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Original Article

Molecular typing of dengue virus in Mizoram, Northeast India

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Abstract

Introduction: Dengue is an emerging vector-borne public health threat and characterization at the molecular level is important for proper management of the disease. The aim of the study is to examine the diversity of the dengue viral serotypes from a hilly mountainous region of Northeast India.

Methodology: Thirty-six blood samples that were positive for dengue virus IgM antibodies identified by the enzyme-linked immunosorbent assay (ELISA) method were collected and quantified for the IL6 gene expression by using reverse transcriptase polymerase chain reaction (RT-PCR).

Results: All the patients had dengue hemorrhagic fever (DHF); 12 samples had a monotypic infection and 14 samples had dual infection with various dengue virus (DENV) serotypes; one sample had triple infection with DENV-1, DENV-2, and DENV-3.

Conclusions: This study identified DENV-1 as the major serotype in the state of Mizoram and it is the first report on the molecular typing of the dengue virus from the hilly mountainous state located in the Indo-Burma region bordering Myanmar and Bangladesh.

Key words: dengue; serotypes; co-infection; moist tropical; Northeast India.

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Introduction

Dengue is an *Aedes* mosquito-borne viral disease that has spread worldwide and the dengue haemorrhagic fever (DHF) has become a lethal disease in Asian and Latin American countries [1]. Re-emergence of dengue with altered clinical manifestations and severity is linked to change in epidemiology as well as a shift in the infection from young children to adults [2]. There are four antigenically unique serotypes of the virus (DENV-1, DENV-2, DENV-3, and DENV-4) [3] with vast geographic distribution and with high epidemic activity. DHF and dengue shock syndrome (DSS) are key public health issues [4,5,6,7]. Hence, it is important to study the serotype of the dengue virus in different climatic zones to fully understand the etiology of the disease.

Primary infection can induce lifetime protective immunity to a single serotype with only transient protection against the other three serotypes. Sero-epidemiological reports show that secondary infection can be a major risk factor for DHF and DSS through antibody-dependent enhancement [8].

It is important to determine the DENV serotypes from different climatic zones for epidemiological and diagnostic investigations. The incidence of dengue is continually increasing globally and the serotype varies based on geographical region. Between 1990 to 2017 there was an increase in the number of cases (from 23.2 to 104.7 million), as well as age-standardized incidence rate (431.6 to 1371.3 per 100000 population) [9].

The state of Mizoram is a part of the Indo-Burma biodiversity hotspot in the eastern Himalayan range with an average annual rainfall of 2,100-3,500 mm, temperature ranging from 11 to 29 °C, and is in the moist tropical to subtropical climatic zone [10]. Mizoram is one of the eight Northeastern India (NEI) states and shares geographical boundary with Myanmar and Bangladesh. Among the NEI states, DENV activity has been documented from Assam [11], Arunachal Pradesh [12], Nagaland [13], and Manipur [11,14]., Dengue incidence cases have been increasing in NEI and this may be due to an increase in population,

urbanization, and poor waste management [15]. Different serotypes along with coinfections have been reported in NEI. DENV2 and DENV3 were detected in Mizoram [15]. This study was undertaken using molecular typing to examine the diversity of the dengue virus serotypes in blood samples in Mizoram.

