Secondary DENV Infections Along with Concurrent Multiple DENV Serotypes in a Single Dengue Session Contributing to Dengue Severity in Chittagong Bangladesh

MoushumiGhosh Roy[1, 2], Kutub Uddin[3], Din Islam[2], M. Monirul Islam[2*], Anjuvan Singh[4*]

[1]Department of Biochemistry, Lovely Professional University, Punjab, India
[2*]Department of Biochemistry and Molecular Biology, University of Chittagong, Bangladesh
[3]Department of Medicine, Chittagong Medical College Hospital, Bangladesh
[4*]Department of Biotechnology, School of Bioengineering and Bioscience, Lovely Professional University, Punjab, India
***Corresponding Author**
Anjuvan Singh, (anjuvan.15950@lpu.co.in), **M. Monirul Islam**, (islammm@cu.ac.bd)

Abstract

Introduction: Natural anti-DENV antibodies might provide long-lasting immunity against the infecting DENV serotype. However, the rationale for infecting DENV serotypes and anti-DENV antibody responses is still missing. Here, we investigated anti-DENV IgG and IgM antibodies along with infecting DENV serotypes and clinical dengue manifestation in recent dengue outbreaks in Chittagong, Bangladesh.

Methods: The 112 samples investigated were confirmed dengue cases who were hospitalized. The infecting DENV serotyping was done by the ratio of anti-DENV IgG and IgM antibodies determined by ELISA as well as by RT-PCR. A hematology cell counter was used for differential blood cell counts following DENV infection.

Results: DENV serotyping based on anti-DENV IgM/IgG antibodies developed following DENV infection indicated that, first, 35% of primary DENV infections, 65% of sera tested had secondary DENV infections, secondly, among the primary DENV infections 51% had single DENV infection where the most prevailed serotype was DENV4 and 49% had concurrent multiple DENV infections where 70% of cases had DENV1 serotype along with other serotypes. There was no strong correlation between clinical dengue manifestation and infecting DENV serotype. However, patients infected with DENV1 had rapid and progressive loss of platelets in the course of dengue infections (days 0-4).

Conclusion: Very high occurrence of secondary DENV infection cases suggested that Bangladesh has been experiencing recurrent DENV infection being contributed by cross-reacting primary anti-DENV antibodies through ADE. Furthermore, the presence of multiple of DENV serotypes and concurrent multiple DENV infections in a single dengue session might be the reason for increased dengue disease incidence and dengue severity in Chittagong, Bangladesh.

Keywords: DENV serotypes, DENV sero-specificity, Primary DENV infection, Secondary DENV infection, Concurrent multiple DENV infection, Anti-DENV ED3 antibodies.

INTRODUCTION

Dengue viruses (DENVs), the causative agents of dengue disease resulting classical dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are affecting millions of people in tropical and subtropical countries worldwide[1,2]. There are four serologically distinct DENV serotypes, DENV1, DENV2, DENV3 and DENV4 which have very similar sequences and structures [3,4]. However, there is very little information available on recurrence of DENV infections and DENV serotype-specific dengue manifestation and whenever available, the information was controversial [5-7]. For examples, DENV2 had been found very aggressive with increased vascular permeability and shock over DENV1 infections in Nicaragua[7]. Similarly,DENV2 (35%) was most prevailed serotype isolated from DHF/DSS cases, followed by DENV3 (31%), DENV1 (24%), and DENV4 (10%) in Thailand[6]. In contrast, in dengue epidemic in Indonesia and Brazil the DENV3 was the most prevalent serotype associated with severe dengue manifestation [8,9]. However, the patients' sera had primary anti-DENV antibodies when they were infected with DENV1 and DENV3 serotypes, while all the patients infected with DENV2 and DENV4 had secondary anti-DENV antibody responses [5]. These observations clearly suggested high recurrent DENV infections with the same and/or heterotypic DENVs in dengue endemic countries making the DENV-serotype-dengue disease severity association more complicated than previously thought[10-13].

In recent years,dengue burden has been increasing substantially worldwide with reemergence of new prevalent DENV serotypes replacing previous ones[11-14]. In addition, high occurrence of concurrent multiple DENV infections have also been reported in recent dengue outbreaks[13-17]. However, much details on the reemergence of DENV serotypes and appearance of multiple DENV serotypes in single dengue session and details on their association with dengue disease severity are yet to be available [18-20]. Moreover, occurrence of very high number of secondary DENV infection cases in dengue endemic regions have been restricting the investigation of dengue etiology in details, and hence hindering the development of a rationale for infecting DENV serotype and anti-DENV antibody responses following natural DENV infection [19-22].

