PUBLICATIONS AND PERFORMANCE EVALUATIONS OF Dengue NS1 Ag Microlisa

Reputed hospitals and recognised institutions in India and other countries have evaluated Dengue NS1 Ag Microlisa. Independent third-party studies have assessed the product's performance and suitability, with findings reported in established scientific and technical journals. A consolidated compilation of the published articles and independent evaluation studies is provided below for reference.

The below information is provided for the Scientific Community; it is not for commercial or promotional purpose.

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Dengue NS1 antigen kit shows high sensitivity for detection of recombinant dengue virus-2 NS1 antigen spiked with *Aedes aegypti* mosquitoes

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Dengue, caused by the dengue virus (DENV) is a significant vector-borne disease. In absence of a specific treatment and vaccine, dengue is becoming a rising threat to public health. Currently, control of dengue mainly focuses on the surveillance of the mosquito vectors. Improved surveillance methods for DENV in mosquito populations would be highly beneficial to the public health. However, current methods of DENV detection in mosquitoes requires specialized equipment and expensive reagents and highly trained personnel. As an alternative, commercially available dengue NS1 antigen ELISA kits could be used for detection of DENV infection in *Aedes aegypti* mosquitoes. In this study, we explored the utility of commercially available Dengue NS1 antigen kit (J. Mitra & Co. Pvt. Ltd) for the detection of recombinant dengue virus-2 (rDENV-2) NS1 protein and serum of dengue infected patient spiked with *Ae. aegypti* mosquito pools. The kit was found to be highly sensitive and specific towards detection of all serotypes of DENV. Further, it could detect as low as 750 femto gram rDENV-2 NS1 protein. It was also observed that rDENV-2 NS1 antigen spiked with blood-fed and unfed mosquito pools could be detected. In addition, the kit also detected dengue infected patient serum spiked with *Ae. aegypti* mosquito pools. Overall, the Dengue NS1 antigen kit displayed high sensitivity towards detection of recombinant as well as serum NS1 protein spiked with *Ae. aegypti* mosquito pools and could be considered for the dengue virus surveillance after a field evaluation in *Ae. aegypti* mosquitoes.

Dengue, a mosquito-borne viral disease is one of the serious public health problems globally. It has been estimated that dengue infection ranges from 100 to 400 million worldwide annually and 70% of the disease burden occurs mainly in Asia[1,2]. Dengue virus (DENV) is a member of the Flaviviridae family belonging to the genus Flavivirus. It is transmitted to humans through the common mosquito vectors, *Aedes aegypti* and *Aedes albopictus*[3]. Four serologically and genetically distinct serotypes namely, DENV-1, DENV-2, DENV-3 and DENV-4 can cause dengue[4]. DENV is an enveloped virus with a single positive-strand RNA genome that encodes for three structural proteins (capsid [C] protein -; pre-membrane [prM] protein—and envelope [E] protein) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5)[4]. Among the non-structural (NS) proteins, NS1 is of great scientific interest as it plays pathogenic roles in haemorrhage and vascular leakage in dengue patients[5]. The protein is found at different cellular locations as cell surface-bound, membrane-associated, or secreted form. The secreted form of NS1 is present in the patients' sera from the onset of illness at a concentration of 50 µg/ml sera[6,7]. These properties of NS1 lead to the development of an array of commercial rapid dengue diagnostic tests.

In absence of specific therapy and vaccines, prevention of dengue virus is mainly focused on vector management strategies. Improvements in vector surveillance methods for DENV in wild mosquito populations would be highly beneficial to public health. Nevertheless, prevention of epidemic dengue in endemic countries or prevention of its introduction into uninfested regions is very difficult with the current vector surveillance systems and

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control strategies[8]. To guide vector control measures, there was a call from the World Health Organization for development of integrated national vector surveillance and health information systems[9]. A positive association between dengue virus infection in human subjects and mosquitoes has been observed. Dengue was reported in human cases about a week after the manifestation of DENV-positive *Ae. aegypti* mosquitoes[10,11]. Thus, monitoring the DENV infection in *Ae. aegypti* mosquito vectors will help in risk analysis and initiating vector prevention methods using rapid DENV diagnostic tools. Indeed, surveillance of DENV prevalence in wild mosquito populations is imperative for detection of changes in vector abundance, obtaining spatial and temporal information of vector populations, evaluation of vector control programmes, as well as facilitation of appropriate evidence-based interventions[12]. In addition, surveillance of DENV prevalence in natural mosquito populations can help in preventing the onset of dengue outbreaks and implementation of effective vector control measures for instance habitat destruction, source reduction, adulticiding, and also community participation to limit possible outbreak especially in dengue endemic areas[13–15].

Current methods of DENV detection in the field-caught mosquitoes follows tedious procedures such as isolation and propagation of virus in cell culture system and finally detection by ELISA or viral RNA extraction from the mosquitoes and confirmation using RT-PCR. These methods suffer from foremost limitations such as highly trained personnel and the need of specialized equipments. In addition, these methods are time consuming, expensive and are not feasible in developing regions of the world. In most of the countries such facilities are located in centralized facilities that require coordinated collection of mosquitoes and transport for further processing[16]. Alternatively, commercial NS1 antigen-based ELISA kits can be explored for detection of DENV-NS1 antigen in the filed-caught mosquitoes. Previously, dengue NS1 ELISA kits such as dengue early ELISA kit (Panbio), Platelia Dengue NS1 Ag (BioRad Labs) and SD Bioline NS1 Ag kit (Standards Diagnostics, Korea) have been tested for detection of DENV infection in *Ae. aegypti*[14,17,18]. In the current study, we have investigated Dengue NS1 Ag Microlisa kit (J. Mitra & Co. Pvt. Ltd, India) for detection of dengue NS1 antigen in *Ae. aegypti* mosquito population.

Materials and methods

All methods were performed in accordance with the relevant guidelines and regulations. After receiving the Dengue NS1 Ag Microlisa kit, the purity and integrity of control proteins were checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%) following standard protocol[19]. In brief, protein samples (20 µl) were mixed with separating gel buffer and dissolved properly. After adding sample loading buffer (0.1% bromophenol blue containing SDS, glycerol and β-mercaptoethanol), the samples were incubated at 90 °C for 10 min. These sample preparations were loaded in 12% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie Brilliant blue R-250 (0.15%) for 2 h and de-stained overnight. Similarly, the NS1 antigens of other flaviviruses were also assessed on 12% SDS-PAGE.

Determination of minimum detection limit of Dengue NS1 Ag Microlisa kit for detection of recombinant NS1 antigen. The detection limit of the Dengue NS1 Ag Microlisa kit was determined using recombinant dengue virus-2 (rDENV-2) NS1 antigen (Recombinant Viral Dengue Virus 2 NS1 Protein, CF Catalog No. 9439-DG R&D Systems, Inc. Canada). The protein concentration of rDENV-2 NS1 antigen was estimated using Bicinchoninic acid kit (Micro BCA protein assay kit from Thermo Scientific, Rockford, IL, USA). The recombinant protein was reconstituted in phosphate-buffered saline (PBS) as per the manufacturers' instructions. Two stocks (750 picogram and 250 picogram) of rDENV-2 were prepared in 0.5 M PBS. From these stocks, subsequent dilutions were prepared (750 pg to 75 pg, 7.5 pg and 750 fg and from 250 to 25 pg, 2.5 pg and 250 fg respectively). The preparations were tested using the Dengue NS1 Ag Microlisa kit as per the manufacturer's instructions. In brief, 50 µl of diluent was added to each well followed by 50 µl of kit controls (negative control, positive control, and calibrator control) and recombinant dengue virus-2 NS1 antigen. Later, 100 µl of working conjugate was added and the Microlisa plate was incubated for 90 min at 37 °C. After washing the wells, working substrate (150 µl) was added to each well and incubated further. After adding stop solution, the absorbance was read at 450 nm in the ELISA Reader (Thermo scientific). The assay was considered valid only if the optical density (OD) of negative control was < 0.3, positive control > 1.0 and mean calibrator OD ≥ 0.35. From the sample OD, the dengue NS1 antigen units were calculated and result was interpreted as negative when dengue NS1 antigen units are < 9, equivocal if the dengue NS1 antigen units are between 9–11 and positive when the antigen units are > 11.

Detection of sensitivity and specificity of the Dengue NS1 Ag Microlisa kit. To determine the sensitivity of the Dengue NS1 Ag Microlisa kit, recombinant NS1 antigen of all four DENV serotypes (DENV-1 to 4) were tested (The Native antigen company, Oxfordshire, UK. SKU: REC31778 to 1781). In this experiment, three concentrations (75 pg, 7.5 pg and 750 fg) of recombinant NS1 antigen were tested as per the standardized protocol of the kit. To determine the specificity, recombinant NS1 of flaviviruses such as, Japanese encephalitis virus, Tick-borne encephalitis virus, West Nile virus and Yellow fever virus (The Native antigen company, Oxfordshire, UK. SKU: FLAVX4-NS1-100) were tested at 750 pg, 250 pg and 75 pg. The antigen unit for each concentration is calculated, and the result was interpreted in terms of dengue NS1 antigen units.

Detection of rDENV-2 NS1 antigen in *Ae. aegypti* mosquito pools. The spiking experiment was carried out to check whether the kit can detect the rDENV-2 NS1 antigen in the mosquito pools. For this, *Ae. aegypti* mosquitoes were collected from the Rearing and Colonization laboratory of ICMR-VCRC, Puducherry. In this experiment, 25 mosquitoes per pool were homogenized following two procedures (Fig. 1). In the 1st procedure (A), the mosquitoes were homogenized in a Tissue Lyser II (Qiagen, Germany) at 24 frequencies for

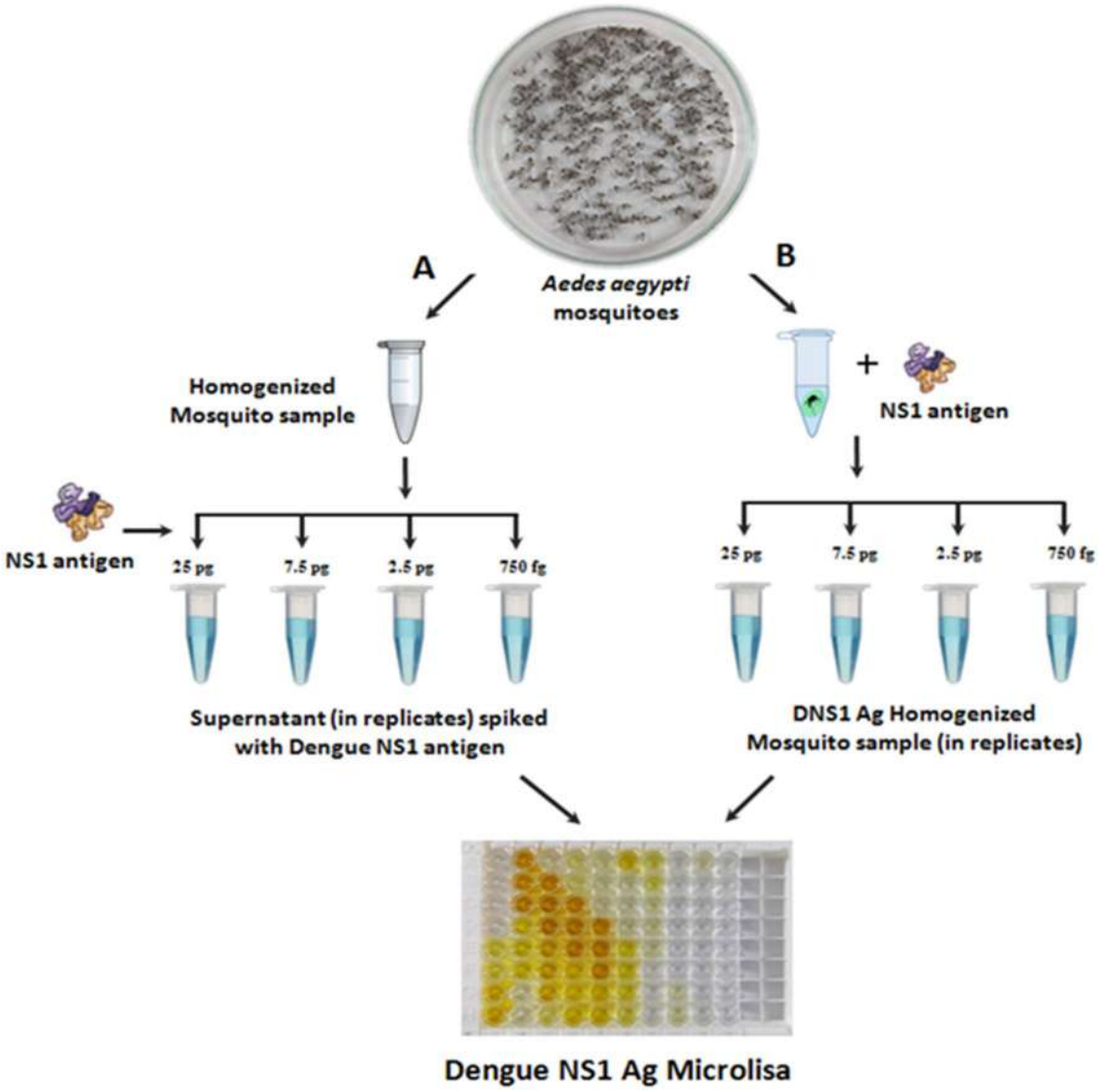


Figure 1. Design of spiking experiment. (**A**) The rDENV-2 NS1 antigen was added to the mosquito homogenate or (**B**) the mosquitoes were homogenized with rDENV-2 antigen and ELISA was performed.

5 min and centrifuged at 5000 rpm (5 min.). Recombinant dengue NS1 antigen (25 pg to 750 fg) was spiked in the mosquito homogenate and ELISA was carried out. In the 2nd procedure (B), the mosquitoes were spiked with the recombinant NS1 antigen at different concentrations (25 pg to 750 fg), homogenized and afterwards ELISA was performed following manufacturer's protocol. Both the procedures were carried out in replicates.

Detection of rDENV-NS1 antigen in blood-fed *Ae. aegypti* mosquitoes. This experiment was carried out to check whether the kit can detect the recombinant NS1 antigen in the blood-fed *Ae. aegypti* mosquito pools. Based on the results of the previous experiment, two concentrations of recombinant NS1 antigen (7.5 pg and 25 pg) were spiked with pools of 25 blood-fed and unfed homogenized mosquitoes. The homogenate was tested using the Dengue NS1 Ag Microlisa kit.

Detection of NS1 antigen of dengue infected human serum in *Ae. aegypti* mosquito pools. For collection of serum sample from the DENV infected patient, approval from the Institutional Human Ethics Committee (ECR 681/Inst/Py/2014/RR-17) of ICMR-Vector Control Research Centre, Puducherry was obtained. The patients enrolled for the study has signed an informed written consent. To simulate the condition of detection of secreted NS1 protein in the mosquitoes, serum of the DENV infected patient was spiked with *Ae. aegypti* mosquito pools. Initially, the dengue positive patient's sera samples (50 µl) tested by the kit to confirm the presence of NS1 antigen displayed OD > 6. Therefore, the sera samples were further diluted (1:50 and 1:100) in PBS and re-tested. Both the dilutions showed OD > 6 again. Based on this, homogenized *Ae. aegypti* mosquitoes (10 and 25 mosquitoes per pool) were spiked with both dilutions of serum (1:50 and 1:100 in PBS) and tested using the kit.

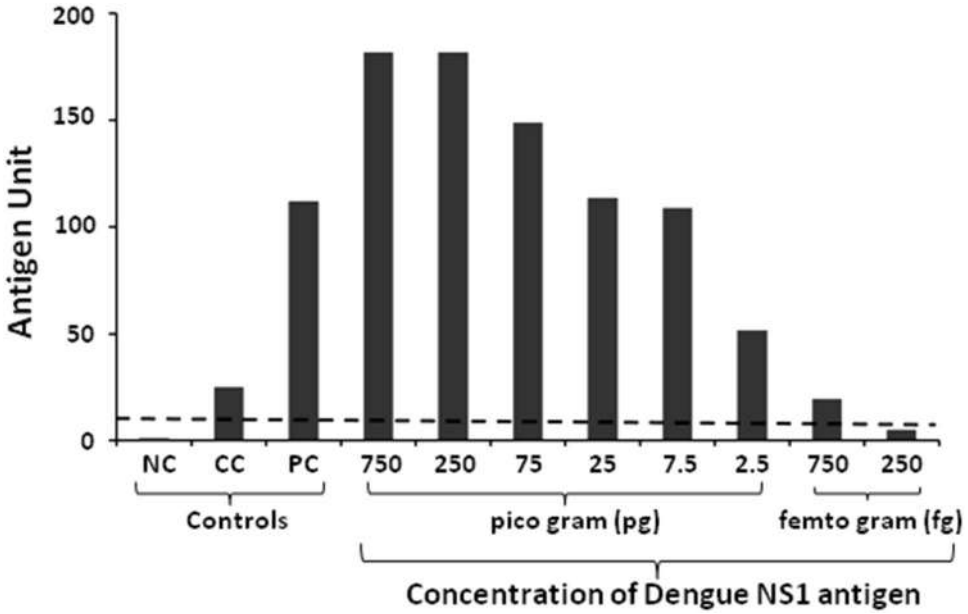


Figure 2. Determination of rDENV-2 NS1 detection limit of dengue NS1 Ag Microlisa kit. Recombinant Dengue NS1 antigen at different concentration was added to the wells of Microlisa plate and the assay was performed as per the instructions of the manufacturer. Absorbance was read at 450 nm in ELISA reader. *NC* Negative control, *CC* Calibrator control, *PC* Positive control. Dotted horizontal line indicates the cut-off value.

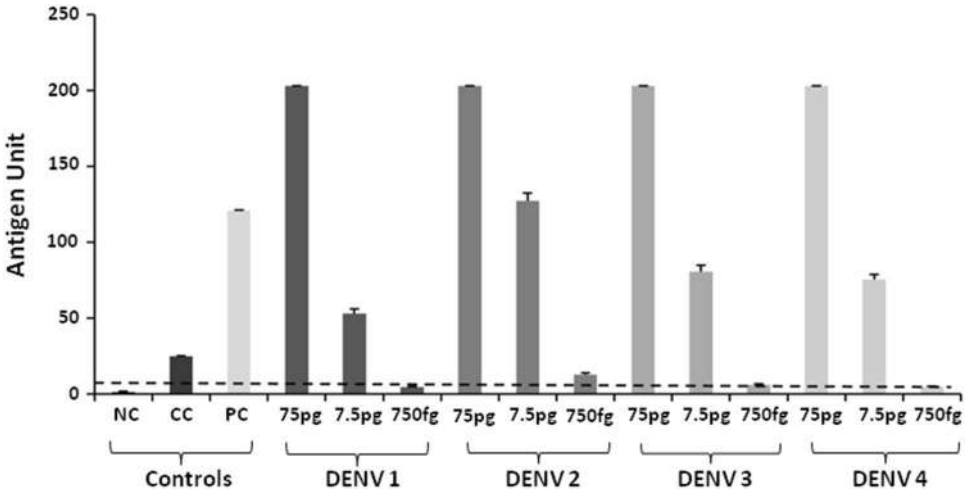


Figure 3. Detection of NS1 antigen of dengue serotypes. Recombinant Dengue NS1 antigens from all serotypes of dengue (DENV1 to DENV4) were tested at different concentration following manufacturers' instructions. Cut-off value is indicated by a horizontal line. *NC* Negative control, *CC* Calibrator control, *PC* Positive control, *DENV* Dengue virus.

Results

Dengue NS1 Ag Microlisa kit detects rDENV-2 NS1 antigen at low concentration. The purity and integrity of Dengue NS1 Ag Microlisa kit checked on 12% SDS-PAGE indicated that the positive control, calibrator control and enzyme conjugate were pure and intact (Fig S1). The kit displayed higher OD values (> 6) for rDENV-2 NS1 tested at 750 pg and 250 pg which was beyond the detection limit of microplate ELISA reader. After converting the OD values into antigen units, it was observed that the kit is highly sensitive to detect rDENV-2 NS1 antigen as it could detect as low as 750 fg of rDENV-2 NS1 antigen (Fig. 2).

Dengue NS1 Ag Microlisa kit is sensitive to detect all serotypes of dengue. The NS1 antigens of flaviviruses checked on SDS-PAGE indicated that the proteins were pure and intact (Fig S2). The kit detected all the DENV serotypes suggesting that it is highly sensitive for detecting any serotype of DENV (Fig. 3). However,

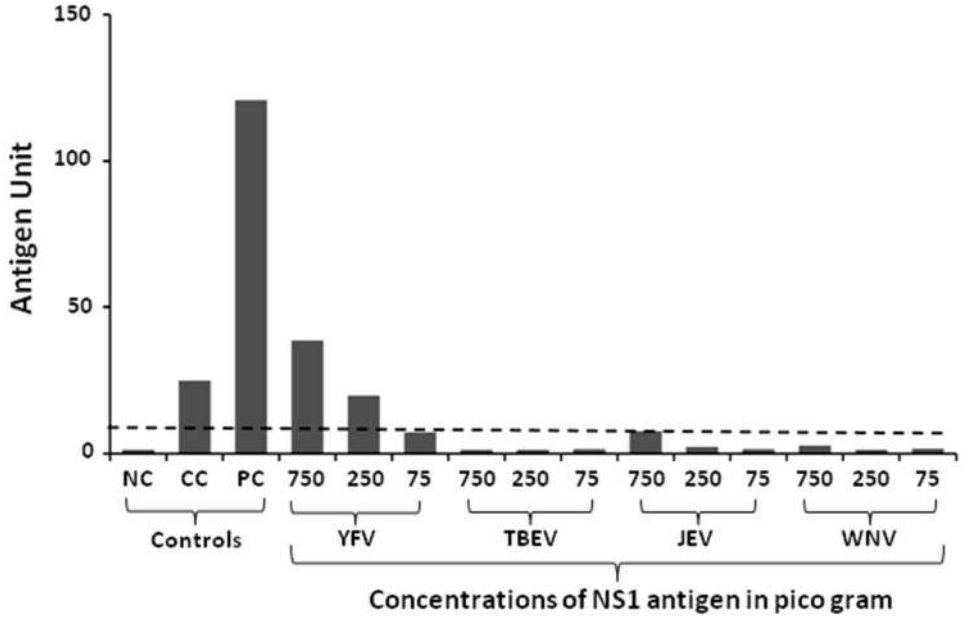


Figure 4. Detection of NS1 antigen of flaviviruses other than dengue serotypes. Recombinant Dengue NS1 antigen from the flaviviruses other than dengue was tested at different concentrations. *YFV* Yellow fever virus, *TBEV* Tick-borne encephalitis virus, *JEV* Japanese encephalitis virus, *WNV* West Nile virus, *NC* Negative control, *CC* Calibrator control, *PC* Positive control. Dotted horizontal line indicates the cut-off value.

it was observed that the antigen units of the DENV2 were comparatively higher than the other serotypes (at 7.5 pg and 750 fg respectively). It was also observed that the detection of rDENV-2 NS1 increased with increased antigen concentration.

Dengue NS1 Microlisa kit is highly specific for detection of only dengue NS1 antigen. Based on our previous experiment that the kit is highly sensitive to detect all the serotypes of dengue, we further determined the specificity of the kit using the recombinant NS1 antigen of flaviviruses such as yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), Japanese encephalitis (JE) and West Nile virus (WNV) (Fig. 4). Only YFV showed positive results (antigen units > 11) for 750 pg and 250 pg antigen concentrations but not for 75 pg (antigen unit is 38.9, 19.9 and 7.3 respectively). In contrary, NS1 antigens of TBEV, JEV, and WNV could not show antigen units even at 750 pg antigen concentrations (antigen units < 9). This highlights that the kit is highly specific for detection of dengue NS1 only.

Dengue NS1 Microlisa kit is sensitive to detect rDENV-2 NS1 antigen in *Ae. aegypti* mosquito pools. This study was carried out to check whether rDENV-2 NS1 antigen spiked with *Ae. aegypti* mosquitoes can be detected using the Microlisa kit. It was observed that the kit is sensitive to detect rDENV-2 NS1 antigen in the mosquito pools up to 2.5 pg by both the procedures (Fig. 5). However, it was noteworthy that the antigen units of rDENV-2 NS1 in the mosquito pools decreased with decreased antigen concentration and none of the procedure could detect rDENV-2 NS1 antigen at 750 fg concentration.

Dengue NS1 Microlisa kit is sensitive to detect rDENV-2 NS1 antigen in blood-fed *Ae. aegypti* mosquito pools. Based on our previous experiment in unfed *Ae. aegypti* mosquitoes, we were further interested in checking whether blood constituents may influence the sensitivity of the kit for detection of rDENV-2 NS1 spiked with blood-fed *Ae. aegypti* mosquito pools. It was observed that blood-feeding does not necessarily interfere with the assay sensitivity as the kit displayed similar absorbance values in unfed and blood-fed mosquito pools. This evinced that the kit is equally sensitive for detection of rDENV-2 NS1 antigen in the blood-fed mosquito (Fig. 6).

Detection of NS1 antigen of DENV infected human serum in *Ae. aegypti* mosquito pools. The Microlisa kit in DENV infection confirmed patient was tested before spiking the serum with the mosquito pools. The serum sample tested at both the dilutions (1:50 and 1:100) displayed OD > 6, which is beyond the limit of detection of the ELISA reader (Fig S3). The same serum sample was used to spike in the *Aedes aegypti* mosquito pools to simulate NS1 protein secretion in the mosquitoes. It was observed that the kit is highly sensitive to detect the NS1 antigen of the serum sample spiked in the *Ae. aegypti* mosquito pools (Fig. 7).

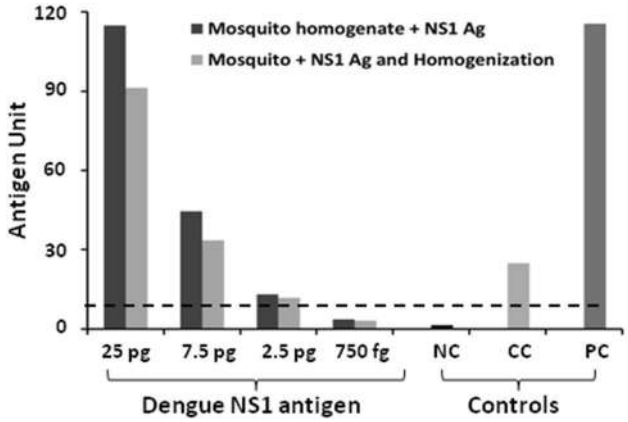


Figure 5. Detection of rDENV-2 NS1 antigen in *Aedes aegypti* mosquito pools. The mosquitoes were either homogenized with different concentrations of rDENV-2 NS1 or the antigen was added to homogenized mosquito samples and ELISA was performed. Antigen units were calculated as described earlier. *NC* Negative control, *CC* Calibrator control, *PC* Positive control.

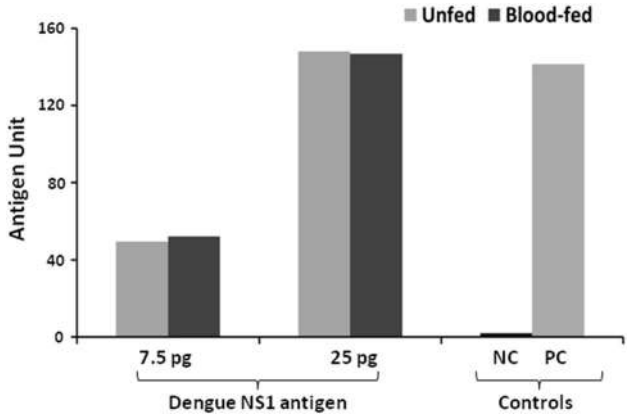


Figure 6. Determination of sensitivity of Microlisa kit for detection of rDENV-2 in blood fed *Ae. aegypti* mosquito pools. The mosquito homogenates were spiked with recombinant dengue NS1 antigen and tested. The absorbance was read in the ELISA reader at 450 nm. *NC* Negative control, *PC* Positive control.

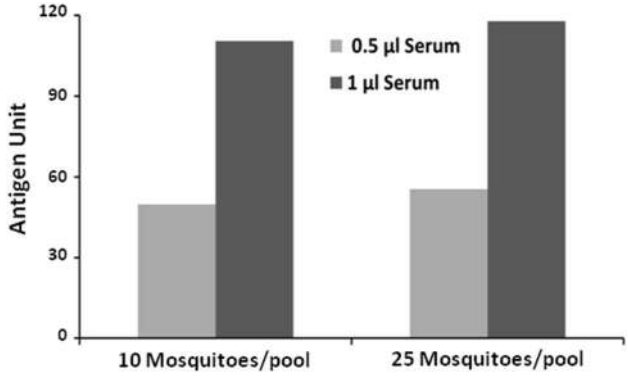


Figure 7. Detection of NS1 antigen in *Aedes aegypti* mosquito homogenate spiked with dengue patient serum. The dengue confirmed patient serum was spiked with mosquito homogenate and ELISA was performed. OD measured at 450 nm was interpreted in terms of antigen unit as per manufacturer's instructions.

Discussion

Current methods of DENV detection in the field-caught mosquitoes suffer from one or the other limitations. To overcome such limitations, commercially available NS1 antigen-based ELISA and Rapid diagnostic tests have been tested. The possible use of Dengue NS1 Ag Strip (BioRad Laboratories) for field surveillance of infected dengue vectors was attempted in Singapore[15]. PanBio dengue early ELISA kit was tested for surveillance of DENV infected mosquitoes in Australia and India[14,20]. In another study, a commercially available Platelia Dengue NS1 Ag kit was evaluated to detect DENV in infected *Ae. aegypti* and found to be more convenient to test mosquitoes with DENV infection[21]. SD Bioline NS1 Antigen kit was used to test dengue antigen in adult Aedes mosquitoes in Selangor, Malaysia[18]. Our group has recently evaluated the DENV Detect™ NS1 ELISA kit (InBios International, Inc.) to detect rDENV-2 NS1 protein in *Ae. aegypti* mosquitoes (manuscript communicated to J Vector Borne Dis). As an extension to this investigation, we tested the utility of Dengue NS1 Ag Microlisa (J. Mitra & Co. Pvt. Ltd) to detect rDENV-2 NS1 spiked with *Ae. aegypti*. The Microlisa kit is highly sensitive and specific (99.5% and 100% respectively as mentioned in the kit insert) for diagnosis of dengue. Therefore, the kit was explored for detection of rDENV-2 NS1 antigen as well as dengue NS1 antigen positive sera in pools of *Ae. aegypti* mosquito samples.

Since dengue is caused by four serotypes of dengue virus (DENV1 to DENV4), it is necessary that the kit should be sensitive for detection of all forms of dengue NS1 antigen. Therefore, we tested NS1 antigen of all serotypes of DENV. The kit detected all the DENV serotypes indicating that it is highly sensitive for detecting any serotype of DENV. As NS1 antigen is common to all flaviviruses, the kit may show the cross-reactivity of DENV NS1 antigen with other flaviviruses, leading to false-positive results. Therefore, the Microlisa kit was tested with recombinant NS1 antigens of four flaviviruses such as JEV, TBEV, WNV, and YFV. The major purpose of testing NS1 antigens of flaviviruses other than dengue virus was to check the specificity of the test (100% as mentioned by the manufacturer). We observed that the kit could not detect NS1 antigen of JEV, TBEV and WNV suggesting that the kit is highly specific for detection of dengue. Although, NS1 antigen of YFV cross-reacted at 750 pg and 250 pg antigen concentrations, it is noteworthy that India is free from yellow fever virus[22].

Based on these observations, we tested rDENV-2 NS1 antigen detection limit of dengue NS1 Ag Microlisa Kit and found that the kit is highly sensitive as it can detect the NS1 antigen in femto gram level. A spiking experiment was designed to check the sensitivity of the kit to detect rDENV-NS1 in *Ae. aegypti* mosquito pools (25/pool) employing two procedures. It was observed that the kit was sensitive to detect the rDENV in these mosquito pools having minimum 2.5 pg rDENV-2 NS1 antigen. This suggests that the kit could be used for the detection of NS1 antigen even up to pico gram levels. Though the antigen unit at 25 pg and 7.5 pg was higher for the 1st procedure, the antigen units remained almost the same at low antigen concentration (2.5 pg). This indicated that NS1 antigen could be detected in field-caught mosquitoes. It was also observed that the kit was equally sensitive to detect rDENV-2 NS1 antigen in both the mosquito pools.

Further, to simulate the condition of detection of NS1 antigen in the dengue infected human blood-fed mosquitoes, the serum sample of a dengue infected patient was spiked with the *Ae. aegypti* mosquito pools (10 and 25/pool). It was observed that the kit was highly sensitive to detect the NS1 antigen in both the pool sizes. This observation indicates that the kit can be used for the detection of DENV infection in the field-caught *Ae. aegypti* mosquitoes also.

A recent study on the comparative assessment of commercial ELISA tests used for DENV diagnosis in India showed that J. Mitra Dengue NS1 Ag Microlisa was more efficient than WHO-approved Panbio Dengue Early ELISA for the detection of all the four DENV serotypes in India[23]. Although like other commercial dengue NS1 antigen diagnostic kits, the Microlisa kit is also intended for dengue diagnosis in humans, this kit has not been explored for detection of NS1 protein antigen in DENV infected *Ae. aegypti* mosquitoes. In the current study, we attempted for the first time to test this kit for detection of rDENV-2 NS1 as well as NS1 antigen from the serum of DENV infected patients in pools of *Ae. aegypti* mosquitoes. In both cases, the kit was found to be highly sensitive to detect dengue virus NS1 antigen. This investigation warrants further studies on DENV infected *Ae. aegypti* mosquitoes and field-collected mosquitoes towards exploring the utility of this kit as a tool for the dengue vector surveillance.

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Author contributions

P.R.A., N.P.K. designed the study. B.R., performed the experiments. P.R.A., B.R. analyzed the data. P.R.A., B.R., N.P.K. wrote the paper and N.P.K., A.K. revised the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Article

Emergence of a Novel Dengue Virus Serotype-2 Genotype IV Lineage III Strain and Displacement of Dengue Virus Serotype-1 in Central India (2019–2023)

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Abstract: Dengue fever remains a significant public health concern in tropical regions, including Central India, where outbreaks are frequent and associated with high morbidity and mortality. This study investigated the dynamics of dengue virus transmission and evolution in Central India from 2019 to 2023, focusing on the emergence of new strains and their impact on outbreak patterns. For this, 40 mosquito pools and 300 patient samples were recruited for the study. Phylogenetic and Bayesian evolutionary analyses performed on CPrM region and whole genome sequences generated by Sanger and Illumina sequencing, respectively, revealed the emergence and predominance of a novel DENV-2 genotype IV lineage III strain in the 2019 and 2023 outbreaks, which displaced the previously circulating DENV-1 genotype responsible for the 2016–2017 outbreak. Despite pre-existing DENV-1 neutralizing antibodies in the community (67 healthy volunteers), the novel DENV-2 strain exhibited higher viral loads and a greater reproduction number (R0), contributing to rapid disease spread. Molecular clock and Shannon entropy analyses suggest that DENV evolution occurred within the mosquito vector, driven by natural selection. Our findings highlight the importance of continuous DENV surveillance, including genetic characterization in both vectors and hosts, to understand viral evolution and predict future outbreaks. Rapid urbanization and inadequate sanitation in densely populated regions like India create ideal breeding grounds for mosquitoes, facilitating the introduction and establishment of novel DENV strains. Interrupting the vector–DENV–host cycle through targeted interventions is crucial for effective dengue control.