Methodology

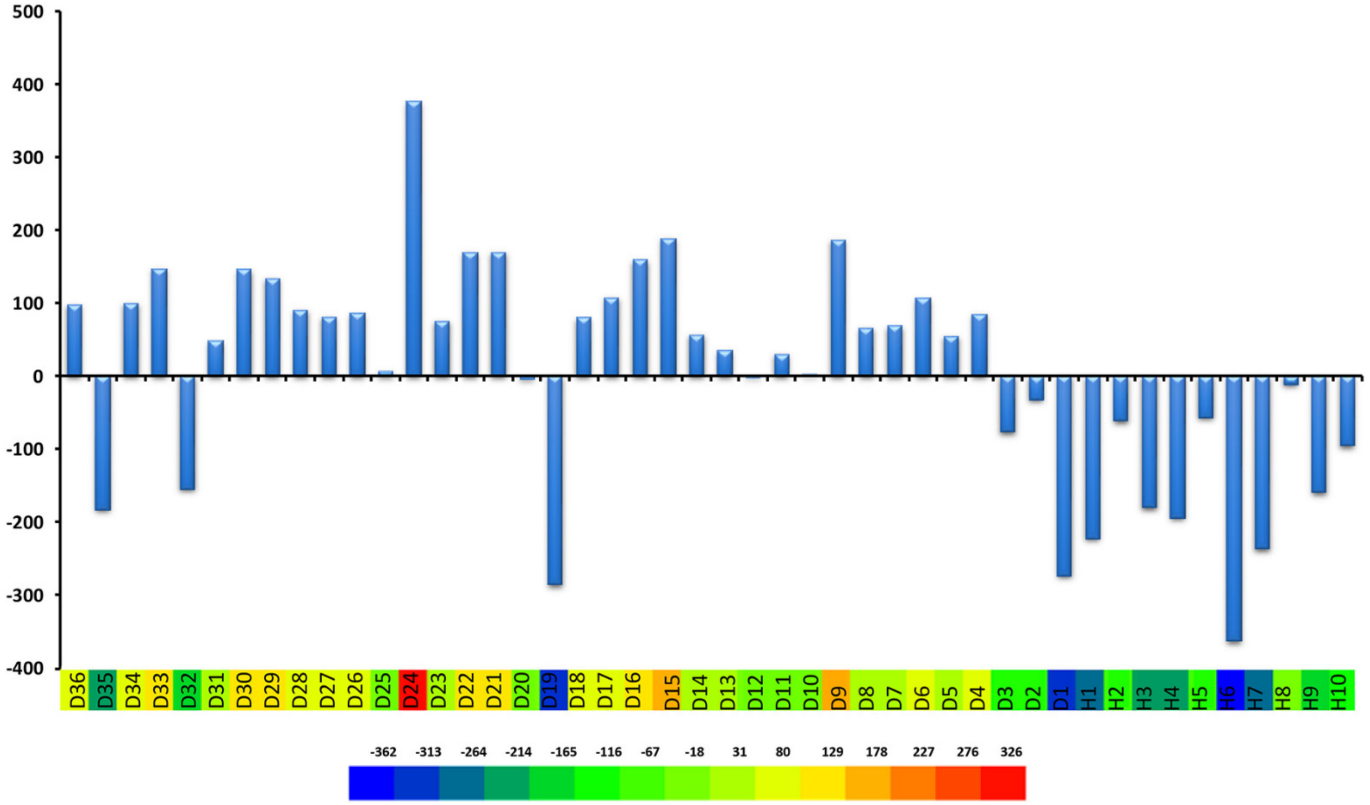
Ethics

Ethical clearance was obtained from Institutional Human Ethical Committee, Mizoram University (MZU/IHEC/2017/004 dated 31 August 2017).