At early stages of DENV infections (day 1-7 of symptom onset) DENV is typically present in blood and virus-specific IgM antibodies appear and thus positive anti-DENV IgM and IgG antibodies in infected sera have been used for the confirmation of recent DENV infections[23]. The anti-DENV IgM is thought to develop following recent primary DENV infections while high anti-DENV IgG could be an indication for secondary DENV infection [19]. Accordingly, the ratio of anti-DENV IgM and IgG antibodies may be used to differentiate primary and secondary dengue infections [24,25]. Recently, we reported that DENV4 was the most prevailed serotype by RT-PCR in recent dengue outbreaks in Chittagong, Bangladesh [26]. Here we report anti-DENV IgM and IgG antibodies in natural DENV infections along

with clinical dengue manifestation and observed that, first, DENV serotyping by anti-DENV IgM (also IgG) antibodies might be very difficult as anti-DENV antibodies were highly sero-cross-reacting. Secondly, almost 49% of primary DENV infected cases had concurrent multiple DENV and finally, almost 65% of total DENV infections might have secondary DENV infections. The co-existence of all four DENV and high prevalence of concurrent multiple DENVs in a single dengue session may not only complicated the understanding dengue disease in details but also might be contributing to increased dengue incidences and dengue severity in dengue endemic countries, like Bangladesh.

MATERIALS AND METHODS:

Study setting and design

This is a retrospective observational study performed with a total of 112 dengue serum specimens obtained from Chittagong Medical College Hospital, Chittagong, Bangladesh. The serum specimens were collected from patients who were primarily admitted for dengue treatment at the hospital and diagnosed (NS1-based DENV diagnosis [23]) as dengue patients from July-August 2019. More precisely, patients with sign and symptoms of dengue infection and dengue confirmation through NS1-based dengue diagnosis (day 0-7 of disease onset) and hospitalized at Chittagong MedicalCollege Hospital, Chittagong, Bangladesh were enrolled in this study. On the day of hospitalization (considered as day 0) blood samples were collected for clinical investigation (using fresh samples) and anti-dengue antibody determination (using frozen sera). Blood parameters were furthermore investigated daily for 4/5 days at Chittagong Medical College Hospital facilities and recorded.

The ED3 in dengue research: DENV ED3 design, expression and purification

The envelop protein domain 3 (ED3) has been reported to contain all the putative epitope residues conferring DENV-serospecificity[27] and has long been considered as a simplified model system for DENV serospecificity[28].Furthermore, the commercial monoclonal anti-DENV antibodies showed very similar serospecific recognition of serotype-specific ED3s [29]. Therefore, DENV ED3s may serve as alternative simplified models of DENVs in dengue research. The ED3 sequences of DENV1 (1ED3), DENV2 (2ED3), DENV3 (3ED3), and DENV4 (4ED3) were retrieved from Uni-Prot IDs P17763, P14340, P27915.1 and P09866, respectively (Figure 1). The nucleotide sequences encoding ED3s along with a thrombin cleavage site and a His_6-tag were cloned into pET15bexpression vector using *NdeI* and *BamHI* restriction enzymes as reported previously [28-30].

All four DENV ED3s were overexpressed through IPTG induction in JM109(DE3)pLysS cell line as inclusion bodies[30].Cells were collected through centrifugation, lysed in lysed in lysis buffer (150 mMNaCl, 0.5% sodium deoxycholate, and 1% SDS in 50 mMTris-HCl pH 8.5) and lysis wash buffer (lysis buffer plus 1% v/v NP-40) through sonication keeping on ice. The Cys residues were air oxidized in 6M guanidine hydrochloride followed by extensive dialysis in 50 mMTris-HCl, pH

8.7at 4ºC and purification through Ni-NTA (Wako, Japan) chromatography. Finally, the N-terminal His_6-tag was cleaved by thrombin proteolysis [29]and removed by a second round of Ni-NTA chromatography and ED3s were purified by reversed-phase HPLC. Protein identities were confirmed by MALD-TOP mass and preserved as lyophilized powder at -30ºC until used.