Keywords: dengue; DENV; mosquitoes; Bayesian analysis; outbreak prediction

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1. Introduction

Dengue is the most prevalent acute febrile illness transmitted by arthropods, caused by the dengue virus, with approximately 390 million new cases reported annually [1]. Female *Aedes* mosquitoes carrying the dengue virus (DENV) are the main vector of transmission [2]. There are four different serotypes of DENV: DENV-1, DENV-2, DENV-3, and

DENV-4. Based on genetic and antigenic differences, these serotypes are further divided into different genotypes [3,4] with a ~30% inter-serotype variability [5,6]. Clinical signs and symptoms of DENV infections range from low-grade fever to severe hemorrhagic fever and even death [7]. The symptoms usually appear 5–8 days after an incubation period of 3–15 days. Clinical manifestations of dengue can range from an infection with no symptoms to a serious, potentially fatal illness if left untreated [8]. Expanded dengue syndrome, dengue fever, dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS), undifferentiated febrile disease, and atypical dengue are the classifications used to describe cases [9]. Children, expectant mothers, and the elderly, particularly those with concomitant conditions like diabetes or hypertension, are among the groups most at risk of contracting dengue.

Diverse climatic conditions make the Indian subcontinent an epicenter for dengue disease, with 33 million infections [1], while in Central India, dengue epidemics have been occurring since 2009 [10–12]. Although previous studies have highlighted the importance of distinct serotypes and genotypes in determining the likelihood of an epidemic, little is known about the underlying genotype and serotype of the dengue outbreak [13].

Antibody-dependent enhancement (ADE) remains underexplored in this endemic region. Nonetheless, studies have demonstrated that subsequent infections with distinct DENV serotypes raise the chance of developing severe disease by three to four times, and various DENV serotypes co-circulate in endemic locations [14]. Higher plasma viremia has been linked to severe dengue sickness [15]. However, some studies showed that viremia is not the main driver of inflammation after a dengue infection, as it was higher in individuals with a primary infection [16].

There is still some uncertainty surrounding how new dengue virus strains emerge and how viremia levels in both humans and mosquito vectors influence disease severity. To better understand the frequency and impact of emerging viral shifts, thorough research is essential. Until an effective vaccine becomes widely available, controlling mosquito populations remains the primary method for reducing dengue transmission. Variations in the dengue virus's genetic makeup contribute to differences in virulence and transmission between serotypes, leading to diverse patterns of disease severity. Consequently, monitoring the molecular characteristics of circulating DENV genotypes and serotypes is vital for predicting and managing outbreaks. Additionally, studying the molecular epidemiology of DENV in both human and mosquito populations is critical for improving epidemic forecasting and guiding public health interventions.

From September 2019 to 2023, this study examined the seroprevalence and genetic epidemiology of DENV in both hosts and vectors in Bhopal, a dengue-endemic region in Central India. This research aimed to bridge the knowledge gap regarding how high viremia, driven by a high reproductive number (R0) in hosts/vectors, and neutralizing antibody responses may predict future dengue outbreaks in endemic areas. Molecular characterization revealed the emergence of new genotypes and serotypes in vectors, influencing strain competition and transmission dynamics. This contributes to the cyclical outbreaks observed every two to three years, enhancing our understanding of improved outbreak management.

2. Materials and Methods

2.1. Study Area, Study Period, and Sample Sources

This study complied with the Declaration of Helsinki and was approved by the Institutional Human Ethics Committee (IHEC Ref No-2019/PhD/Jan/19/04) at AIIMS, Bhopal, India. After obtaining written informed consent, 3 mL of blood from 300 patients showing suspected dengue symptoms was collected in a serum separator tube (SST/yellow

vial) and in an EDTA vial from the OPD (Out Patient Department). The samples were transported to the laboratory for centrifugation to separate the serum and stored at −80 °C for further analysis. Of the 300 samples, serum was successfully isolated from 280 samples, and 92 of them tested positive for the DENV NS1 antigen. Two samples were disqualified because they were co-infected with both DENV and Chikungunya virus. The city of Bhopal in Madhya Pradesh, Central India, with a population of 2,553,000, was chosen for the current study. Gulmohar Colony, Bagsewaniya, Arera Colony, Baghmugaliy, Barkheda, Saket Nagar/Shakti Nagar, Kasturba Nagar, Bharat Heavy Electrical Limited (BHEL), and Maharana Pratap Nagar represents ward number 52 to 61 (Figure 1) and were identified as hot-spots for this study due to the high dengue case density in these areas. A total of 15% (0.37 million) of the city's population lives in these locations, which were chosen as catchment areas to gather samples from dengue patients, vector samples, and samples for neutralizing antibodies testing.

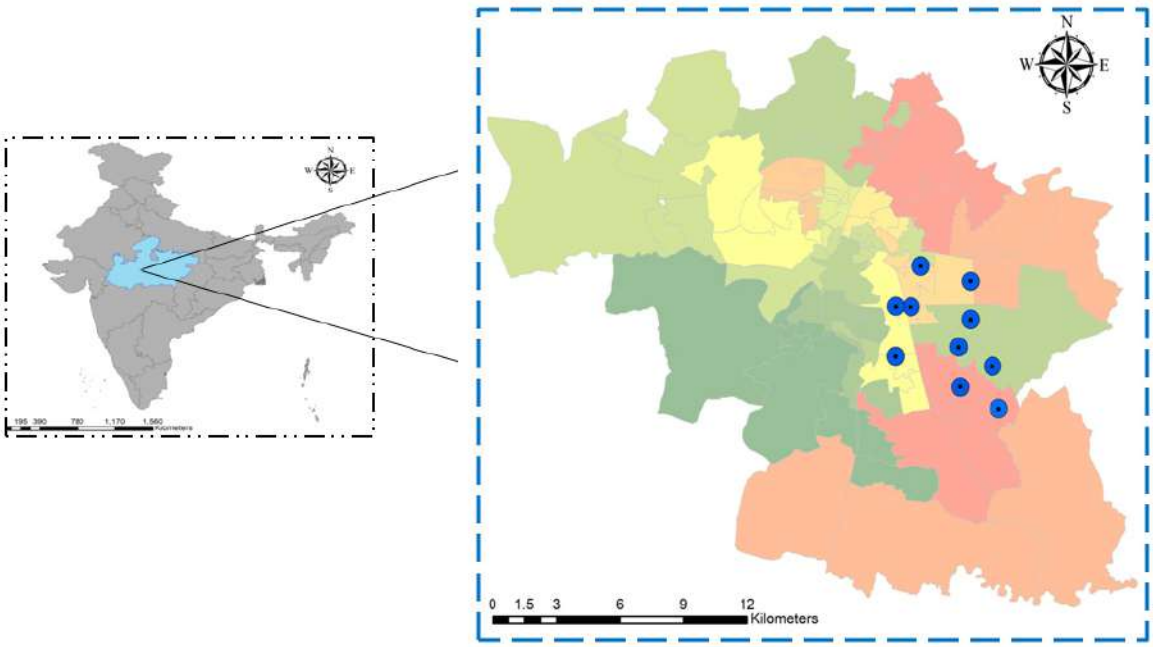


Figure 1. Map of Bhopal City showing hot-spot wards (52–61) for dengue cases and for mosquito sample collection (Blue dot represents the sample collection wards).

2.2. Patients Clinical Details

After considering the inclusion and exclusion criteria, 300 patients with warning signs of a headache, myalgia, fever, arthralgia, rash, vomiting/nausea, retro-orbital pain, hematocrit (%), and thrombocytopenia were recruited in the study. A Complete Blood Count (CBC) and a biochemical parameter (LFT) were also performed for the patients (Supplementary Table S1).

2.3. Serological Diagnosis of Dengue Virus

An ELISA was performed to detect the presence of dengue NS1 protein in 280 serum samples by using a commercially available kit, Dengue NS1 Ag Microlisa ELISA kit (J. Mitra & Co. Pvt. Ltd., New Delhi, India; Ref. No. IR031096), following the manufacturer's protocol (Figure 2).

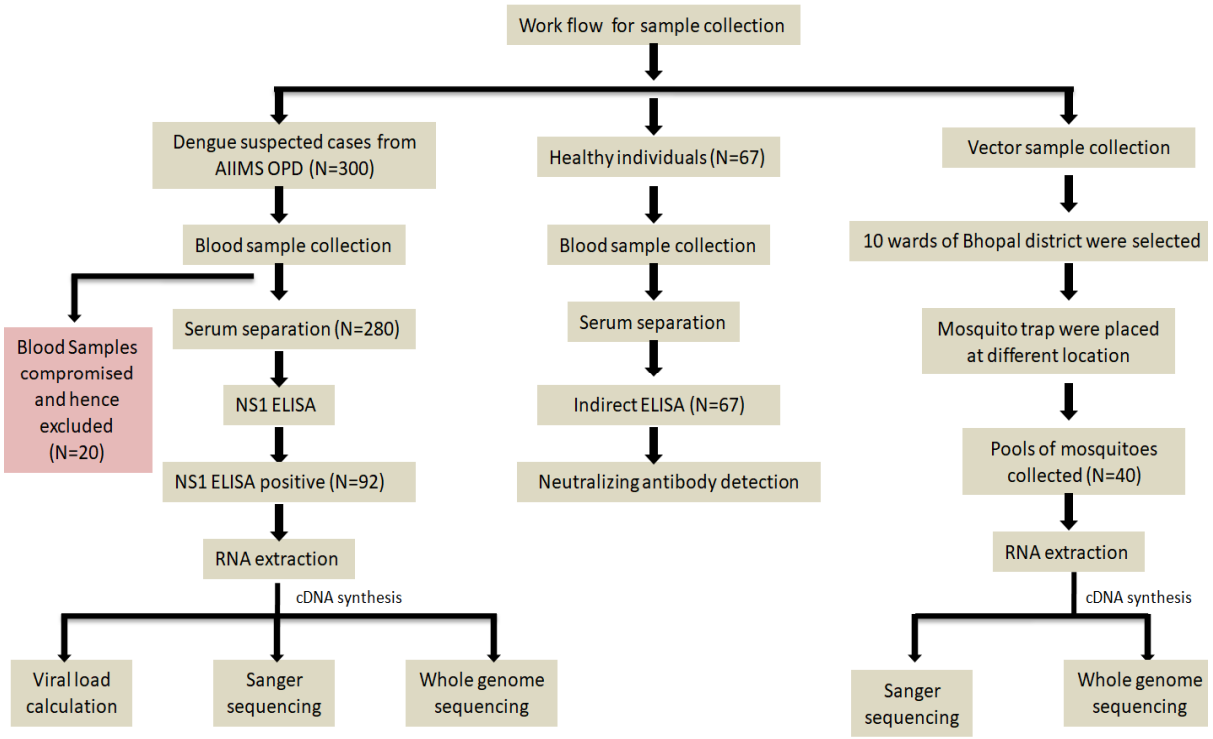


Figure 2. Flow chart of the sample recruitment.

2.4. Mosquito Sample Collection and Processing

Mechanical aspirators and the Biogents BG-Sentinel-2-Mosquito-Trap (Biogents, Regensburg, Germany) were used to collect mosquito pools. After being captured and brought to the lab, mosquitoes were identified at the species level using common taxonomy characteristics [17]. From August 2019 to September 2021, adult female *Aedes* mosquitoes were gathered from ten distinct places in Bhopal's wards no. 52–61. All of the female mosquitoes were separated into 40 pools (15–18 mosquitoes per pool) and stored at −80 °C until further use. Each mosquito pool received 1.5 mL of 4% bovine serum albumin in phosphate buffer saline (PBS, pH 7.8) at the time of processing, using a pestle and mortar (Figure 2) [18].

2.5. In-Vitro Culture of Dengue Virus in Vero E6 Cell Line

Vero-E6 cells were maintained in MEM media containing 10% FBS, and viral infection in Vero cells was performed using a method mentioned in a previous study [19]. Briefly, 100 µL of liquid from mosquito processing was added to 70% confluent cultured Vero cells. After CPE was observed, the virus-containing cell culture supernatant was kept at −80 °C until the RNA extraction, cDNA conversion, and qRT PCR.

2.6. Establishing the Positive Control and qRT-PCR for Detection of Dengue Virus

PCR-positive clone generation was performed according to the method published previously [20]. Briefly, the cloning of the 112 base pair region of DENV-1 and 79 base pair region of DENV-2 was performed using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA) and T4 DNA ligase. The positive clones were then confirmed by colony PCR (Supplementary Figure S1). After confirmation, the cloned plasmids having the desired segment of dengue virus were isolated using a plasmid isolation kit (MACHEREY-NAGEL GmbH & Co. KG. NucleoSpin Plasmid Kit, REF 740588.50; Düren, Germany). Successive dilutions of the plasmid from 10^9 to 10^2 copies/mL were made after isolation.

Serotyping and quantification were performed using a commercial kit: Takyon Master Mix by Eurogentec (Eurogentec, Liège, Belgium; Cat.#UF-LPMT-B0701). Multiplexing qRT-PCR was performed to identify all four dengue virus serotypes (Figure 2) [21].

2.7. CPrM Region Sanger Sequencing and Whole Genome Sequencing of DENV Virus

From the serum samples that tested positive for NS1, total RNA was extracted using TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA; Cat. # T3934) according to the manufacturer's protocol. Then, using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA; Cat. #1708897), 10 µL of the extracted RNA was converted into cDNA. DENV-specific RT-PCR primers were used to amplify the 511 base pair region of the CPrM gene [22]. This 511 bp of CPrM region was sequenced using the Sanger sequencing method (Supplementary Figure S2). The whole genome of the dengue virus was sequenced using Illumina sequencing by the method described previously (Figure 2) [23].

2.8. Phylogenetic Analysis of the CPrM Regions and the Whole Genome Sequences

The sequence alignment was performed using the MUSCLE program incorporated into the MEGA 10 software [24]. The maximum-likelihood approach was used to build the phylogenetic tree for the CPrM regions as well as for the whole genome of all host and mosquito pool samples. A total of 1000 replicates were used to calculate the bootstrap values (Figure 2).

2.9. Shannon Entropy Analysis for the WGS of DENV Virus

HIV sequences database software (https://www.hiv.lanl.gov/cgi-bin/ENTROPY/entropy.cgi, accessed on 20 May 2024) was used to examine the Shannon entropy of DENV-1 and DENV-2. The probability of disorder or variation at a certain amino acid position in protein sequences is increased by the amino acid's entropy [25]. The entropy graph was then created by manually editing values from an online software database into Microsoft Excel sheets.

2.10. Molecular Clock Analysis of the WGS Using Bayesian MCMC

Molecular clock analysis was performed using a dataset that included eight whole genome sequences of DENV-2 (a total of sixty-two sequences) and two whole genome sequences of DENV-1 (a total of fifty-two sequences) from this investigation. The Bayesian MCMC approach, provided in BEAST v1.8.4 [26], was used to calculate the nucleotide substitution rates and time to their most recent common ancestor (TMRCA) of the DENV-1 and DENV-2 strains. The Bayesian information criterion (BIC) was used to determine the best-suited nucleotide substitution model using jModelTest 2 [27]. Based on the lowest BIC (Bayesian Information Criterion) scores, the GTR + G model for DENV-1 and the TrN93 + G + I model for DENV-2 were determined to be the most appropriate models for Bayesian Markov Chain Monte Carlo (MCMC) analysis. We employed a strict molecular clock model with constant size for both viruses. The MCMC chain was run in two stages: 100,000 for DENV-1 and 30,000 for DENV-2. Every 1000 steps, parameter values were sampled. Log-Combiner v1.8.1 was used to merge the log files from the two different MCMC investigations, with 10% burn-in sets for each run. Log data were viewed using Tracer v1.6 in order to confirm chain convergence and ascertain whether an effective sample size (>200) had been achieved for all parameters. HPD intervals of 95% were assessed to ascertain the degree of uncertainty in the parameter estimates. TreeAnnotator v1.8.1 was used to generate a maximum clade credibility tree. Figtree v1.4.2 was used to display the generated tree file.

2.11. Neutralizing Antibody Assay for Control Samples

Serum samples of 67 apparently healthy volunteers from wards No. 52–61 and contacts of dengue patients staying in the same household who came into the AIIMS OPD for routine checkups were studied for DENV-neutralizing antibodies (Figure 2). For this, indirect DENV IgG enzyme-linked immunosorbent assay (IgG-ELISA) was performed using a recombinant DENV envelope (Domain-III) for serotype 1, 2, 3 and 4 with specific antigens (DEN-005 for DENV-1, DEN-007 for DENV-2, and DEN-008 for DENV-3, and DEN-009 for DENV-4) used (ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel). Each antigen was used as 1 µg/well (1 µg/100 µL/well). The neutralization test was performed for in-vitro viral inhibition (cytopathic effect inhibition) with some modification [28]. All 67 heat-inactivated human serum samples were diluted 1:20 in MEM (2% heat-inactivated FBS) and incubated with DENV in a CO_2 incubator at 37 °C for one hour. This mixture was added to the cells, and the 96-well plates were incubated at 37 °C for five days. The test was performed with the following setups: (i) Vero cells without DENV, (ii) Vero cells with DENV served as a positive control (DENV 1–4), and (iii) Vero cells with the mixture (virus and diluted serum from the healthy individual) (Supplementary Figure S3).

3. Results

From a total of 280 serum samples collected from suspected dengue patients, 92 samples tested positive for NS1. Of these positive cases, the CPrM gene was successfully sequenced in 45 samples using the Sanger sequencing method, while 8 samples underwent whole genome sequencing (WGS) (Figure 3).

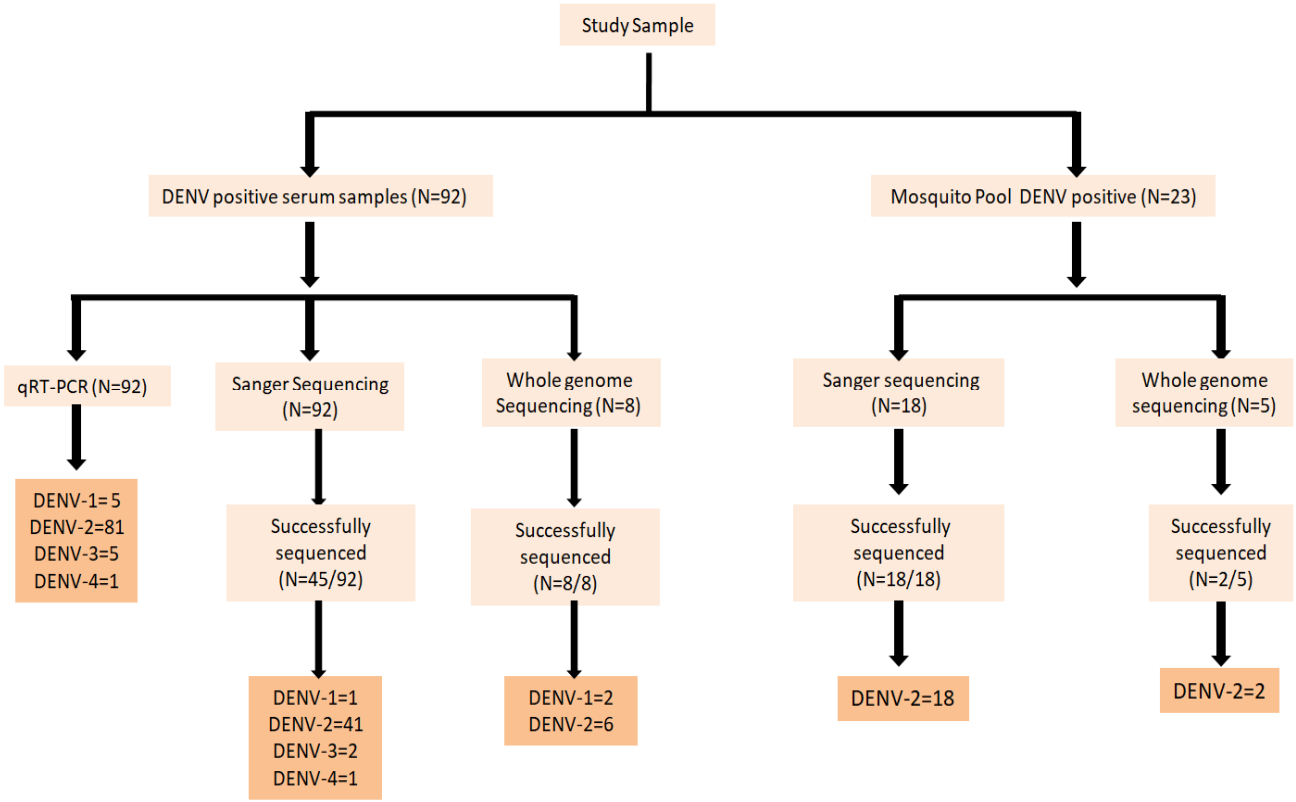


Figure 3. Details of samples used in this study.

The clinical profiles of suspected DENV cases, including those who tested NS1 negative, revealed that patients ranged in age from 18 to 65 years, encompassing both males and females. Common symptoms reported were low to high-grade fever persisting for 5–6 days, along with myalgia, arthralgia, thrombocytopenia in approximately 19–20% of cases, and abnormal liver function tests (LFT) (Table 1).

Table 1. Clinical history of DF and DHF/hospitalized dengue patients.

Clinical and Demographic Characteristics		NS1 ELISA Positive		NS-1 Negative (*n* = 188)
		DF * (*n* = 72)	DHF * (*n* = 20)	
Age (years)	18–30	23	6	88
	30–50	34	9	32
	50–65	15	5	68
Sex (Male/female)		45/27	15/5	110/78
Headache		12 (16%)	10 (50%)	56 (29%)
Myalgia		13 (18%)	20 (100%)	40 (21%)
Fever		65 (90%)	16 (80%)	165 (88%)
Arthralgia		10 (13%)	16 (80%)	89 (47%)
Rash		9 (12.5%)	4 (20%)	32 (17%)
Vomiting/Nausea		6 (8.33%)	3 (15%)	23 (12%)
Retro-orbital pain		5 (6.94%)	13 (65%)	15 (8%)
Hematocrit		14 (19%)	8 (40%)	25 (13%)
Thrombocytopenia		15 (20%)	18 (90%)	15 (8%)
Number of patients with increased aminotransferase level		32 (44%)	16 (80%)	8 (4%)

* DF = Dengue fever; * DHF = dengue hemorrhagic fever.

A total of 735 adult female *Aedes* mosquitoes (532 *Ae. aegypti* and 203 *Ae. albopictus*) were collected from wards of Bhopal, representing a population of 7–8 thousand per ward of the study area. A total of 40 pools of vector were processed, and among them, 23 pools from cell culture supernatant were positive for DENV RNA based on qRT-PCR (Supplementary Table S2). Eighteen pools were sequenced using Sanger sequencing for the CPrM gene, and two pools were sequenced using whole genome sequencing.

3.1. Serotype Specific qRT-PCR

qRT-PCR was conducted to determine the serotype and viral load of DENV in host samples collected during the study period. Viral load analysis showed a range of 0.2 to 7.7 Log viral RNA copies/mL in all 92 samples. Among the 20 patients of DHF or those requiring hospitalization, a significant difference ($p < 0.0001$) was observed in the average viral load, which was 4.93 Log_{10} viral RNA copies/mL, while the remaining 72 samples of DF had an average of 1.72 Log_{10} viral RNA copies/mL (Figure 4 and Supplementary Table S3)

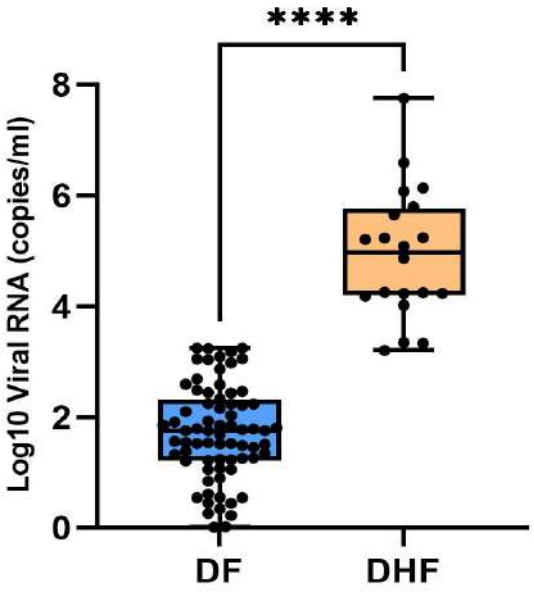


Figure 4. Viral load difference between DF and DHF patients (**** $p < 0.0001$).

3.2. Phylogenetic Analysis

3.2.1. DENV Genotype Distribution for Vector Pool

Phylogenetic analysis revealed that eighteen DENV CPrM gene sequences from mosquitoes generated using Sanger sequencing belonged to serotype 2 (Figure 5). Analysis of the representative serotype-2 CPrM gene sequences from India and other countries clearly showed that all 18 sequences were clustered with non-Indian lineage of DENV-2 (Cosmopolitan genotype 4, lineage III). Other isolates obtained from Bhopal in 2016 (GenBank accession nos. MH051272-MH051275) were grouped into distinct clades within the most prevalent cosmopolitan 4 genotype lineage II.

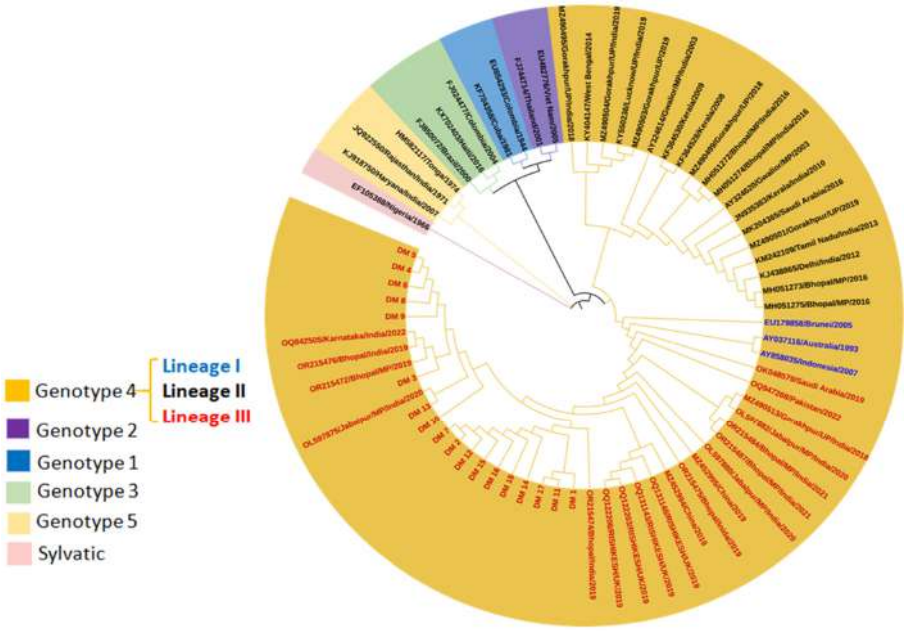


Figure 5. Phylogenetic tree based on CPrM gene of DENV-2 from a vector (n = 18) generated using Sanger sequencing. Each strain is identified by its GenBank accession number, country/state/city of origin, and the year of isolation. The analysis of DENV 2 was performed with the study isolates using the maximum likelihood and Tamura–Nei method in MEGA 10 software (Bootstrap = 1000).

3.2.2. DENV Genotype Distribution for Host Serum Samples

Sequencing of 45 samples for the CPrM gene shows 1 patient was infected with DENV-1, 41 with DENV-2, 2 patients with DENV-3, and 1 with DENV-4. Although we found that all serotypes were in circulation in Bhopal from September 2019 to November 2023, among them DENV-2 was the predominant serotype with 41 isolates.

To determine the genotype distribution of DENV within each serotype, CPrM gene sequences obtained during this study and sequences from different geographical locations across the globe were retrieved from the NCBI database, aligned, and used for phylogenetic analyses.

Phylogenetic analysis identified one sequence from this study, generated through Sanger sequencing, as belonging to DENV-1 and classified it within clade C of genotype 5 (refer to Supplementary Figure S4). Notably, this finding aligns with previous observations of isolates from 2016 circulating in Bhopal (MH051267-MH051271), which also belonged to the same clade. Additional members of this genotype were traced to sequences originating from Thailand (1980), Singapore (2004–2015), and China (2014).

As evident, DENV-2 sequences were categorized into six genotypes [29]. The studied isolates generated using Sanger sequencing during the period of September 2019 to November 2023 (n = 41) were classified as Cosmopolitan or genotype 4 (lineage III) (Figure 6). This genotype contains three geographically distinct lineages: lineage I (Australian, Indonesian, Brunei), lineage II (isolates from Southeast Asia, China, and Oceania), and lineage III (isolates mostly from the Indian subcontinent). Present DENV-2 sequences from Bhopal were attributed to lineage III, clustering mostly with Indian strains. Conversely, other isolates (OR215476, OR215472, OL597875, OR215475, OR215474, OL597880, OL597882, OR215487, and OR215484) were also circulated in the same region and grouped in the same lineage (lineage III), while isolates from 2016 (MH051272-MH051275) were clustered in genotype lineage II.

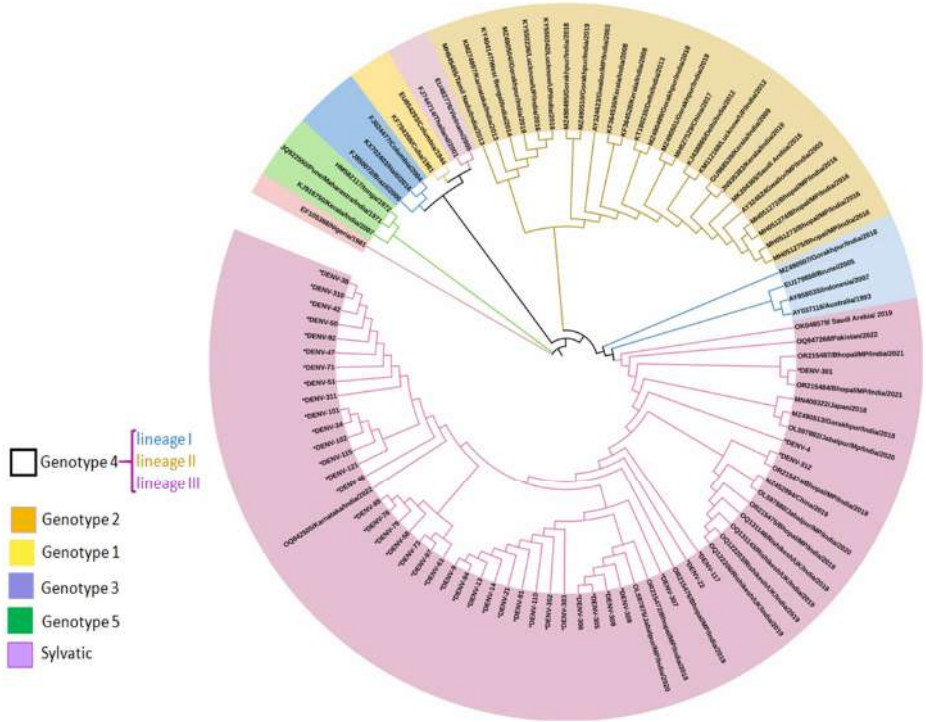


Figure 6. Phylogenetic tree-based CPrM gene of DENV-2 from hosts (n = 41) generated using Sanger sequencing. Each strain is identified by its GenBank accession number, country/state/city of origin, and the year of isolation. The analysis of DENV 2 was performed with the study isolates by using the maximum likelihood and Tamura–Nei method in MEGA 10 software (Bootstrap = 1000).

Phylogenetic analysis demonstrated that the study isolates which were generated using Sanger sequencing were clustered within genotype III of DENV-3 (n = 2) (Supplementary Figure S5). Genotype III was further subdivided into five lineages. The current DENV-3 sequences were closely related to isolates from India (2008–2019), China (2013), and Singapore (2009, 2012). The study isolates from 2019 to 2021 were clustered within lineage III, similar to previously reported isolates from 2016 in Bhopal (MH051276, MH051277) and other isolates from Madhya Pradesh (MK829114, MK829116, AY770511).

DENV-4, a rare serotype in India, was detected in the early 1960s in Vellore, Tamil Nadu [30], and responsible for a few outbreaks in Kanpur (1968–1969) [31] and Andhra Pradesh (2007) [32]. Serotype 4 is divided into five genotypes, and only study isolates from Bhopal were clustered within genotype I (Supplementary Figure S6), alongside isolates from other Asian countries (Sri Lanka, Thailand, Singapore, Cambodia, China, Pakistan, and the Philippines).

3.2.3. Whole Genome Sequence Analysis

Phylogenetic Analysis

To assess the above observations based on partial sequencing (CPrM gene), some representative samples were subjected to whole genome sequencing (Illumina platform) for the in-depth study. Phylogenetic analysis was performed separately for both serotypes (DENV-1 and DENV-2), and the respective reference sequences were retrieved from GenBank (KF289073 for DENV-1 and EU854293 for DENV-2). The clean reads of isolates (two sequences of DENV-1 and eight sequences of DENV-2; six were from human serum, and two were from the mosquito pool) were aligned to the reference sequences. All isolates have a full-length sequence of 10,138 (DENV-1) and 10,136 (DENV-2) nucleotides, which were used for the phylogenetic study. Similar results were found from whole genome sequences of DENV-1 (n = 2) belonging to the clade C of genotype V isolated from Bhopal during 2019–2023 (Supplementary Figure S7); the DENV-2 sequences from Bhopal during the period of 2019–2023 (n = 8) were clustered within genotype IV (Lineage III) (Supplementary Figure S8).

Shannon Entropy Analysis

A relatively high degree of intra-type and inter-type sequence conservation was observed, with low entropy values, generally below on average 0.25, indicating a relatively low level of viral evolution and diversity [33]. The identification of the sites that are prone to mutation in the whole proteome of the DENV was performed for the datasets with n = 58 (n = 2 from the present study with 56 sequences retrieved from NCBI) (DENV-1) and n = 62 (n = 8 from the present study and 54 sequences retrieved from NCBI) (DENV-2). For the selection of a variable site, a value of 0.2 was set as the threshold. The inter-type entropy difference between both the serotypes is relatively not so large ($\pm$0.05). However, inter-type all structural proteins are relatively conserved with low entropy value (capsid~0.04, PrM~0.067, Envelop~0.04, NS1~0.05), while all the non-structural proteins were showing substantial divergence with relatively high entropy values (NS2A~0.413, NS2B~0.37, NS3~0.35, NS4A~0.35, 2K peptide~0.43, NS4B~0.34, and NS5~0.33) (Figure 7).

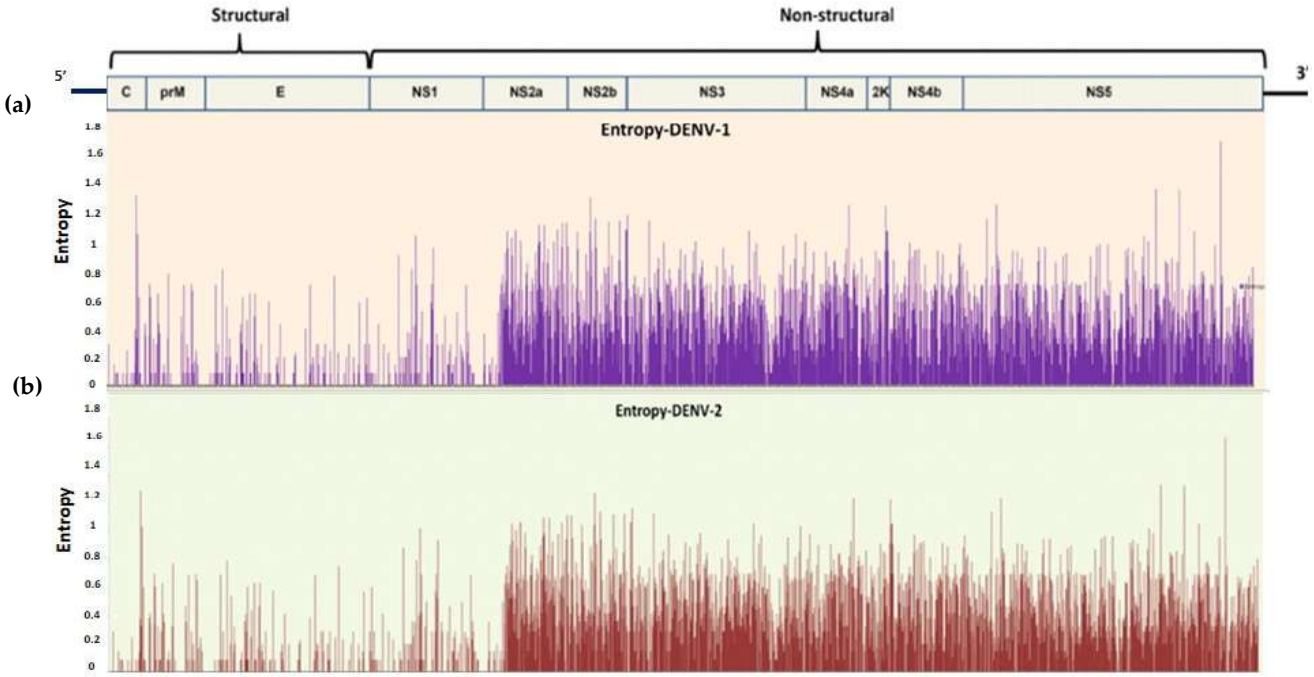


Figure 7. Shannon entropy plot of the whole proteome of dengue virus. (**a**) DENV-1 and (**b**) DENV-2.

