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Temporal stability of human serum for the diagnosis of dengue virus by ELISA

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Abstract

Every year, millions of people are affected by the mosquito-borne dengue virus. Early and accurate diagnosis of the disease plays a crucial role in proper disease management, and prevention. The detection of immunoglobulin M (IgM) by enzyme-linked immunosorbent assay (ELISA) is a widely used method for the diagnosis of dengue fever. Analytical variables like the post-collection procedure of the blood sample can influence the performance of serological results. This study addresses the temporal effect on the detection of dengue-specific IgM through ELISA. Blood samples were collected from patients with fever cases suspected of dengue infection. Serum was separated by centrifugation. The present study was carried out on samples of active dengue cases. Thereafter, serum was stored and analysed every hour for a maximum of 4 hours. The IgM levels against dengue were measured using a commercial ELISA kit, and the optical density (OD) values were recorded at 450nm. The OD for the IgM values of dengue positive cases were steadily elevated and significantly different from the initial readings compared to each of the time points, with the highest peak at the 3rd hour. All the negative samples demonstrated a lower baseline IgM level of less than 30 units during the 0,1, 2 and 4 hours of the study. There was an increased activity recorded at 3 hours in negative samples. These results underscore the necessity to standardise analytical handling protocols during serological diagnostics to reduce variability in these tests. By understanding these temporal dynamics, we may improve the quality of IgM testing for dengue.

Keywords- Dengue, ELISA, IgM, cross-reactivity

1.Introduction

Dengue fever is transmitted by the Aedes mosquito and causes a major public health issues in the tropical countries [1,2]. Dengue virus (DENV) belonging to the genus *Flavivirus* within the family *Flaviviridae*. It comprises four genetically and antigenically distinct serotypes (DENV-1 to DENV-4). Transmission occurs primarily via the bites of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes. Both the vector serve as competent vectors due to their anthropophilic feeding behavior and adaptation to urban environments in India [3,4].

The estimated annual incidence of dengue worldwide is 390 million infections, of which approximately 96 million result in clinically apparent disease [1]. In India, the dengue disease burden is significant, with an annual incidence of over 100,000 cases reported; however, the actual burden is much higher [5]. One of the most affected states, Rajasthan, shows seasonal outbreaks, more so during the monsoon, and major cities like Jaipur and Jodhpur report large numbers of cases [6]. [i]

Accurate and early diagnosis is crucial for effective disease management and epidemiological surveillance. The dynamics between the host immune system and antigenic property of virus directly affect the diagnostic window. Reverse transcription polymerase chain reaction (RT-PCR) and nonstructural (NS1) antigen assays demonstrates high sensitivity during the febrile phase (0–5 days after onset of fever) [7,8]. RT-PCR remains the gold standard for detecting viral RNA because of its high sensitivity and specificity to detect and differentiate between DENV serotypes [9]. However, molecular methods' technical complexity, cost, and infrastructure requirements limit their applicability in many endemic regions [10].

Enzyme-linked immunosorbent assays (ELISA) is more accessible and widely used for diagnosing dengue. Detection of antiviral immunoglobulin M (IgM) antibodies is a key component of serological diagnostics. IgM antibodies usually appear at around day 4–5 post-infection and rise to a peak over 7–10 days [11].

Pre-analytical variables, especially the delay between blood draw, serum separation, and analysis of the sample, play an important role in diagnostic performance [12]. However, in most resource-constrained settings where there are often delays in processing samples, these aspects may introduce variability in the performance of the assay and thereby affect the reliability of a diagnosis [13].

2. Materials and Methods

2.1 Study design and site

This prospective sero-surveillance study was conducted in Jodhpur, Rajasthan, from September to November 2024. The study was carried out for three months in the virology laboratory. Blood samples were collected from patients presenting with febrile illnesses at District Hospital, Government Satellite Swami Prabhutanand Hospital, and Mahila Bagh Hospital, Jodhpur, Rajasthan, India.

2.2 Study Population

Patients with clinically suspected dengue fever (DF) attending outpatient (OPD) and inpatient (IPD) departments were included.