Immunological assay

Detection anti-DENV ED3 antibodies in dengue-infected sera

Anti-DENV ED3 IgG and IgM antibodies (hereafter as anti-ED3 IgG and anti-ED3 IgM) in naturally DENV infected human sera were tested by ELISA in 96-well microtiter plates (Nunc), as reported previously[28,30]. The plates were coated overnight at room temperature at 2.5 µg/mL of purified ED3s in phosphate buffered saline (PBS, pH7.4; 100 µL/well). Unbound ED3s were washed out with PBS, and the plates were blocked with 1% BSA in PBS for 45 min at 37°C. After washing with PBS, anti-dengue sera were applied at an initial dilution of 1:50 in 0.1% BSA in PBS, followed by a 3-fold serial dilution and incubated at 37°C for 2 hours. Unbound antibodies were removed by thoroughly washing three times with PBS−0.05% Tween-20, and once with PBS. Microtiter plates were blot dried, and a 100µL/well of anti-human IgG-HRP conjugate (at 1:4000 dilution) and anti-human IgM-HRP conjugate (at 1:10000) in 0.1% BSA in PBS-0.05% Tween-20 and incubated at 37ºC for 90 minutes. The unbound conjugates were removed by washing three times with PBS−0.05% Tween-20 and once with PBS. Coloring was performed by adding the substrate OPD (*o*-phenylenediamine) at 0.4 mg/ml concentration supplemented with 4 mM H_2O_2 (100 µL/well) and the color intensity was measured after 20 minutes of incubation at 450 nm (OD_{450nm}) using a microplate reader (DynatechMicroplate Reader).Antibody titers were calculated from the power fitting of OD_{450nm} vs the reciprocal of the antisera dilution using a cutoff of OD_{450nm} = 0.1 and 0.4 above the background values for IgM and IgG, respectively [30].

Differentiation of DENV infections

Categorization of DENV infections into primary and secondary infections was done based on the anti-ED3 IgM and IgG ratio, as previously reported [25, 31-33]. To be more precise, anti-ED3 IgG and IgM antibody titers of anti-dengue sera were determined against ED3s of four DENVs. Then anti-ED3 IgM titers were divided by anti-ED3 IgG titers and if the anti-ED3 IgM antibody titers were≥1.5 fold over anti-ED3 IgG titers the DENV infections were categorized as primary DENV infection and otherwise as secondary DENV infections (Figure 2A). Similarly, ≥1.5 fold higher anti-ED3 IgM titers over anti-ED3 IgG titers against a single DENV ED3 and against multiple DENV ED3s were considered as single DENV serotype and concurrent multiple DENV serotype infections, respectively. Such DENV serotyping based on anti-ED3 IgG and IgM antibodies was furthermore validated with RT-PCR based DENV serotyping data [26].

RESULTS AND DISCUSSION

DENV Serospecificity of ED3s

First, all four DENV ED3s were non-toxic, and immunogenic in Swiss albino mice (Figure:1).all four ED3s generated mostly ED3-specific IgG responses, very similar to what have been observed with DENV-specific commercial monoclonal anti-DENV antibodies, very similar to our previous observations [29]. To be precise, anti-2ED3, anti-3ED3 and anti-4ED3 were mostly serotype-specific while anti-1ED3 was 1ED3-2ED3-3ED3 sero-cross-reactive (*personal communication*). However, their immunogenicity was different, 2ED3 and 3ED3 were highly immunogenic over 1ED3 and 4ED3 was the least immunogenic (Figure 1c). These observations suggested that DENV ED3s are worth considering as alternatives to whole live DENV for evaluating anti-dengue responses in natural dengue infection.

DENV infection status in Chittagong, Bangladesh

The ratio of anti-ED3 IgM and anti-ED3 IgG titers showed that only 39out of 112 samples tested had over 1.5 fold higher anti-ED3 IgM titers over anti-ED3s IgG titers, indicating that only 35% of samples had been primarily infected with DENVs (primary DENV infections) while the remaining 73 samples (65%) were secondarily infected with DENVs (secondary DENV infections; Figure: 2a). Interestingly, among the primary DENV infected samples 20 (51%) samples were positive for single DENV serotype infections while the remaining 19 (49%) were positive for multiple DENV serotype infections (Figure: 2b). Moreover, among the primary DENV infected samples, the most prevailed DENV serotype was DENV4 (26%), followed by DENV1 (13%), DENV2 (8%), DENV3 (5%), respectively (Figure:2b), which was in contrast to the DENV seroprevalence observed in Dhaka, Bangladesh in 2019[14,34-35], but almost fully corroborated with our previous study based on RT-PCR of the same samples (Figure 2C) [26].

On the other hand, both the RT-PCR and ELISA-based DENV serotyping indicated that 10 and 19 out 112 samples (24%versus49%) samples were concurrently infected multiple DENV serotypes (Figure.2b-c). Moreover, among the 10 concurrently multiple DENV infected samples 9 samples had DENV1 infections (Figure.2c). Although the exact reason for the high occurrence of DENV1 serotype in multiple DENV infections is still not known, the high sequence similarities among DENV1, DENV2 and DENV3[6,36]and DENV1-DENV2-DENV3 sero-cross-reactivity of anti-DENV1 ED3 sera cross-reacting IgG both in mice models***(personal communication***), and natural DENV infections might together contribute in such concurrent multiple DENV infections.