Bayesian MCMC Analysis

The best-fit nucleotide substitution model for the DENV-1 (n = 58) dataset includes all of its six genotypes. The best-fit nucleotide substitution model for the dataset was chosen as GTR + G (gamma categories = 4). A strict constant size clock tree prior was chosen as the best-fit model in the molecular clock analysis. The maximum clade credibility (MCC) tree for DENV-1 sequences was generated in Fig tree v1.4.1 using the best-fit model (Figure 8).

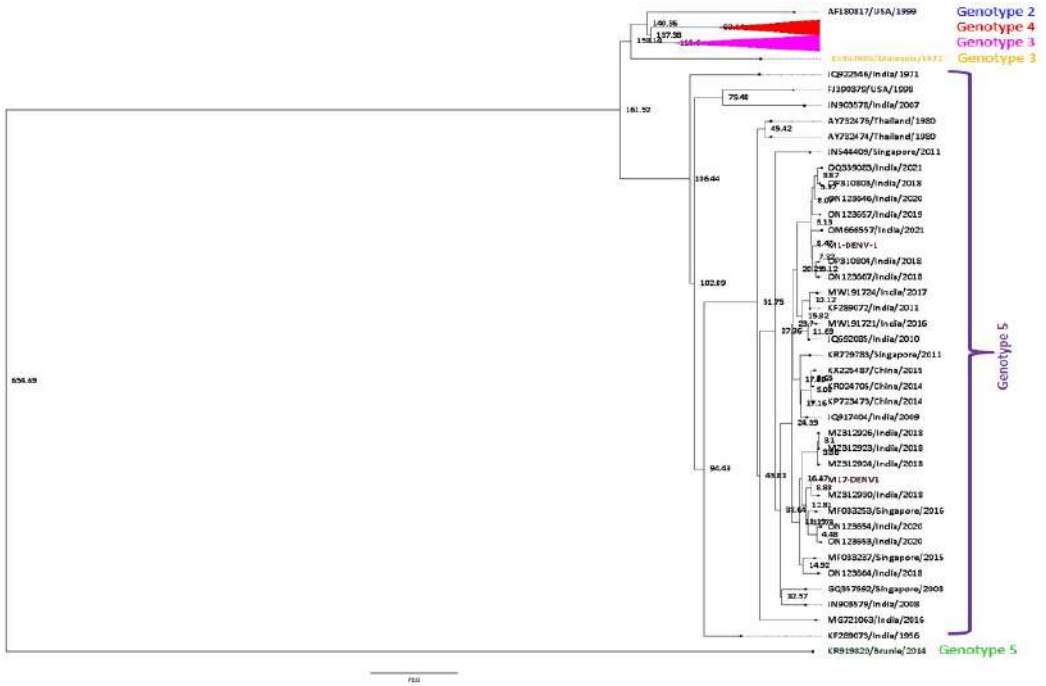


Figure 8. Maximum clade credibility tree of DENV-1 (n = 58). The tree was generated with the best-fit strict clock, the Bayesian skyline model. Node ages are denoted at each node.

The mean nucleotide substitution rate under the strict clock was detected to be 3.77×10^{-4} substitutions per site per year (95% HPD (3.40×10^{-4} to 4.12×10^{-4} s/s/y)). The age from the root was estimated to be approximately 654 years (95% HPD (582–735 years)), 1366 (95% HPD (1287–1337)). Similarly, the time to the most recent common ancestor (tMRCA) for genotype 5 was about 161 years (95% HPD (152–175 years)). Further, the study strains of DENV-1 clustered in genotype 4 were found to be approximately 8 years old (95% HPD (6–10 years)). Likewise, tMRCA for genotypes 2, 4, 3, and 1 was estimated to be around 140, 83, 153, and 137 years old, respectively.

The best-fit nucleotide substitution model for the dataset for DENV-2 (n = 62) was chosen as TrN 93 + I + G (gamma categories = 4). A strict constant size clock tree prior was chosen as the best-fit model in the molecular clock analysis. The maximum clade credibility (MCC) tree of DENV-2 sequences was generated in Fig tree v1.4.1 using the best-fit model (Figure 9).

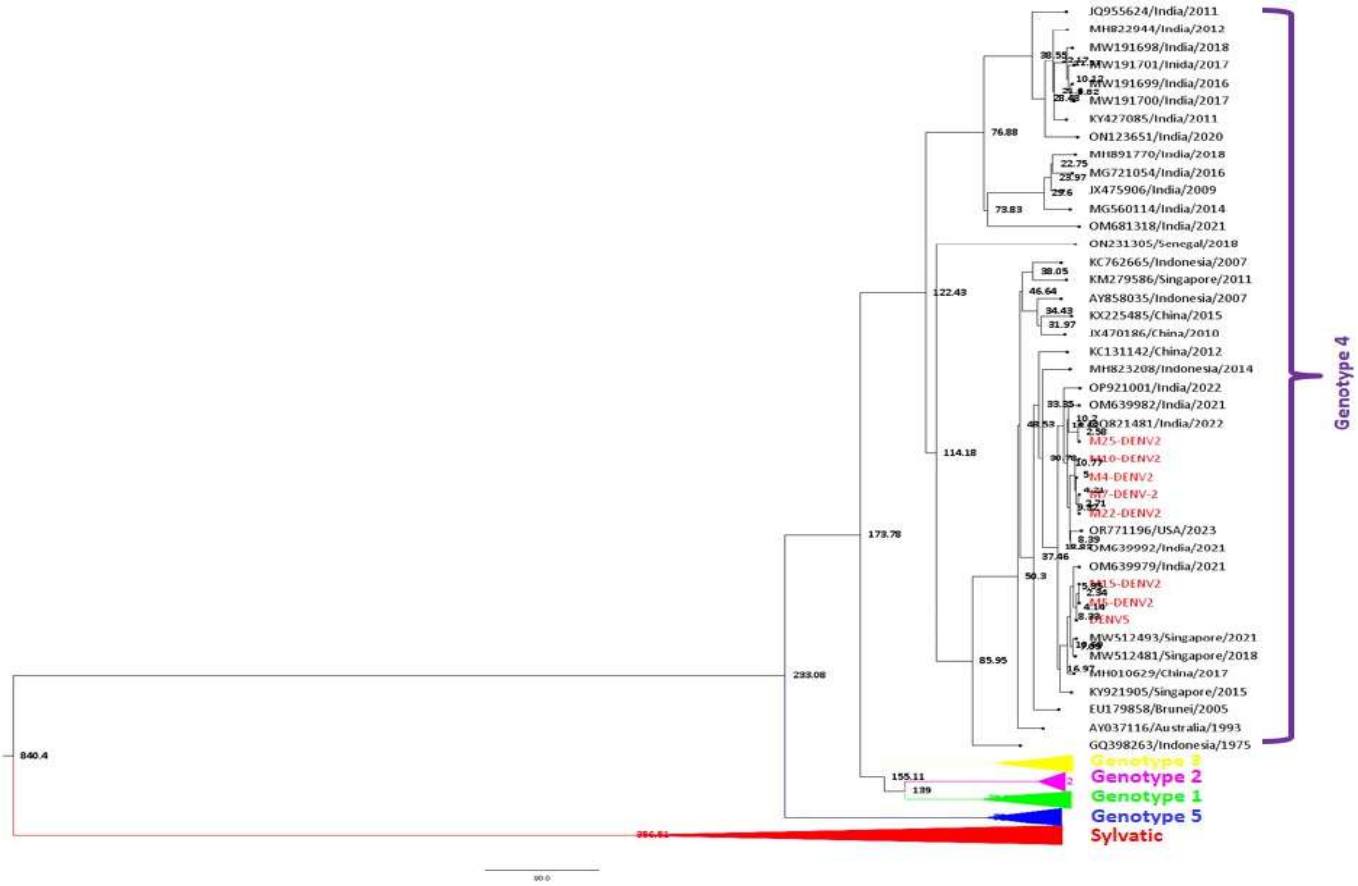


Figure 9. Maximum clade credibility tree of DENV-2 (n = 62). The tree was generated with the best-fit strict clock and constant size model. Node ages are denoted at each node.

A total of 3.62×10^{-4} substitutions per site per year (95% HPD (2.68×10^{-4} to 4.69×10^{-4} s/s/y)). The age from the root was estimated to be approximately 841 years (95% HPD (731–981 years)), 1180 (95% HPD (1040–1290)). Similarly, the time to the most recent common ancestor (tMRCA) for genotype 4 was about 174 years (95% HPD (156–191 years)). Further, the study strains of DENV-1 clustered in genotype 4 were found to be approximately 8 years old (95% HPD (6–10 years)). Likewise, tMRCA for genotypes 5, 1, 2, and 3 was estimated around 76, 80, 35, and 69 years, respectively.

3.3. Analysis of Neutralizing Antibody Assay for Control Samples

To assess the seroprevalence of dengue antibodies, serotype-specific ELISA was performed, followed by a neutralizing assay using Vero cell lines to observe the inhibition of CPE. The ELISA results revealed that 28.4% of healthy controls had IgG antibodies for DENV-1 infection, followed by DENV-2 (12.4%), DENV-3 (10.8%), and (10.1%) (Figure 10). However, we also found heterotypic or multitypic infection in some of the samples. The results of inhibition of cytopathic effect (CPE) using the Vero cell line revealed a serotype heterogeneity with seroprevalence detected for DENV-1, DENV-2, DENV-3, and DENV-4 in the studied wards (Supplementary Figure S9).

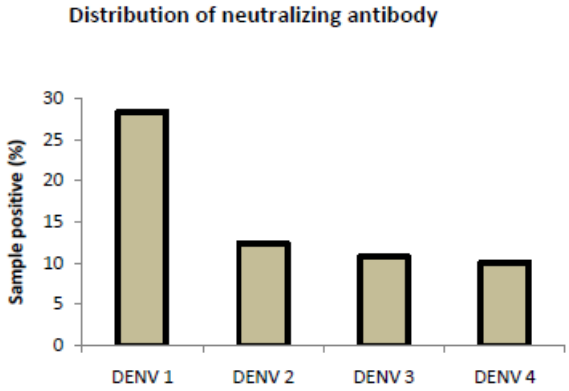


Figure 10. Plot representing the distribution of neutralizing antibodies in the population.

4. Discussion

Bhopal, one of the largest cities in terms of both population and area in Central India, an endemic dengue zone, has seen frequent DENV outbreaks over the past 20 years [10–12]. Dengue outbreaks, occurring every 2–3 years, result in significant morbidity and mortality in the region. Despite extensive research on dengue, knowledge gaps remain in understanding the circulating DENV variants, transmission dynamics, and factors influencing disease severity. Understanding dengue's etiology and progression requires monitoring of genomic alterations, primarily through the evolutionary dynamics of sequences. To address these gaps, this study utilized genomic sequencing, phylogenetic analysis, and Bayesian MCMC to investigate the molecular epidemiology of DENV in hosts and vectors. Additionally, DENV seroprevalence was assessed in a seemingly healthy population to understand disease dynamics.

The present investigation revealed that DENV-2 was predominant in both the host and vector, followed by DENV-1, DENV-3, and DENV-4. The DENV-2 isolates recovered belong to genotype-IV (lineage III), dominating both host and vector samples, indicating an epidemiological relationship between DENV serotype mosquitoes and human cases. This study revealed a displacement of the native DENV-1 genotype-V (clade C) strain by the DENV-2 genotype-IV (lineage-III) strain in the Bhopal region. This transition was notable during the periods of 2016–2017 and 2019–2021, reflecting a competitive replacement between the serotypes (supplementary Figure S10). The new genotypic and lineage variants of DENV-2 circulating in the vectors may likely contribute to the higher incidence of dengue cases. According to earlier research, "stochastic events attributable to the low rate of virus transmission during the inter-epidemic period" could be the cause of the rise of dominant serotype [34]. Experimental infection trials within *Aedes aegypti* infected with either DENV-1, DENV-4 (mono-infection), or both viruses (co-infection) indicate that DENV-4 had a competitive advantage in a co-infection scenario. Although, no significant differences in transmission or dissemination were observed [35]. These findings emphasize the ability of

mosquitoes in endemic areas to harbor multiple DENV serotypes, with natural selection driving the replacement of one genotype by another due to vector-mediated dynamics [36].

This study suggests that the DENV-2 serotype likely achieved greater viral loads in vectors compared to native DENV-1, enabling its rapid proliferation and dominance in the region. Seroprevalence statistics suggested that immunity against DENV-1 in the population provided limited protection against the newly emerged DENV-2 genotype. This lack of neutralizing antibodies against DENV-2 facilitated the outbreak.

The comparison of the viral loads (viremia) in host patients revealed an additional finding that the DENV NS1-positive participants who needed hospitalization for thrombocytopenia, high-grade fever, or abnormal liver function tests had high viral loads. These results are consistent with research from Vietnam showing that elevated viremia is linked to severe dengue manifestations such as DHF or DSS [15]. However, contrasting results from another study have been observed showing that viremia in DENV infection is not associated with a cytokine storm [16]. The findings suggest that a high viral load or reproduction number (R0) plays a crucial role in establishing a transmission chain, as it enables the host to maintain elevated viral loads early in the infection; these conditions facilitate the displacement of native strains and the propagation of new DENV genotypes. When mosquitoes feed on infected hosts with high viremia, it enables the transmission of emerging strains. This process underscores the epidemiological impact of viremia in driving the emergence and spread of novel DENV variants. The phenomenon of genetic variants and the competitive displacement of the original strain of the virus have had a substantial impact on the epidemiology and global pathogenicity of dengue illness [35,37].

Genetic analysis found a predominance of synonymous amino acid mutations, which do not alter the protein structure or function, suggesting sequence conservation across the viral proteome. This is further supported by the Shannon entropy analysis, indicating minimal variation in protein-coding regions. This stability has implications for vaccine development, as monitoring proteomic changes can help predict viral behavior and inform vaccine design strategies [38]. This study also signifies the use of non-structural genes as similar diversity to structural DENV genes; this may advocate distinct evolutionary pressures [39].

Bayesian evolutionary analysis estimated that DENV-1 and DENV-2 have their respective average nucleotide substitution rates of 3.77×10^{-4} substitutions per site year and 3.62×10^{-4} substitutions per site per year, respectively. This suggested that DENV-2 evolved more slowly than DENV-1 and experienced more early evolutionary events. The estimated mean root age of DENV-2 was 841 years (95% HPD 731–981 years), while the inferred mean root age of DENV-1 in 2021 was 654 years (95% HPD 582–735 years). These results are in line with research that has already been published [4,40]. It is mentioned that the earliest known cases of an illness similar to dengue fever were recorded in 992 during the Chin dynasty in China, which lasted from the third to the fifth century [41]. This year is also rather near to our mean estimate for the TMRCA of all serotypes. The broadest estimate suggests a composite TMRCA of DENV was about 1670 years ago [42]. This period corresponds with the first documented medical report of "water poison", a dengue-like disease that was transmitted by mosquitoes in third-century China [26]. This study predicted a major divergence in cosmopolitan genotype stemmed from the emergence of an Indian subcontinent lineage approximately 123 years ago.

Vectors play a crucial role in the emergence of new genotypes, as viral replication in mosquitoes influences the evolutionary trajectory of DENV. This study also provides crucial information on how high viremia in hosts and the emergence of new DENV in vectors by natural selection maintain the transmission cycle in endemic areas. However, predicting the outbreak or emergence of a new DENV strain remains a difficult task despite advances

in understanding the host–virus pathophysiology, tracing the evolution of the viruses, and genetic epidemiology. A key limitation of this study was the inability to evaluate differences between primary and secondary DENV infections, which could provide deeper insights into disease dynamics. Until the proper antiviral medication or vaccines are developed, routine surveillance of DENV serotypes and genotypes in endemic areas will remain essential for forecasting large-scale outbreaks. Early detection of shifts in circulating strains can inform public health interventions, minimizing the impact of dengue epidemics.

5. Conclusions

According to the current study, DENV-2 was the most prevalent serotype during the dengue outbreaks in the Bhopal region of Central India in 2019 and 2021, followed by other serotypes. Compared to earlier studies, tMRCA was found earlier, and low substitution rates based on the entire genome were noted. The current study investigated how the high viral load emerging DENV-2 genotype-IV (lineage-III) replaced the circulating native strain of DENV-1 and DENV-2, despite some disagreement on the origin time estimate of DENV. It may be brought on by the presence of monotypic protective antibodies against a particular serotype or by the competitive displacement of novel strains with high viral loads. In this endemic region, new serotypes and genotypes may emerge as a result of future outbreaks, as the study also found that all serotypes are co-circulated at the vector level. It is unknown which serotype will ultimately manifest in the vector's midgut to initiate an outbreak. Molecular characterization, epidemiology, and vector surveillance, however, can greatly enhance predictions for severe dengue outbreaks in the future and break the cycle of outbreaks that recur every two to three years, which is linked to high death rates in the region. Continuous vector, virus, and DENV molecular epidemiology surveillance would help medical professionals make the necessary preparations for future outbreaks in the absence of a dengue vaccine in endemic areas.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v17020144/s1, Figure S1: 112 base pair band of dengue serotype 1 and 78 base pair band of dengue serotype 2 was cloned and confirmed on 2.5% agarose gel; Figure S2: 511 base pair band of dengue was confirmed on 1.5% agarose gel; Figure S3: Schematic representation of neutralizing antibody generation against DENV-1; Figure S4: Phylogenetic tree based on CPrM gene of DENV-1 from host (n = 1). Each strain is identified by its GenBank accession number, country/state/city of origin and the year of isolation. The analysis of DENV 1 was done with the Study isolates by using Maximum likelihood & Tamura-Nei method in MEGA 10 software (Bootstrap = 1000); Figure S5: Phylogenetic tree based on CPrM gene of DENV-3 from host (n = 2). Each strain is identified by its GenBank accession number, country/state/city of origin and the year of isolation. The analysis of DENV 3 was done with the Study isolates by using Maximum likelihood & Tamura-Nei method in MEGA 10 software (Bootstrap = 1000); Figure S6: Phylogenetic tree based on CPrM gene of DENV-4 from host (n = 1). Each strain is identified by its GenBank accession number, country/state/city of origin and the year of isolation. The analysis of DENV 4 was done with the Study isolates by using Maximum likelihood & Tamura-Nei method in MEGA 10 software (Bootstrap = 1000); Figure S7: Phylogenetic analysis of DENV-1 complete genome sequences (n = 2): Each strain is indicated by GenBank accession number followed by country and year of isolation. Numbers at the nodes are support values for the major branches (bootstrap; 1000 replicates). The sequences obtained in this study are marked in filled-colored circles. The scale bar indicates the number of base substitutions per site; Figure S8: Phylogenetic tree based on the whole genome of DENV-2 (n = 8). Each strain is identified by its GenBank accession number, country/state/city of origin and the year of isolation. The analysis of DENV 2 was done with the Study isolates by using Maximum likelihood & Tamura-Nei method in MEGA 10 software (Bootstrap = 1000); Figure S9: in vitro viral inhibition assay showing vero cell lines post infection of (Day 5) A. control cells, B. Vero

cells infected with DENV-1 + DENV2 IgG antibody serum samples showing partial inhibition of viral growth, C. Vero cells lines infected with DENV-2 + DENV2 IgG antibody serum samples showing no inhibition of viral growth D. Vero cells infected with DENV3 + DENV2 IgG antibody serum samples showing partial inhibition of viral growth E. Vero cells infected with DENV-4 + DENV2IgGantibody serum samples showing complete inhibition of viral growth; Figure S10: Schematic representation of Dengue outbreak situation in Bhopal city during 2014–2016 and 2019–2021; Table S1: Patients' clinical history at the time of OPD visit;. Table S2: Mosquitoes pools infected with different serotypes; Table S3: Serotype-specific viral load of DENV in host samples.

Author Contributions: Conceptualization, R.C.; methodology, R.C. and A.K.Y.; software, A.K.Y., and A.G.M.; validation, A.K.Y., A.K. and S.K.; formal analysis, A.K.Y. and A.G.M.; investigation, S.K.G. and R.J.; resources, R.C., J.R.K. and S.K.G.; data curation, A.K.Y., A.P. and A.G.M.; writing—original draft preparation, A.K.Y., A.K., and R.C.; writing—review and editing, A.K.Y., A.G.M., A.S. and R.C.; visualization, A.K.Y., A.G.M. and R.C.; supervision, R.C. and D.B.; project administration, R.C.; funding acquisition, R.C., J.R.K. and S.K.G. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained within the article or Supplementary Material. The Whole genome and Sanger sequenced sequences were submitted to NCBI (PP817678, PP817679, PP817680, PP817681, PP817682, PP817683, PP817684, PP817685, PP817686, PP817687, PP817688, PP817689, PP817690, PP817691, PP817692, PP817693, PP817694, PP817695, OR888734, OR888735, PP795941, PP795942, PP795943, PP795944, PP795945, PP795946, PP795947, PP795948, PP795949, PP795950, PP795951, PP795952, PP795953, PP795954, PP795955, PP795956, PP795957, PP795958, PP795959, PP795960, PP795961, PP795962, PP795963, PP795964, PP795965, PP795966, PP795967, PP795968, PP795969, PP795970, PP795971, PP795972, PP795973, OR492473, OR492474, OR492475, OR888730, OR888731, OR888732 OR888733, MZ841823, MZ841824, MZ841825, MZ841826, MZ841827, MZ841828, MZ841829,MZ841830).

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

RC: Rashmi Chowdhary; AYK: Ashish Kumar Yadav; AK: Ashok Kumar; SK: Sagar Khadanga; RL: Rajnish Joshi; AP: Abhijit Pakhare; JRK: Jagar Rakesh Kanwar; AS: Arshi Siddiqui; SKG: Sudhir K Goel; DB: Debasis Biswas; AGM: Anvita Gupta Malhotra.

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Original Article

A comparative study of NS1 and IgM using enzyme-linked immunosorbent assay and immunochromatography test for detection of dengue

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Abstract

Background: Dengue is the most common arboviral infection that spreads by *Aedes aegypti* and *Aedes albopictus* mosquitoes, and is quickly gaining prominence as a major mosquito-borne viral disease. One of the major public health issues is dengue fever (DF), which can also cause dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Therefore, this study focused on comparison of dengue antigen non-structural protein (NS1) and immunoglobulin M (IgM) using enzyme-linked immunosorbent assay (ELISA) and immunochromatography test (ICT) for detection of dengue. **Method:** In a Tertiary Care Hospital (TCH), sociodemographic status of probable dengue cases from February 2021 to February 2022 was studied. The results of the Dengue Antigen NS1 and IgM ICT, Dengue NS1 Microlisa, and Dengue IgM Microlisa were compared in order to determine the effective one at managing patients and preventing complications like DHF and DSS. **Results:** In distribution of 100 ICT reactive samples, 50% were NS1 reactive and 50% were IgM reactive. One hundred ICT reactive samples were further processed for IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) for both NS1 and IgM. Sensitivity and specificity of NS1 ICT were 89.3% and 71.4% and that of IgM ICT was 88% and 64.5%. As a result of antigenic cross-reactivity, false positive cases were reported. Platelet count of the patients was correlated with an optical density (OD) value of ELISA for both NS1 and IgM. In the present study, patients having low platelet count showed high OD value. **Conclusion:** In cases of severe thrombocytopenia (platelet count <50000), early diagnosis by screening ICT and confirmation by ELISA (NS1 and IgM) would reduce the complications like DHF and DSS.

Keywords: Dengue haemorrhagic fever, dengue shock syndrome, enzyme-linked immunosorbent assay, iummnochromatography test, viral disease

Introduction

Flaviviridae a mosquito borne virus causes Dengue Fever (DF) which is caused by the single stranded Ribonucleic acid (RNA) virus known as dengue virus.[1,2] Tropic and subtropic countries around the world are mostly affected by dengue virus infection. In India, dengue fever outbreaks are spreading even into the rural area[3] and is the most common arboviral infection spread by *Aedes albopictus*, and *Aedes aegypti* mosquitoes, which is quickly gaining prominence as a major mosquito-borne viral disease.[4] One of the significant public health issues is DF, along with dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS).[5] Every year, the disease affects 50 to 390 people, involving 2.5 billion people at risk.[6] DHF is officially notified in approximately 2,50,000 cases and the real cases are undoubtedly much higher.

DF and a severe version of the disease, DHF/DSS, have emerged as the most serious arthropod-borne viral infections

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of humans in the last 20 years.[7] The incidence of dengue virus (DENV) infection has increased 30 times in the previous 50 years, and more than 50% of the world's population now resides in areas at risk of infection.[8] Between January and November, in 2021, Mumbai has recorded 821 cases and 3 deaths of dengue over 129 cases and 3 fatalities. In 2019, there were 920 cases and in 2018, 1008 of dengue.[9] Therefore, this study compared non-structural protein (NS1) and immunoglobulin (IgM) using enzyme-linked immunosorbent assay (ELISA) and immunochromatography test (ICT) for detection of dengue which will benefit both the primary care physicians and patients detected with dengue for early diagnosis and treatment.

Methodology

After approval from the Institutional Ethical Committee with reference number N-EC/2021/SC/01/06 and according to World Health Organization (WHO) standards, 400 patients of DF in total were involved from a tertiary care hospital (THC) after obtaining written informed consent for the study. Samples were centrifuged at 3000 rpm for 5 minutes to separate the serum following which the clear supernatant was then examined. The samples were not frozen and thawed repeatedly. J Mitra's Dengue Day 1 Test was used to perform ICT for the detection of Dengue NS1 antigen and IgM antibodies. The kit consists of two tools: one to detect dengue IgM/IgG antibodies and the other to detect dengue NS1 antigen in human plasma or serum. Two lines made up the antigen device: a Control Line (C line), an IgM test Line (M line), and an IgG test Line (G line).

As a reactive test, a clear pink line at "T" or "M"/"G" was used. ICT was performed in 400 clinically suspected patients for dengue, and out of this 100 ICT reactive samples were further taken for the study. Out of 100, 50 were reactive for NS1 antigen and 50 were reactive for IgM antibody. One hundred ICT reactive samples were further processed for ELISA using Dengue IgM Microlisa, and Dengue NS1 Ag Microlisa kit by J Mitra with 50 samples which demonstrated that 25 Non-reactive for NS1 antigen and 25 Non-reactive for IgM antibody negative samples were served as controls. According to the kit's literature, the test samples were combined with the kit's positive and negative control. A total of 100 ICT reactive samples were tested simultaneously using two kits each having 96 microwells from the same manufacturer.

The direct sandwich principle is the foundation of the Dengue NS1 Microlisa, which is a solid phase ELISA. Monoclonal anti-dengue NS1 antibodies were used to coat the microwells, and the Dengue IgM Microlisa was an enzyme immunoassay based on "MAC-Capture ELISA". Micro-titre wells have anti-human IgM antibodies coated on them. Controls and specimens were added to the microtitre wells following which they were incubated. A yellow colour that was finally read at 450 nm demonstrated a positive reaction.

Results

Of the 400 clinically suspected dengue patients that were admitted to the THC between February 2021 and February 2022, 100 samples were positive for the dengue IgM antibodies and dengue NS1 antigen in the ICT. Out of 81 ELISA positive, 65 samples were from male and 16 samples were from female, which represents the socio-demographic distribution of male and female. The age distribution of all reactive dengue fever cases was 9 patients between 0-20 years, 42 patients in 21–40 years, 18 patients in the 41–60 years, and 12 patients who were found to be >60 years old.

Out of total number of 400 clinically suspected samples of dengue patients, 81 samples were found to be reactive by gold standard method. So the incidence rate of dengue in the hospital was 1.23%. Out of 100 reactive samples that were taken, 50 were reactive for NS1 and 50 were reactive for IgM. The peak of serologically positive cases in the current study occurred from June to October, and the other seasonal months, from November to April, showed a significant reduction. Fifty ICT reactive samples for NS1 antigen and IgM antibody further processed for ELISA. Twenty-five non-reactive ICT samples also processed which serve as control. In Dengue NS1 Microlisa, 42 out of 50 ICT reactive samples came positive and 20 out of 25 negative samples came negative. The specificity and sensitivity of the Dengue NS1 ICT were 89.3% and 71.4%, respectively, with a negative predictive value (NPV) of 80%, and positive predictive value (PPV) of 84%. In Dengue IgM Microlisa, 39 out of 50 ICT reactive sample came positive and 20 out of 25 negative samples came negative. PPV for the Dengue NS1 ICT was 78%, NPV was 80%, and the NS1 ICT's sensitivity and specificity were 64.5% and 88%, respectively. The distribution of IgM ICT reactive and IgM ELISA is described in Figure 1.

The distribution of NS1 ICT reactive and NS1 ELISA is described in Figure 2.

According to Figure 3, the peak period for serologically positive patients in the current study was from June to October, and from November to April, the number of cases significantly decreased.

In NS1 ELISA, the present study, out of 50 NS1 positive patients samples, OD value of 13 patients was observed to be between 3.761 and 3.970 and the platelet count of the those patients was <50000 which shows that there is a correlation between severe thrombocytopenia and an increase in the OD value of the patients; 19 patients showed the OD value of Dengue NS1 Microlisa between 3.561 and 3.760 with the platelet count between 100000 and 150000; and in 15 patients, platelet count was between 150000 and 200000 with the OD value of 3.360-3.560. In IgM ELISA, as shown in the current study, out of 50 NS1 positive patients samples, OD value of 11 patients was observed to be between 3.661 and 3.840 and the platelet count of the those patients were <50000 which shows that there is a correlation between severe thrombocytopenia and an increase in the OD value of the patients; 16 patients showed the

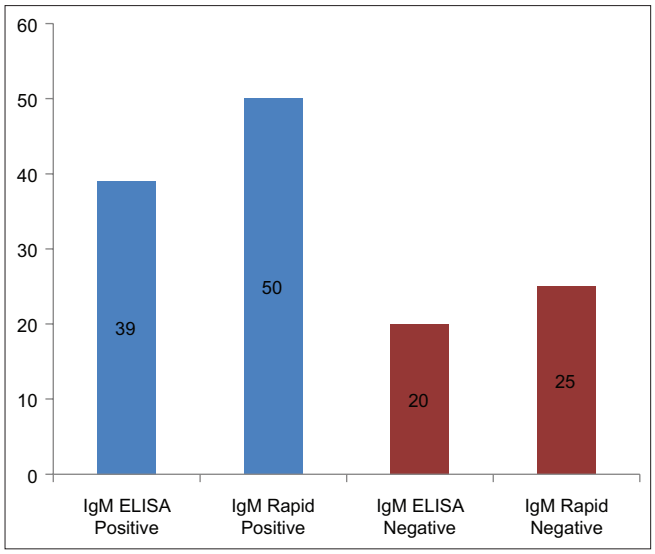


Figure 1: Distribution of IgM ICT reactive and IgM ELISA. IgM = immunoglobulin M, ICT = immunochromatography test, ELISA = Enzyme-linked immunosorbent assay

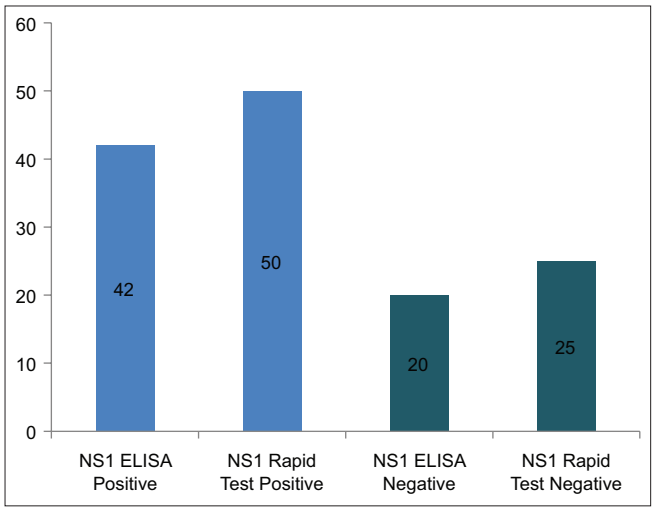


Figure 2: Distribution of NS1 ICT reactive and NS1 ELISA. NS1 = Dengue antigen NS1, ICT = immunochromatography test, ELISA = Enzyme-linked immunosorbent assay

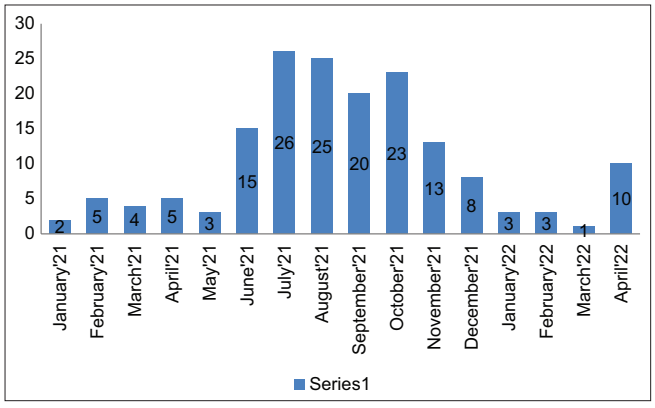


Figure 3: Seasonal variation of dengue fever

OD value of Dengue NS1 Microlisa between 3.461-3.660 with the platelet count between 100000 and 150000; in 17 patients, platelet count was between 150000 and 200000 with the OD value of 3.150-3.460.

Discussion

Dengue fever has become a Global threat along with no sign of decreasing and has a significant direct economic and social impact on the endemic regions. Various studies around the world have methodologically proved Dengue's ultimatum to travellers. Dengue has recently made a resurgence on a global scale due to the growth in the quantity and size of densely populated metropolitan areas, which encourages the disease's spread, as well as the adaption and reproduction of the Aedes aegypti mosquito. Over the past three decades, there is an exponential increase of the people around the cities. The increased transmission of the virus has effect in the genetic expansion of the virus.[10]

Serologically positive instances peaked from June to October, and from November to April, which are the other seasonal months, showed a sharp fall. According to Chakravarti and Kumaria,[11] dengue infections mainly happened following the monsoon season, when rainfall was below average. Rain, relative humidity, and temperature were highlighted in the study as important and major climatic factors. According to the present study, most of the population affected were between 21 and 40 years, whereas according to Gupta *et al*.,[12] dengue is most prevalent in people between the ages of 21 and 30. In the present study, male predominance was 77% compared to female as 33%. Most of the findings are consistent with those of another study.

Mehta *et al*.[13] conducted a cross-sectional investigation in which 1628 samples were examined and 403 IgM positives and 1225 NS1 positives were found. Male predominance in their study was observed which was 71.4% compared to female. In the current investigation, 400 clinically suspected samples underwent a quick examination using a standard kit with good sensitivity and specificity. One hundred samples were taken for the study after being reactive for one or more dengue parameters. One hundred ICT reactive samples were further processed for MAC-ELISA (for both NS1 and IgM) which demonstrated that 50 were reactive for NS1 and 50 were reactive for IgM only.