Sampling and total RNA from blood sample

Blood samples were collected from dengue patients in Synod Hospital, Durtlang, Mizoram, Northeast India. All the patients exhibited DHF and were IgM positive (Supplementary Table 1). The red blood cells (RBCs) were lysed using a hypotonic buffer (ammonium bicarbonate and ammonium chloride; Himedia, Thane West, India) and the lymphocytes were separated. Three volumes of Red Blood Cells (RBC) lysis buffer was added to the blood sample, vortexed and inverted 5 minutes, and centrifuged (Eppendorf 5415R, Hamburg, Germany) at 2000 g for 10 minutes. The pellet was further treated with RBC lysis buffer and mixed well, centrifuged 2-3 times until a clean white pellet was obtained. The pellet was resuspended in 1 mL of TRIzol (ThermoFisher, Waltham, USA) and 10 µL proteinase K (10 mg/mL stock; Himedia, Thane West, India). The sample was vortexed 5 times for 30 seconds for lysis, with 5 minute incubation in ice in each interval. Next, 200 µL of chloroform was added, mixed well by vortexing, and centrifuged at 15,000 g (at 4 °C) for 10 minutes, and the supernatant was transferred to a fresh tube containing equal volumes (1:1) of chilled isopropanol (Merck, Kenilworth, USA) and incubated at -20 °C overnight, followed by centrifugation at 10,000 g at (4 °C) for 20 minutes. The pellet was washed in 70% ethanol containing DEPC treated water, followed by centrifuging at 10,000 rpm for 10 minutes. The pellet was air-dried and suspended in 30 µL RNAse-free MilliQ water; the purity and concentration were determined by quantitative spectrophotometric assay (Cary UV-visible spectrophotometer, Agilent Technologies, Santa Clara, USA). The total RNA was checked using denaturing agarose gel and Qubit (Eppendorf, Hamburg, Germany) [16].

Figure 1. Expression of IL-6 gene using semi-quantitative PCR analysis.



Densitometric analysis of intensity of each amplified PCR product using Image J software. X- axis: Sample number; Y axis: relative mRNA expression.

cDNA Preparation and PCR amplification

First-strand cDNA synthesis was carried out (Revert Aid kit, Thermo Scientific, Waltham, USA) and specific primers were used for the amplification of the DENV serotypes. The standard 20 µL PCR reactions were performed (125 nM of each primer, 10× PCR buffer, 1.5 mM $MgCl_2$, 1U *Pfu* DNA polymerase, 0.5 mM dNTP) for 35 cycles with appropriate annealing temperatures (Supplementary Table 2) [17].

Semi-quantitative reverse transcriptase PCR (RT-PCR)

Total cellular RNA (5 µg) was used for each reverse transcription reaction for the synthesis of the first-strand cDNA. Primers targeting exons 4 and 5 of IL6 (5'-CCAGCTATGAACTCCTTCTC-3' and 5'-GCTTGTTCCTCACATCTCTC-3') and exons 1 and 3 of β-actin (5'-ACCATGGATGATGATATCGC-3' and 5'- ACATGGCTGGGGTGTTGAAG-3') were used [18]. The PCR program was 35 cycles consisting of 94 °C for 30 seconds, 1-minute at the annealing temperature using a touchdown protocol (4 cycles each at 62 °C and 60 °C; 30 cycles at 58 °C), and 72 °C for 5 minutes. Polyacrylamide gel (10%) electrophoresis and ethidium bromide staining were used to visualize the products [19,20].

Table 1. PCR based serotypic pattern of dengue in Mizoram.

Sample No.	DENV 1.1/ DENV 1.2 / DENV 2.1 / DENV 2.2 / DENV 3 / DENV4 Infectious type (mono/multi serotype)
D1	S3
D2	-
D3	S1.2/S2.1
D4	-
D5	S1.1/S2.1/S2.2
D6	S1.2/S2.1/S3
D7	S2.2/S3
D8	S1.2/S3
D9	S1.2/S2.1
D10	S1.2/S2.1/S3
D11	-
D12	-
D13	S2.2/S3
D14	S2.1
D15	S1.2/S3
D16	S1.2
D17	S1.2/S3
D18	S1.2
D19	S1.2
D20	S1.2
D21	S1.2
D22	S1.1/S1.2
D23	S1.2
D24	S1.2
D25	S1.2
D26	-
D27	S1.2/S2.1
D28	S1.2/S2.1
D29	S1.2
D30	S1.1
D31	S1.2/S2.1
D32	S1.2
D33	S1.1/S1.2/S2.1/S2.2
D34	S2.2
D35	-
D36	-

Densitometric analysis

The banding patterns and molecular mass of the RT-PCR results were analyzed using Syngen G-Box gel image analysis software (Sacramento, CA, USA) and ImageJ ver. 1.53 (https://imagej.nih.gov/ij/).