DENV serotyping: RT-PCR versus anti-DENV IgM/IgG

RT-PCR is considered as gold standard in DENV serotyping for confirming the presence of viral RNA in the infected sera[37], but during the acute phase of infection. However, at very earlyand very late days of infection it may suffer from high false negative prediction [38-40] .Therefore, RT-

PCR alone cannot be used for dengue diagnosis. Here, we report the anti-DENV ED3 IgG and IgM antibodies in DENV infected sera and compared the DENV serotyping by RT-PCR[26]. Both RT-PCR and anti-DENV antibody based serotyping indicated that the DENV4 was the most prevailed serotype in the recent dengue outbreaks in Chittagong (31% versus 26%). However, the percentagesof single and multiple DENV serotypes were (76% and 24%) and (51% and 49%) by RT-PCR and anti-DENV IgM/IgG, respectively (Figure 2; Table 1).Such large discrepancies were most likely to be originated from the fact that RT-PCR is solely based on the presence of unstable damage prone viral RNA while anti-DENV antibody testing relies on the presence of stable antibodies developed following present and previous DENV infections. For the purpose of discussion, let us have a case-by-case comparison hereafter:

First, 9 samples found DENV positive by RT-PCR were also positive anti-DENV IgM/IgG antibody testing (Figure 3a), however, in most cases anti-DENV IgM antibodies confirmed the presence of multiple DENV infections instead of mostly single DENV serotype infection depicted by RT-PCR (Figure 2c). Similarly, 27 samples shown positive for single serotype (20) and multiple serotypes (7) by RT-PCR were found secondarily DENV infected following anti-DENV antibody testing (Figure 3b) suggesting that all 27 samples could have been previously infected with DENVs in addition to be re-infected in the recent outbreaks;all of them might experience a heterotypic secondary DENV infection.

The samples included in this study were confirmed dengue cases, however, 70 out of 112 samples were negative by RT-PCR, but positive by anti-DENV IgM/IgG antibodies (Figure 3c). These observations clearly suggested that RT-PCR alone cannot be used for true dengue surveillance, and the occurrence of secondary and multiple concurrent DENV infections in Bangladesh might by much higher than previously thought and reported[14,34].Therefore, we suggest that RT-PCR along with NS1-based diagnosis and anti-DENV antibody testing are worth considering to explore the real scenario of recurrent dengue infectionfor accurate DENV surveillance, seroprevalence and DENV infection status in dengue endemic regions.

Serotype-specific clinical dengue manifestation

Increase in vascular permeability, plasma leakage and RBCs and decrease of platelets and leucocyte counts have been reported to be associated with clinical dengue[15]. Investigation of DENV serotype-specific clinical dengue manifestation indicated that irrespective of the infecting DENV serotypes, blood platelet counts started to decrease soon after hospitalization and started replenishing after day 2-3 of admission to the hospital (Figure 4). However,dropped in platelet counts continued on day 4 (perhaps for few more days) with DENV1 infection(Figure 4a). These observations suggested that DENV1 may generated severe dengue through increased thrombocytopenia[41].On the other hand, no DENV serotype-specific changes were observed in the hematocrit value and

leukocyte counts (Figure 4b-c), suggesting that there may not have any association among DENV serotypes, and erythropoiesis and leukocytosis [17]. All four serotypes resulted typical dengue fever in all cases. Interestingly, DENV1 and DENV2 infections generated very similar sign and symptoms of typical dengue diseases. On the other hand, higher percentages of study population infected with DENV3, DENV4 and multiple DENVs in a single session showed Ache-Pain, Myalgia and nausea (Figure 5). However, DENV serotype specific data analyzed in each category were very limited and inclusions of more samples are worth considering for a definitive conclusion on DENV serospecific clinical dengue manifestation in future.

CONCLUSION

DENV serotyping by RT-PCR and anti-DENV IgM/IgG antibodies clearly showed that all four DENV serotypes co-circulated during the last (August 2019) dengue outbreak in Chittagong, Bangladesh. Alarmingly, very high percentage of secondary infection cases (24-49%) along with high concurrent multiple DENV infections (24-49% of total infections) in a single dengue session might be contributing to dengue disease severity in Bangladesh and as well as in other dengue endemic tropical countries. This is perhaps for the first time in Bangladesh where we report DENV4 as the most prevalent DENV serotype in Bangladesh, which is contrary to the previous reports where DENV3 had been reported as the most prevalent serotype [14]. Emergence of DENV4 as the most prevailed serotype supported the idea for re-emergence of new prevalent serotype over time asobserved in other tropical dengue endemic countries [4,42]. Therefore, inclusion of RT-PCR with NS1-based diagnosis and anti-DENV antibody testing are worth considering to explore the real scenario of recurrent dengue infection, for accurate DENV surveillance, seroprevalence and DENV infection status in DENV endemic regions [43].