2.3 Sample Collection and Testing

Blood samples were collected aseptically and transported under controlled conditions maintaining 4°C to the ICMR- National Institute of Health Research, Jodhpur (Formerly ICMR- National Institute for Implementation Research on Non-Communicable Diseases). Blood samples were drawn into sterile serum separation tubes (SST) of Becton Dickinson (BD) make. The sample was immediately stored at 4°C and transported to the ICMR- National Institute of Health Research, Jodhpur (Formerly ICMR-National Institute for Implementation Research on Non-Communicable Diseases) in a temperature-controlled box to ensure optimal preservation for further analysis.

2.4 Sample Processing and Experimental Conditions

The serum was separated f by centrifugation at 3,000 rpm for 5 minutes. The serum was then evenly aliquoted into five cryovials, each designated for analysis at a specific time. To evaluate temporal variations in IgM reactivity, the aliquoted serum samples were maintained at 37°C until tested. ELISA was performed at 0, 1-, 2-, 3-, and 4 hours post-incubation at 37C. Each aliquot was used only once for its respective time point to minimize variability.

2.5 Detection of Dengue-Specific IgM by MAC-ELISA

Serum was separated by centrifugation at 3,000 rpm for 5 minutes. Dengue-specific IgM antibodies were detected using the J. Mitra Dengue IgM ELISA kit (J. Mitra & Co. Pvt. Ltd., India), following the manufacturer's protocol. The ELISA microplates were pre-coated with dengue antigens.100 µL diluted serum and controls were added to the microplate wells. Microplates added with serum were incubated for 60 minutes at 37C. Thereafter, unbound ºmaterials were removed through five washes using an automated Wellwash™ Microplate Washer (Thermo Scientific™). A horseradish peroxidase-conjugated anti-human IgM antibody was added and incubated for another 60 mins at 37C, followed by another five washes for removing the ºunbound antibodies. The reaction was initiated with tetramethylbenzidine (TMB) substrate and terminated with a stop solution containing sulfuric acid, changing color from blue to yellow. The optical density was measured at 450 nm on an ELISA Microplate Reader. Results were interpreted based on the cut-off values provided by the kit. This modified sample handling method allowed for evaluating IgM reactivity under non-standard conditions.

2.6 RNA Extraction

Dengue Viral RNA extraction was carried out using the QIAamp Viral RNA Mini Kit (Qiagen, Germany). Protocol of the manufacturer was followed for the extraction. A 140 µL serum sample was mixed with buffer AVL containing carrier RNA to lyse viral particles, followed by ethanol addition to enable RNA binding to the silica column. The column was washed with buffers AW1 and AW2 to eliminate impurities, and the RNA was eluted in 60 µL of buffer AVE. The eluted RNA was stored at -80°C for stability and further analysis.

2.7 qRT-PCR

The qRT-PCR for determining Dengue serotypes was carried out using the RealStar dengue PCR kit 1.0 (Altona Diagnostics, Hamburg, Germany). Each reaction mixture contained 5 µL of master mix A,15 µL of master mix B 10 uL RNA template were added to every reaction mixture. RT-PCRs were carried out again using the CFX96 Real-Time thermal cycler, Biorad, with cycling conditions as follows: 55 °C for 20 min (RT step), 95 °C for 2 min, 45 cycles at 15 s at 95 °C, 45 s at 55 °C and 15 s at 72 °C. The threshold was set automatically by the software.

3. Results

The IgM ELISA assay was carried out on ten dengue-positive and seven negative serum samples to evaluate temporal variations in IgM levels at 37°C over a period of 4-hours. Positive samples (P1– P9) exhibited a gradual increase in IgM units from 0 to 3 hours. An elevated level of IgM was observed at the 3rd hour of incubation (Figure 1). The IgM units (±SD) at 0,1,2, 3 and 4 hours after incubation at 37°Cwas 61.87 (±49.86), 55.6 (±37.46), 90.72 (±66.77), 126.85 (±93.06) and 74.99 (±69.82). The ODs at all 4 points of time were found to be varied. ODs of the negative samples were also found to be increased with the incubation duration. After 1 hour of incubation, 16% sample turned positive, and the same happened after 2 hours of incubation. 83% of the total sample turned positive after 3 hours of incubation. After 4 hours, 50% samples again turned negative and 33% samples turned positive in comparison to the results of the 3-hour (Figure-2). Negative samples (P15–P21) demonstrated a lower baseline IgM level of less than 16 units during the 0,1, 2 and 4 hours of the study. There was an increase in the activity recorded at 3 hours (Figure-2).