Results

Identification of expression of IL-6 from blood samples

Twenty-six samples showed positive amplification of the IL-6 gene, out of which 11 samples had intensity between 100-200 RFU. The intensity of D24 was more than 300 RFU which indicates higher expression of IL-6 when compared with the other positive samples (Figure 1).

Detection of serotypes from blood samples

PCR amplification of the dengue patient's DNA showed that DENV Serotype 1 (1.1 and 1.2) were absent in 12 samples (D1, D2, D4, D7, D11 to D14, D26, D34 to D36) and the serotype 1.2 was present in 23 Samples (D3, D6, D8 to D10, D15 to D25, D27 to D29, D31 to D33). Samples D5, D22, D30 and D33 have serotype 1 (1.1) of DENV.

PCR amplification of the samples showed that D3, D6, D9, D10, D14, D27, D28 and D31 were DENV-2.1 positive and samples D7, D13 and D34 were DENV-2.2 positive. The samples D5 and D33 also showed positive amplification in both DENV-2.1 and 2.2. Serotype-DENV 3 was detected in samples D1, D6, D7, D8, D10, D13, D15 and D17 and serotype DENV 4 was not detected in any sample (Supplementary Figure 1). Most of the DENV infections in patients who were 4-40 years of age were multi serotype infections (Table 1, Figure 2).

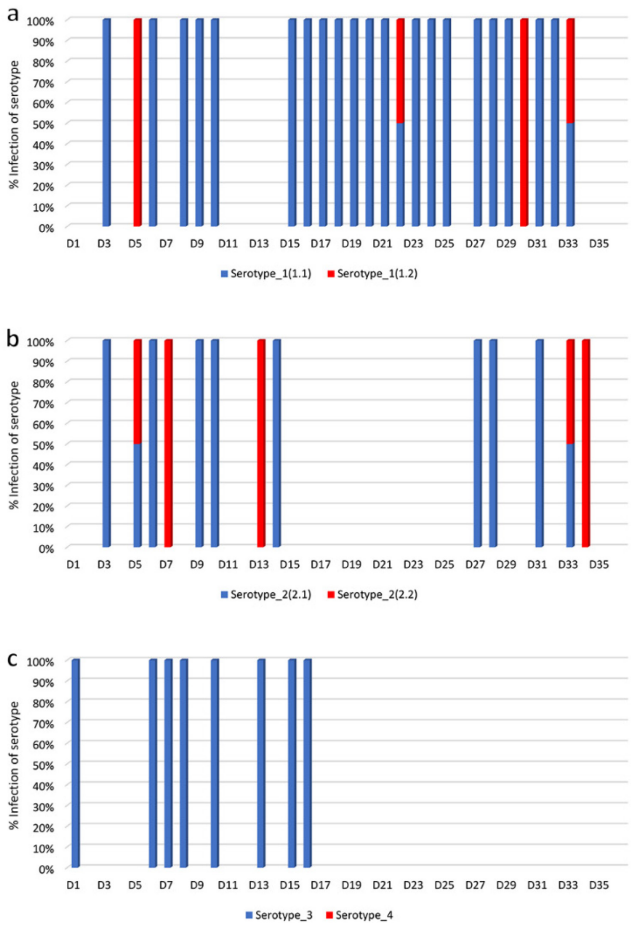
Discussion

Identification of DENV serotypes is important for informed planning of community health programs as the incidence of the disease is increasing. DENV serotypes 1 and 4 were reported from India in 1964 [26,27] and serotype 3 was reported in 1968 [28]. Simultaneous infections with DENV serotypes were detected in 2006 in New Delhi, India [29]. Mizoram, is situated in the southernmost part of Northeast India at

an altitude of 500-2157 m. The state experiences wet summers (18–33 °C) and dry winters (11–24 °C) influenced by the southwest monsoon [30] and has become a dengue-endemic zone with outbreaks of DF and DHF. Dengue is now gradually spreading and establishing in the rural and urban areas of Mizoram, as well as in other parts of Northeast India, and there is monitoring of serotypes using molecular detection. Consequenty there are few reports on the spread of dengue as an epidemic or sporadic outbreaks in the region.