In NS1 ICT, sensitivity was 89.3%, specificity was 71, PPV was 84%, and NPV was 80%. In IgM ICT, sensitivity was 88%, specificity was 64.5%, PPV was 78%, and NPV was 80%. Kulkarni *et al*.[14] tested 2104 samples in their study by using the Rapid test kit of same manufacturer. 320 samples came reactive for one or more dengue parameters. Out of 320, 95 were reactive for NS1, 161 were reactive IgM while 9 showed reactive for IgG only. Fifty-five samples showed reactive for more than one dengue parameters.

When comparison was made between the present study and Mehta *et al*.[13] study, the total number of cases tested was 400 and 1628, number of reactive cases were 100 and 563, number of NS1 reactive cases were 50 and 363, number of IgM reactive cases were

50 and 200, thrombocytopenia in total reactive cases was 59 and 220, thrombocytopenia in NS1 reactive cases was 32 and 70, and thrombocytopenia in IgM reactive cases was 27 and 96, respectively.

It has been acknowledged that antigenic cross-reactivity across Flaviviruses is a challenge for the rapid identification of DENV infection. NS1 is frequently employed as an early diagnostic marker for Flavivirus infection, including dengue. A study given by Tan *et al*.[15] demonstrated that when employing DENV NS1 Ag-based fast testing, Zika virus (ZIKV) and other Flaviviruses produce false positive signals designed for dengue diagnosis. Additionally, Sri Masyeni *et al*.[16] showed the inter-reactivity between dengue and Covid-19 patients. Singapore reported cases of dengue and COVID-19 cross-reactivity with Thailand and Indonesia.[15] Cross-reactivity was the reason that some dengue patients had false positive Covid-19 serology results.

The limitations of the study consisted of ICT-based tests are less sensitive in comparison to ELISA. Therefore, comparison of ELISA with ICT-based tests for detection of dengue could have given the chance to comment on the efficacy of ICT. Dengue frequently occurs in remote, underdeveloped places where ICT-based diagnostics may be the only accessible support. ICT is a great instrument for managing this potentially lethal, epidemic-prone infection that has emerged as a significant public health issue in our nation because of its simplicity, speed, and dependability. Hence, application of both the tests for detection of dengue will enable primary care physicians for early diagnosis and treatment for patients detected with dengue consequently saving and enhancing the quality of life.

Conclusion

The study concludes that when comparison of NS1 and IgM using enzyme-linked immunosorbent assay and immunochromatography test for detection of dengue was performed, both the techniques demonstrated positive results. Early diagnosis or screening by Immunochromatography test and confirmation by ELISA would help in the early treatment and prevention of complications of dengue fever. In all clinically suspected samples, where the NS1 or IgM showed positive or negative result by immunochromatographic method, confirmation by ELISA should be performed, as it is a gold-standard method for detection of dengue.

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Conflicts of interest

There are no conflicts of interest.

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Quick Response Code:

Comparative assessment of commercial enzyme-linked immunosorbent assay & rapid diagnostic tests used for dengue diagnosis in India

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***Background & objectives*: Dengue diagnosis is routinely carried out by detection of dengue virus (DENV) antigen NS1 and/or anti-DENV IgM antibodies using enzyme-linked immunosorbent assays (ELISAs) and rapid diagnostic tests (RDTs). This study was aimed at evaluation of quality of diagnostic assays currently in use in India for the identification of DENV infection.**

***Methods*: During 2016 dengue season (July-November) in Pune, India, comparative assessment of a few immunoassays was undertaken using (*i*) WHO-approved Panbio-Dengue-Early-(NS1)-ELISA and Panbio-Dengue-IgM-Capture-ELISA as reference tests, and (*ii*) Bayesian latent class analysis (BLCA) which assumes that no test is perfect. The assays included J.Mitra-Dengue-NS1-Ag-MICROLISA (JME-NS1), J.Mitra-Dengue-IgM-MICROLISA (JME-IgM), and two RDTs, namely, J.Mitra-Dengue-Day-1-Test (JM-RDT) and SD-BIOLINE-Dengue-Duo (SDB-RDT). Serum samples from patients seeking dengue diagnosis (n=809) were tested using the diagnostic kits. The presence of NS1 and/or IgM was taken as evidence for dengue-positive diagnosis.**

***Results*: Panbio-NS1/IgM-ELISAs identified 38.6 per cent patients as dengue positive. With Panbio-ELISA as reference, all the tests were less sensitive for IgM detection, while for NS1, JM-RDT was less sensitive. For combined diagnosis (both markers), sensitivity of all the tests was low (55.7-76.6%). According to BLCA, Panbio-ELISA was 84 per cent sensitive for NS1, 86 per cent specific for IgM and 87 per cent specific for combined diagnosis. Accordingly, performance of the other tests was substantially improved with BLCA; however, sensitivity of both the RDTs for IgM detection remained unacceptable. The NS1 ELISAs and RDTs detected all four DENV serotypes, JME being most efficient. All IgM tests exhibited higher sensitivity in secondary infections.**

***Interpretation & conclusions*: These results confirmed superiority of ELISAs, and testing for both NS1 and IgM markers for dengue diagnosis, and emphasized on improvement in sensitivity of RDTs.**

Key words Dengue virus - enzyme-linked immunosorbent assay - IgM - NS1 - rapid diagnostic test

Dengue, a mosquito-borne viral disease caused by infection with any of the four serotypes of dengue virus (DENV 1-4), is a major public health problem worldwide and is endemic/hyperendemic in India[1,2].

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DENV infection is diagnosed by detection of the virus (virus isolation and immunofluorescence assay), viral RNA [reverse transcriptase-polymerase chain reaction (RT-PCR)] and viral antigen (NS1) or anti-DENV IgM antibodies (immunoassays) in acute-phase patient serum[3]. On account of NS1/IgM dynamics during infection, variations among serotypes and occurrence of secondary infections, dengue diagnosis remains complicated and challenging. Timely diagnosis is important for clinical management of patients, surveillance and prevention/control of epidemics.

Immunoassays for NS1 and IgM offer a convenient format for dengue diagnosis, and several enzyme-linked immunosorbent assay (ELISA) and rapid diagnostic tests (RDTs) are commercially available. Performance of such diagnostic kits has been assessed globally using well-characterized serum panels[4-9]. In a multicountry evaluation by the World Health Organization and others[6-8], eight NS1/IgM ELISAs and seven RDTs were assessed, of which two NS1 and four IgM ELISAs were approved for patient diagnosis while RDTs were recommended only for outbreak confirmation in resource-restricted countries[6-8]. India was not part of this assessment.

The present study was aimed at evaluation of currently used diagnostic assays in the identification of DENV infections caused by recently circulating strains in Indian population. Such an evaluation would require a well-characterized panel of recently collected serum samples. In the absence of such an exclusive panel, testing of a large number of samples from suspected dengue patients was done using different kits and the results were compared with a reference test. One of the WHO-approved ELISAs[6,7] Panbio ELISA was included as a reference test. Às this reference test itself is not 100 per cent specific and sensitive[6,7], an unbiased Bayesian latent class analysis (BLCA) was carried out for determination of accuracy of diagnostic tests in the absence of a gold standard[10,11].

Material & Methods

This study was conducted at the department of Communicable Diseases, Interactive Research School for Health Affairs (IRSHA), departments of Medical Microbiology and Immunohaematology & Blood Transfusion, Bharati Medical College and Research Center, Pune, India, during the 2016 dengue season (July-November) in Pune, India. A total of 809 patients suspected to have DENV infection were included. Blood sample (4-5 ml) was collected within one week of symptom onset; exact day was recorded only for 300 patients. 434 patients were referred by a private tertiary care hospital, Bharati Hospital and Research Centre and tested at the hospital's laboratory (accredited by National Accreditation Board for Testing and Calibration Laboratories) using Dengue Day 1 Test (JM-RDT, J.Mitra and Co. Pvt. Ltd., New Delhi, India). The remaining 375 patients were tested at two private laboratories using SD BIOLINE Dengue Duo (SDB-RDT, Standard Diagnostics, Inc., Republic of Korea). For 127 of these patients, a repeat ELISA test was requested by the clinicians and accordingly was carried out at the respective laboratories using J.Mitra Dengue NS1 Ag MICROLISA (JME-NS1) and J.Mitra Dengue IgM MICROLISA (JME-IgM).

Left-over serum samples from these patients (n=809) were collected and brought to the laboratory at department of Communicable Diseases, IRSHA, and stored in at least two aliquots at −80°C, till testing. These samples were retested using JME-NS1, JME-IgM and the Panbio Dengue Early (NS1) ELISA (PBE-NS1) and Panbio Dengue IgM Capture ELISA (PBE-IgM) (Alere Inc., Australia). For comparing RDTs, 375 samples which were tested previously by SDB-RDT, were retested by using JM-RDT. However, the 434 samples tested earlier by JM-RDT could not be retested using SDB-RDT due to insufficient sample quantity.

Patients positive for NS1 and/or IgM (Panbio ELISA) were diagnosed as dengue-positive. Based on quantity, samples of 192 dengue-positive patients were subjected to Panbio Dengue IgG Capture ELISA (Alere Inc., Australia), for differentiating primary and secondary infections. As per manufacturer's instructions, samples showing Panbio units >22 were categorized as indicative of secondary infection. Among these, patients with IgM/IgG absorbance ratio of >1.2 were classified as primary dengue, and those with ratio <1.2 as secondary dengue, as per the WHO criteria[12].

Detection of dengue virus (DENV) RNA in NS1-discordant samples: Twenty one samples showing discordant results in NS1 ELISA were subjected to detection of DENV RNA using RT-PCR, as described previously[13].

Serotyping of NS1-positive samples: Fifty two NS1-positive serum samples serotyped previously[13] were included for assessing efficiency of the diagnostic tests in detecting different serotypes.

The study was approved by the Ethics Committee (No. IEC/2017/04) of the Bharati Vidyapeeth (Deemed to be University), Pune.

Statistical analysis

Panbio ELISAs as reference tests: Sensitivity, specificity, positive and negative predictive values (PPV & NPV) of the diagnostic tests were assessed against the PBE-NS1 and PBE-IgM, respectively for NS1 and IgM detection. Agreement between the two tests was measured in terms of Kappa statistic. Uncertainty was expressed by 95 per cent confidence intervals (95% CI). Proportions were compared using McNemar Chi-square test or *Z*-test as appropriate. The analyses were conducted using RStudio version 3.4.1 (RStudio, Inc., Boston, MA, USA).

Bayesian latent class analysis (BLCA): Bayesian approach was used for creating latent class models based on the outcomes of all laboratory test results. The model building was done using expectation maximization algorithm on each type of laboratory test. The algorithm was optimized on the basis of lowest values of Akaike information criterion[14] and Bayesian information criterion[15]. The models were evaluated with metrics such as sensitivity, specificity, PPV and NPV. The analysis was done using RStudio version 3.5.1 (RStudio, Inc., MA, USA) using BayesLCA (*https://cran.r-project.org/web/packages/BayesLCA/index.html*) and epiR (*https://cran.r-project.org/web/packages/epiR/index.html*) packages.

Results

Detection of dengue virus (DENV) infection among suspected dengue patients: Table I describes results of individual tests for NS1 and IgM. For each patient sample, detection of NS1 and/or IgM was considered as the evidence for dengue-positive diagnosis.

Enzyme-linked immunosorbent assay (ELISAs): Of the 809 samples tested, PBE-NS1 and JME-NS1 detected NS1 in 158 (19.5%) and 208 (25.7%) samples, respectively. For IgM detection, 231 (28.6%) and 144 (17.8%) samples were tested positive, respectively, by PBE-IgM and JME-IgM. Based on NS1 and/or IgM positivity, 312 (38.6%) and 275 (34.0%) patients were diagnosed as dengue positive, respectively, using PB and JM ELISAs.

Rapid diagnostic tests: JM-RDT identified 181 of 809 (22.4%) samples as NS1 reactive, 89 (11.0%) as IgM positive and 226 (27.9%) patients were diagnosed as dengue positive. SDB-RDT detected 101 (26.9%) and 76 (20.3%) of 375 as positive, respectively, for NS1 and IgM, and 143 (38.1%) patients were classified as dengue positive (Table I).

Comparison of diagnostic tests and test formats

ELISAs: Almost perfect agreement was noted among the ELISAs for NS1 detection (93.3%, Kappa value: 0.81) (Table II). For IgM, moderate agreement (84.8%, Kappa value: 0.58) was observed, and for combined diagnosis (NS1 and/or IgM), substantial agreement (86.5%, Kappa value: 0.71) was noted.

As the proportion of NS1 positives was high with JME-NS1 (208 vs. 158), and that of IgM with PBE-IgM (231 vs. 144), further analyses of the discordant samples were undertaken. Of the 52 samples reactive for NS1 by JME-NS1 alone, 31 were positive for IgM by PBE-IgM, while DENV RNA was detected in three of the remaining 21 samples by RT-PCR. Likewise, of the 105 PBE-IgM alone positive samples, 34 were positive for NS1 by JME-NS1, of which 30 were also positive by PBE-NS1.

Rapid diagnostic tests: Agreement among the RDTs was 88.5 per cent (Kappa value: 0.72) for NS1, 74.9 per cent (Kappa value: 0.18) for IgM and 78.7 per cent (Kappa value: 0.55) for combined diagnosis (Table II). Both RDTs showed good agreement with Panbio-ELISA for NS1, but not for IgM or combined diagnosis.

Table I. Diagnosis of dengue virus infection using the evaluated tests

Diagnostic test	Total number of serum samples tested	Number of samples positive for DENV infection		
		NS1, n (%)	IgM, n (%)	Combined diagnosis NS1 and/or IgM, n (%)
Panbio ELISA	809	158 (19.5)	231 (28.6)	312 (38.6)
J.Mitra ELISA	809	208 (25.7)	144 (17.8)	275 (34.0)
J.Mitra RDT	809	181 (22.4)	89 (11.0)	226 (27.9)
SD BIOLINE RDT	375	101 (26.9)	76 (20.3)	143 (38.1)

RDT, rapid diagnostic test; DENV, dengue virus; ELISA, enzyme-linked immunosorbent assay; SD, standard diagnostics

Comparison of the performance characteristics of diagnostic tests

Panbio ELISA as the reference test: For NS1 detection, JME-NS1 showed significantly higher sensitivity (98.7%) and NPV (99.7%) as compared to the RDTs (sensitivity: 87.3-93.1%, NPV: 96.8-97.4%) (P<0.05) (Table III). The specificity and PPV of JME-NS1 and JM-RDT was lower than SDB-RDT (P<0.05). With PBE-IgM as reference, sensitivity of the remaining IgM tests was markedly low: 54.5 per cent (JME-IgM), 34.4 per cent (SDB-RDT) and 22.5 per cent (JM-RDT) (Table III). JME-IgM showed significantly higher

Table II. Comparison of the tests evaluated with the standard test (Panbio ELISA) for diagnosis of dengue virus infection

Panbio ELISA and J.Mitra ELISA				
Panbio ELISA	J.Mitra ELISA	Number of serum samples		
		NS1	IgM	Combined diagnosis (NS1 and/or IgM)
Positive	Positive	156	126	239
Positive	Negative	2	105	73
Negative	Positive	52	18	36
Negative	Negative	599	560	461
Total		809	809	809
Agreement between the tests				
Per cent agreement		93.3	84.8	86.5
Kappa value (95% CI)		0.81 (0.76-0.86) (P<0.05)	0.58 (0.51-0.65) (P<0.05)	0.71 (0.66-0.76) (P<0.05)

Panbio ELISA, J.Mitra RDT and SD BIOLINE RDT					
Panbio ELISA	J.Mitra RDT	SD BIOLINE RDT	Number of serum samples		
			NS1	IgM	Combined diagnosis (NS1 and/or IgM)
Positive	Positive	Positive	82	22	102
Positive	Positive	Negative	2	14	15
Positive	Negative	Positive	13	44	30
Positive	Negative	Negative	5	112	90
Negative	Positive	Positive	4	2	5
Negative	Positive	Negative	26	28	29
Negative	Negative	Positive	2	8	6
Negative	Negative	Negative	241	145	98
Total			375	375	375
Agreement with Panbio ELISA test					
Per cent agreement			SD: 96.5 J.Mitra: 87.2	SD: 63.7 J.Mitra: 50.4	SD: 69.1 J.Mitra: 58.9
Kappa value (95% CI)			SD: 0.91 (0.86-0.96) (P<0.05) J.Mitra: 0.69 (0.60-0.77) (P<0.05)	SD: 0.28 (0.19-0.38) (P<0.05) J.Mitra: 0.02 (0.0-0.12) (P>0.05)	SD: 0.42 (0.33-0.50) (P<0.05) J.Mitra: 0.22 (0.12-0.31) (P<0.05)
Agreement among the RDTs					
Per cent agreement			88.5	74.9	78.7
Kappa value (95% CI)			0.72 (0.64-0.80) (P<0.05)	0.18 (0.04-0.33) (P<0.05)	0.55 (0.47-0.64) (P<0.05)

Table III. Comparison of performance characteristics of dengue diagnostic tests

Test	Marker	Panbio ELISA as reference test, % (95% CI)				Bayesian latent class analysis, % (95% CI)			
		Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
Panbio ELISA[a]	NS1	100	100	100	100	84 (78-89)	100 (99-100)	99 (96-100)	96 (94-97)
Panbio ELISA[a]	IgM	100	100	100	100	99 (95-100)	86 (83-89)	60 (54-67)	100 (99-100)
Panbio ELISA[a]	Combined	100	100	100	100	97 (94-99)	87 (84-90)	77 (72-81)	98 (97-99)
J.Mitra ELISA[a]	NS1	98.7 (97.0-100)	92.0 (89.9-94.1)	75.0 (69.1-80.9)	99.7 (99.2-100)	100 (98-100)	96 (95-98)	89 (84-93)	100 (99-100)
J.Mitra ELISA[a]	IgM	54.5 (48.1-61.0)	96.9 (95.5-98.3)	87.5 (82.1-92.9)	84.2 (81.4-87.0)	91 (85-95)	98 (96-99)	89 (83-94)	98 (97-99)
J.Mitra ELISA[a]	Combined	76.6 (71.9-81.3)	92.8 (90.5-95.0)	86.9 (82.9-90.9)	86.3 (83.4-89.2)	100 (99-100)	95 (93-97)	90 (86-93)	100 (99-100)
J.Mitra RDT[a]	NS1	87.3 (82.2-92.5)	93.4 (91.5-95.3)	76.2 (70.0-82.4)	96.8 (95.4-98.2)	90 (85-94)	98 (96-99)	92 (87-96)	97 (96-98)
J.Mitra RDT[a]	IgM	22.5 (17.1-27.9)	93.6 (91.6-95.6)	58.4 (48.2-68.7)	75.1 (72.0-78.3)	38 (30-47)	95 (93-96)	61 (50-71)	88 (85-90)
J.Mitra RDT[a]	Combined	58.3 (52.9-63.8)	91.1 (88.6-93.6)	80.5 (75.4-85.7)	77.7 (74.3-81.1)	74 (68-79)	92 (90-94)	81 (75-86)	89 (86-91)
SD RDT[b]	NS1	93.1 (88.2-98.0)	97.8 (96.1-99.5)	94.1 (89.4-98.7)	97.4 (95.6-99.3)	94 (88-98)	100 (98-100)	99 (95-100)	98 (95-99)
SD RDT[b]	IgM	34.4 (27.7-41.1)	94.5 (91.2-97.8)	86.8 (79.2-94.4)	57.9 (52.3-63.5)	58 (49-67)	98 (96-100)	95 (87-99)	83 (78-87)
SD RDT[b]	Combined	55.7 (49.4-62.0)	92.0 (87.5-96.5)	92.3 (87.9-96.7)	54.7 (48.3-61.1)	94 (88-97)	95 (91-97)	92 (86-96)	96 (93-98)

[a]Number of samples tested=809; [b]Number of samples tested=375. PPV, positive predictive value; NPV, negative predictive value

Table IV. Detection of primary and secondary dengue virus infections by the evaluated tests

Type of infection	Number of samples positive (%)												
	No. of samples	Panbio ELISA			J.Mitra ELISA			J.Mitra RDT			No. of samples	SD BIOLINE RDT		
		NS1	IgM	NS1 and/or IgM	NS1	IgM	NS1 and/or IgM	NS1	IgM	NS1 and/or IgM		NS1	IgM	NS1 and/or IgM
Primary	113	72 (63.7)	65 (57.5)	113 (100)	80 (70.8)	33 (29.2)	91 (80.5)	67 (59.3)	14 (12.4)	72 (63.7)	93	51 (54.8)	25 (26.9)	63 (67.7)
Secondary	79	40 (50.6)	73 (92.4)	79 (100)	51 (64.6)	53 (67.1)	67 (84.8)	43 (54.4)	19 (24.1)	47 (59.5)	73	38 (52.1)	23 (31.5)	44 (60.3)
Total	192	112	138	192	131	86	158	110	33	119	166	89	48	107

Table V. Influence of dengue virus serotype on NS1 detection by different diagnostic tests

DENV serotype	Number of samples positive (%)					
	No. of samples	Panbio ELISA	J.Mitra ELISA	J.Mitra RDT	No. of samples	SD BIOLINE RDT
DENV-1	11	10 (90.9)	11 (100)	8 (72.7)	2	2 (100)
DENV-2	20	19 (95.0)	20 (100)	16 (80.0)	9	7 (77.8)
DENV-3	12	12 (100)	12 (100)	12 (100)	3	3 (100)
DENV-4	9	8 (88.9)	9 (100)	7 (77.8)	5	5 (100)
Total	52	49 (94.2)	52 (100)	43 (82.7)	19	17 (89.5)

sensitivity, specificity, PPV and NPV than both RDTs (P<0.05). For combined diagnosis based on both the markers, JME showed higher sensitivity (76.6%) and NPV (86.3%) than both the RDTs (P<0.05). In terms of PPV, the performance of SDB-RDT (92.3%) was superior than both JME-NS1 and JM-RDT (P<0.05). No significant difference was observed in the specificity of different tests.

Bayesian latent class analysis (BLCA): Panbio-ELISA showed a sensitivity of 84 per cent (NS1) and specificity of 86 per cent (IgM) (Table III). For combined diagnosis, a sensitivity of 97 per cent and specificity of 87 per cent were recorded. In relation to these findings, performance of other tests was substantially improved with BLCA. However, sensitivity of both the RDTs remained unacceptable for IgM detection. In spite of the low sensitivity of its IgM component, the SDB-RDT showed comparable performance with JME and was superior to PBE for combined diagnosis.

Detection of primary and secondary dengue virus infections: Among the 113 patients with primary dengue infection, 72 (63.7%) and 80 (70.8%) were NS1 positive, respectively, by PBE-NS1 and JME-NS1 (Table IV). For IgM detection, 65 (57.5%) and 33 (29.2%) were positive, respectively, by PBE-IgM and JME-IgM. The RDTs exhibited lower sensitivity for NS1 (54.8-59.3%), IgM (12.4-26.9%) and combined diagnosis (63.7-67.7%) (Table IV). Of the 79 patients with secondary dengue, 40 (50.6%) and 73 (92.4%) were positive, respectively, by PBE-NS1 and PBE-IgM. NS1 was detected by JME-NS1, JM-RDT and SDB-RDT in 51 (64.6%), 43 (54.4%) and 38 (52.1%) patients, respectively (Table IV). For IgM testing and combined diagnosis, all the tests were less sensitive than Panbio ELISAs.

Performance of the individual tests was compared during primary and secondary dengue infection (Table IV). For IgM detection, all the tests showed higher sensitivity in secondary infections when compared to primary infections (P<0.05). For NS1 and combined diagnosis, no significant difference emerged in the detection of primary and secondary infections by any of the tests.

Comparative detection of infection with different DENV serotypes: Among the ELISAs, JME-NS1 detected all infections with each of the four DENV serotypes (52/52), while PBE-NS1 missed one each of DENV-1, DENV-2 and DENV-4 (49/52) (Table V). Despite detecting all DENV-3 infections (12/12), JM-RDT was significantly less sensitive than JME-NS1 (P<0.006), suggestive of less efficiency in detecting the other three serotypes. Although the number of samples tested by SDB-RDT was small (n=19), it was noted that the RDT identified all of the DENV-1, DENV-3 and DENV-4 infections, while two of DENV-2 serotypes escaped detection (7/9) (Table V).

Discussion

This study compared performance of two RDTs and one ELISA used for dengue diagnosis in Pune, India. One of the WHO-approved kits, Panbio-ELISA, was selected as best possible option for dengue diagnosis for reference-based analysis (RBA). To overcome the bias introduced by the use of an imperfect reference test, the data were also analyzed using BLCA, which allowed comparative evaluation of different diagnostic tests, even in the absence of a gold standard. During the 2016 dengue season in Pune, 38.6 per cent of 809 suspected dengue patients seeking diagnosis were confirmed as dengue-positive by Panbio ELISAs, reflecting the degree of dengue positivity in Pune, and thus providing an opportunity to compare the performance of different diagnostic tests in a field setting. Importantly, all four DENV serotypes were circulating[13], facilitating analysis of serotype-specific performance.

First, the two ELISAs were compared (PBE & JME). RBA revealed JME to be less specific for NS1 and less sensitive for IgM and combined diagnosis that was also reflected in its PPV (NS1) and NPV (IgM). However, with BLCA, PBE was only 84 per cent sensitive for NS1 and 86 per cent specific for IgM, while its PPV for IgM and combined diagnosis appeared to be low (60 and 77%, respectively). Consequently, BLCA-estimated sensitivity of JME for all markers appeared to be higher than that determined by RBA. For combined diagnosis, PPV and NPV for Panbio ELISAs (JME) were 77 per cent (90%) and 98 per cent (100%), respectively. Thus, when BLCA was used, the performance of JME appeared to be superior. It is to be noted that a substantial proportion of JME-NS1 alone positives (~60%) and PBE-IgM alone positives (~30%) were positive, respectively, for IgM (PBE) and NS1 (JME). This suggested true positivity of these discordant samples leading to higher sensitivity of JME-NS1 than PBE-NS1 and that of PBE-IgM than JME-IgM. However, in view of the possibility of non-specific reactions[6,7] and absence of additional tests for confirmation, the validity of these findings remains debatable. These findings emphasize a definite need to test for both NS1 and IgM markers for accurate diagnosis, irrespective of the day of sample collection.

Evaluation of the RDTs employing RBA and BLCA showed comparable results by both analyses for JME-RDT (all 3 markers) and SDB-RDT (NS1 and IgM). Combined diagnosis for SDB-RDT improved substantially when BLCA was used (94% sensitive, 95% specific). The performance of SDB-RDT for combined diagnosis seemed comparable to ELISAs, as per BLCA. Earlier Indian studies employing limited number of stored serum samples have noted good agreement of JM-RDT and SDB-RDT with ELISAs for NS1 detection[16-19]. While confirming these observations, our study showed low sensitivity of the IgM component of both the RDTs. These results were in concurrence with previous reports of poor diagnostic performance of SDB and other RDTs for IgM detection in dengue-endemic regions[20-23]. The need to improve sensitivity of the IgM component of RDTs is obvious.

Another important issue in dengue diagnosis is the efficiency in detecting primary and secondary infections. Contrary to the earlier reports of higher sensitivity of NS1 tests in primary infections[7,24-27], we did not detect any significant difference in NS1 positivity during primary and secondary dengue. Although IgM levels are generally low in secondary infections[28], higher IgM sensitivity was recorded during secondary infections by both ELISAs and RDTs. Similar observations have been reported previously[7] and warrant further investigation to understand the basis for this higher IgM positivity.

Identification of infections by all four DENV serotypes is a major requirement for dengue diagnostic tests. Both ELISAs detected all the four serotypes, although JME-NS1 was more efficient than PBE-NS1. Despite being from the same manufacturer, JM-RDT detected lower proportion of DENV serotypes (except DENV-3) than JME-NS1, probably reflecting influence of the format. Serotype-specific evaluation on a larger sample size would be required for further confirmation of these findings.

In summary, our study confirmed superiority of ELISAs over RDTs and requirement for both NS1 and IgM testing for accurate diagnosis. The results emphasize an urgent need for substantial improvement in RDTs.

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MEDICINE

Evaluation of NS1-Detection-Based Cell Culture Method for Isolation of Dengue Viruses from Clinical Samples

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Abstract

Dengue is the fastest spreading mosquito-borne viral infection across the globe affecting over 40% of world populations. Co-existence of 4 distinct serotypes, disease severity due to secondary infection, and continuous evolution of dengue viruses over the years intensify the need for a better and effective virus surveillance program. In this study, we evaluated NS1 antigen detection method to screen large number of clinical samples for dengue virus isolation. Patient's serum samples were added onto in vitro tissue culture grown C6/36 or Vero cell lines. Seven days post-infections, culture supernatants were harvested for testing of NS1 antigen by ELISA to confirm the presence of dengue viruses. Significantly higher rate of virus isolation was observed in Vero cells at 7 days post-infection in comparison to C6/36 cells. NS1 antigen could be detected earliest at day 3 post-infection in culture supernatants of both C6/36 and Vero cells. Use of Vero cells in a single 35-mm culture dishes resulted in 69.7% DENV isolations from NS1 alone positive samples. The method is economical, easy to perform, and useful in handling large number of clinical samples and will be of value in virus surveillance, especially post-vaccination.

Keywords Dengue virus · Serotypes · Vero · C6/36 · Isolation · Clinical samples

Introduction

Dengue is a mosquito-borne viral disease affecting more than 390 million individuals every year in more than 125 countries across the world [1, 2]. In recent years, dengue infection has spread to non-endemic countries, such as Afghanistan, European Union, Chile, and Japan, where several cases of autochthonous dengue outbreak have been reported [3]. India contributes to 34% of dengue infection, and still, the number of officially reported dengue cases is grossly under-reported [1, 2]. Efforts are therefore needed for accurate diagnosis of dengue infection. The presence of four distinct serotypes and variable circulation of individual serotypes in different areas and over time coupled with continuous evolution of DENV strains necessitate active dengue virus surveillance. Such surveillance is of special significance for dengue infection wherein disease severity is enhanced during secondary dengue infection with a different serotype.

At present, a range of laboratory diagnostic methods are available for dengue diagnosis. Depending on the time lapse between appearance of first clinical symptoms and blood collection, tests for virus/antigen or antibody detection are chosen. These include the following: (a) detection of nonstructural protein 1 (NS1) antigen, (b) detection of viral RNA, (c) isolation of DENV, and (d) detection of anti-dengue IgM and/or IgG antibody in the serum of infected individuals [4]. Of these, RT-PCR continues to be the most commonly used method for serotyping and virological surveillance of DENV [5–8]. Such molecular techniques cannot replace the classical virus isolation methods yielding viruses for in-depth characterization and future studies. An immunofluorescence assay is performed for the detection of viral specific antigens. Usually, few samples are subjected to virus isolation as the methods are time consuming and do not allow simultaneous screening of large number of samples.

With the availability of dengue vaccine in near future, it would be essential to isolate and characterize viruses from immunized or unimmunized suspected dengue patients.

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Requirement of a simple DENV isolation method is eminent. In this study, we evaluated the use of NS1 antigen ELISA as an alternative and convenient approach for cell line–based DENV isolation procedures.

Methods

Clinical Samples

A total of 149 serum samples stored at − 80 °C were used for virus isolations. These represented 113 primary dengue (NS1 and/or IgM-anti-DENV positive) and 35 secondary (capture IgG anti-DENV positive) dengue patients confirmed by serology. All the samples were previously tested for NS1 antigen by Dengue NS1 Ag Microlisa kit (Cat no. IR031096, J Mitra & Company Pvt. Ltd., New Delhi, India). Panbio Dengue IgM and IgG capture ELISA kits (Alere Product code 01PE10 and 01PE20) were used to determine primary and secondary dengue infections. Primary dengue infections were those that did not show any detectable levels of serum IgM and IgG (seronegative) and those that had IgM > IgG by a ratio ≥ 1.2. Secondary dengue was defined as samples that had IgG only or IgG/IgM ≥ 1.2.

Virus Isolation

Mosquito, C6/36 and mammalian, Vero cell lines were used for virus isolation. C6/36 cell line was procured from National Center for Cell Science, reference laboratory in India. Vero cell line (CCL-81) was procured from ATCC, USA. One day prior to infection, 1.5×10^6 C6/36 and 1×10^6 Vero cells per well were seeded in 35-mm culture dishes in complete media (MEM supplemented with 10% fetal bovine serum (FBS) along with penicillin and streptomycin). Culture dishes containing C6/36 cells were incubated at 28 °C in BOD incubator, while Vero cells were incubated at 37 °C in 5% CO_2 incubator. 80–90% confluent cells were infected with 500 µl of undiluted (neat), 1:10 and 1:100 diluted serum samples, in MEM media containing 2% FBS and incubated for 2 h to allow virus adsorption. After incubation, virus inoculum was removed and cells were washed with MEM media. Fresh 2 ml of MEM medium supplemented with 2% FBS was added to each well. C6/36 culture dishes were incubated further at 28 °C in BOD incubator, while Vero culture dishes were incubated at 37 °C in 5% CO_2 incubator. For the comparative time kinetics study in two cell lines, culture supernatants were harvested at day 3, 5, 7, and 10 post-infections to detect NS1 antigen by ELISA. Otherwise, from NS1 antigen positive wells, culture supernatants were harvested at day 7 post-infections, centrifuged at 2000 rpm for 10 min at 4 °C, and aliquots were stored at − 80 °C.

NS1 Antigen ELISA from Culture Supernatants

Dengue virus isolation was done based on the detection of NS1 antigen in culture supernatants by ELISA method. Dengue NS1 Ag Microlisa (Cat no. IR031096, J Mitra & Company Pvt. Ltd., New Delhi, India) was used as per manufacturer's protocol.

Plaque Assay from Virus Quantification

Dengue virus titer was determined by plaque assay. Briefly, 1 day prior to infection, 1×10^5 Vero cells were seeded in each well of 24-well plate and incubated at 37 °C in 5% CO_2 incubator. The following day, 200 µl of 10-fold serially diluted virus samples was added onto 80–90% confluent cell monolayer. Plates were incubated for 2 h at 37 °C to allow virus adsorption. After incubation, cell monolayer was covered with overlay media containing 1% Aquacide-II (Carboxymethyl cellulose, CMC, Calbiochem). Plates were further incubated at 37 °C in 5% CO_2 incubator for 3 days. After 3 days post-infections, overlay media was removed and cells were fixed with 3.7% formaldehyde solution for 30 min at room temperature (RT). Plates were washed 3 times with phosphate buffer saline (PBS), and cells were permeabilized using 0.2% triton X-100 solution for 5 min at RT. Cells were washed thrice with PBST (0.02% Tween-20 in PBS). Then, 1:500 diluted primary antibody, HB112 (D1-4G2-4-15 clone, pan-flavivirus mouse monoclonal antibody, ATCC, USA), was added to the cells and incubated for 2 h at RT. Plates were washed 3 times with PBST. Then, secondary antibody (Goat anti-mouse IgG HRP, 1:1500 dilution) was added onto the cells and incubated for 1 h at RT. Plates were washed 2 times with PBST and 3 times with PBS. True Blue Peroxidase substrate (KPL, Sera Care, MA, USA) was added to each well and incubated in dark for 30 min to develop blue colored virus infected foci. Plates were washed with distilled water and viral foci were counted in each well. Virus titer was calculated as plaque forming units (pfu) per ml.