The specificity of the assay was calculated to be 50%, indicating notable cross-reactivity and potential false-positive results. The sensitivity of the test was 89%, confirming its reliability in detecting dengue-specific IgM. Overall, these findings highlight the critical importance of processing serum samples

within 1 hour of collection to ensure assay accuracy. Storage of samples at 37°C increases the risk of protein degradation, non-specific binding, and altered IgM dynamics, which may compromise diagnostic outcomes. Of the samples with identified serotypes, DENV 2 was the most commonly found (47.17%), followed by DENV 3 (15.09%) and DENV 1 (6.6%). DENV 4 was the least frequently detected (0.94%), while 6.6% of the cases showed mixed serotype infections.

4. Discussion

Rapid and precise diagnostic tools are required in the clinical management of dengue-infected patients. ELISA is a widely utilised serological tool for detecting IgM specific to dengue virus. However, the reliability of its findings is dependent upon the adherence to strict protocols for both sample handling and processing [14,15]. Changes in optical density (OD) occur during the incubation of serum at 37°C. Significant changes were recorded after the third hour of incubation of the sample in the present study. This suggests that dynamic quantitative modifications in serum proteins occurred. This may be potentially mediated by proteolysis, aggregation, or altered antibody interactions, contribute to assay variability. The increase in OD values with time highlights the multifaceted nature of serum as a biological matrix and its sensitivity to intrinsic and extrinsic elements, such as enzyme activity, temperature extremes, and adsorbent of proteins to the surfaces of storage vessels. These changes can amplify non-specific interactions and, consequently, background noise and false positives. The observed variability in OD values across true positive and true negative samples emphasizes the necessity for stringent sample handling to mitigate non-specific interactions.

C-reactive protein (CRP), lipoproteins, and immunoglobulins undergoing structural modifications that may enhance cross-reactivity within immunoassays [16]. Even at low levels, proteolytic activity can generate peptide fragments with unexpected antigenic properties, leading to spurious assay signals [17]. Environmental factors, such as sample evaporation and protein re-solubilization from storage tube surfaces, may further exacerbate these inconsistencies. These findings are consistent with prior studies demonstrating that prolonged storage and suboptimal handling conditions can compromise serological assay specificity and sensitivity. Incomplete blocking of non-specific binding sites, cross-reactivity of assay components, and variable enzymatic activity during signal detection may collectively amplify non-specific interactions. This is particularly problematic in samples from healthy individuals, wherein the absence of dengue-specific IgM should yield minimal OD values, yet time-dependent alterations in serum composition may erroneously suggest seropositivity. Such confounding factors can significantly impact clinical decision-making, leading to unnecessary interventions and misclassifying dengue cases.

This study highlights the necessity of rapid sample processing to mitigate these effects. ELISA should ideally be performed within one hour of serum separation to minimize the risk of protein degradation and non-specific interactions. In cases where immediate analysis is not feasible, serum should be aliquoted and stored under controlled conditions—4°C for short-term preservation or - 20°C to -80°C for long-term storage to maintain sample integrity [18].

5. Conclusion

In conclusion, while ELISA remains an essential tool for dengue diagnostics, its reliability is contingent upon meticulous sample handling and adherence to strict pre-analytical protocols. Understanding the dynamic nature of serum and its biochemical alterations over time is imperative for ensuring assay fidelity. By implementing rigorous sample processing guidelines, the risk of false-positive results can

be minimized, ultimately enhancing diagnostic accuracy and supporting more effective clinical and epidemiological decision-making in dengue-endemic settings.

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Authorship contribution: SSM and RJ conceived and designed the study. SSM, JG, and DC wrote the protocol. RJ and DC generated the data. SSM, RK and DC analyzed the data. SSM, RJ, and JG wrote the report, and all authors contributed to the drafts before submission.