Several factors are associated with dengue incidences and their spread including rainfall, temperature, humidity and host immunity; however, it has been reported that temperature has the strongest effect on its transmission [26]. A study on the meteorological data of Mizoram between 1979-2003 revealed an average annual increase of 0.08% in relative humidity, a 20.45 mm increase in annual rainfall and a ~0.1 °C increase in temperature (since 2007). These climatic factors may have facilitated the rise of dengue cases in the state by favoring the growth of its vector *Aedes aegypti* [27]. Therefore, study of serotypes that are predominant in the state may help develop effective prevention and mitigation strategies.

Figure 2. Graphical representation for the detection of DENV serotypes.



a. presence of serotype DENV (1.1 & 1.2); **b.** samples D3, D6, D9, D10, D14, D27, D28, D31 are DENV-2.1 positive, samples D7, D13, D34 are DENV-2.2 positive and samples D5, D33 are both DENV-2.1 and 2.2 positives; **c.** Presence and absence of Serotype DENV in Samples D1, D6, D7, D8, D13, D15, D16. DENV 3 with 100% efficiency and there is no presence of Serotype-4.

In the present study, all the blood samples reacted to the dengue IgM antibody. The highest number of dengue cases were detected in the age group 20-40 years, followed by the group 10-20 years (Supplementary Table 1). Although fewer samples of suspected dengue cases were detected during the monsoon season (June to October) in Mizoram, most of the samples were positive for dengue IgM antibody by the ELISA method. The dengue cases started in June and peaked in November. This is the monsoon or post-monsoon period and the stagnant waters are favored for the breeding of vector mosquitoes, *Aedes* sp. and their relative abundance is 5–222 larvae/10 dips/m^2 [10].

Semi-quantitative RT-PCR was performed for molecular detection with the 36 positive samples; out of these, 26 samples were IL-6 positive involving DENV 1, DENV 2, and DENV 3. DENV 1 and 2 were predominant in the samples, some samples (D3, D6, D9, D10, D27, D28, D31, and D33) had dual infections with DENV 1 and DENV 2. These samples with dual infection were IgM reactive and IL-6 positive (Figure 1). The samples D7 and D13 had the dual infection of DENV 2 and DENV3, and D8, D15, and D17 samples had a dual infection of DENV1 and DENV3 (Table 1) and they were IgM reactive and IL-6 positive. D10 amplified against DENV1, DENV 2, and DENV 3 and also contained IgM antibody and IL-6. The presence of viral RNA along with IgM antibody may be due to successive infection and IgM production due to the initial infection, as is evident by the intensity of the IL-6 bands (Figure 1). The level of IL-6 was found to be significantly elevated in the severe form of dengue fever [28,29]. In the present study we found an upregulation of the IL-6 expression and all the cases were only IgM positive which indicates primary infection. The downregulation or limited upregulation in a few patient samples may be because they were initial infections. IL-6 shows an increased level of secondary infection (IgM and IgG positive) compared to primary infection (IgM positive) [29].

DHF was found in all the patients with multiple dengue serotypes and was mainly found among the adults (20-40 years) and younger age groups which may

be due to the non-exposure of all serotypes of DENV in them. Although multiple DENV serotypes have been found, further monitoring is needed to determine its endemic nature in the state of Mizoram.