Immunofluorescence Assay

One day prior to infection, each well of 8-well chamber slides (Nunc) was seeded with Vero cells at 1×10^4 cells/well in complete media. Cells were infected with serum samples using MEM media supplemented with 2% FBS as described above. After incubation, virus inoculum was removed and cells were washed twice with MEM media. Fresh 500 µl of MEM medium supplemented with 2% FBS was added to each well. Culture supernatants were harvested at day 3, 5, 7, and 10 post-infections, and cell monolayer was fixed with 3.7% formaldehyde solution for 20 min. After fixing the cells were

washed twice with PBST (0.02% Tween-20 in PBS) and permeabilized with 0.2% triton X-100 in PBS for 5 min. After incubation, the cells were washed with PBST. Blocking was done by incubating the cells with 3% BSA for 1 h at RT. Cells were washed twice with PBST and incubated with primary antibody, 1:250 diluted B1427M (Anti Dengue virus NS1 glycoprotein antibody, Abcam) for 2 h at RT. Then, cells were washed twice with PBST and incubated in dark with 1:500 diluted Alexa-fluor Plus 488 conjugated Goat anti-mouse IgG (H+L) secondary antibody (Molecular Probes, Thermofisher Scientific, USA) for 1 h at RT. Cells were washed twice with PBST. Cell nuclei were counter-stained with 1:10,000 diluted DAPI and incubated in dark for 5 min. Then, cells were washed twice with PBS, mounting fluid was added, and cover slip was mounted onto the chamber slides. Images were acquired using EVOS FLoid Cell Imaging Station (Thermofisher Scientific, USA) at × 20 objective and superimposed digitally for merge images.

Statistical Analysis

T test was performed for paired samples. For time-point kinetics analysis, proportion test was performed to compare number of virus isolates obtained in C6/36 and Vero cells. Spearman correlation rank test was performed to analyze the data between OD values obtained in NS1 ELISA with either log10 transformed virus titers or percentage of NS1 protein expressing cells.

Results

For virus isolation, patient's serum samples were added onto in vitro tissue culture grown C6/36 or Vero cell lines and culture supernatants were harvested for testing of NS1 antigen by ELISA to confirm the presence of dengue viruses.

Time Kinetics Study of Virus Isolation in C6/36 and Vero Cell Lines

Initially, 43 acute phase sera from dengue patients were used for virus isolation in C6/36 cells. Undiluted and 1:10 diluted serum samples were found to be toxic to the cells. Therefore, 1:100 diluted serum samples were used for virus isolation in C6/36 cells. Based on NS1 ELISA, DENV isolation rates were 2.3% ($n = 1$) at day 3, 6.97% ($n = 3$) at day 5, 13.9% ($n = 6$) at day 7, and 34.9% ($n = 15$) at day 10 (Fig. 1a). The respective isolation rates in Vero cells using same samples were 11.6% ($n = 5$) at day 3, 18.6% ($n = 8$) at day 5, and 37.2% ($n = 16$) at day 7. No further increase in the number of isolates was observed at day 10 in Vero cells (Fig. 1b). At day 7, a significantly higher rate of virus isolation was recorded in Vero cells (16/43) than in C6/36 cells (6/43) ($p = 0.026$).

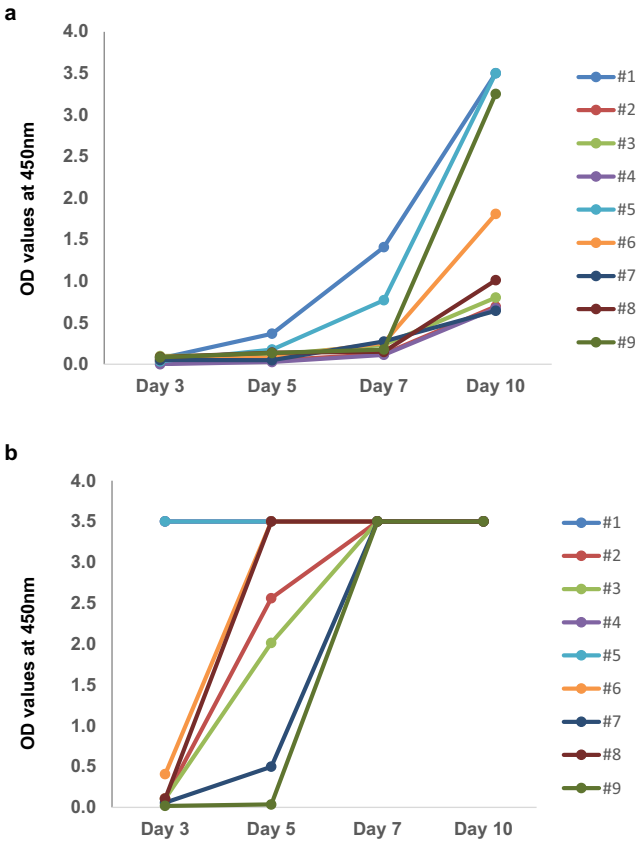


Fig. 1 NS1 ELISA positivity in culture supernatants on addition of patient's serum samples at different days post-incubation in (**a**) C6/36 and (**b**) Vero cell lines

Among these, 12 isolates obtained in both C6/36 and Vero cell lines, belonged to DENV-1 (1), DENV-2 (3), and DENV-3 (8) serotypes. The virus titers were comparable in isolates either obtained from C6/36 or Vero cells (Fig. 2, $p = 0.61$). Three isolates were only obtained in C6/36 cells and all of them belonged to DENV-3 serotype. Four isolates were only

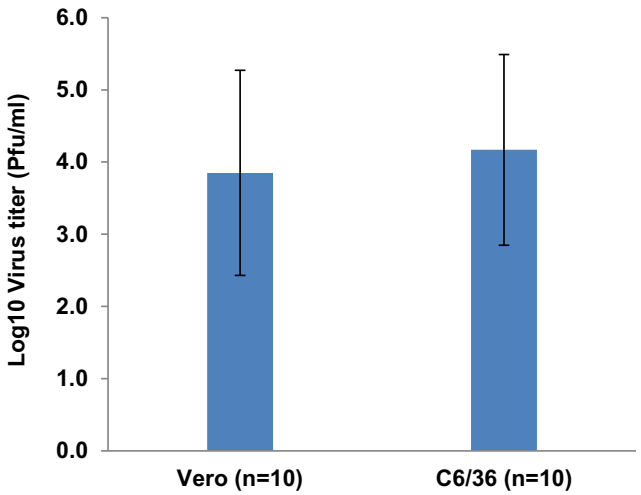


Fig. 2 21pt?>Virus titers of passage no. 1 isolates obtained in Vero and C6/36 cell lines

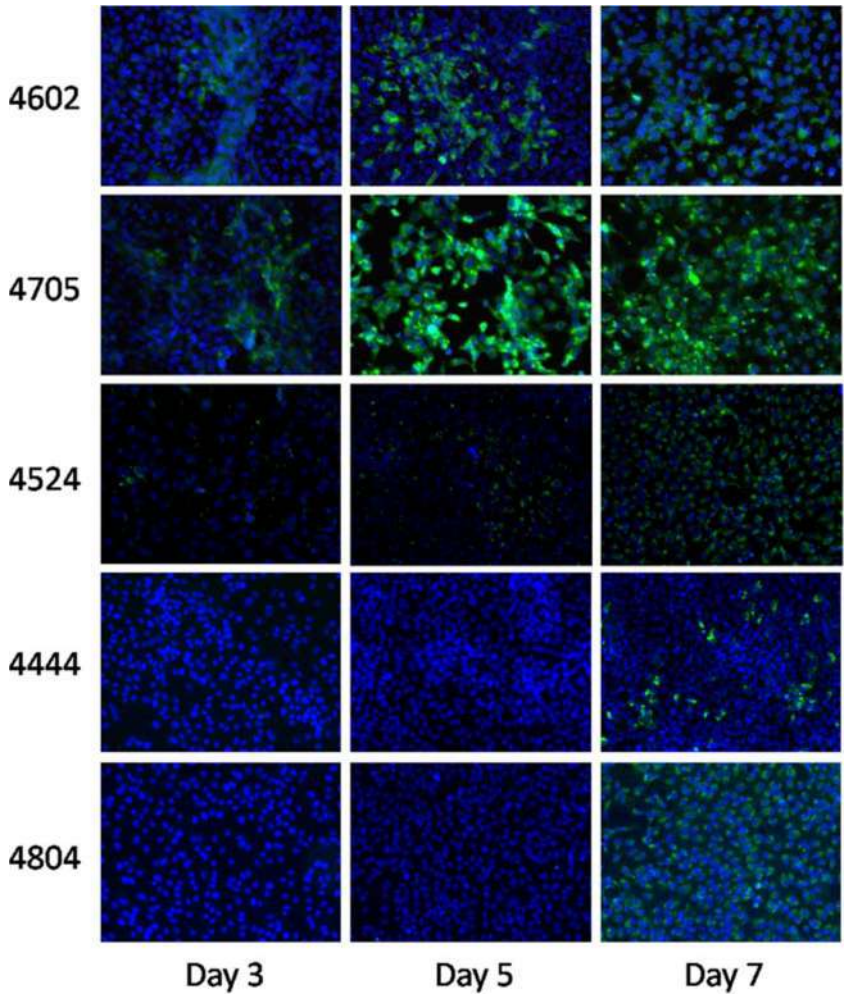


Fig. 3 Immunofluorescence analysis of Dengue specific NS1 protein (green color) in Vero cells at different days post-infections after addition of patient's serum samples. Cell nuclei were stained with DAPI (blue color). Representative images of NS1 expressing cells after incubation with 5 individual patient's serum samples are shown

obtained in Vero cells, of which 1 isolate belonged to DENV-2 and 3 isolates belonged to DENV-3. Based on the observed superiority of Vero cells in DENV isolation, we opted for Vero cell lines for further evaluations.

Use of Single Dilution of Serum Samples for Virus Isolation in Vero Cells

We did not observe any toxicity when 1:10 diluted serum samples were added onto Vero cells allowing us to use concentrated virus with higher probability of isolations. A total of 103 acute phase sera from dengue patients were examined for virus isolation. Of these, 63 (61.2%) serum samples yielded DENV isolations as indicated by NS1 ELISA at day 7 post-infection. These virus isolates were obtained from 62/89 NS1+IgM− (69.7%) and 1/14 NS1+IgM+ (7.1%) samples collected at 2–5 days post-onset of fever. Majority of the isolates, 62/82 (75.6%), were from primary dengue infection, and only 1/21 (4.8%) were from secondary dengue cases (Panbio units above 22). The isolates included all the four serotypes with higher frequency of DENV-3 ($n = 28$, 44.4%) and DENV-1 ($n = 26$, 41.3%) followed by DENV-2 ($n = 6$, 9.5%) and DENV-4 ($n = 3$, 4.8%). Rate of virus isolation was much higher among NS1+IgM− and primary dengue infected patients' sera. All the isolates were obtained from dengue patients with warning signs (WS), according to WHO guidelines, 2009 [9].

IFA Confirmation of Virus Isolation

Next, we compared NS1 antigen specific ELISA and immunofluorescence assay (IFA) in identifying dengue viral antigens in Vero cell cultures infected with ten serum samples obtained from dengue patients. After infection with three serum samples, on day 3 post-infection, NS1 antigen was detected in supernatants (ELISA) and in infected cells (IFA) and remained positive till day 7 post-infection. For 2 infected cultures, simultaneous detection by both the methods was seen on day 7 post-infection (Fig. 3). The number of virus infected cells increased gradually with concurrent rise in the OD values in ELISA (Spearman rank correlation coefficient, $r = 0.93$, Fig. 4a). We observed increasing trend of virus titers when

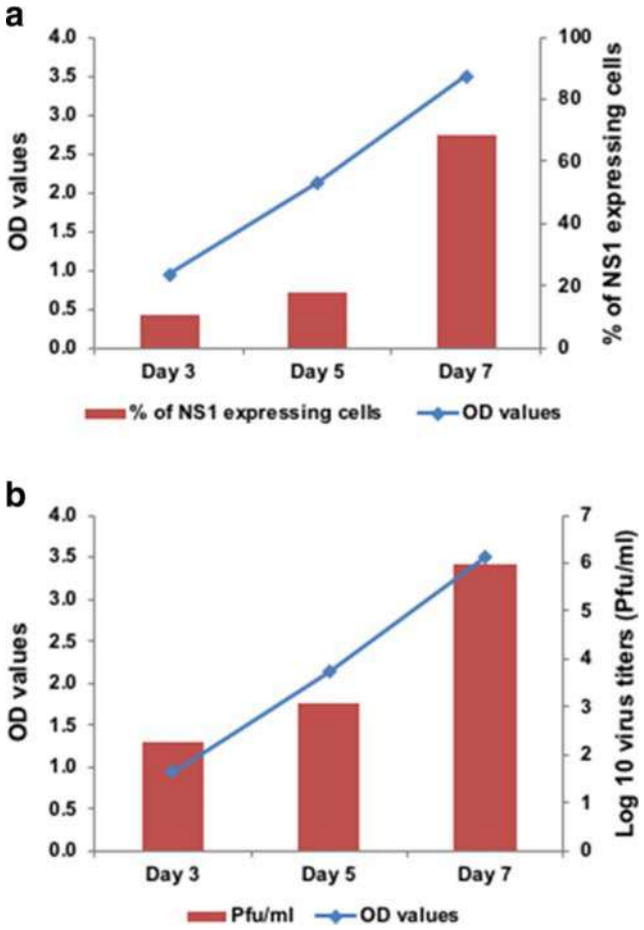


Fig. 4 Correlation of OD values in NS1 antigen ELISA with (**a**) percentage of NS1 expressing cells and (**b**) virus titers (pfu/ml) in culture supernatants at different days post-infections after addition of patient's serum samples

the infectious serum samples were allowed to incubate for longer duration (Spearman rank correlation coefficient, $r = 0.96$, Fig. 4b).

Discussion

Dengue remains a complex viral infection because of the existence of 4 distinct serotypes, secondary infections leading to severe dengue, and continuously evolving dengue viruses. Clearly, active virus surveillance is essential. In view of the possible serious complications of dengue vaccines, this information has become more relevant. Despite the availability of nucleic acid–based tests, virus isolation remains the central method. A simple, cost-effective protocol allowing handling of large number of specimens is essential for more laboratories to be able to undertake this activity. Our study demonstrates that when we use Vero cells for virus isolation even in 35-mm culture dishes and employ ELISA for the detection of NS1 antigen in the cell-culture supernatant as a marker for virus replication, it is possible to screen large number of clinical samples for virus isolation. We could isolate DENV from 61.2% (63/103) of clinical samples. If we restrict virus isolation attempts to only NS1 positives, the isolation rate would be 69.7% (62/89).

The discovery of secretion of NS1 protein by cells supporting DENV replication [10, 11] has revolutionized dengue diagnosis by allowing identification of IgM antibody-negative, virus-positive patients during early disease phase. In view of the ADE response associated with the whole virus-based vaccines, NS1 could be an appropriate vaccine candidate [12]. Secretion of NS1 by mammalian/insect cell lines infected with known dengue virus isolates was shown thereafter [13–15]. However, utility of this observation in isolation of DENV from clinical specimens was not explored. Further, since NS1 protein is highly conserved in all four dengue serotypes, infection with any of the viral serotypes can be identified. In the present series, all the four serotypes were detected. The study confirms earlier findings of direct correlation of proportions of NS1 expressing cells and OD values for NS1 present in culture supernatants as detected by ELISA (Fig. 4a) [15]. Importantly, the OD values correlated with infectious virus titers (Fig. 4b).

We could detect NS1 protein earliest at day 3 post-infection in culture supernatants of both C6/36 and Vero cells suggestive of high viral load in the clinical sample. Our data revealed superiority of Vero cells yielding all the isolations by day 7. This method allows screening of clinical specimens for virus isolation by laboratories without a fluorescent microscope, and hence, the surveillance network can be extended to smaller places as well. However, use of serotype-specific antibodies for IFA allows simultaneous serotyping of isolated DENV. Lack of such possibility is a limitation of NS1-based detection method. Nonetheless, serotype-specific monoclonal antibodies are expensive and PCR-based methods can be applied to virus isolates. Of note, for DENV surveillance of *Aedes albopictus* mosquitoes in Tokyo metropolis, NS1 detection strips were used for the evidence of viral replication [3]. On the other hand, Teramoto et al. (2019) monitored DENV2 replication in BHK-21 and Vero by NS1 protein–based IFA [16]. Thus, utility of NS1 in assessing in -vitro DENV replication is being increasingly recognized; we are providing data for its utility in routine surveillance programs.

Recent observations of Dengvaxia vaccine containing DENV-4-genotype-II exhibiting greater efficacy against vaccine matched strain than circulating DENV4-genotype I virus in subjects who experienced DENV-4 breakthrough infections are noteworthy [17]. Clearly, dengue vaccine efficacy needs to be evaluated with respect to continued evolution of different serotypes. Importantly, DENV strains used in vaccine formulation are composed of genotypes that are extinct and no longer circulate in human populations [18]. Ongoing active virus surveillance remains an integral part of dengue vaccine evaluations and NS1-detection-based isolation method will prove

useful. Use of NS1-based ELISA to differentiate DENV-exposed and unexposed individuals for prevaccination screening during clinical trials was not found to provide added advantage when the performance of ELISA and proportion of severe dengue patients in antibody positive and negative vaccine recipients during follow-up studies were considered [19].

In conclusion, we provide data for utility of NS1-detection-based ELISA for easy identification of DENV isolations from clinical samples and suggest that this method may be applied for extensive DENV surveillance programs including post-vaccination studies.

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Authors' Contributions AS and MG carried out all the experiments including virus infection, plaque assay, ELISA, and immunofluorescence assay. DT was involved in serotyping of virus isolates. ACM and VAA contributed reagents and materials. SS, ACM, and VAA conceived the study and participated in study design, writing and editing the manuscript. SS contributed to data analyses. All authors have read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

Ethical Approval The study was approved by Institutional Ethics Committee of Bharati Vidyapeeth, Pune (IEC/2017/04). Written informed consent was obtained from all subjects.

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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Incidence of lab-confirmed dengue fever in a pediatric cohort in Delhi, India

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Abstract

Background

Our aim was to estimate the overall and age-specific incidence of lab-confirmed dengue fever using ELISA based assays among children 6 months to 15 years in Delhi.

Methods

We enrolled a cohort of 984 children aged 6 months to <14 years in South Delhi and followed-up weekly for fever for 24 months or till 15 completed years of child-age. Households of the enrolled children were geo-tagged. NS1, IgM and IgG assays were conducted using ELISA method to confirm dengue fever in children with ≥3 consecutive days of fever. Molecular typing was done in a subset of NS1 positive cases to identify the circulating serotypes.

Principal findings

We had a total of 1953 person-years (PY) of follow up. Overall, there were 4208 episodes of fever with peaks during June to November. The overall incidence (95%CI) of fever was 215/100 PY (209 to 222). A total of 74/1250 3-day fever episodes were positive for acute dengue fever (NS1 and/or IgM positive). The overall incidence (95%CI) of acute dengue fever was 37.9 (29.8 to 47.6) per 1000 PY; highest among children aged 5 to 10 years (50.4 per 1000 PY, 95% CI 36.5 to 67.8). Spatial autocorrelation analysis suggested a clustering pattern for the dengue fever cases (Moran's Index 0.35, z-score 1.8, p = 0.06). Dengue PCR was positive in 16 of the 24 specimens tested; DEN 3 was the predominant serotype identified in 15/24 specimens.

Conclusions

We found a high incidence of dengue fever among under 15-year children with clustering of cases in the community. DEN 3 was the most commonly circulating strain encountered. The findings underscore the need for development of affordable pre-vaccination screening strategy as well as newer dengue vaccines for young children while continuing efforts in vector control.

Competing interests: The authors have declared that no competing interests exist.

Author summary

South Asian countries especially the Indian subcontinent contributes the highest to the global burden of dengue. The number of dengue cases reported in India is likely an underestimate of the actual disease burden and there is a clear lack in the availability of population-based data on incidence of dengue in India. In our pediatric cohort of 984 children aged 6 months to 15 years from Delhi, India, we found a high incidence of lab-confirmed dengue fever, with the highest burden among 5 to 10 year old children. Dengue fever was observed to be highest in the post-monsoon months with significant clustering of cases in the community. DEN 3 was the most commonly circulating strain encountered. Given the high burden in children, the findings highlight the need for strengthening efforts to developing newer dengue vaccines for younger children.

Introduction

The World Health Organization listed dengue as one of the top 10 potential threats to global health in 2019. Global burden of dengue was estimated to be 104 million (95% CI 64 to 159) cases in 2017 [1] with the highest burden in South Asia of 3546.9 cases (95% Uncertainty interval 2128.5 to 5429.5) per 100,000 population [1]. The number of dengue related deaths, globally, is estimated to increase from 6957 (95% UI 7613 to 30091) in 1990 to 40467 (95% UI 17620 to 49778) in 2017 [1]. Clinically apparent dengue can affect all age groups but is most frequently observed in the age group 5–15 years, globally and the highest mortality is in under-five children [2]. Modeling of surveillance data suggest, India contributes to around one-third of the global burden of symptomatic dengue infections [3].

In India, during last two decades, large and frequent dengue outbreaks and urban to rural spread with increasing proportion of severe cases, along with hyper endemicity in urban areas are reported [4]. In 2017, National Vector Borne Disease Control Program (NVBDCP) reported 188,401 laboratory confirmed cases of dengue and 325 deaths through its network of 646 sentinel surveillance hospitals [5, 6]. It is speculated that the number of dengue cases is highly underreported in India [7]. A recent systematic review in 2018 estimated that the proportion of laboratory confirmed dengue cases on testing of 213,285 clinically suspected patients from 180 studies in India was 38.3% (95% CI 34.8% to 41.8%)[8]. The overall seroprevalence of dengue fever in India based on 7 studies was 56.9% (95% CI 37.5% to 74.4%). The systematic review observed a clear lack in the availability of population-based data on incidence of dengue in India and highlighted the need of community-based studies for burden estimation [8].

Dengue prevention and control has been largely based on principles of vector control. However, currently several new dengue vaccines are in development. In December 2015, the first dengue vaccine (CYD-TDV, or Dengvaxia) was registered and is now licensed in several countries including Mexico and Philippines. The WHO in its 2016 position paper on vaccination against dengue recommends its use only in children 9 years and above in high burden countries. With newer indigenous vaccines being developed, it is imperative to have population-based incidence data on dengue for conducting vaccine studies and designing vaccination strategies. Our study was planned with the primary objective to estimate the incidence of lab-confirmed acute dengue fever using ELISA based assays in children under 15 years of age. In addition, we planned to report the age-categorized incidence in children aged <5 years, 5–10 years, and 10–15 years of age. As an exploratory objective we reported the most common circulating serotype in the population in a subsample.

Methods

Ethics statement

The cohort study was approved by the Ethics Review Committee, Centre for Health Research and Development Society for Applied Studies, New Delhi, India in 2017.

Study population

The study was conducted as a sub-cohort within the Surveillance of Enteric Fever in India (SEFI) cohort among children in Delhi (CTRI/2017/09/009719) [9], in two contiguous blocks of Sangam Vihar, a low-middle income urban and peri-urban neighborhood in the southern district of Delhi [10]. In the study area, more than 80% of the population are Hindu by religion, more than half of the women have secondary school education, with an average household monthly income between 10,000 to 20000 INR, similar to the National average [11].

A door-to-door survey was conducted in the two blocks to line list the households with potentially eligible children. Families were screened to determine likelihood of moving out of the study area and/or adhering to study procedures specially blood specimen collection. Eligible children aged between 6 months and <14 years who were likely to stay in the study area for two years were enrolled consecutively in the cohort from contiguous households until the sample size was attained. All enrolled children were followed up for 24 months or until 15 years of age, whichever was earlier. The upper age limit at enrollment was restricted to <14 years so that each enrolled child in the cohort be followed up for at least 12 months. Prior to screening, written informed consent for participation and storage of specimens for future research was obtained from the parents after explaining study processes. We obtained verbal assent from children aged 7 to 12 years and written assent from children aged above 12 years. (Fig 1).

Acute febrile illness surveillance

We conducted active weekly contacts either through phone or home visit with at least one face-to-face contact every four weeks to collect information on fever, illness or hospitalizations. A monthly mobile recharge of INR 50 was provided to all enrolled families to encourage early telephonic reporting of fever to the study team. Families were provided with a digital thermometer and fever diary card to document temperature. Fever was defined as caregiver reported or a recorded temperature of ≥38˚C (100.4˚F). Upon reporting of fever, our study team visited the participant's home as early as possible. Participant was visited daily during the fever episode. If fever lasted for ≥3 days, the child was referred to the study fever clinic at Hakeem Abdul Hameed Centenary Hospital (HAHCH) for physician evaluation and blood specimen collection (not later than 6 days of fever onset) to test for enteric fever (blood culture) and dengue fever. Management of the fever episode was as per the advice of the physician in the study fever clinic or any other preferred place of care-seeking. In case the participant visited other health facilities for care-seeking, information related to the fever episode was collected during the weekly contacts.

Laboratory methods

Blood specimens were transported to Clinical and Research Laboratories, Society for Applied Studies (CRL SAS), New Delhi, within 2 hours of collection. Serum was separated in CRL SAS and used for the laboratory assays for dengue using the automated EVOLIS Twin Plus system (BioRad, California, USA). Dengue NS1 Ag Microlisa, dengue IgM Microlisa and dengue IgG Microlisa (J. Mitra & Co. Pvt. Ltd., New Delhi, India) kits were used.

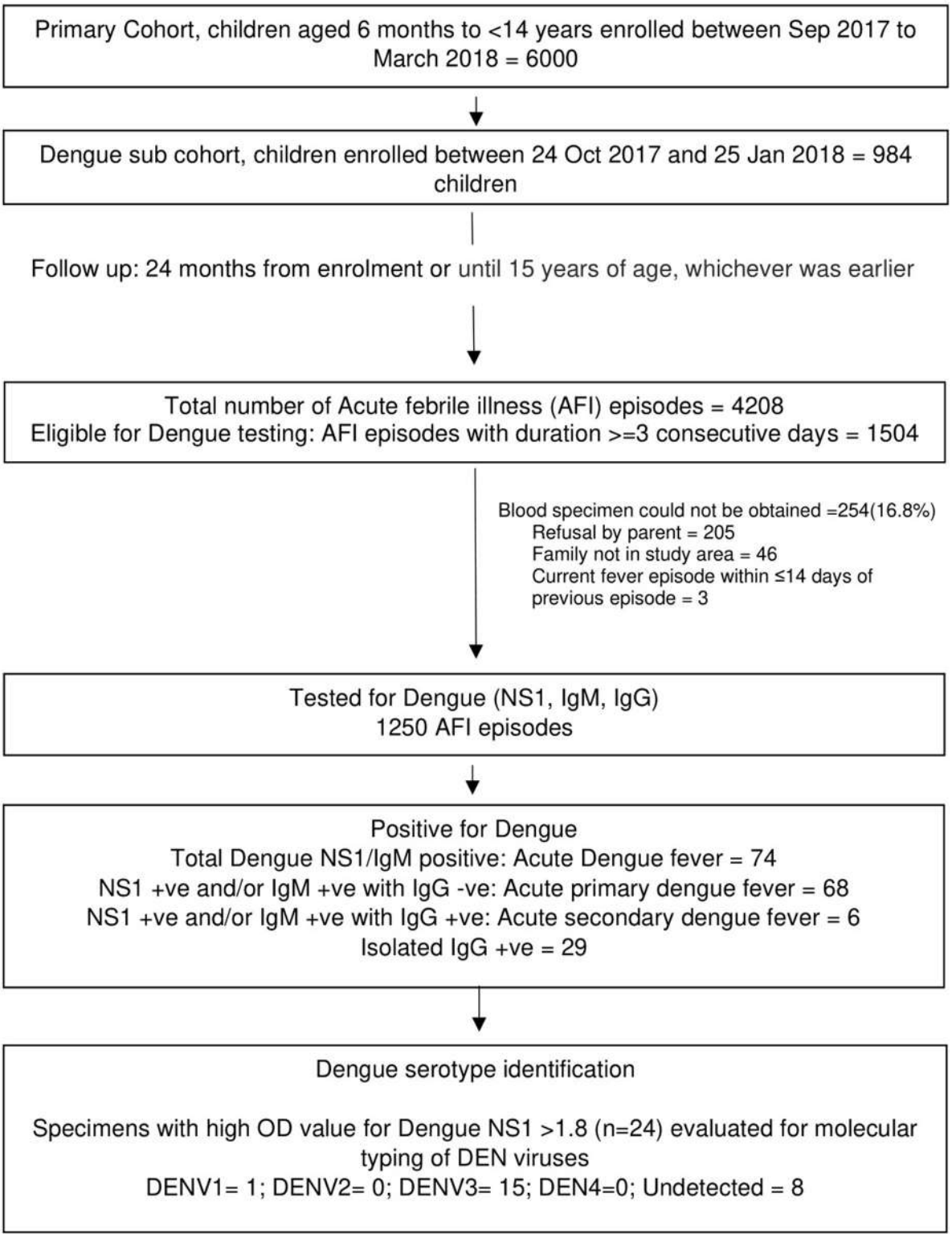


Fig 1. Study Flowchart.

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To study the circulating serotypes of dengue viruses, the serum specimens positive for NS1 antigen with an optical density (OD) value of ≥1.8 were further used for molecular typing. This was based on prior experience that it is difficult to get a positive signal for a specific dengue serotype in multiplex PCR assays in specimens with lower NS1 OD values. The molecular typing of dengue viruses was conducted at the Virology lab of All India Institute of Medical Sciences, New Delhi using the Geno-Sen's dengue 1–4 (Rotor Gene) real time PCR quantitative kit (Genome Diagnostics Pvt. Ltd.).

Data management

Data was collected on tablets using android application package with built-in Geographic Information Systems (GIS) developed in house by the SEFI team and stored in a secured Amazon cloud-based server. Data was monitored using a dashboard-based system; weekly reports were generated and reviewed. A comprehensive audit trail was maintained. Quality assurance was done by an independent team of experts.

Statistical analysis

To capture an incidence of acute dengue fever as low as 3 per 100 person-years (PY) of follow up among children under 15 years of age with 95% confidence, 30% relative precision, and 25% loss to follow up a total of 1841 PY of follow up was deemed to be sufficient. Based on our previous experience and considering each child will contribute to around 1.9 years of follow up, a total of 969 eligible children were deemed to be required.

Analysis was done using STATA 16.0 MP (TX, USA). The Stata command "xtset" was used to declare the dataset to be panel data. The characteristics of the study population was presented as proportions (%) and mean (SD). 'Person-year' for each child was calculated from date of enrolment to censorship (end of study, withdrawal, death, or no face-to-face contact for ≥90 days). During the follow-up, if a participant was not contactable for ≥2 consecutive weeks, the information from the previous 2-weeks from the date of subsequent contact was captured and considered in person-years of follow up and the intervening time period was not considered in analysis.

Fever episodes positive for dengue NS1 and/or IgM were defined as lab-confirmed acute dengue fever episodes. In addition to NS1 and/or IgM, fever episodes that were positive for dengue IgG were identified as acute secondary dengue fever and those negative for IgG were acute primary dengue fever. Children were divided into 3 age categories i.e., 6 months to <5 years, 5 years to <10 years and 10 to 15 years. The age was calculated from date of birth verified from birth certificate or immunization card. The age-specific incidence rates of acute dengue fever or acute febrile illness was calculated as the number of new events in the specific age-interval divided by total number of person-years of follow-up contributed by all children at risk in this interval with allowing for children to move to higher age categories. Confidence intervals were calculated using the "poisson" option in Stata. The cases of lab-confirmed dengue fever were plotted using ArcGIS 10.8 on a base map using Sentinel 2 satellite data with 10 m spatial resolution available from Copernicus Open Access Hub (https://scihub.copernicus.eu/) of 2021 for the study area. After preparing the GIS maps, Moran's Index of spatial autocorrelation was estimated to identify any clustering pattern of the dengue fever cases.

Results

We followed up a fixed cohort of 984 children from 755 households from the date of first enrollment, 24th October 2017 to date of completed follow up, 13th February 2020. In this cohort we had a total of 1953 person-years (PY) of follow up, of which, 535, 854 and 564 PY

were contributed by children aged 6 months to <5 years, 5 to <10 and 10 to 15 years, respectively (Fig 1). During this follow-up period a total of 16 episodes of hospitalizations were reported in this pediatric cohort.

Characteristics of the study population

Around two-third (72%) of the children enrolled belonged to nuclear families with median monthly income of 143 USD; 77% of these households were overcrowded. Piped water from the Delhi municipal corporation (81%) and bottled water (12%) were the major sources of household drinking water (Table 1).

Acute febrile illness surveillance

In this cohort, there were 4208 episodes of fever with peaks during the months of June to November (Table 2 and Fig 2). The overall incidence (95%CI) of fever was 215/100 PY (209 to

Table 1. Characteristics of the study population.

Baseline characteristics of enrolled children	n/N (%) [a]
Sex of the Child: Female	474/984 (48.17)
Age at enrollment	
6 months to < 5years	342/984 (34.76)
5 years to <10 years	434/984 (44.11)
10 years to 15 years	208/984 (21.13)
Sociodemographic parameters	
Type of Family	
Nuclear	540/755 (71.52)
Three generation	47/755 (6.23)
Joint	168/755 (22.25)
Family Size: Mean (SD)	5.83 (2.11)
Highest education in family as years of schooling: Mean (SD)	11.05 (3.17)
Type of House [b]	
Pucca	749/755 (99.21)
Mixed	6/755 (0.79)
Kuccha	0/755 (0.00)
Overcrowding present (>2.5 persons per living room)	583/755 (77.22)
Separate kitchen available	535/755 (70.86)
Primary fuel used for cooking in household	
LPG Gas	754/755 (99.87)
Others	1/755 (0.13)
Monthly income in USD [c]	
Median (IQR)	142.86 (114.29, 214.29)
Mean (SD)	192.76 (159.64)
Source of Drinking water	
Piped water in household	609/755 (80.66)
Bottled water	88/755 (11.66)
Public tap/standpipe	34/755 (4.50)
Tube well	7/755 (0.93)
Tanker-truck	16/755 (2.12)
Others	1/755 (0.13)

[a] This is a cohort of 984 children from 755 households–the denominators are mentioned accordingly

[b] A pucca house is one, which has walls and roof made of bricks, stones packed with cement. Kutcha houses are made of material other than those mentioned above, such as bamboos, mud, grass, reeds, thatch, etc.

[c] 1 US dollar (USD) = 70 Indian Rupee (INR)

[d] Methods of water treatment include boiling, chlorination, and filtration

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Table 2. Incidence of fevers by age-categories in the cohort (n = 984).

Age group	Person years of follow up	Number of Fever episodes	Incidence (95%CI) of fever per 100 PY	Number of ≥3-day fever episodes	Incidence (95% CI) of ≥3-day fevers per 100 PY
6m to <5y	535.08	3251	607.6 (587.5 to 628.2)	572	106.9 (98.4 to 115.9)
5y to <10y	853.84	547	64.1 (58.8 to 69.6)	589	68.9 (63.5 to 74.7)
10y to 15y	563.70	410	72.7 (65.9 to 80.1)	343	60.8 (54.6 to 67.6)
All children	1952.62	4208	215.5 (209.1 to 222.0)	1504	77.0 (73.2 to 81.0)

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222), with highest incidence in under-five children 607.6/100 PY (587.5 to 628.2). Median (IQR) duration of a fever episode was 2 (1–4) days. In 34.7% (1460/4208) of the fever episodes an antibiotic was prescribed which was initiated around day 2 (IQR 1 to 3) of fever onset. Oral beta-lactam, cephalosporin and macrolides were the most prescribed antibiotics. Of the 1504 episodes of 3-day fevers, dengue tests were conducted in 1250 episodes (83.1%, Fig 1). The median (IQR) time of blood collection was on day 3 (3 to 4) from onset of fever. In the other 3-day fever episodes (254/1504) dengue tests could not be performed due to refusal by parent for blood collection or family unavailable in the study area.

Acute dengue fever

A total of 74/1250 3-day fever episodes were positive for acute dengue fever (Dengue NS1 and/or IgM positive); 68/74 were primary acute dengue fever, and 6/74 were acute secondary dengue fever episodes (Table A in S1 Appendix). The positivity rate (95% CI) for dengue NS1 and/or IgM in our cohort was 5.9% (4.6% to 7.4%). Spatial autocorrelation analysis suggested a clustering pattern for the dengue fever cases in the study area with a 6% likelihood that the observed clustered pattern could be due to random chance (Moran's Index 0.35, z-score 1.8, p = 0.06, Fig 3). We observed higher number of acute dengue fever episodes during August to December (monsoon and post-monsoon seasons) with the highest risk in the month of October. Number of dengue fevers were higher in 2018 compared to 2019 (Fig 2).