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Conflict of Interest: The authors declare no conflict of interest

Approval of IEC: The study protocol was approved by the Institutional Ethics Committee of the National Institute for Implementation Research on Non-Communicable Diseases (Formerly known as Desert Medicine Research Centre, Jodhpur). (Approval number-IEC-NIIRNCD/2023/ER/004, dated 16-10-2023)

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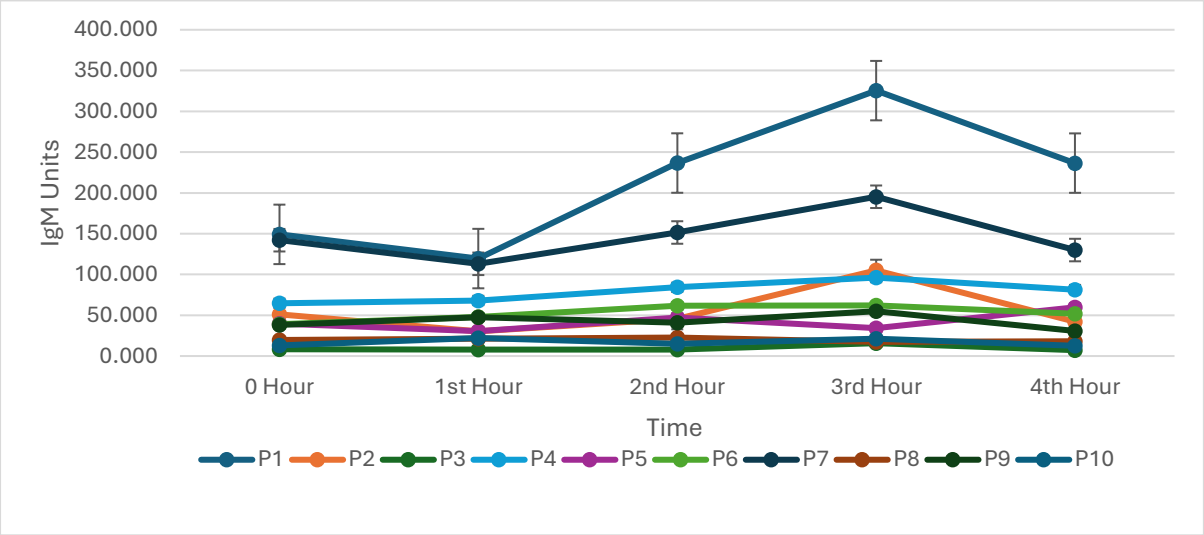


Fig.1 Temporal Variation in IgM Units of True Positive Dengue Samples at 37°C

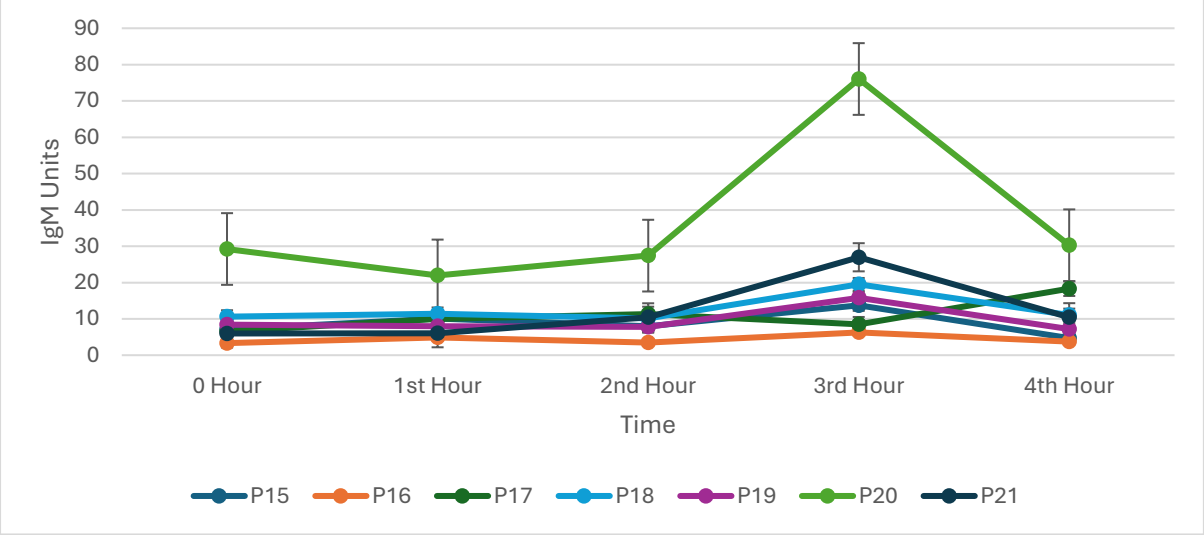


Fig.2 Temporal Variation in IgM Units of True Negative Dengue Samples at 37°C