Earlier studies have reported dengue in the Northeastern States including Manipur and Arunachal Pradesh [11]. The first incidence of dengue-like fever was reported at ten different localities of the Aizawl district in Mizoram between August and December 2016. The mosquito (*Aedes aegypti*) was the potential disease-causing vector species from the 11 localities of the Aizawl district in Mizoram. There was no preference toward gender or age groups and the study revealed a high density of vector mosquitoes [30].

This study adds to the report on co-circulation of the DENV serotypes from the Mizoram state [15]. The region is still remote, and with the increasing migration of the population towards urban areas, there is a great dependency on water resources. This also leads to more pockets of water bodies in urban settings generating new breeding grounds for the dengue viral hosts [25]. The DENV serotypes may evolve into new serotypes with higher severity and infectivity and will depend on the climatic conditions and environmental changes. The present study highlights the importance of continuous monitoring of DENV using molecular typing to understand the circulation and presence of the various serotypes in the Northeastern Indian states, as distinct genotypes were found which may be associated with different clinical outcomes and could be used for designing effective mitigation strategies [30].

Conclusions

This study identified DENV-1 as the main serotype. There is a need for molecular typing and systematic monitoring of the serotypes in relation to environmental alterations and geographic regions. This study also highlights the need for large-scale sampling and identification of the *Aedes* mosquito species across geographical boundaries.

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

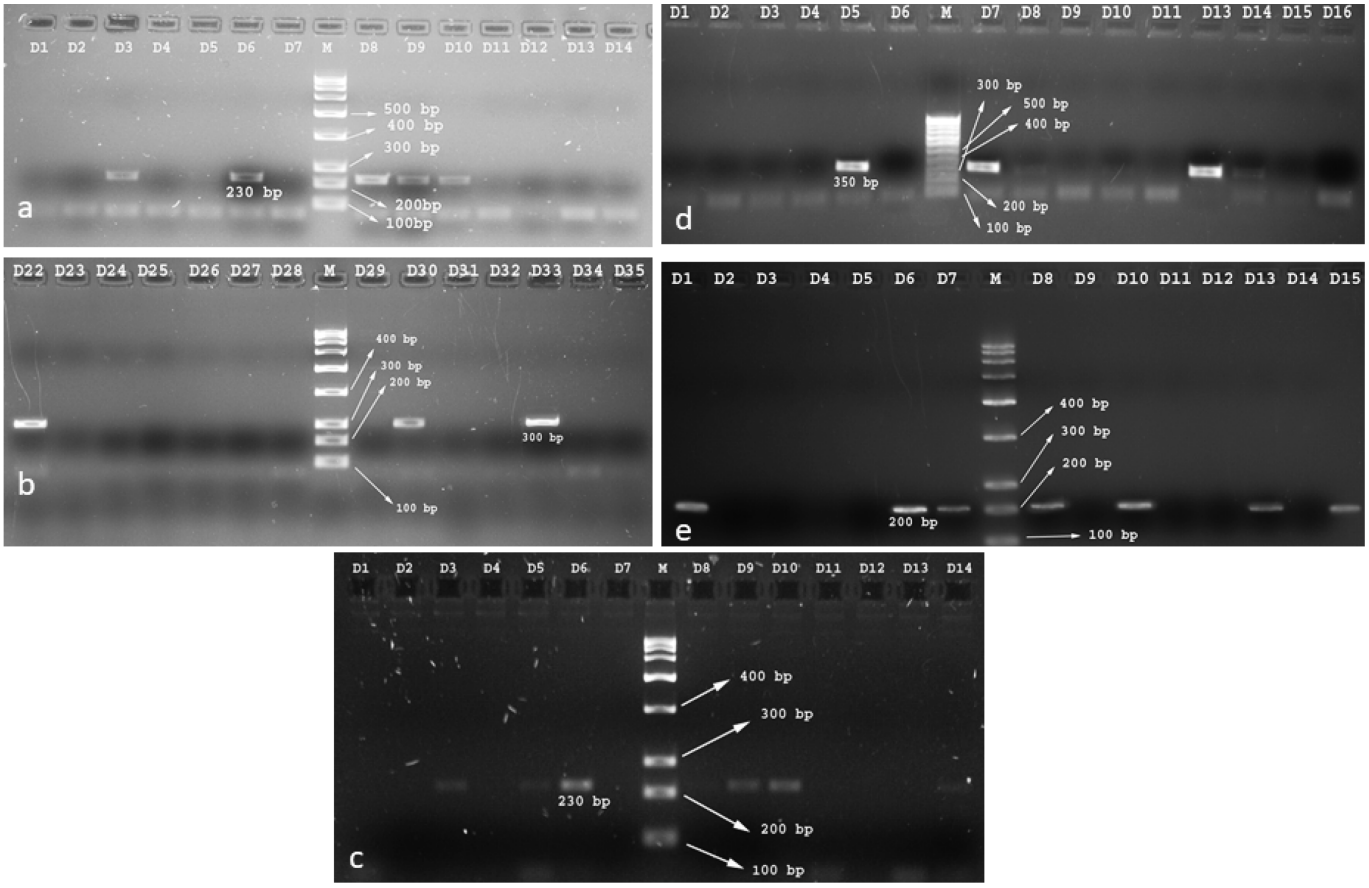
Supplementary Table 1. List of samples collected for dengue viral characterization.