Overall incidence (95%CI) of acute dengue fever was 37.9 (29.8 to 47.6) per 1000 PY, primary dengue fever was 34.8 (27.0 to 44.2) per 1000 PY and secondary dengue fever was 3.1

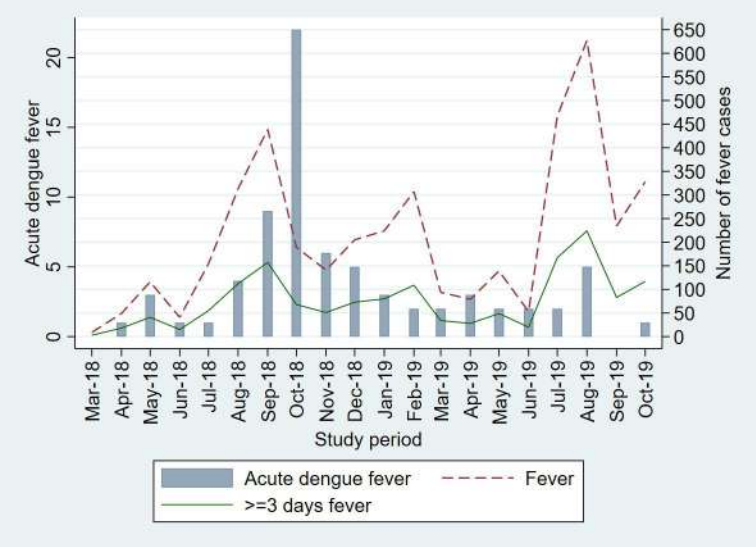


Fig 2. Acute febrile illness and dengue fever during the study period, by month.

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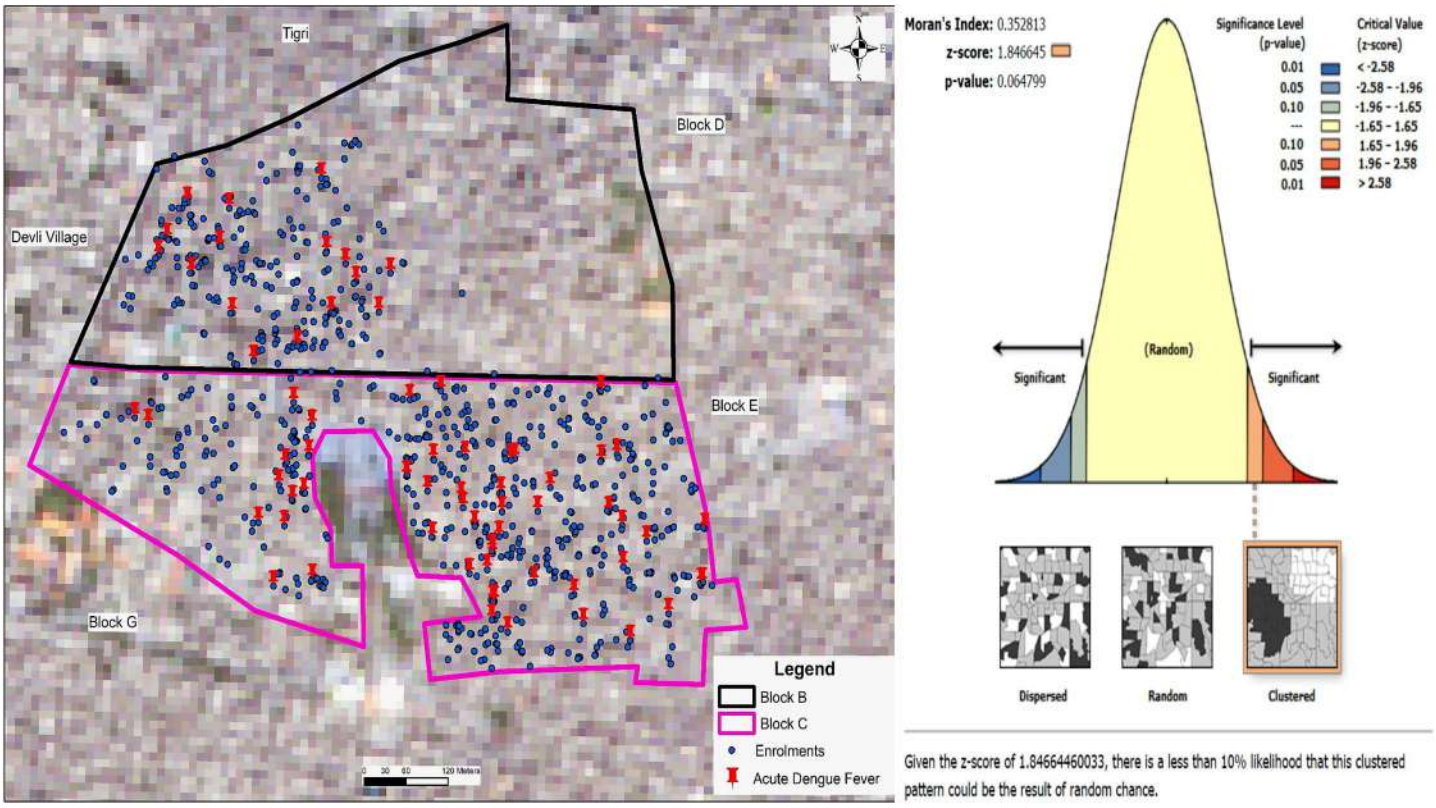


Fig 3. Geographic distribution of acute dengue fever cases in the study area. Figure legend: Moran's Index = 0.353, Variance = 0.04, z-score = 1.85, p-value = 0.06. Given the z-score of 1.85, there is a less than 10% likelihood that this clustered pattern could be the result of random chance.

https://doi.org/10.1371/journal.pntd.0010333.g003

(1.1 to 6.7) per 1000 PY. Incidence of acute dengue fever was observed to be highest among children aged 5 to 10 years (50.4, 95% CI 36.5 to 67.8 per 1000 PY, Table 3). The mean duration of acute dengue fever episode was 6.9 (1.7) days. Only one child had two episodes of acute dengue fever at ages of 5 year 3 months and 6 year 5 months. Two children with acute dengue fever episodes were hospitalized, and mean duration of hospitalization was 8 days. All children with dengue fever recovered; no dengue fever associated deaths were observed (Table B in S1 Appendix).

Of all the acute dengue fever episodes, in 24 cases OD value of dengue NS1 was found to be >1.8 and were subjected to molecular typing of dengue virus (DENV). Dengue PCR was positive in 16 of the 24 specimens (66.7%). The median viral load of dengue in the specimens measured by PCR was estimated to be 138 (IQR 9.25 to 505) x 10^4 copies/ml. In the tested specimens, the predominant serotype identified was DEN3 in 62.5% (15/24), followed by DEN1 in 4.2% (1/24). We did not identify any DEN2 or DEN4 serotype (Fig 1).

Table 3. Incidence of dengue fever by age-categories in the cohort (n = 984).

Age group in years	Person years	Acute dengue fever		Acute dengue fever–primary		Acute dengue fever–secondary	
		No. of episode	Incidence (95%CI) per 1000 PY	No. of episode	Incidence (95%CI) per 1000 PY	No. of episode	Incidence (95%CI) per 1000 PY
6m to <5y	535.08	20	37.38 (22.83 to 57.72)	19	35.51 (21.38 to 55.45)	1	1.87 (0.05 to 10.41)
5y to <10y	853.84	43	50.36 (36.45 to 67.83)	40	46.85 (33.47 to 63.79)	3	3.51 (0.73 to 10.27)
10y to 15y	563.70	11	19.51 (9.74 to 34.91)	9	15.97 (7.30 to 30.31)	2	3.55 (0.43 to 12.82)
All children	1952.62	74	37.90 (29.76 to 47.57)	68	34.83 (27.04 to 44.15)	6	3.07 (1.13 to 6.69)

https://doi.org/10.1371/journal.pntd.0010333.t003

Beyond dengue, in the 1250 AFI episodes, 18 were found to be positive for Salmonella Typhi, 7 were found to be positive for Salmonella Paratyphi and 7 were found to be positive for Chikungunya.

Discussion

Our pediatric cohort showed high burden of acute dengue fever in low-middle income urban settings in Delhi. The incidence of dengue fever was highest among children aged 5 to 10 years. All children recovered without any associated major complications or death. Acute dengue fever episodes were higher in the months of August to December; more dengue fever episodes were recorded in 2018 against 2019. We observed a clustering pattern in the dengue fever cases in the community. In our study sample, DEN3 was the most encountered strain.

The estimates of dengue fever in children from our study seems to be similar or higher compared with community-based cohorts from other countries [12–15]. In a prospective cohort of 3000 Latin American children aged 9–16 years followed up during 2010–2011 the reported incidence of dengue fever was 41 (95% CI 32 to 54) per 1000 person-years [13]. The incidence of dengue fever in the Nicaraguan pediatric cohort study of 5,545 children aged 2–14 years was 16.1 (95% CI 14.5 to 17.8) per 1,000 person-years [15]. In 2012–13, a prospective cohort of subjects $\geq$6 months old in Cebu City, Philippines reported incidence of symptomatic dengue to be 16.2 per 1000 person-years [14]. Between November 2008 and January 2010, in a cohort of 800 children from urban Colombo, Sri Lanka the incidence of dengue fever per 1000 children per year was 33.8 (95% CI 22.4 to 48.8) [16].

But there are limited community-based studies that reported incidence of dengue fever in Indian children. Similar to our effort, the Vellore site of the SEFI cohorts reported the annual incidence rate of dengue in 2017–2018 to be 49.5 per 1000 PY in children aged 6 months to 14 years with fever >3 days [17]. Using the serial sero-survey methodology during 2014–2016, a study in a rural area of Pune, Maharashtra estimated the incidence rate of primary dengue infections as 54.2/1000 PY (95% CI 43.0 to 67.3) among children aged 5 to 15 years [18]. Despite some heterogeneity due differences in methodology, pooled estimates of our study along with above two studies indicate that the population-based incidence of symptomatic dengue fever may vary from around 35 to 60 per 1000 PY among Indian children under 15 years of age across different geographies [12–15]. However, multicentric studies with improved diagnostics and standard methodologies across sites may be helpful to generate more precise country-specific estimates.

For diagnosis of dengue fever, rt-PCR is currently considered as the gold standard although NS1 has high sensitivity and specificity. But even rt-PCR may have its own challenges. It has been seen that rt-PCR may be false negative in cases where dengue virus RNA is detected using transcription-mediated amplification (TMA) technique, and where NS1 is found to be positive. Therefore, it has been suggested that NS1 antigen assay is a valuable method for diagnosis of dengue independent of rt-PCR [19]. In limited-resource settings, some researchers even consider NS1 ELISA assay to be superior to rt-PCR due to its high sensitivity, low cost, ease of performance and rapidity [19, 20]. NS1 in combination with IgM assay may offer the most sensitive and cost-effective diagnostic modality for dengue diagnosis [20]. Previous longitudinal studies in Latin America [13] and in India [17] have used NS1 along with IgM assay to estimate incidence of dengue fever, which is similar to our study. Nonetheless, we acknowledge that rt-PCR in addition to NS1 and IgM could enhance the accuracy of the estimates.

The National Centre for Disease Control reported a higher number of cases in 2018 compared to 2019 in Delhi as was also observed in our study [6]. The higher burden of dengue cases as observed during the post-monsoon months of September to December can be

attributed to climatic conditions such as mean temperatures and precipitation levels which shortens the extrinsic incubation period of the dengue virus [17, 21]. Beyond the seasonal peak, we observed cases of dengue throughout the year suggesting sustained endemic transmission similar to other Southeast Asian countries [22]. All four strains of the dengue virus are known to circulate during epidemics in India [23]. The community-based study from Vellore, Tamil Nadu, in 2017–18 reported DEN 1 as most common subtype in their population [17]. DEN 1 and 2 has been previously reported to be the commonest circulating strains in North India [24]. We found DEN 3 as the most common strain in our study sample. Our study was not powered to determine the serotype-specific incidence of dengue and therefore should be interpreted with caution. Nonetheless, the findings corroborate to a recent study in Delhi [25] and may indicate an epidemiological shift. The changing trend of the virus type underscores the importance of continuous disease surveillance.

The study findings have important implications. The high burden of dengue fever specially in the 5 to 10 year old children highlights their vulnerability given the current WHO recommendation to use the available dengue vaccine only in those with a documented past infection or in children above 9 years of age in select areas of high seroprevalence >80% [26]. In addition to continued efforts towards development of newer dengue vaccines and evaluating its effectiveness in younger children, it is crucial to develop affordable pre-vaccination screening strategy with high sensitivity and specificity. The significant clustered pattern of cases in our study area may be related to local mosquito breeding patterns in these sites and highlight the importance of promoting vector surveillance and control.

This study, to the best of our knowledge is the first report related to community-based burden of dengue fever among children under 15 years of age estimated using a pediatric cohort design in low-middle income settings in Delhi, North India. Our study has several limitations. First, our strategy for blood culture precludes capture of dengue fevers with <3-day febrile illness. Second, there were challenges to conduct blood tests in 20% (254/1250) of the eligible 3-day fever episodes. Based on the 5.9% dengue positivity rate in our cohort, it is possible that around 14/254 of these missed 3-day fever episodes could have tested positive for dengue, giving an overall projected incidence rate of 45/1000 PY. Third, the study was not powered for ascertainment of severity of disease. Fourth, given the median duration of a fever episode of 2 days, a biweekly contact would have allowed better capture of fevers at the cost of operational feasibility. We used a combination of active weekly contacts along with incentivization in form of monthly phone recharge for promoting passive reporting of fever. With our approach, we may have missed some fever episodes as many of these households have a common mobile phone, not always available with the primary caregiver of the child to report fever. Fifth, although rt-PCR is the gold standard for dengue diagnosis, our case ascertainment was based on NS1 and/or IgM positivity. But, given the high sensitivity and specificity (>95%) of the kits compared to rt-PCR, we believe this is not a major source of bias. Lastly, our study population is restricted to children <15 years belonging to low-middle income urban area in Delhi, North India and may not be generalizable to adults or other populations with different characteristics or dissimilar geographies.

In conclusion, this cohort study provides robust evidence on the high burden of dengue fever among children in urban, peri urban low middle-income populations in Delhi, India and can be applicable to other similar settings with contextual relevance. Given that India has large variations in geographical and climatic conditions, multicentric studies with standard methodology across sites may help to capture the within country variation in dengue incidence. We think improved methods for disease surveillance along with research for development of newer vaccines particularly for children, together can help to bring down morbidity and mortality related to dengue in our country.

Supporting information

S1 Appendix. Table A: Distribution of different dengue assay results and its classification. Table B: Clinical characteristics and outcomes of acute primary, acute secondary and past dengue infection.
(DOCX)

S1 Strobe checklist. STROBE Statement—Checklist of items that should be included in reports of cohort studies.
(DOCX)

S1 Data. Dataset used for analysis.
(XLSX)

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Writing – review & editing: Bireshwar Sinha, Nidhi Goyal, Mohan Kumar, Aashish Choudhary, Deepak More, Temsunaro Rongsen-Chandola.

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Microbiology Section

NS1 Antigen Capture ELISA an Effective Method for Diagnosis of Early Dengue Infection - Report of an Outbreak at Angul District, Odisha, India

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ABSTRACT

Background: An outbreak of dengue infection occurred in Angul district of Odisha in the month of August & September, 2011. The study was undertaken to detect NS1 antigen positivity among the study population, to compare IgM capture ELISA with NS1 antigen detection for diagnosis of dengue and to identify the predominant genotype of Dengue virus responsible for the outbreak.

Materials and Methods: Total 1020 serum samples were collected from clinically suspected cases of dengue from the outbreak. All were subjected for NS1 antigen detection, 92 were selected based on their clinical severity of illness (fever, rash, bleeding manifestation, arthralgia) for further study of IgM ELISA and platelet count and 148 NS1 positive samples were selected from different Blocks of Anugul district for RT-PCR at NIV, Pune, India.

Results: Five hundred and thirteen (50.2%) samples were positive for NS1 antigen (highly significant p-value <0.0001, C.I - 95%) with 88% positivity during 1-5 days. The NS1 Ag positivity was peaked to 86.9% on days 3 to 5 (Sensitivity & NPV - 100% each) & declined to 6.2% during 6-10 days with a low sensitivity of 7.14% but 100% specificity & PPV. However, the IgM antibody positivity was 81.2% on days 6 to 10 and 87.5% after 10 days (Sensitivity- 100%, Specificity-13.33%,PPV-7.14% & NPV - 100%). RT-PCR resulted 32.4% positivity (6-DEN1, 39 - DEN 2 & 3- DEN 3) among which 20% were in IgM +ve & 68% in IgM -ve cases.

Conclusion: Therefore, early diagnosis of dengue could be mainly by NS1 antigen detection whereas Ig M ELISA is a better tool during the later stage of infection &RT-PCR is more effective in IgM -ve cases.The predominant genotype responsible for the outbreak was found to be DEN-2.

Keywords: Antigen detection, Dengue outbreak, IgM ELISA

INTRODUCTION

Dengue virus is a flavi virus transmitted by aedes mosquito. There are four closely related but antigenically distinct serotypes of Dengue viruses (DEN-1-4). Infection with any serotype causes a spectrum of clinical features ranging from asymptomatic infection, undifferentiated fever and classical dengue fever (DF) to life threatening manifestation like dengue haemorrhagic fever (DHF) to dengue shock syndrome (DSS). Globally dengue is an emerging disease that causes an estimated 50-100 million infections, 500,000 dengue haemorrhagic fevers and 22,000 deaths annually [1]. Since the symptoms of dengue virus infection are insufficient for clinical differentiation from other viral illnesses, definitive diagnosis relies on laboratory tests. A rapid and accurate diagnosis of dengue in the acute phase of illness is important for initiation of therapy as well as for early enhancement of epidemic control measures especially in low endemic areas. Detection of specific IgM antibody by ELISA forms the mainstay for diagnosis. However, IgM antibodies develop after 4 to 5 days of infection. Viral isolation is the gold standard for diagnosis and serotyping of dengue virus infection, but this method is time consuming and requires sophisticated laboratory [2]. Molecular diagnosis such as RT- PCR requires experienced personnel and specialized laboratory equipments. As an alternative the detection of NS1 antigen of dengue virus has been identified as highly conserved glycoprotein expressed on either membrane bound or secreted form [3].

The present study was aimed -

1. To detect NS1 antigen positivity among the study population.
2. To compare IgM capture ELISA with NS1 antigen detection for diagnosis of dengue.
3. To identify the predominant genotype of Dengue virus responsible for the outbreak in Angul district of Odisha in the year 2011.

MATERIALS AND METHODS

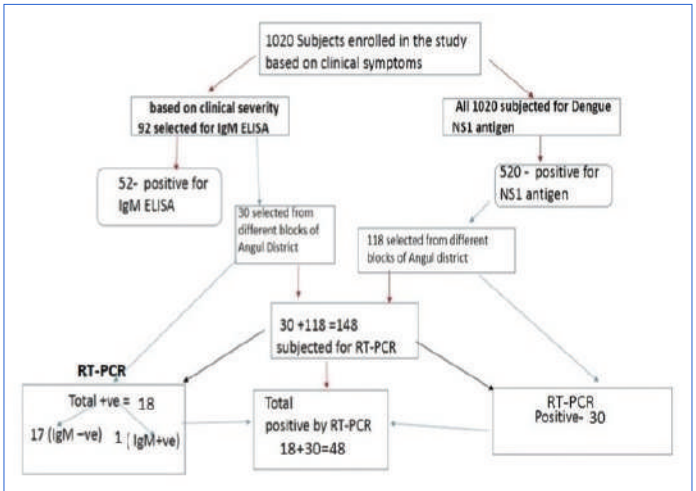
Type of study - survey

One thousand and twenty serum samples were collected from clinically suspected cases of dengue fever during the outbreak in Angul district of Odisha during the month of August- September, 2011. Blood samples were collected from the patients of the epidemic affected area and sent to the Department of Microbiology, S.C.B Medical College, Cuttack, which is the referral hospital for eastern Odisha. Majority of the samples were collected during the acute phase of illness i.e. 1-5 days of fever. The samples were grouped into 3 categories according to the days of fever - 1-5,6-10, >10 d. Serum was separated from all the samples and subjected for NS1 Ag detection.

As IgM ELISA & genotyping by RT-PCR was not possible for all samples in our resource constraint laboratory, 92 out of 1020 samples were selected, based on their clinical severity of illness (fever, rash, bleeding manifestation, arthralgia), for further study of IgM ELISA and platelet count & 148 NS1 positive samples were selected from different Blocks of Anugul district for RT-PCR at NIV, Pune which also included 30 out of the 92 samples for Ig M ELISA [Table/Fig-1].

Control- Twenty blood samples from healthy individuals were collected as control& subjected for detection of both NS1 Ag and dengue specific IgM.

NS1 Ag assay: The test was performed using NS1Ag MICROLISA (J. Mitra& Co, New Delhi) test kit as per manufacturer's instruction.



[Table/Fig-1]: Flow chart followed for detection of dengue

Detection of IgM: The Dengue IgM capture ELISA (Panbio, Australia) was used & interpreted as per the recommendations of the manufacturers.

Platelet Count: EDTA blood samples were collected & the platelet count was done in the Department of Pathology, S.C.B Medical College, Cuttack and interpreted as normal, when the count was between 1, 50, 000- 4, 50, 000 and DHF, when the count was ‹ 1, 00, 000/ cmm(WHO cutoff for platelet count for DHF).

RESULTS

Out of the 1020 blood samples screened, 513 (50.2%) were positive for dengue NS1 Ag with a two tailed p-value of < 0.0001 (Z-statistics), among which majority were males 427(83.2%) between the age group of 21- 40 y [Table/Fig-2]. Among the 513 NS1Ag +ve cases 88% were detected during 1-5 days of fever.

When detection of NS1Ag and IgM were taken into account together for diagnosis, 81.2% & 47.19% respectively were found to be positive during 1- 5 days, then 65.6% & 81.2% during 6-10 days & only 6.2%& 87.5% after 10days. Both tests were positive in 25% cases during 1-5 days and 56.8% were positive for only NS1Ag which were missed by IgM capture ELISA. However, after 10 days of fever, 81.2% were positive by IgM ELISA and all were negative for NS1Ag [Table/Fig-3]. The IgM positivity was 47.1% with a sensitivity & specificity of 30.6 & 100% within 5 days, increased to 81.2% (sensitivity-90.48% & specificity 36.6%) during 6-10

Age Group	Male	Female	Total (%)
0-10	4	5	9(1.7)
11-20	52	9	61(11.8)
21-30	169	34	203(39.5)
31-40	111	21	132(25.7)
41-50	70	13	83(16.1)
›50	21	4	25(4.8)
Total	427(83.2)	86(16.7)	513(100)

[Table/Fig-2]: Age and sex distribution of the NS1Ag positive cases. (N=513)

(N=92) Sample age	NS1 +ve IgM +ve	NS1 +ve IgM -ve	NS1 –ve IgM +ve	NS1 –ve IgM -ve	Total-58 (NS1 +v)	Total Ig M+ve
D_{1-5} (n=44)	11(25%)	25 (56.8%)	0(0.0%)	8 (18.2%)	36 (81.2%)	11 47.1%)
D_{6-10} (n=32)	19 (59.3%)	2(6.2%)	7 (21.8%)	4 (12.7%)	21 (65.6%)	26 (81.2%)
D_{10} (n=16)	1(6.2%)	0 (0%)	13(81.2%)	2(12.6%)	1 (6.2%)	14 (87.5%)

[Table/Fig-3]: Comparison of NS1Ag detection and Ig M ELISA for diagnosis of dengue during different days of fever

NS1 Ag +ve cases 30+118=148	+ve for genotype -1	+ve for genotype -2	+ve for genotype -3	Total No(%)
NS1+ve IgM -ve (25)	2	15	0	17 (68) n =25
NS1+ve IgM+ve (5)	0	0	1	1(20) n =5
NS1+ve (118)	4	24	2	30(25.4)
Total	6	39	0	48(32.4) n=148

[Table/Fig-4]: Distribution of RT-PCR positivity in NS1 antigen positive cases

days & peaked to 87.5% (sensitivity -100 %, specificity -13.33%) after 10 days [Table/Fig-4]. All the 20 samples from controls were negative by both the assays. RT-PCR resulted 32.4% positivity out of the 148 selected NS1Ag positive samples. Among them 20% were +ve out of (5) IgM +ve & 68 % out of (25) IgM -ve cases. The genotypes 1,2 &3 were identified in 6,39&3 number of cases respectively [Table/Fig-4].

DISCUSSION

Dengue is an endemic viral disease affecting tropical and subtropical regions around the world [4]. Dengue infection presents with non specific fever that mimics other viral illnesses. However, according to WHO case definition dengue fever (DF) is defined as acute febrile illness with two or more manifestations among headache, retro-orbital pain, myalgia, athralgia, rash, haemorrhagic manifestations and leucopenia. Dengue haemorrhagic fever (DHF) is defined as 2- 7 days of acute febrile illness with bleeding, thrombocytopenia and evidence of plasma leakage. When all the features of DHF are present along with circulatory failure then the patient is categorized as dengue shock syndrome (DSS) [5]. Bleeding manifestation was not a common feature in our study, majority of the patients presented with fever, headache, myalgia and generalized rash except four cases who presented with DHF and two cases with DSS which correlates well with other authors [6]. But others had reported higher incidence of bleeding manifestations in their study [7,8]. The major diagnostic methods currently available are viral culture, viral RNA detection by RT-PCR and serological tests such as IgM capture ELISA. Current strategy under National Vector Disease Control Program(NVBDCP) suggests that diagnosis of a particular flavi virus should always be made taking into account the clinical presentation, the performance of serological tests and the knowledge of the flavi virus circulating in that particular geographic region. Tests for antigen and antibody detection are widely used for confirmation of dengue infection. However, performance of tests depends upon specificity and sensitivity of the test kits used. High specificity is important if the tests are being used to confirm dengue as the cause of the outbreak whereas sensitivity is very important if the tests are to be used to support clinical diagnosis and for case management [9]. Following the bite of an infected *aedes mosquito*, dengue virus replicates quickly before the development of signs and symptoms. NS1 Ag circulates uniformly in all serotypes of dengue virus and circulates at high level during the 1st few days of illness [10]. This is the reason for high detection rate of NS1Ag in acute phase sera, there after decreases gradually and antibody detection becomes more prominent after 5 days.

In the present study 50.2% samples were found to be positive by NS1Ag detection with a highly significant p-value (<0.0001). Majority was in the age group of 21-40 & detected during 1-5 days with a mean day of 5. Similar male preponderance was observed by various authors in previously reported outbreaks [11,12]. Srivastav et al., reported only 26% NS1Ag positivity but Dutta and Wattal had reported higher percentage of NS1Ag positivity (71.42%) in acute phase sera [13,14].

Earlier studies found the presence of NS1Ag in 82-83% of patients with dengue infection from day 1 up to 9 to 18 days after the onset

of fever [15,16]. We detected maximum NS1Ag positivity (81.2%) in 1 to 5 days of fever. But low sensitivity of NS1 protein tests was evidenced during a dengue type 2 virus outbreak in Santos, Brazil, in 2010 [17]. Among the 92 subjects selected for IgM capture ELISA, only 47.1% were positive during 1 -5 days of fever. But after 10 days IgM positivity was 87.5%. During 6-10 days, though 81.2% could be detected by Ig M in comparison to 65.6% +ve for Ns1 Ag, but this difference is not significant statistically. But after 10th day 81.2% were positive by IgM ELISA and all were negative for NS1Ag [Table/Fig-3]. The 4 cases of DHF and DSS were also positive for IgM ELISA with low platelet count. Aditi A et al., had reported IgM capture ELISA to be 98.4% specific and 96.4% sensitive for the diagnosis of dengue infection [4].

Our study found 90.4-100% sensitivity of Ig M ELISA after 6th day onwards but the specificity is low. During 1-5 days, 56.8% were positive for only NS1Ag which were missed by IgM capture ELISA because, development of dengue specific IgM depends on the time taken for an infected person's immune response to produce antibodies. Thus IgM ELISA does not provide early diagnosis of dengue infection. Moreover, a single serological detection of IgM is merely indicative of recent infection and should not be interpreted as confirmatory [13]. In this context NS1Ag detection has the added advantage of giving good detection rates up to 7 days of illness. However, some authors emphasize the use of combination kit for detection of additional cases of dengue, which are negative for NS1 antigen but positive for IgM and/or IgG antibodies [18].

RT-PCR, though a sensitive and specific method, could detect only 32.4% of the 148 selected NS1Ag positive samples in our study. This low positivity may be due to failure of maintenance of stringent condition for storage and transportation of the samples. Moreover, RNA detection could be possible only in 20% of IgM +ve in contrast to 68% IgM -ve cases. The possibility for this may be due to neutralization of viral components after appearance of IgM antibodies. The predominant serotype identified was serotype – 2 (39/48) followed by serotype- 1(6/48) and serotype- 3(3/48). The major serotype reported from PGI, Chandigarh during the outbreak in the year 2007 was serotype-1, but in 2005 it was serotype-2 [11]. A study from Delhi had reported concurrent prevalence of all the serotypes during 2008 [19]. Major dengue epidemics have been observed in the Indian subcontinent since the 1980s & researchers from the Pune-based NIV found that Indian DENV-2 strains isolated from 1956 to 2005 belonged to only two of the six genotypes — American and Cosmopolitan [20].

CONCLUSION

NS1Ag is an effective method in comparison to MAC ELISA for diagnosis of dengue virus infection, especially within first five days of illness. The morbidity and mortality of dengue fever can be reduced by early diagnosis and symptomatic management. However, from sixth day onwards combination of MAC ELISA and NS1Ag assay would increase the sensitivity of diagnosis and after 10 days IgM ELISA should be the method of choice for diagnosis. RT-PCR is mandatory for genotyping to ascertain the cause of the current outbreak.

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Seroprevalence and Evaluation of IgM and NS1 Ag Detection for Early Diagnosis of Dengue Cases in a Tertiary Care Hospital

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Dengue fever, a mosquito borne illness, have grown to pose a serious danger to public health systems in developing nations. The Dengue virus (DENV), a member of the Flavivirus genus has four serotypes that cause the disease which range in severity from subclinical infections to severe disease with high morbidity & mortality. This study aims to assess the seroprevalence of dengue fever cases in a tertiary care hospital from East- Delhi region.
Material & Methods: This study was a retrospective observational study conducted in the Department of Microbiology at UCMS & GTBH. The data was collected from laboratory records from a period of Jan 2019 to December 2019.
Results: A total of 1449 clinically suspected cases of dengue were tested for the presence of NS1 antigen and IgM antibodies by capture ELISA. This study showed 32.22% (467/1449) seropositivity of dengue infection. Out of 1272, a total of 456 samples were positive for NS1 Ag ELISA. Whereas, out of 177, a total 11 samples were positive for IgM ELISA.
Observation: The study highlights the importance of early diagnosis and prompt management of dengue cases to prevent severe disease and reduce morbidity and mortality. This underscores the need for increased awareness, early detection, and appropriate management of dengue fever in endemic areas. especially during the peak months of October and November.

Keywords: Aedes mosquito; dengue virus; enteric fever; seroprevalence; IgM.

1. INTRODUCTION

Dengue fever, a mosquito borne illness, have grown to pose a serious threat to public health systems in developing nations. The vector borne viral disease is primarily transmitted by two most common *Aedes* mosquito (*Aedes aegypti* and *Aedes albopictus*) [1]. The Dengue virus (DENV), a member of the *Flavivirus* genus has four serotypes that cause the disease which range in severity from subclinical infections to severe disease with high morbidity & mortality. Despite the fact that the most of dengue fever episodes are mild and self-limiting, it can nevertheless result in deadly complications such as DHF (Dengue Hemorrhagic Fever) and DSS (Dengue Shock Syndrome) [2]. The asymptomatic cases in the earlier stages of infection play a major role in the maintaining transmission of the disease in the community resulting in outbreak [3]. Typical symptoms of dengue include a high-grade fever, headache, discomfort in the muscles and joints, aches, retroorbital pain, and skin rashes [3]. Severe dengue symptoms result from plasma leakage, hemo-concentration leading to hemorrhagic shock, and multiple organ failure with high fatality. The National Centre for Vector Borne Diseases Control (NCVBDC) has provided case definition for the disease based on symptoms & laboratory finding and is a notifiable disease under Integrated Disease Surveillance Project (IDSP) disease survey [4].

Serology, Virus isolation and molecular methods like Nucleic Acid Amplification Test (NAAT) are some of the diagnostic modalities currently available to detect dengue infection. NAATs and viral culture are expensive & specialized infrastructure is needed therefore have limited diagnostic use. Advance molecular techniques such as RT-PCR, Multiplex RT-PCR, Realtime RT-PCR, and LAMP are available to detect the virus during acute phase of infection [4]. However, for early diagnosis and intervention in resource limited settings, cost effective tests like ELISA are routinely used to identify dengue infection [5].

As per NVBDCP, India reported a total case 157315, 44585 and 193245 during the year of 2019, 2020 and 2021, respectively. The total death reported during the period was 166, 56 and 346, respectively. Delhi reported a total case 5077, 1269 and 13089 during the year of 2019, 2020 and 2021, respectively and only 23 deaths in 2021 [6]. The present study aim to assess the seroprevalence of dengue in the patients treated at a tertiary care hospital from East- Delhi region during 2019.

2. MATERIALS AND METHODS

A retrospective observational study was conducted in the Department of Microbiology at UCMS & GTB Hospital. The demographic and laboratory data were retrieved from the department for year 2019. Serological examination based on the days of fever (either NS1 or IgM) was routinely performed in the serum samples which were collected from the

dengue suspected patients. Serology test were performed for NS1Ag and IgM antibodies by using dengue NS1Ag MICROLISA (IR031096) and MACELISA kit (Panbio Dengue IgM Capture ELISA) respectively. Demographic data were entered in MS excel (version 2016). Statistical analysis was performed using SPSS software.

3. RESULTS

A total of 1449 clinically suspected dengue cases were tested for the presence of NS1 antigen and IgM antibodies by capture ELISA in the year 2019. This study showed 32.22% (467/1449) seropositivity of dengue infection. Out of 1272, NS1Ag ELISA 456 samples were positive. Whereas, out of 177 sample total 11 samples were positive for IgM ELISA.

Table 1 showed that from the total positive cases, Males (51.82%, 242/467) were more affected than females (48.17%, 225/467). Most common affected age group was 21-40 years (43.89%, 205/467), followed by 6-15 years (25.26%, 118/467) of age group (Table 2). Majority of positive dengue patients were from IPD (93.79%, 438/467) than OPD (6.20%, 29/467) (Table 3). Table 4 depicts the monthly distribution of positive cases. Maximum cases were recorded in October (30.40%, 142/467) followed by November (27.19%, 127/467).

4. DISCUSSION

Dengue NS1 antigen, a highly conserved glycoprotein which is produced in both membrane-associated and secretion forms, is abundant in the serum of patients during the early stages of DENV infection [5]. It's been discovered to be helpful in recognizing the presence of acute dengue infections. It is a simple test that is specific and shows high sensitivity. Early case detection with NS1, or when the infection is in the viremic stage is important form an epidemiological perspective to prevent transmission of the infection. For DENV, the NS1 ELISA-based antigen assay is commercially available, and the sensitivity and specificity of this assay has been assayed previously. Moreover, the specificity of NS1 assay helps in differential diagnosis of various flaviviruses [5]. Considering the cost effectiveness of this test and ease of performance as compared to molecular diagnostic test, the Mac ELISA gives a practical advantage in resource limited settings.

