from -1 to 1, where values  $\geq 0.6$  indicate substantial agreement, 0.2-0.4 indicate fair agreement, and values  $< 0$  suggest agreement worse than chance

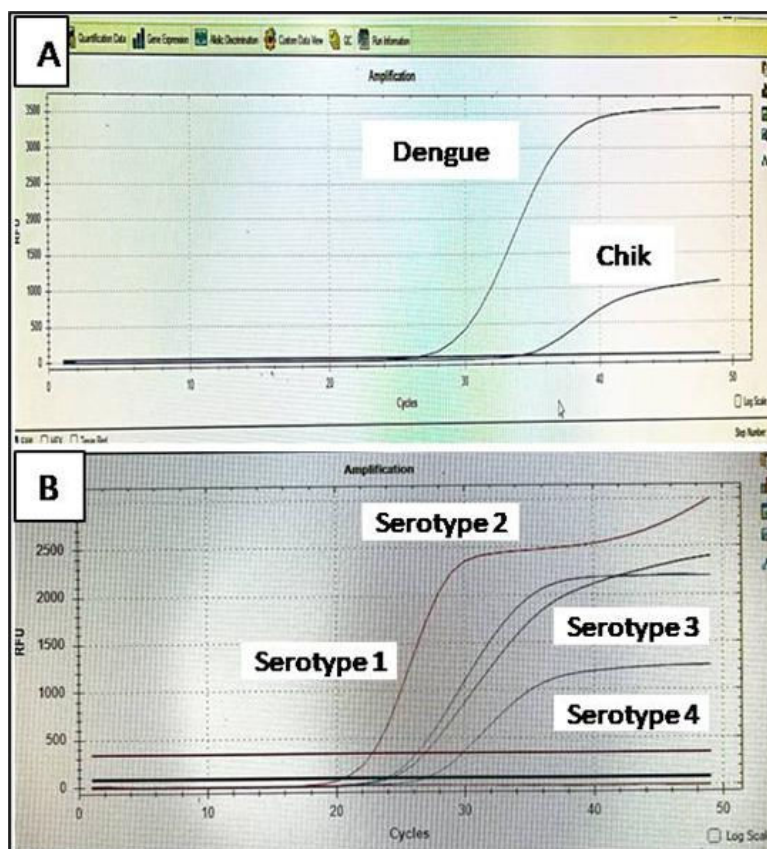
**Table 4:** Cohen's Kappa Analysis of Concordance Between Diagnostic Tests for Dengue and Chikungunya.



**Figure 1:** The screening and selection workflow for serum samples analyzed in this study.



**Figure 2:** Representative nanodrop spectrum illustrating RNA concentration and purity.



**Figure 3:** Representative RT-PCR amplification graphs for each gene (A) Multiplexing: Dengue and Chikungunya gene(B) Dengue serotype genes.

## Discussion

This study evaluated the diagnostic performance and inter-assay agreement of multiplex RT-PCR and ELISA-based assays (NS1 antigen and IgM antibody) for the detection of dengue and Chikungunya viruses, with additional analysis of dengue virus serotype distribution.

Multiplex RT-PCR results (Table 1) revealed that 54.5% of the samples were positive for dengue alone, 31.8% for Chikungunya alone, and 9.1% exhibited co-infection. A subset of 22.7% tested negative for both viruses. Interestingly, only 2 (9.1%) of the 22 samples tested positive for both Chikungunya and Dengue viruses by RT-PCR, despite all 22 yielding dual positivity for Chikungunya and Dengue IgM antibodies via ELISA. This discrepancy highlights the potential serological cross-reactivity of IgM responses, likely due to antigenic similarities between the two arboviruses, which may compromise the specificity of IgM-based sero-diagnostic assays. This leads to significant overestimation of coinfection rates, which has critical clinical and public health implications, including inappropriate treatment decisions, inaccurate surveillance data, and misallocation of healthcare resources [12,13]. While molecular methods such as Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) offer higher specificity through direct detection of viral RNA, their widespread use remains limited in many endemic areas due to cost and infrastructure constraints [14]. These findings underscore the diagnostic value of multiplex RT-PCR in distinguishing between clinically similar arboviral infections and identifying co-infections, which may have implications for patient management and public health response strategies [17].

Dengue virus serotyping (Table 2) indicated that serotype 2 (41.67%) was the most prevalent, consistent with prior studies [18,19,20], followed by serotypes 1 (33.33%), 3 (16.67%), and 4 (8.33%). The predominance of DENV-2 is particularly relevant, as this serotype has been associated with more severe clinical outcomes, including increased risk of dengue hemorrhagic fever and dengue shock syndrome [18]. These findings emphasize the importance of ongoing molecular surveillance to monitor serotype shifts, which may influence epidemic dynamics and vaccine effectiveness.



Concordance analysis between diagnostic modalities (Table 3) revealed the highest agreement between NS1 and IgM ELISA for dengue (84.6%), with a corresponding Cohen's Kappa value of 0.69, indicating substantial agreement. This suggests a strong correlation between viral antigen and the host immune response when tested within the appropriate diagnostic window period. By contrast, concordance between RT-PCR and NS1 ELISA was lower at 77.3%, with a  $\kappa$  value of 0.33, reflecting only fair agreement. Notably, 22.7% of NS1-positive samples were negative by RT-PCR, suggesting the possibility of false positives or cross-reactivity in the NS1 ELISA, a recognized limitation in serological testing, particularly in regions with co-circulating flaviviruses and alphaviruses [21].

In addition, five samples were negative by RT-PCR but positive for both NS1 and IgM ELISA, raising concerns about false-positive results potentially arising from prior flaviviral exposure or concurrent infections, all of which can contribute to non-specific serological reactivity [22,23]. These findings highlight the necessity of cautious interpretation of serological results, particularly in endemic regions.

The poor concordance between Chikungunya RT-PCR and IgM ELISA (31.8%), along with a negative Cohen's Kappa value ( $\kappa = -0.36$ ), indicates agreement worse than expected by chance (Table 4). This discrepancy likely reflects a temporal mismatch between the detection windows of the two assays, possibly due to differences in test timing or sensitivity. RT-PCR is optimized for detecting viral RNA during the early acute phase (typically within the first five days of symptom onset), whereas IgM antibodies begin to appear around day 4-5 and peak during the second week of illness [24]. Consequently, samples collected outside the narrow overlapping window may yield discordant results. Additionally, cross-reactivity in IgM ELISA assays, especially in regions with multiple circulating arboviruses, may further compromise specificity. Variability in host immune responses, particularly in individuals with prior arboviral exposure, may also delay or reduce IgM production, contributing to the observed diagnostic inconsistency [25,26].

## Conclusion

This study reinforces the diagnostic utility of combining NS1 antigen and IgM antibody testing for dengue, particularly in settings where molecular assays are inaccessible. However, the limited agreement and risk of false positives associated with serological testing-especially for Chikungunya highlights the need for confirmatory RT-PCR testing where feasible. These findings underscore the importance of enhancing assay specificity and adopting integrated diagnostic strategies in regions with co-circulating arboviruses to improve diagnostic accuracy and inform effective clinical and public health decision-making.

## Conflict of Interest

The authors have declared no conflict of interest.

## Funding

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