Case No.	Age of the patients (years)	Diagnostic test
D1	32	IgM positive
D2	36	IgM positive
D3	38	NS-1 & IgM positive
D4	10	IgM positive
D5	7	NS-1 & IgM positive
D6	14	IgM positive
D7	17	NS-1 & IgM positive
D8	32	IgM positive
D9	36	IgM positive
D10	26	IgM positive
D11	31	NS-1 & IgM positive
D12	38	NS-1 & IgM positive
D13	5	IgM positive
D14	16	IgM positive
D15	23	IgM positive
D16	35	NS-1 & IgM positive
D17	10	IgM positive
D18	4	IgM positive
D19	17	IgM positive
D20	56	IgM positive
D21	46	NS-1 & IgM positive
D22	27	IgM positive
D23	63	IgM positive
D24	29	IgM positive
D25	6	IgM positive
D26	19	IgM positive
D27	28	IgM positive
D28	43	IgM positive
D29	14	IgM positive
D30	48	IgM positive
D31	28	NS-1 & IgM positive
D32	25	IgM positive
D33	26	IgM positive
D34	51	IgM positive
D35	37	IgM positive
D36	8	IgM positive

Supplementary Table 2. Primers used for the serotyping of dengue virus.

Primer	Primer Sequence (5'-3')	Annealing temperature	Extension time
DNV.1.1	F- CAGACTAGTGGTTAGAGGAGA R- GGAATGATGCTGTAGAGACA	47 °C	40 seconds
DNV.1.2	F- CAAACCATGGAAGCTGTACG R- TTCTGTGCCTGGAATGATGCT	58 °C	30 seconds
DNV.2.1	F- CAACCATGGAAGCTGTACG R- CATCATGAGACAGAGCGAT	56 °C	40 seconds
DNV.2.2	F- TTCCAACAAGCAGAACAACAT R- TTCTGTGCCTGGAATGATGCCT	58 °C	30 seconds
DNV.3	F- ATATGCTGAAACGCGTGAG R- CATCATGAGACAGAGCGAT	57 °C	40 seconds
DNV.4	F- TTCCAACAAGCAGAACAACAT R- GCTACAGGCAGCACGGTTT	58 °C	30 seconds

Supplementary Figure 1. PCR amplification of dengue serotypes.



Agarose gel showing the presence of the dengue serotypes: **a.** DENV1 - 230 bp product; **b.** DENV1 - 300 bp product; **c.** DENV2- 230 bp product; **d.** DENV2 - 350 bp product; **e.** DENV3 - 200 bp product