Table 1. Gender-wise distribution of dengue cases

	NS 1 Ag ELISA		IgM Ab ELISA	
GENDER	**Total number**	**Positive**	**Total number**	**Positive**
Male	499	236 (51.75%)	62	6 (54.55%)
Female	773	220 (48.25%)	115	5 (45.45%)
Total	**1272**	**456**	**177**	**11**

Table 2. Age-wise distribution of dengue cases

AGE	NS 1 Ag ELISA		IgM Ab ELISA	
	Total Number	**Positive**	**Total Number**	**Positive**
0-5	53	17 (3.75%)	12	01 (9.09%)
6-15	236	115 (25.21%)	23	03 (27.27%)
16-20	169	66 (14.47%)	24	01 (9.09%)
21-40	658	201 (44.07%)	93	04 (36.36%)
>41	156	57 (12.5%)	21	02 (18.19%)
Total	**1272**	**456**	**177**	**11**

Table 3. Incidence of dengue cases from the IPD& OPD wards

Department	NS 1 Ag ELISA		IgM Ab ELISA	
	Total number	**Positive**	**Total number**	**Positive**
IPD	1175	428	170	10
OPD	97	28	07	01
Total	**1272**	**456**	**177**	**11**

Table 4. Month-wise distribution of dengue infection

MONTHS	IgM Ab ELISA		NS1 Ag ELISA	
	Total Samples	Positive Rate	Total Samples	Positive Rate
Jan	19	06 (31.57%)	132	23 (1.74%)
Feb	00	00	34	02 (5.88%)
Mar	17	00	01	00
Apr	36	00	00	00
May	40	00	00	00
Jun	11	00	44	01 (2.27%)
July	00	00	105	25 (23.8%)
Aug	00	00	140	26 (18.57%)
Sep	00	00	251	105 (41.83%)
Oct	00	00	293	142 (48.46%)
Nov	00	00	253	127 (50.19%)
Dec	54	05 (9.25%)	19	05 (26.31%)
Total	**177**	**11 (6.21%)**	**1272**	**456 (35.84%)**

IgM-Capture (MAC-ELISA) detect the anti-dengue IgM antibody develops within 5 days of fever though it varies between patients. Detectable IgM may persist in some primary infections for up to 90 days, but in the majority of patients, it declines to undetectable levels by 60 days [5]. Nevertheless, it verifies the presence of infection in the person at some point in the recent past months. MAC-ELISA is now widely used as a potential tool for DF/DHF surveillance. Hospitalized patients, who are frequently admitted at the end of their illness and have detectable IgM levels in their blood, can benefit the most from it [5].

The study shows a high seropositivity rate (32.22%) for dengue infection among clinically suspected cases during 2019. This result was higher compared to the study done by Kumar e al, which shows the seropositivity of dengue infection in 23% cases [7]. This highlights the higher burden and need for accurate and timely diagnosis of dengue fever, especially in endemic areas.

In the present study, the male to female ratio of dengue cases was 1.07:1 (242/225) with a male (51.82%) predominance than female (48.17%). However, the difference between the two groups was not statistically significant and this result is similar to the Indian studies which also showed no difference in the gender distribution of dengue cases [8,9].

The most commonly affected age group was 21-40 years, followed by 6-15 years. Indirectly the need for targeted interventions to prevent and manage dengue fever in these high-risk groups. A study done by Badoni G *et al*, found that the most positive cases for DENV are in the 21–30-year age group [10].

The predominance of IPD dengue cases (93.79%) compared to OPD cases (6.20%) raises concerns about the severity of the disease. It suggests that a significant number of patients require specialized care and monitoring. Similarly, a study done by Singh T et al, also reported that out of 108 positive cases, most of the cases from the IPD ward (66.66%, 72/108) and rest of the positive cases from OPD 33.33%,36/108) [11]. The difference in the burden of dengue cases in IPD vs OPD emphasizes the need for an early detection and prompt treatment, in order to reduce its impact on healthcare facilities.

This study also noted that the majority of positive cases occurred during the months of October and November. A seasonal variation as reported from other endemic areas, where transmission peaks during the rainy season. Post-monsoon months are more favorable for the vector to breed due to the stagnation of rainwater. The findings underscore the importance of targeted surveillance and control measures during the peak transmission season. This result was also represented by many other Indian studies done by Badoni G *et al*, Kalpana S *et al* and Badoni G, Gupta P et al [10,12,13].

The NVBDCP data reveals the fluctuating nature of dengue cases in Delhi, with a high number of cases in 2019, a decrease in 2020, and a significant increase in2021 [6]. Total 23 deaths were reported in2021 as per government data [6], which is lesser than the previous year's and thus it highlights the need for continued efforts to

combat dengue, including mosquito control measures, public awareness campaigns, and accessible healthcare services to further reduce the impact of this mosquito-borne disease on the population.

The death rate associated with dengue in Delhi, India can vary from year to year and depends on various factor such as the severity of the outbreak, access to healthcare, and the effectiveness of preventive measures [6]. It is crucial for the government and healthcare authorities to continuously monitor and respond to outbreaks to minimize the impact on public health.

5. CONCLUSION

In conclusion, the study demonstrated a high seropositivity rate for dengue infection among clinically suspected cases, and NS1 antigen ELISA test was more effective than IgM ELISA test for early diagnosis. The findings also suggest that males and younger age groups are more prone to dengue infection. Additionally, the study highlights the importance of early diagnosis and prompt management of dengue cases to prevent severe disease and reduce morbidity and mortality. This implies the need for increased awareness, early detection, and appropriate management of dengue fever in endemic areas. especially during the peak months of October and November.

CONSENT

As per international standard or university standard, patient(s) written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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RESEARCH ARTICLE

The Combined Antigen and Antibody Testing Approach Improved the Sensitivity of Early Detection of Dengue Virus Infection

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ABSTRACT:

The viral infection has become an important issue for public health within India. It is the foremost common mosquito-borne viral infection in people due to elevated mortality as well as morbidity rates caused by dengue. Hence, it is crucial that a laboratory diagnosis of dengue be carried out early and as quickly as possible. This study examined the possible application of IgM and IgG antibodies, as well as the dengue NS1 antigen, for their sensitivity in the early detection of dengue. All patients with fevers lasting less than four days who visited a medical OPD between April and November 2023 are included in the study. Out of which 155 subjects were positive for the dengue virus were included in the present study. IgM, IgG, and NS1 antigen were measured in the collected specimens by immunochromatographic testing (card test). All samples were subjected to detection of NS1 Ag by ELISA. In all positive cases, dengue-specific parameters like NS1, IgM, and IgG were compared against platelet counts. This study included 155 cases, of which 82 (52.9%) were males and 73 (47.10%) were females. The positive detection rates of combined IgM antibody and NS1 antigen, NS1 antigen alone, and antibody (IgM/IgG) were 69.67%, 16.77%, and 13.56%, respectively. When only NS1 was considered, the sensitivity and specificity of rapid test kits compared to ELISA were 92.06% and 82.76%, respectively. NS1 antigen detection was the most highly sensitive in the early days of infection, but in the latter stages, IgM detection was more sensitive. In the current investigation, NS1 positivity (69.6%) was more commonly associated with the occurrence of thrombocytopenia than the antibodies IgG and IgM (30.43%). For diagnosing dengue, the most economical and sensitive method is to combine the rapid test of NS1 antigen with the IgM/IgG antibody test. It is recommended to combine the NS1 antigen card test with the ELISA assay for the most reliable diagnosis of dengue fever.

KEYWORDS: Dengue, NS1 antigen, Immunochromatographic rapid test, ELISA assay, Antibody.

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INTRODUCTION:

The Arbovirus, which belongs to the Flavivirus family, causes Dengue, a mosquito-borne disease that belongs to the Flavivirus genus. It is often referred to as break-bone fever as well. Female *Aedes aegypti* and *Aedes albopictus* mosquitoes are the predominant carriers of this virus[1]. In accordance with the World Health Organisation (WHO), the dengue virus infects around fifty million individuals every year, of which roughly three billion reside in regions where the virus is

prevalent globally. These viral infections represent about 500,000 hemorrhagic dengue fever cases, and the disease claims the lives of 25,000 individuals worldwide each year[2]**.** The main signs and symptoms of a dengue virus infection include a fever and rash that cover the entire body, an increase in body temperature that lasts for two to seven days, excruciating pain in the tendons, muscles and joints and retro-orbital pain, headaches, nausea, and vomiting[3]. Skin haemorrhages, or bleeding beneath the skin's surface, can cause purple or red dots form on the entire body in dengue fever patients[4]. The fever is extremely high during this period, and the number of thrombocytes in the blood decreases over the course of the next few days[5]. Based on types of antigens, dengue is transmitted by four diverse serotypes of the virus: DENV-1, DENV-2, DENV-3, and DENV-4[6,7]. Out of four known serotypes of dengue, serotype 4 (DENV-4) was the first to be discovered in Brazil.

A precise diagnosis is crucial for selecting the correct method of treatment. A high body temperature persisting for at least seven days is required for a clinical diagnosis in accordance with World Health Organisation (WHO) guidelines[8]. The methods that are employed for confirmatory dengue laboratory diagnosis include the detection of ribonucleic acid (RNA) of dengue virus isolation in culture, serological assays for NS1, IgM, or IgG seroconversion, and antigen detection[9,10]. These diagnostic techniques are costly, time-consuming, and require specialised staff and apparatus. These factors make them potentially impracticable, particularly in times of epidemics. IgM/IgG antibody detection can be done with commercially available immunochromatographic and ELISA techniques, which can provide results in the span of minutes to hours[11,12]. However, an individual affected by dengue cannot recognise antibodies until 4-5 days after the disease onset. The dengue virus infection is characterised by an increase in the level of a highly conserved glycoprotein identified as dengue NS1 antigen, which produce in both secretory form and also attached with membrane in the early stages of dengue infection in all serotypes[13,14]. The NS1 antigen can be detected before the formation of antibodies[15,16]. From the first day following the start of the fever until Day 9, the dengue specific antigen NS1 can be detected in the blood[17,18]. Conversely, three to five days following the onset of symptoms, an increase in particular immunoglobulin M (IgM) levels is indicative of a primary dengue virus infection. IgG levels are detectable for the entirety of an individual's life and begin to rise even after 10 to 14 days[19].

In order to treat the disease quickly, an early diagnosis of the dengue virus is necessary, as is ruling out other possible causes. Rapid immunochromatographic tests (RITs) are an appropriate choice for early detection in low-resource settings, can be utilized at the point of treatment, and do not require specialised infrastructure or technical skills[20]. RITs are currently being used more frequently in developing nations for identifying infectious epidemics, where they can be carried by medical professionals in clinics or by untrained health workers in communities[21]. This study aims to determine and evaluate the diagnostic effectiveness, sensitivity, and specificity of the RITs for dengue fever in relation to ELISA tests combining NS1 Ag and IgM/IgG antibody rapid diagnostic tests.

MATERIALS AND METHODS:

Population study and collection of sample:

The present examination was conducted from April 2023 to November 2023 in the Department of Medical Laboratory Technology at the Shri Alpesh N. Patel PG Institute of Science and Research, Anand (Gujarat) and collaborated with the Department of Pathology, Rytham Hospital, Khambhat, Anand (Gujarat) to undertake a comparative study. The Institutional Ethical Committee at the Shri A. N. Patel PG Institute of Science and Research, Anand (India) approved the study protocol by the Declaration of Helsinki (Approval Date: March 2, 2023; number: P-02). No private or confidential information was collected. Local Institutional Ethics Committee. Documented consent was received from all patients following study explanation. A total of 155 acute-phase blood samples were collected from children, adults and aged ranging in age from 0 to 60 years. During the research period, all patients (adults, children, and elderly) with fever who fit the new World Health Organisation (WHO) definition of dengue were included. Formal consent was obtained from the parents or caretakers of patients. In the laboratory, each patient was given two blood samples along with their full medical history, which included their IP number, name, and surname. For instance, one tube should be used for both the peripheral smear for the platelet count and the total blood count/picture. A haematology analyser (Mindray BC 5140 5 Part) was used every day to perform whole blood counts or platelet counts[22].

Immunochromatographic card assay.

After serum had been separated from all of the specimens, kits were utilised to test the presence of antibodies IgM/IgG and dengue specific antigen NS1. The objective of the Dengue fever rapid IgG/IgM immunochromatographic test is to qualitatively identify and separate specific IgM and IgG antibodies to the virus load present in human serum. This test is effective for distinguishing both primary and secondary dengue infections by detecting anti-dengue IgM and IgG antibodies. Tests that show only the control line (C) are regarded as negative. A result showing only the control line (C) is considered negative. A primary dengue virus

infection is indicated if two lines (IgM and control) appear in the result window. A positive diagnosis for a secondary dengue virus infection is indicated if three lines—IgM, IgG, and control—are displayed in the result window. Dengue NS1 Ag Strip (The Bioline™ Dengue Duo kit) was used for the detection of NS1 antigen from serum. The reaction is carried out on an immunochromatographic strip by applying serum or plasma with a migration buffer[23].

NS1 Antigen Detection ELISA Assays:

The dengue NS1 Ag ELISA is the method used to detect the presence of NS1 antigen found on dengue virus in serum. This test is called solid-phase enzyme-linked immunosorbent assay to detect dengue NS1 antigen[24]. The assay was performed strictly following the instructions provided by Abbott Bioline Dengue NS1 Ag Test Kit manual. Microwells with strong reactivity for the Dengue NS1 antigen are coated with anti-Dengue NS1 antibodies. The wells are filled with the serum sample. Then, an enzyme conjugate (monoclonal anti-dengue NS1 antibodies connected to HRPO) was introduced. Wash buffer is introduced to remove the unbound conjugate. The quantity of bound peroxidase is directly correlated with the sample's concentration of dengue NS1 antigen. A yellow colour appears when a stop solution is given to limit the enzyme-substrate reaction, and this colour is ultimately measured spectrophotometrically at 450nm.

Statistical analysis:

The statistical study was carried by utilising SPSS version 21 software. The result was considered statistically significant when P was less than 0.05.

RESULTS:

This study included 155 cases in total, of which 82 (52.9%) were male and 73(47.10%) were female. In this study, the patients' ages ranged from 3 to 56 years. The age-wise distribution has been tabulated in Table 1. The majority of dengue cases were reported in the 21-30 age group, with 68 cases. This was followed by the 31-40 age group, which had 38 cases. The fewest cases were observed in the 41-60 age group, with only 10 cases. Table 2 displays the frequency of clinical findings and total number of dengue-positive cases.

Table 1: Age and sex wise distribution of dengue positive patients

Age group (Years)	Male (n)	%	Female (n)	%	Total (n)
0-10	7	8.52	5	6.85	12
11-20	15	18.29	12	16.44	27
21-30	32	39.02	36	49.31	68
31-40	22	26.83	16	21.92	38
41-60	6	7.32	4	5.48	10
Total (n)	82		73		155

Table 2: Number of cases according to clinical sign and symptom in dengue positive cases

	Number of cases (n)	Percentage (%)
Fever	155	100
Headache	132	85.16
Myalgia	128	82.58
Rash	52	33.54
Pain Abdomen	75	48.39
Bleeding Manifestation	23	14.84
Vomiting	24	15.48
Diarrhea	14	9.03

IgM, IgG, and dengue NS1 antigen have been determined in all serum samples obtained for this investigation. Based on the presence of the IgM antibody and the NS1 antigen, 69.67% (108/155) of the 155 examined samples were determined to be positive for dengue virus infection. This was followed by the presence of the NS1 antigen (16.77%) (26/155), as shown in Table 3. Only one sample was found to have an IgG-positive antibody for dengue virus.

Table 3: Comparison of efficacy of various dengue specific parameters in the diagnosis of dengue infection using rapid test kit method on the day (Day 1) of admitted in hospital

Method of detection	Total number of cases (n)	Percentage (%)
NS1 only	26	16.77
NS1 + IgM	108	69.67
IgM only	12	7.75
IgM + IgG	8	5.16
IgG only	1	0.65

A total of 155 clinically suspected dengue cases (positive by one or more of the following tests: NS1, IgM, and IgG) were assessed further from day 1 to day 7 of fever. Figure 1 displays the sensitivities of IgM/IgG antibody and NS1 antigen detection in relation to the number of days following the beginning of fever. The sensitivity of NS1 antigen detection increased from day 1 to day 4 (145 cases showed positivity out of 155 on 4th day). Afterwards, the sensitivity of NS1 declined up to the 7th day. The sensitivity towards the positivity of the IgM antibody in all suspected dengue cases increased from day 1 to the 3rd day and started to decline up to the 7th day. The presence of IgG antibody positivity was very low in all cases.

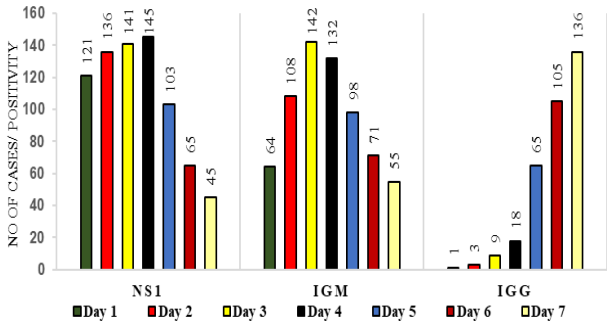


Figure 1. NS1, IgM, and IgG positivity in all suspected dengue cases from the infection's onset date.

The sensitivity of the IgG antibody was drastically

increased from the 5th day of the onset of dengue fever. Five samples that tested positive by the NS1 rapidly immunochromatographic test method but negative by ELISA were considered to be false negatives. Ten samples tested positive by ELISA but negative by rapid RIT for NS1 Ag. Based on findings from the RIT approach, the Dengue NS1 Ag MICROLISA test was used to determine the accuracy, specificity, positive predictive value, and negative predictive value for the Dengue NS1 antigen. The assay showed an 82.76% specificity and a 92.06% sensitivity. Furthermore, there was a 95.87% positive predictive value and a 70.59% negative predictive value. (Table 4).

Table 4: Comparison of two method for the detection of NS1 Antigen by RIT (rapid Immuno-chromatography test method and NS1 antigen detection by ELISA method.

	NS1 ELISA Positive (n=126)	**NS1 ELISA Negative (n=29)**
NS1 RIT positive (n=121)	116	05
NS1 RIT Negative (n=34)	10	24
Sensitivity	92.06% (95% CI= 85.89% to 96.13%)	
Specificity	82.76% (95% CI= 64.23% to 94.15%)	
Positive Predictive Value	95.87%	
Negative Predictive Value	70.59%	

In the present study, we investigate the association of dengue diagnostic parameters with platelet count on the 1st day of hospital admission. For this analysis, we considered 155 dengue positive cases that showed positivity of NS1antigen only, combined NS1 and IgM antibody, and IgM/IgG antibody. Out of these 155 cases, 46 subjects had a platelet count less than 1.0lakh/ml, and 109 subjects had a platelet count greater than 1lakh/ml. The rate of NS1 antigen positivity was significantly higher in patients (n = 21, 80.76%) with platelet count less than 1.0 lakh/ml when compared to (n = 5, 19.24%) platelet count more than 1.0lakh/ml as presented in Figure 2. When NS1 was found, thrombocytopenia was more frequently connected than when antibodies were found (P value <0.001).

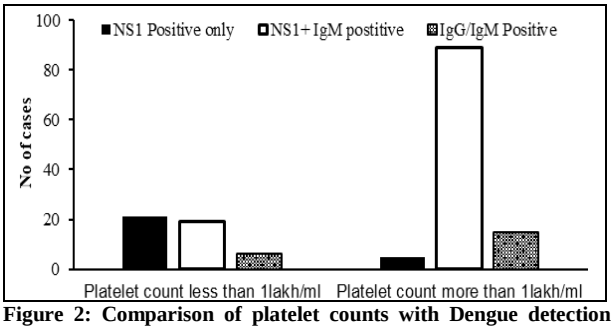


Figure 2: Comparison of platelet counts with Dengue detection parameters.

DISCUSSION:

Dengue has evolved to be a serious health issue for the public across the Indian subcontinent and its surrounding nations over the past few decades[25]. Establishing an early detection of a dengue infection within a few days of the onset of symptoms is crucial for both early and effective healthcare control of dengue epidemics as well as timely and accurate patient therapy. In this study, we used patient data to investigate the association between age and laboratory-confirmed clinical dengue. The highest number of dengue virus affected patients were in the age group of 21–30 years, and the second most common affected age was 31–40 years. There is a correlation between age and a higher chance of developing clinical dengue[13]. Throughout the period, the percentage of dengue cases was greater in women than in men in the age range 21-30 year. A dengue virus infection can cause a wide range of symptoms, from a slight fever that goes away on its own to a severe sickness that can be fatal. A sickness known as "symptomatic dengue" is defined as a temperature exceeding 40°C, accompanied by rashes, myalgia, arthralgia, headache, nausea, vomiting, and retroorbital pain[26]. The symptoms of rash and dyspnea demonstrated a positive association with dengue, whereas joint, bone, and muscle pain, rhinorrhea, and a confluence of nausea and diarrhea exhibited a negative relationship. Headaches and myalgia were more frequently reported in the dengue-positive group[27].

Dengue infection can be identified within the laboratory setting through the utilization of a diverse range of methodologies, encompassing the detection of RNA by real time RT-PCR or nested RTPCR[28], the examination of antigens, the evaluation of antibodies and fluorescence immunoassay (FIA)[29]. We use an immunochromatographic card assay to examine the patterns of circulating antigen NS1, antibodies IgM and IgG during the progression of the disease in order to evaluate the combined diagnostic value of fast assays. While ELISA assay can provide a precise diagnosis of dengue infection but it is an expensive, time-consuming procedure that is out of the reach of even the majority of tertiary care hospitals[30]. The 5-minute Rapid Card test is an easy test that may be completed without complex equipment, making it possible to administer it even in places without a lot of laboratory space[29].

Serum containing the highly specific NS1 antigen can be found one to nine days after the start of a fever during dengue virus infection[31,32]. In clinical practice, point-of-care rapid diagnostic tests (RDTs) have been widely utilised as the primary diagnostic technique for dengue NS1 antigen and dengue-specific IgM detection[33]. Dengue IgM begins increasing on day 4, following the commencement of the disease. In this investigation, blood samples from 155 dengue patients were examined,

and dengue-specific IgM or IgG antibodies as well as dengue NS1 antigen were confirmed in the lab by RDT. Based on the results of the combined NS1 antigen and the IgM antibody, 69.67% (108/155) of the sera were found to be positive for DENV infection, and 5.16% of the sera tested positive for both IgM and IgG antibodies on the day of hospital admission (Day 1). Compared to RDTs that relied solely on antibody or antigen detection, these assays showed a significant increase in accuracy over the course of the patient presentation period by integrating the findings of both NS1 antigen and IgM antibody detection[34,35]. Combining antigen- and antibody-based testing has definite benefits, and it should be used in reference assay and RDT formats alike.

The current investigation examined the changing patterns of NS1 antigen detection and IgG/IgM antibody kinetics in all dengue-positive patients from the first day of hospital admission to the seventh day. As hospitalization and treatment progressed, we noticed a tendency toward a decrease in NS1 antigen detection and an increase in antibody IgG/IgM detection. The results of the present investigation showed a correlation between the number of days after the beginning of fever and the sensitivity of NS1 antigen and antibody detection[36,37].

This study evaluated the dengue ELISA test and the rapid immunochromatographic test for dengue NS1 antigen detection in order to facilitate early dengue diagnosis. A total of 126 cases were positive for NS1 antigen by ELISA method, whereas 116 cases were positive by the rapid card test. Thus, only 10 cases (7.9%) of NS1 positivity were missed by the rapid immunochromatographic card test. In this study, sensitivity and specificity were 92.06% and 82.76%, respectively, when NS1 antigen was considered on rapid immunochromatographic test kits when compared to ELISA test kit[38,39]. The combination of the NS1 antigen card test and ELISA assay provides the most reliable and cost-effective diagnosis of dengue[40,41]. Rapid ICTs, on the other hand, have the benefit of being quick, easy to perform, and requiring minimal expertise. They can be completed in a matter of minutes. An attempt has been made to determine the relationship between thrombocytopenia and the dengue serological marker in this study. If the platelet count is less than 100,000/mL, it is considered thrombocytopenia[42].The study revealed that NS1 positivity (80.76%) was higher in thrombocytopenia patients than IgM/IgG positivity (30.43%), indicating a strong association amongst NS1 and thrombocytopenia in contrast to antibodies IgG and IgM[43,44]. NS1 antigen detection exhibited the maximum sensitivity in the early stages, although IgM detection was more sensitive in the latter stages. In terms of cost, convenience of use, and speed, NS1 RDT and ELISA are both beneficial for early dengue diagnosis.

CONCLUSION:

The current investigation demonstrated a highly substantial correlation between thrombocytopenia and dengue parameter NS1-positive cases. Determining dengue NS1 may shorten the window of opportunity within the first several days of illness. Testing for both IgM antibodies and dengue NS1 antigen enables higher diagnostic rates.

CONFLICTS OF INTEREST OF EACH AUTHOR:

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS:

Concept, Design, proof reading – HVP; Supervision & Funding – PNJ; Execution of experiment, Data collection– DA.; Literature search & Writing – LP and RZ; Analysis and/or interpretation of Data – NKS

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Evaluations

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NIV/DEN/EVA 1/14-15

July 20, 2014

To,
J Mitra and Co. Pvt. Ltd.
A180, 181 Okhala Industrial area,
Phase I, New Delhi 110020

Dear Sir,

I am forwarding the report of the tests done on the Kit (Dengue NS1 Ag screening Micro-plate ELISA test system) for qualitative detection of dengue virus NS1antigen in human serum or plasma manufactured by J Mitra and Co. Pvt. Ltd, supplied by you for evaluation. A total of 15 known NS1 antigen negative and 15 NS1 antigen positive sera were tested.

1. Lot No.: EDA040414,
   Manufactured On 4/2014
   Expiry On 03/2015
   Sensitivity: 100 %,
   Specificity: 93.3 %
   Efficiency: 96.6 %

With regards,
Yours sincerely,

Dr. P.S. Shah
Scientist 'D'
Dengue gr.

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EVALUATION REPORT OF DENGUE NS1 Ag MICROLISA

Name of the kit evaluated : Dengue NS1 Ag Microlisa
Name of the manufacturer : J. Mitra & Co. Pvt Ltd.
A-180-181, Okhla Industrial Area, Phase I,
New Delhi – 110020
Lot Number : EDA-061013
Date of Manufacture : 2013-10
Date of Expiry : 2014-09

The performance of Dengue NS1 Ag Microlisa kit was evaluated for its sensitivity and specificity using a total of 108 samples including both confirmed Dengue NS1 positive and negative samples.

SENSITIVITY

The sensitivity of Dengue NS1 Ag Microlisa kit was evaluated on 58 retrospective serum samples from patients with dengue infection confirmed by Dengue RT-PCR. The samples were from the four different dengue serotypes (Den1, Den 2, Den 3, Den 4).

The sensitivity of the Dengue NS1 Ag Microlisa kit when compared with Dengue RT-PCR is as follows:

Serotype	Dengue RT PCR Positive Samples	Result of Dengue NS1 Ag Microlisa	Sensitivity of Dengue NS1 Ag Microlisa (%)
Den 1	25	25	100%
Den 2	8	7	87.5%
Den 3	22	22	100%
Den 4	3	3	100%

The Overall sensitivity of Dengue NS1 Ag Microlisa kit on the above panel was found to be 98.28%.

SPECIFICITY

The specificity of Dengue NS1 Ag Microlisa kit was evaluated on 50 known Dengue negative samples. All the 50 samples were negative in Dengue NS1 Ag Microlisa Kit resulting in a specificity of 100%.

RESULT	SENSITIVITY	SPECIFICITY
	98.28%	100%

Navin Khanna PhD
Group Leader, Recombinant Gene Products Laboratory

November 14, 2013

अखिलभारतीयआयुर्विज्ञानसंस्थान, नागपुर

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, NAGPUR
Address: Plot No2, Sector 20, MIHAN, Nagpur-441108
Department of Microbiology

AIIMS-NAG/ Micro/Misc/2021 Date: 14/12/2021

Evaluation Report of Dengue NS1 Ag Microlisa

OBJECTIVE : To evaluate the performance of the Dengue NS1Ag Microlisa

Name of Kit : **DENGUE NS1 Ag MICROLISA**

Lot Number : **EDA020821**

Expiry on : **2023-01**

Manufactured by : JMitra & Co. Pvt. Ltd., New Delhi

SAMPLE SOURCE : 72 Fresh and stored serum samples from clinically dengue suspected patients were used for evaluation of Dengue NS1 Ag Microlisa. To check the performance of the kit with regards specificity is tested with cross reacting samples; 2 Malaria positive and 1 Typhoid positive.

The status of the samples is already confirmed by commercially available Biorad Dengue NS1 Ag Elisa.

INTERPRETATION : Results are noted at the end of test procedure & results were interpreted as per the instructions given in the instruction manual.

14/12/2021
Dr. Meena Mishra
Professor & Head
Department of Microbiology
AIIMS, Nagpur

RESULTS :

No.Of Sample	Sample Attribute	Dengue NS1 Ag Microlisa		Biorad Dengue NS1 Ag Elisa	
		Positive	Negative	Positive	Negative
72	Screened Positive for Dengue NS1	67	0	67	0
	Screened Negative for Dengue NS1	0	5	0	5

CONCLUSION:

1) Dengue NS1 Ag Microlisa gave comparable results.

2) Dengue NS1 Ag Microlisa gave 100% sensitivity and 100% specificity.

3) The detailed results are enclosed in the following Annexure.

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Department of Microbiology,
AIIMS, Nagpur

Annexure

Sample No.	Dengue NS1 Ag Microlisa Unit	ResultInterpretation	Biorad Dengue NS1 AgIndex value	ResultInterpretation	Correlation
Smp11	59.44	Positive	4.87	Positive	Yes
Smp12	78.10	Positive	3.13	Positive	Yes
Smp13	85.80	Positive	3.56	Positive	Yes
Smp14	87.16	Positive	3.65	Positive	Yes
Smp15	84.78	Positive	4.12	Positive	Yes
Smp16	80.53	Positive	3.91	Positive	Yes
Smp17	0.08	Negative	0.03	Negative	Yes
Smp18	75.69	Positive	6.91	Positive	Yes
Smp19	78.90	Positive	8.18	Positive	Yes
Smp110	82.70	Positive	7.56	Positive	Yes
Smp111	41.32	Positive	5.32	Positive	Yes
Smp112	80.47	Positive	7.58	Positive	Yes
Smp113	74.98	Positive	7.98	Positive	Yes
Smp114	76.13	Positive	8.12	Positive	Yes
Smp115	82.68	Positive	8.95	Positive	Yes
Smp116	79.61	Positive	7.32	Positive	Yes
Smp117	84.52	Positive	7.55	Positive	Yes
Smp118	77.93	Positive	6.25	Positive	Yes
Smp119	86.27	Positive	9.38	Positive	Yes
Smp120	85.81	Positive	9.22	Positive	Yes

Senior Resident
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AIIMS, Nagpur

Smp121	79.65	Positive	8.65	Positive	Yes
Smp122	70.25	Positive	6.21	Positive	Yes
Smp123	86.52	Positive	7.92	Positive	Yes
Smp124	81.07	Positive	7.85	Positive	Yes
Smp125	78.47	Positive	8.31	Positive	Yes

Sample No.	Dengue NS1 AgMicrolisa Unit	ResultInterpretation	Biorad Dengue NS1 Index value	ResultInterpretation	Correlation
Smp126	78.73	Positive	8.55	Positive	Yes
Smp127	84.53	Positive	9.37	Positive	Yes
Smp128	77.16	Positive	6.51	Positive	Yes
Smp129	86.16	Positive	7.99	Positive	Yes
Smp130	80.03	Positive	9.12	Positive	Yes
Smp131	62.99	Positive	5.32	Positive	Yes
Smp132	65.53	Positive	7.31	Positive	Yes
Smp133	73.61	Positive	7.86	Positive	Yes
Smp134	81.37	Positive	8.29	Positive	Yes
Smp135	84.74	Positive	9.35	Positive	Yes
Smp136	87.80	Positive	7.62	Positive	Yes
Smp137	86.42	Positive	8.15	Positive	Yes
Smp138	83.61	Positive	7.81	Positive	Yes
Smp139	86.35	Positive	8.11	Positive	Yes
Smp140	45.03	Positive	5.19	Positive	Yes

14/12/21
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Smp141	85.96	Positive	9.11	Positive	Yes
Smp142	85.58	Positive	7.92	Positive	Yes
Smp143	77.97	Positive	6.33	Positive	Yes
Smp144	85.81	Positive	7.88	Positive	Yes
Smp145	86.53	Positive	8.21	Positive	Yes
Smp146	84.30	Positive	9.15	Positive	Yes
Smp147	87.13	Positive	8.18	Positive	Yes
Smp148	82.80	Positive	7.26	Positive	Yes
Smp149	0.27	Negative	0.08	Negative	Yes
Smp150	55.42	Positive	4.91	Positive	Yes

Sample No.	Dengue NS1 Ag Microlisa Unit	ResultInterpretation	Biorad Dengue NS1 Ag Index value	ResultInterpretation	Correlation
Smp151	83.40	Positive	7.65	Positive	Yes
Smp152	86.25	Positive	7.96	Positive	Yes
Smp153	83.72	Positive	8.95	Positive	Yes
Smp154	31.05	Positive	4.26	Positive	Yes
Smp155	79.17	Positive	6.25	Positive	Yes
Smp156	0.15	Negative	0.05	Negative	Yes
Smp157	41.14	Positive	5.27	Positive	Yes
Smp158	56.59	Positive	6.11	Positive	Yes
Smp159	32.12	Positive	2.18	Positive	Yes
Smp160	76.64	Positive	8.12	Positive	Yes

Senior Resident
Department of Microbiology,

Smpl61	83.82	Positive	8.97	Positive	Yes
Smpl62	78.44	Positive	8.35	Positive	Yes
Smpl63	65.33	Positive	7.16	Positive	Yes
Smpl64	0.06	Negative	0.01	Negative	Yes
Smpl65	68.60	Positive	6.21	Positive	Yes
Smpl66	85.22	Positive	9.15	Positive	Yes
Smpl67	78.24	Positive	8.33	Positive	Yes
Smpl68	84.74	Positive	7.65	Positive	Yes
Smpl69	80.21	Positive	9.11	Positive	Yes
Smpl70	0.11	Negative	0.04	Negative	Yes
Smpl71	58.18	Positive	6.22	Positive	Yes
Smpl72	83.14	Positive	9.15	Positive	Yes
Malaria	0.06	Negative	0.02	Negative	Yes
Malaria	0.07	Negative	0.03	Negative	Yes
Typhoid	0.09	Negative	0.06	Negative	Yes

Sample No.	Dengue NS1 Ag Microlisa Unit	ResultInterpretation	Biorad Dengue NS1 Ag Index value	ResultInterpretation	Correlation
NC	0.002	PC1	2.9825	-	-
CL1	0.6586	PC2	2.8611	-	-
CL2	0.6367	NC1	0.0025	-	-
CL3	0.6343	NC2	0.0035	-	-
PC	3.0075	NC3	0.0022	-	-

14/11/24
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Result Interpretation Criteria of Dengue NS1 Ag Microlisa

Dengue NS1 Ag positive if Unit is > 11

Dengue NS1 Ag Negative if Unit is < 9

Dengue NS1 Ag Equivocal if Unit is 9-11

Result Interpretation Criteria of Biorad Dengue NS1 Ag Elisa

Dengue NS1 Ag Positive if Index value is > 0.5

Dengue NS1 Ag Negative if Index value is < 0.5

Verified By: 14/12/21

(Dr. Pooja Shende)
Senior Resident
Department of Microbiology,
AIIMS, Nagpur

14/12/2021

Dr. Meena Mishra
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10-05-2016

EVALUATION REPORT

Name of the kit	: DENGUE NS1 Ag MICROLISA
LOT number	: EDA050814
Expiry	: 01.07.2015
Imported and Marketed by	: J Mitra & Co. Pvt. Ltd
Panel used for testing	: Serum samples
Sensitivity	: 100%
Specificity	: 90.91%
Positive Predictive Value	: 90.48%
Negative Predictive Value	: 100%

The above stated kit gave concordant result with the panel of 81 samples used and is fit for diagnostic use.

Thanking you

Yours sincerely

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DENGUE VALIDATION REPORT NS1 ELISA VS RTPCR

Name of the Kit: Dengue NS1Ag Microlisa

Manufacturer: J.Mitra&.Pvt.Ltd, NewDelhi, India.

Lot No.: EDA010321

Expiry Date: 2022-08

Name of the kit : HI-PCR Dengue Serotyping Probe PCR kit.

Manufacturer : HIMEDIA Laboratories Pvt.Ltd. India.

Lot No: 0000506949

Expiry Date: 2022-11

Methodology : A total of 100 stored clinical sample suspected Dengue are taken from the in patients admitted at Govt General hospital, Ananthapurum department of General medicine and department of pediatric and tested on Dengue NS1 Ag by Jmitra & Co.pvt.Ltd company Elisa kit and the results correlated with HI-PCR Dengue Serotyping probe PCR kit manufactured by HIMEDIA Company.

Intended Use: Dengue NS1Ag Microlisa is an enzyme immunoassay based on Sandwich ELISA principle designed for in-vitro qualitative detection of Dengue NS1Ag (Dengue serotype 1,2,3,4) in human serum or plasma and is used as a screening test of collected blood samples suspected for Dengue.

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Dengue virus is a single – Standard RNA virus comparing mainly four distinct serotypes (DENV-1 to– 4), Nucleic acid amplification based assays or Polymerase Chain Reaction (PCR) is an alternative method of Dengue Serotyping that allows for sensitive and specific detection of Dengue RNA. Real-time PCR technique is considerably simple and fast with respect to the standard PCR technique. This technique has been successfully used for the rapid detection and identification of a variety of infections pathogens and recommended for sensitive and specific detection of DEN 1-4 serotype in clinical Samples from suspected from dengue patients.

Results: Results observed are as given below;

Sample Type	**No. of samples Tested**	**Results with J.Mitra Dengue NS1Ag Microlisa**		**Results with HI-PCR Dengue Serotyping probe PCR kit**	
	100	**Dengue NS1 Ag Positive**	**Dengue NS1 Ag Negative**	**Dengue RTPCR Positive**	**DengueRTPCR Negative**
Dengue NS1Ag Positive		**30**		**32**	
Dengue NS1Ag Negative			**70**		**68**

RTPCR

ELISA

	+VE	--VE
+	30	0
-	02	68

Sensitivity= TP/TP+FN 30/30+2 30/32 = 93.75%

Specificity = TN/TN+FP 68/68 68/68 = 100 %

Conclusion:

The evaluation of study shows that the Dengue NS1Ag Microlisa from J.Mitra&Co.Pvt.Ltd has shown an overall sensitivity 93.75% and specificity 100% respectively in correlation with the results of Dengue HI-PCR Dengue Serotyping probe PCR with the tested samples.

Thanking You
Consultant Microbiologist

ADIREDDI PARADESI NAIDU

ADIREDDI PARADESI NAIDU
M.Sc., Medical (PhD)
Tutor in Dept. of Microbiology
Govt. Medical College,
ANANTHAPURAMU.

Date: 04-02-2022

Enclosure Detailed Annexure: 1

S.No	SAMPLE TYPE	Dengue NS1Ag (J.Mitra Co) Microlisa Units Cut of value 0.371	Results	Hi-Media Serotype probe RTPCR values	RTPCR Results With Serotyping	Result correlation /yes/No
PhD 01	SERUM	3.02	Negative	NA	Negative for DENV 1-4	YES
PhD 02	SERUM	3.41	Negative	NA	Negative for DENV 1-4	YES

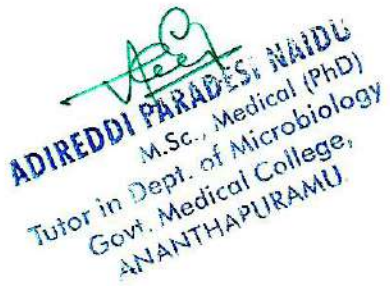
PhD 03	SERUM	2.48	Negative	NA	Negative for DENV 1-4	YES
PhD 04	SERUM	3.14	Negative	NA	Negative for DENV 1-4	YES
PhD 05	SERUM	2.03	Negative	NA	Negative for DENV 1-4	YES
PhD 06	SERUM	102.89	Positive	36.27	Positive for DENV-2	YES
PhD 07	SERUM	3.13	Negative	NA	Negative for DENV 1-4	YES
PhD 08	SERUM	102.09	Positive	24.13	Positive for DENV-2	YES
PhD 09	SERUM	3.5	Negative	NA	Negative for DENV 1-4	YES
PhD 10	SERUM	3.65	Negative	NA	Negative for DENV 1-4	YES
PhD 11	SERUM	3.26	Negative	NA	Negative for DENV 1-4	YES
PhD 12	SERUM	3.02	Negative	NA	Negative for DENV 1-4	YES
PhD 13	SERUM	2.27	Negative	NA	Negative for DENV 1-4	YES
PhD 14	SERUM	2.87	Negative	NA	Negative for DENV 1-4	YES
PhD 15	SERUM	88.8	Positive	26-08	Positive for DENV-2	YES
PhD 16	SERUM	3.98	Negative	NA	Negative for DENV 1-4	YES
PhD 17	SERUM	5.47	Negative	NA	Negative for DENV 1-4	YES
PhD 18	SERUM	2.04	Negative	NA	Negative for DENV 1-4	YES
PhD 19	SERUM	90.45	Positive	35.97	Positive for DENV-2	YES
PhD 20	SERUM	2.12	Negative	NA	Negative for DENV 1-4	YES
PhD 21	SERUM	1.56	Negative	NA	Negative for DENV 1-4	YES
PhD 22	SERUM	89.08	Positive	22.56	Positive for DENV-2	YES
PhD 23	SERUM	1.02	Negative	NA	Negative for DENV 1-4	YES
PhD 24	SERUM	1.80	Negative	NA	Negative for DENV 1-4	YES
PhD 25	SERUM	1.32	Negative	NA	Negative for DENV 1-4	YES

PhD 26	SERUM	1.94	Negative	NA	Negative for DENV 1-4	YES
PhD 27	SERUM	88.16	Positive	22.10	Positive for DENV-2	YES
PhD 28	SERUM	90.72	Positive	34.95	Positive for DENV-2	YES
PhD 29	SERUM	1.18	Negative	NA	Negative for DENV 1-4	YES
PhD 30	SERUM	1.18	Negative	NA	Negative for DENV 1-4	YES
PhD 31	SERUM	33.96	Positive	34.85	Positive for DENV-1	YES
PhD 32	SERUM	1.56	Negative	NA	Negative for DENV 1-4	YES
PhD 33	SERUM	1.59	Negative	NA	Negative for DENV 1-4	YES
PhD 34	SERUM	1.50	Negative	NA	Negative for DENV 1-4	YES
PhD 35	SERUM	8.67	Negative	NA	Negative for DENV 1-4	YES
PhD 36	SERUM	90.05	Positive	22.35	Positive for DENV-2	YES
PhD 37	SERUM	1.05	Negative	NA	Negative for DENV 1-4	YES
PhD 38	SERUM	1.024	Negative	NA	Negative for DENV 1-4	YES
PhD 39	SERUM	0.08	Negative	NA	Negative for DENV 1-4	YES
PhD 40	SERUM	1.34	Negative	NA	Negative for DENV 1-4	YES
PhD 41	SERUM	2.42	Negative	NA	Negative for DENV 1-4	YES
PhD 42	SERUM	12.96	Positive	34.39	Positive for DENV-2	YES
PhD 43	SERUM	92.61	Positive	28.91	Positive for DENV-3	YES
PhD 44	SERUM	2.34	Negative	NA	Negative for DENV 1-4	YES
PhD 45	SERUM	1.29	Negative	NA	Negative for DENV 1-4	YES
PhD 46	SERUM	1.34	Negative	NA	Negative for DENV 1-4	YES
PhD 47	SERUM	0.99	Negative	36.33	Positive for DENV-2	NO
PhD 48	SERUM	0.91	Negative	NA	Negative for DENV 1-4	YES

PhD 49	SERUM	1.18	Negative	NA	Negative for DENV 1-4	YES
PhD 50	SERUM	1.59	Negative	NA	Negative for DENV 1-4	YES
PhD 51	SERUM	0.970	Negative	NA	Negative for DENV 1-4	YES
PhD 52	SERUM	2.075	Negative	NA	Negative for DENV 1-4	YES
PhD 53	SERUM	1.37	Negative	NA	Negative for DENV 1-4	YES
PhD 54	SERUM	1.07	Negative	NA	Negative for DENV 1-4	YES
PhD 55	SERUM	1.889	Negative	NA	Negative for DENV 1-4	YES
PhD 56	SERUM	88.22	Positive	35.16	Positive for DENV-2	YES
PhD 57	SERUM	1.07	Negative	NA	Negative for DENV 1-4	YES
PhD 58	SERUM	2.58	Negative	NA	Negative for DENV 1-4	YES
PhD 59	SERUM	92.74	Positive	28.29	Positive for DENV-2	YES
PhD 60	SERUM	12.74	Positive	36.42	Positive for DENV-2	YES
PhD 61	SERUM	1.32	Negative	NA	Negative for DENV 1-4	YES
PhD 62	SERUM	1.59	Negative	NA	Negative for DENV 1-4	YES
PhD 63	SERUM	1.48	Negative	NA	Negative for DENV 1-4	YES
PhD 64	SERUM	1.56	Negative	NA	Negative for DENV 1-4	YES
PhD 65	SERUM	84.55	Positive	32.16	Positive for DENV-2	YES
PhD 66	SERUM	88.43	Positive	23.32	Positive for DENV-1	YES
PhD 67	SERUM	89.38	Positive	30.08	Positive for DENV-2	YES
PhD 68	SERUM	91.69	Positive	24.94	Positive for DENV-2	YES
PhD 69	SERUM	88.62	Positive	18.86	Positive for DENV-3	YES
PhD 70	SERUM	1.37	Negative	NA	Negative for DENV 1-4	YES
PhD 71	SERUM	86.95	Positive	28.54	Positive for DENV-2	YES

PhD 72	SERUM	90.48	Positive	29.07	Positive for DENV-2	YES
PhD 73	SERUM	9.514	Eqvivocal	NA	Negative for DENV 1-4	YES
PhD 74	SERUM	1.42	Negative	NA	Negative for DENV 1-4	YES
PhD 75	SERUM	4.25	Negative	NA	Negative for DENV 1-4	YES
PhD 76	SERUM	1.48	Negative	NA	Negative for DENV 1-4	YES
PhD 77	SERUM	1.23	Negative	NA	Negative for DENV 1-4	YES
PhD 78	SERUM	2.80	Negative	NA	Negative for DENV 1-4	YES
PhD 79	SERUM	90.02	Positive	24.38	Positive for DENV-2	YES
PhD 80	SERUM	93.42	Positive	26.10	Positive for DENV-3	YES
PhD 81	SERUM	99.02	Positive	20.46	Positive for DENV-2	YES
PhD 82	SERUM	1.32	Negative	37.92	Positive for DENV-2	NO
PhD 83	SERUM	1.88	Ngative	NA	Negative for DENV 1-4	YES
PhD 84	SERUM	73.68	Positive	32.14	Positive for DENV-2	YES
PhD 85	SERUM	1.26	Negative	NA	Negative for DENV 1-4	YES
PhD 86	SERUM	8.76	Negative	NA	Negative for DENV 1-4	YES
PhD 87	SERUM	91.21	Positive	21.16	Positive for DENV-2	YES
PhD 88	SERUM	1.40	Negative	NA	Negative for DENV 1-4	YES
PhD 89	SERUM	3.126	Negative	NA	Negative for DENV 1-4	YES
PhD 90	SERUM	8.65	Negative	NA	Negative for DENV 1-4	YES
PhD 91	SERUM	92.12	Positive	29.22	Positive for DENV-3	YES
PhD 92	SERUM	2.29	Negative	NA	Negative for DENV 1-4	YES
PhD 93	SERUM	2.075	Negative	NA	Negative for DENV 1-4	YES
PhD 94	SERUM	1.48	Negative	NA	Negative for DENV 1-4	YES

PhD 95	SERUM	92.77	Positive	20.03	Positive for DENV-2	YES
PhD 96	SERUM	9.32	Eqvivocal	NA	Negative for DENV 1-4	YES
PhD 97	SERUM	1.85	Negative	NA	Negative for DENV 1-4	YES
PhD 98	SERUM	91.15	Positive	22.84	Positive for DENV-2	YES
PhD 99	SERUM	90.78	Positive	32.16	Positive for DENV-2	YES
PhD 100	SERUM	3.072	Negative	NA	Negative for DENV 1-4	YES



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Evaluation of Dengue NS1Ag Microlisa

Name of the Kit: Dengue NS1Ag Microlisa

Manufacturer: J.Mitra &Co.Pvt.Ltd, NewDelhi,India.

Lot no & Expiry: EDA041017 Exp 2019-03

Methodology : A total of 100 clinical samples; 40 Positive and 60 negative samples were tested by Dengue NS1Ag Capture ELISA from Panbio and were compared with Dengue NS1Ag Microlisa from J.Mitra & Co.Pvt.Ltd as per the test procedure given by the manufacturer.

Intended Use: Dengue NS1Ag Microlisa is an enzyme immunoassay based on Sandwich ELISA principle designed for in-vitro qualitative detection of Dengue NS1Ag in human serum or plasma and is used as a screening test of collected blood samples suspected for Dengue.

Results: Results observed are as given below;

Sample Type	No. of samples Tested	Results with J.Mitra Dengue NS1Ag Microlisa	Results with Panbio Dengue NS1 Early ELISA
Dengue NS1Ag Positive	40	40	40
Dengue NS1Ag Negative	60	60	60

Sensitivity-100 % **Specificity-100%**

Conclusion:

The evaluation study shows that the Dengue NS1Ag Microlisa from J.Mitra &Co.Pvt.Ltd has shown an overall sensitivity and specificity of 100% and there is good correlation with the results of Dengue Early NS1Ag ELISA from Panbio with the tested samples.

Thanking you

26-03-2018

Dr Satish Kumar Reddy
Department of Microbiology
Apollo BGS Hospital Mysore

Enclosed : Detailed annexure

Dr. Satishkumar Reddy. G
Microbiologist
Apollo BGS Hospitals, Mysore

ANNEXURE-1(STUDY REPORT)

Sample ID	Sample Type	Results with Panbio Dengue Early NS1Ag ELISA	Results with J.Mitra Dengue NS1Ag Microlisa	Results
		Panbio units <9 –Negative 9-11-equivoca >11-Positive	IgM units <9 –Negative 9-11-equivocal >11-Positive	
1	Serum	11.65	33.00	Positive
2	serum	2.15	3.02	Negative
3	Serum	1.80	2.08	Negative
4	serum	54.69	51.34	Positive
5	Serum	5.12	3.02	Negative
6	serum	3.15	0.10	Negative
7	Serum	11.8	13.1	Positive
8	serum	56.21	54.22	Positive
9	Serum	3.90	2.36	Negative
10	serum	2.45	1.34	Negative
11	Serum	16.4	15.8	Positive
12	serum	2.0	1.55	Negative
13	Serum	1.22	0.985	Negative
14	serum	53.36	52.87	Positive
15	Serum	7.28	6.43	Negative
16	serum	6.44	5.32	Negative
17	Serum	4.50	4.10	Negative
18	serum	56.36	50.16	Positive
19	Serum	3.75	3.10	Negative
20	serum	0.87	0.65	Negative
21	Serum	1.28	1.07	Negative
22	serum	54.41	52.81	Positive
23	Serum	12.89	20.95	Positive
24	serum	21.5	20.8	Positive
25	Serum	0.48	0.32	Negative
26	serum	21.15	24.02	Positive
27	Serum	2.2	1.9	Negative
28	serum	2.8	3.4	Negative
29	Serum	3.1	3.9	Negative
30	serum	2.0	1.3	Negative
31	Serum	56.36	48.83	Positive
32	serum	41.91	56.65	Positive
33	Serum	55.15	55.04	Positive
34	serum	3.8	3.02	Negative
35	Serum	1.2	2.1	Negative

Dr. Satishkumar Reddy. G
Microbiologist
Apollo BGS Hospitals, Mysore

Sample ID	Sample Type	Results with Panbio Dengue Early NS1Ag ELISA	Results with J.Mitra Dengue NS1Ag Microlisa	Results
		Panbio units <9 –Negative 9-11-equivocal >11-Positive	IgM units <9 –Negative 9-11-equivocal >11-Positive	
36	Serum	55.80	49.55	Positive
37	serum	6.14	5.45	Negative
38	Serum	5.60	7.80	Negative
39	serum	42.71	46.71	Positive
40	Serum	56.26	52.06	Positive
41	serum	3.90	2.30	Negative
42	Serum	1.29	3.82	Negative
43	serum	7.55	6.43	Negative
44	Serum	8.12	6.41	Negative
45	serum	2.44	3.08	Negative
46	Serum	5.01	4.72	Negative
47	serum	4.84	3.05	Negative
48	Serum	54.76	50.69	Positive
49	serum	6.07	5.12	Negative
50	Serum	1.02	0.86	Negative
51	serum	54.78	47.02	Positive
52	Serum	47.58	51.38	Positive
53	serum	2.08	2.19	Negative
54	Serum	0.52	0.30	Negative
55	serum	3.84	3.41	Negative
56	Serum	20.93	12.59	Positive
57	serum	51.80	21.24	Positive
58	Serum	1.38	2.32	Negative
59	serum	2.38	1.63	Negative
60	Serum	3.2	2.11	Negative
61	serum	1.22	0.35	Negative
62	Serum	5.26	3.73	Negative
63	serum	1.38	0.48	Negative
64	Serum	3.15	0.10	Negative
65	serum	37.5	40.46	Positive
66	Serum	42.97	28.59	Positive
67	serum	28.5	10.61	Positive
68	Serum	4.36	2.24	Negative
69	serum	1.11	0.28	Negative
70	Serum	8.23	3.02	Negative

Dr. Satishkumar Reddy. C
Microbiologist
Apollo BGS Hospitals, Mys

Sample ID	Sample Type	Results with Panbio Dengue Early NS1Ag ELISA Panbio units <9 –Negative 9-11-equivoca >11-Positive	Results with J.Mitra Dengue NS1Ag Microlisa IgM units <9 –Negative 9-11-equivocal >11-Positive	Results
36	Serum	55.80	49.55	Positive
37	serum	6.14	5.45	Negative
38	Serum	5.60	7.80	Negative
39	serum	42.71	46.71	Positive
40	Serum	56.26	52.06	Positive
41	serum	3.90	2.30	Negative
42	Serum	1.29	3.82	Negative
43	serum	7.55	6.43	Negative
44	Serum	8.17	6.41	Negative
45	serum	2.44	3.08	Negative
46	Serum	5.01	4.72	Negative
47	serum	4.84	3.05	Negative
48	Serum	54.76	50.69	Positive
49	serum	6.07	5.12	Negative
50	Serum	1.02	0.86	Negative
51	serum	54.78	47.02	Positive
52	Serum	47.58	51.38	Positive
53	serum	2.08	2.19	Negative
54	Serum	0.52	0.30	Negative
55	serum	3.84	3.41	Negative
56	Serum	20.93	12.59	Positive
57	serum	51.80	21.24	Positive
58	Serum	1.38	2.32	Negative
59	serum	2.38	1.63	Negative
60	Serum	3.2	2.11	Negative
61	serum	1.22	0.35	Negative
62	Serum	5.26	3.73	Negative
63	serum	1.38	0.48	Negative
64	Serum	3.15	0.10	Negative
65	serum	37.5	40.46	Positive
66	Serum	42.97	28.59	Positive
67	serum	28.5	10.61	Positive
68	Serum	4.36	2.24	Negative
69	serum	1.11	0.28	Negative
70	Serum	8.23	3.02	Negative

Dr. Satishkumar Reddy. G
Microbiologist
Apollo BGS Hospitals, Mysore

METROPOLIS
EXCELLENCE IN DIAGNOSTICS
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EVALUATION REPORT

Service Provider: Metropolis Healthcare Limited

Client: J Mitra PVT LTD

Kit Details: Dengue Ns1 Antigen microlisa

Lot No. - EDA 050913

Exp Date: 2014/08

Validated during: November 2013

Method: ELISA

Reference Range:

Negative	Below 9.0
Equivocal	9.0 – 11.0
Positive	Above 11.0

Evaluation Summary:

 Forty one cases (24 Positive and 17 Negative) which were earlier analyzed for dengue NS1 antigen using BIO RAD kit were analyzed for dengue NS1 antigen using J Mitra PVT LTD dengue NS1 antigen microlisa kit to assess the sensitivity and specificity of the assay.

Results:

Lot No: EDA 050913	**Quality Parameters**	**Results**
1	Sensitivity	True Positive (TP) = 24 False Negative (FN) = 2 Sensitivity = (TP/TP+FN)x100 = 92.3%
2	Specificity	True Negative (TN) = 17 False Positive (FP) = 0 Specificity = (TN/TN+FP)x100 = 100%

REMARKS: J Mitra PVT LTD Dengue NS1 antigen microlisa shows sensitivity of 92.3% and specificity of 100% this kit can be used for *in vitro* detection of dengue NS1 antigen in the clinical specimens.

Signature of Technical Head

Name & Designation
Pratip Patiyane
Operational Head

Date: 5/12/2013

Signature of Lab Head

Name & Designation
Dr Rajesh Bendre
Chief of Laboratory

Date: 5/12/13

Approved By:

Ms. Seema Jadhav
Associate Vice President – QA
Date: 5/12/2013

Evaluation of Dengue NS1Ag Microlisa

Name of the Kit: Dengue NS1Ag Microlisa

Manufacturer: J.Mitra &Co.Pvt.Ltd, NewDelhi, India.

Lot No.: EDA020821

Expiry Date: 2023-01

Methodology : A total of 65 stored clinical samples; 35 Dengue Positive ,20 negative & 10 Chikungunya Antibody positive (cross reacting samples) are taken and tested on **Dengue NS1Ag Capture ELISA** from **Panbio** and compared with **Dengue NS1Ag Microlisa** from **J.Mitra & Co.Pvt.Ltd** as per the test procedure given by the manufacturer. To check the reproducibility in test results of Dengue NS1Ag Microlisa , 1 negative ,1 weak positive and 1 strong positive sample was run in 4 different runs by one analyst.

Intended Use: Dengue NS1Ag Microlisa is an enzyme immunoassay based on Sandwich ELISA principle designed for in-vitro qualitative detection of Dengue NS1Ag (Dengue serotype 1,2,3,4) in human serum or plasma and is used as a screening test of collected blood samples suspected for Dengue.

Results: Results observed are as given below;

Sample Type	No. of samples Tested	Results with J.Mitra Dengue NS1Ag Microlisa		Results with Panbio Dengue NS1 Early ELISA	
		Dengue NS1 Ag Positive	Dengue NS1 Ag Negative	Dengue NS1 Ag Positive	Dengue NS1 Ag Negative
Dengue NS1Ag Positive	35	35	0	35	0
Dengue NS1Ag Negative	20	-	20	-	20
Chikungunya Antibody Positive	10	-	10	-	10

Sensitivity-100 % **Specificity-100%**

107680

Note : This report is subject to terms and conditions mentioned overleaf

Conclusion:

The evaluation study shows that the Dengue NS1Ag Microlisa from J.Mitra &Co.Pvt.Ltd has shown an overall sensitivity and specificity of 100% and there is good correlation with the results of Dengue Early NS1Ag ELISA from Panbio with the tested samples. Absolutely there was no cross reactivity with the Chikungunya Antibody positive samples.

T. Kishore

Thanking You
Consultant Microbiologist

YES LABS Hyderabad

Place Hyderabad

Date 3/12/21

Enclosure : Detailed Annexure - 1

107681

Note : This report is subject to terms and conditions mentioned overleaf

Annexure- 1 (Detailed observations of Dengue NS1 Ag MICROLISA)

LABORATORY TEST REPORT

S.No.	Lab Sample Number	Sample Type	JML DENGUE NS1Ag Microlisa Units Cut of value : 0.338	Results	PanBio DENGUE NS1Ag Early ELISA Units. Cut of value : 0.329	Result	Result correlation /Yes/No
1	YLBS DEN S1	Serum	42 Units	Dengue NS1 Ag Positive	48 Units	Dengue NS1 Ag Positive	Yes
2	YLBS DEN S2	Serum	36 Units	Dengue NS1 Ag Positive	40 Units	Dengue NS1 Ag Positive	Yes
3	YLBS DEN S3	Serum	96 Units	Dengue NS1 Ag Positive	88 Units	Dengue NS1 Ag Positive	Yes
4	YLBS DEN S4	Serum	46 Units	Dengue NS1 Ag Positive	54 Units	Dengue NS1 Ag Positive	Yes
5	YLBS DEN S5	Serum	26 Units	Dengue NS1 Ag Positive	28 Units	Dengue NS1 Ag Positive	Yes
6	YLBS DEN S6	Serum	58 Units	Dengue NS1 Ag Positive	55 Units	Dengue NS1 Ag Positive	Yes
7	YLBS DEN S7	Serum	34 Units	Dengue NS1 Ag Positive	36 Units	Dengue NS1 Ag Positive	Yes
8	YLBS DEN S8	Serum	29 Units	Dengue NS1 Ag Positive	25 Units	Dengue NS1 Ag Positive	Yes
9	YLBS DEN S9	Serum	42 Units	Dengue NS1 Ag Positive	47 Units	Dengue NS1 Ag Positive	Yes
10	YLBS DEN S10	Serum	55 Units	Dengue NS1 Ag Positive	59 Units	Dengue NS1 Ag Positive	Yes
11	YLBS DEN S11	Serum	70 Units	Dengue NS1 Ag Positive	64 Units	Dengue NS1 Ag Positive	Yes
12	YLBS DEN S12	Serum	82 Units	Dengue NS1 Ag Positive	88 Units	Dengue NS1 Ag Positive	Yes
13	YLBS DEN S13	Serum	22 Units	Dengue NS1 Ag Positive	24 Units	Dengue NS1 Ag Positive	Yes
14	YLBS DEN S14	Serum	118 Units	Dengue NS1 Ag Positive	121 Units	Dengue NS1 Ag Positive	Yes
15	YLBS DEN S15	Serum	16 Units	Dengue NS1 Ag Positive	18 Units	Dengue NS1 Ag Positive	Yes
16	YLBS DEN S16	Serum	52 Units	Dengue NS1 Ag Positive	59 Units	Dengue NS1 Ag Positive	Yes
17	YLBS DEN S17	Serum	84Units	Dengue NS1 Ag Positive	92 Units	Dengue NS1 Ag Positive	Yes
18	YLBS DEN S18	Serum	66 Units	Dengue NS1 Ag Positive	54 Units	Dengue NS1 Ag Positive	Yes
19	YLBS DEN S19	Serum	32 Units	Dengue NS1 Ag Positive	30 Units	Dengue NS1 Ag Positive	Yes

107837

Note : This report is subject to terms and conditions mentioned overleaf
PARTIAL REPRODUCTION OF THE REPORT IS NOT PERMITTED

S.No	Lab Sample Number	Sample Type	JML DENGUE NS1Ag Microlisa Units Cut of value : 0.338	Results	PanBio DEN NS1Ag Early ELISA Units. Cut of value : 0.329	Result	Result ation /Yes/No
20	YLBS DEN S20	Serum	88 Units	Dengue NS1 Ag Positive	80 Units	Dengue NS1 Ag Positive	Yes
21	YLBS DEN S21	Serum	42 Units	Dengue NS1 Ag Positive	48 Units	Dengue NS1 Ag Positive	Yes
22	YLBS DEN S22	Serum	22 Units	Dengue NS1 Ag Positive	28 Units	Dengue NS1 Ag Positive	Yes
23	YLBS DEN S23	Serum	42 Units	Dengue NS1 Ag Positive	36 Units	Dengue NS1 Ag Positive	Yes
24	YLBS DEN S24	Serum	26 Units	Dengue NS1 Ag Positive	27 Units	Dengue NS1 Ag Positive	Yes
25	YLBS DEN S25	Serum	98 Units	Dengue NS1 Ag Positive	92 Units	Dengue NS1 Ag Positive	Yes
26	YLBS DEN S26	Serum	62 Units	Dengue NS1 Ag Positive	56 Units	Dengue NS1 Ag Positive	Yes
27	YLBS DEN S27	Serum	42 Units	Dengue NS1 Ag Positive	46 Units	Dengue NS1 Ag Positive	Yes
28	YLBS DEN S28	Serum	28 Units	Dengue NS1 Ag Positive	34 Units	Dengue NS1 Ag Positive	Yes
29	YLBS DEN S29	Serum	89 Units	Dengue NS1 Ag Positive	92 Units	Dengue NS1 Ag Positive	Yes
30	YLBS DEN S30	Serum	106 Units	Dengue NS1 Ag Positive	108 Units	Dengue NS1 Ag Positive	Yes
31	YLBS DEN S31	Serum	2.0Units	Dengue NS1 Ag Negative	2.8 Units	Dengue NS1 Ag Negative	Yes
32	YLBS DEN S32	Serum	0.212 Units	Dengue NS1 Ag Negative	0.20 Units	Dengue NS1 Ag Negative	Yes
33	YLBS DEN S33	Serum	3.15 Units	Dengue NS1 Ag Negative	2.8Units	Dengue NS1 Ag Negative	Yes
34	YLBS DEN S34	Serum	1.2Units	Dengue NS1 Ag Negative	1.4Units	Dengue NS1 Ag Negative	Yes
35	YLBS DEN S35	Serum	4.8Units	Dengue NS1 Ag Negative	3.Units	Dengue NS1 Ag Negative	Yes
36	YLBS DEN S36	Serum	1.8 Units	Dengue NS1 Ag Negative	1.2 Units	Dengue NS1 Ag Negative	Yes
37	YLBS DEN S37	Serum	0.341Units	Dengue NS1 Ag Negative	0.45 Units	Dengue NS1 Ag Negative	Yes
38	YLBS DEN S38	Serum	0.46 Units	Dengue NS1 Ag Negative	1.2 Units	Dengue NS1 Ag Negative	Yes
39	YLBS DEN S39	Serum	3.7Units	Dengue NS1 Ag Negative	2.4Units	Dengue NS1 Ag Negative	Yes

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S.No.	Lab Sample Number	Sample Type	JML DENGUE NS1Ag Microlisa Units Cut of value : 0.338	Results	PanBio DEN NS1Ag Early ELISA Units. Cut of value : 0.329	Result	Result ation /Yes/No
40	YLBS DEN S40	Serum	1.1Units	Dengue NS1 Ag Negative	2.6Units	Dengue NS1 Ag Negative	Yes
41	YLBS DEN S41	Serum	0.21 Units	Dengue NS1 Ag Negative	.9Units	Dengue NS1 Ag Negative	Yes
42	YLBS DEN S42	Serum	2.8Units	Dengue NS1 Ag Negative	2.3Units	Dengue NS1 Ag Negative	Yes
43	YLBS DEN S43	Serum	3 Units	Dengue NS1 Ag Negative	4.6 Units	Dengue NS1 Ag Negative	Yes
44	YLBS DEN S44	Serum	0.4 Units	Dengue NS1 Ag Negative	0.9 Units	Dengue NS1 Ag Negative	Yes
45	YLBS DEN S45	Serum	2.5Units	Dengue NS1 Ag Negative	1.8Units	Dengue NS1 Ag Negative	Yes
46	YLBS DEN S46	Serum	2.2 Units	Dengue NS1 Ag Negative	1.2 Units	Dengue NS1 Ag Negative	Yes
47	YLBS DEN S47	Serum	4.5Units	Dengue NS1 Ag Negative	5 Units	Dengue NS1 Ag Negative	Yes
48	YLBS DEN S48	Serum	0.9 Units	Dengue NS1 Ag Negative	2.6 Units	Dengue NS1 Ag Negative	Yes
49	YLBS DEN S49	Serum	2.8Units	Dengue NS1 Ag Negative	4.1Units	Dengue NS1 Ag Negative	Yes
50	YLBS DEN S50	Serum	1.3Units	Dengue NS1 Ag Negative	2.5Units	Dengue NS1 Ag Negative	Yes
51	YLBS DEN S51	Serum	1.1Units	Dengue NS1 Ag Negative	0.7Units	Dengue NS1 Ag Negative	Yes
52	YLBS DEN S52	Serum	2.1 Units	Dengue NS1 Ag Negative	3.7 Units	Dengue NS1 Ag Negative	Yes
53	YLBS DEN S53	Serum	0.23 Units	Dengue NS1 Ag Negative	1.8Units	Dengue NS1 Ag Negative	Yes
54	YLBS DEN S54	Serum	1.1 Units	Dengue NS1 Ag Negative	2.6 Units	Dengue NS1 Ag Negative	Yes
55	YLBS DEN S55	Serum	0.6Units	Dengue NS1 Ag Negative	1.8Units	Dengue NS1 Ag Negative	Yes
56	Chikunguya Positive	Serum	2.2Units	Dengue NS1 Ag Negative	2.5Units	Dengue NS1 Ag Negative	Yes
57	Chikunguya Positive	Serum	0.11Units	Dengue NS1 Ag Negative	0.7Units	Dengue NS1 Ag Negative	Yes
58	Chikunguya Positive	Serum	1.6Units	Dengue NS1 Ag Negative	2.1 Units	Dengue NS1 Ag Negative	Yes
59	Chikunguya Positive	Serum	1.1Units	Dengue NS1 Ag Negative	2.8Units	Dengue NS1 Ag Negative	Yes

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S.No.	Lab Sample Number	Sample Type	JML DENGUE NS1Ag Microlisa Units Cut of value : 0.338	Results	PanBio DEN NS1Ag Early ELISA Units. Cut of value : 0.329	Result	Result ation /Yes/No
60	Chikunguya Positive	Serum	1.6Units	Dengue NS1 Ag Negative	1.5Units	Dengue NS1 Ag Negative	Yes
61	Chikunguya Positive	Serum	0.6Units	Dengue NS1 Ag Negative	0.8Units	Dengue NS1 Ag Negative	Yes
62	Chikunguya Positive	Serum	.455 Units	Dengue NS1 Ag Negative	0.487 Units	Dengue NS1 Ag Negative	Yes
63	Chikunguya Positive	Serum	2.6Units	Dengue NS1 Ag Negative	2.1Units	Dengue NS1 Ag Negative	Yes
64	Chikunguya Positive	Serum	0.7Units	Dengue NS1 Ag Negative	1.5Units	Dengue NS1 Ag Negative	Yes
65	Chikunguya Positive	Serum	0.9Units	Dengue NS1 Ag Negative	1.1Units	Dengue NS1 Ag Negative	Yes

Result Interpretation of Dengue NS1Ag Microlisa & Panbio Dengue NS1 Early ELISA

If Dengue NS1 Ag Units >11 then interpret as Dengue NS1Ag positive
If Dengue NS1 Ag Units <9 then interpret as Dengue NS1Ag Negative
If Dengue NS1 Ag Units are between 9-11 then interpret as Equivocal

Reproducibility:

Sample No.	1st Run (Dengue NS1 Ag Units)	2nd Run (Dengue NS1 Ag Units)	3rd Run (Dengue NS1 Ag Units)	4th Run (Dengue NS1 Ag Units)	CV%
YLBS DEN S52	2.1	2.3	2.1	2.2	3.81%
YLBS DEN S20	88	86	85	90	2.20%
YLBS DEN S15	16	16.5	17	18	4.9 %

Results: The results of Dengue NS1Ag Microlisa for all 3 samples are comparable in inter assay variation and CV% for all 3 samples is less than 6%. Hence the test results of Dengue NS1Ag Microlisa are reproducible.

Checked BY: G. Kishore

Consultant Microbiologist

Date : 3/12/21

Hyderabad YES LABS

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