Figure Legends

Figure I: Sequences and structures of DENV ED3 variants.(a) The sequences 1ED3, 2ED3, 3ED3 and 4ED3 were retrieved from the Uni-Prot IDs P17763, P14340, P27915.1 and P09866, respectively. (b) The surface structure models of 1ED3, 2ED3, 3ED3 and 4ED3 were generated from Protein Data Bank IDs 3g7t.pdb[44], 4utc.pdb[45], 3vtt.pdb[29]and 3we1.pdb[36], respectively. The putative epitope residues (308 and 382) are shown in blue spheres. (c) Anti-ED3 IgG titers. Artificial immunization using four ED3s generated ED3-specific IgG responses in Swiss albino mice model. Anti-ED3 IgG titers against the respective ED3s are shown.

Figure II: DENV infection status in Chittagong, Bangladesh.(a) The anti-ED3 IgM and anti-ED3 IgG antibody titers are shown. Only 39 samples have over 1.5 higher IgM titers over IgG titers, the primarily DENV infected samples. On the other hand, 71 samples had low anti-DENV ED3 IgM over IgG, suggesting that 65% of the samples had been secondarily DENV infected. (b) DENV serotyping based on anti-DENV ED3 IgM/IgG ratio. Among the 112 samples tested 39 were positive for primary DENV infection. Among the 39 samples tested

DENV positive, 20 (51%) were infected with single DENV serotypes while the remaining 19 samples (49%) had been concurrently infected with multiple DENV serotypes. (c) DENV serotyping done by RT-PCR (results reproduced from our previous report)[26]. Among the 112 samples tested 42 were positive for DENV serotypes, where 76% samples were infected with single DENV serotype while remaining 24% were concurrently infected with multiple DENV serotypes. DENV4 was the most prevailed serotype in Chittagong, Bangladesh during the study period.

Figure III: Comparison DENV serotyping by RT-PCR and anti-DENV IgM/IgG antibodies. (a) Anti-DENV IgM responses (by ELISA) versus infecting DENV serotypes (by RT-PCR) are shown. 9 samples positive for DENVs by RT-PCR were also positive for DENVs by anti-DENV ED3 IgM/IgG ratio. However, 7 samples positive for single DENV serotype by RT-PCR, but were positive for multiple DENV serotypes by anti-DENV ED3 IgM-IgG. These observations suggested a very high prevalence of multiple DENVs in single dengue session. (b) 27 samples, which were DENV positive by RT-PCR positive, were found positive for secondary DENV infection by anti-ED3 IgM/IgG ratio. These suggested that these 27 samples had been previously infected with DENVs and recently they might experience secondary DENV infections. (c) Among the 70 samples tested DENV negative by RT-PCR, 11, 12 and 47 were single DENV, multiple DENVs and secondary DENV positive, respectively by anti-DENV ED3 IgM/IgG antibodies.

Figure IV: Serotype-specific clinical manifestation of DENV infections. Effects of infecting DENV serotypes on (a) platelet counts (per microliter of blood) (b) hematocrit counts (%) and (c) leucocyte counts (per microliter of blood) are shown. Legends are the same for all panels and are shown at the top of the panels. Irrespective of infecting DENV serotypes platelet counts dropped soon after infection, however, started replenishing from day 4 of infection, except for DENV1 infection where dropped in blood platelet continued over time. On the other hand, effects of infecting DENV serotypes on hematocrit values and leukocyte counts were minimal. (d) Effects of infecting DENV serotypes on the sign and symptoms of dengue disease.

Figure V: DENV serotype-specific effects on dengue disease. Effects of infecting DENV serotypes on the sign and symptoms of dengue disease are shown. Infecting DENV serotypes with number of cases (n) are shown within the panel. All four serotypes resulted typical dengue fever in all cases. Interestingly, DENV1 and DENV2 infections generated very similar sign and symptoms of typical dengue diseases. However, higher percentages of study population infected with DENV3,DENV4 and multiple DENVs in a single session showed Ache-Pain, Myalgia and nausea. Although the numbers of cases analyzed in each category were very limited, inclusion of more samples may help developing a rationale for infecting DENV serotypes and clinical dengue manifestation in future

Table I: Comparative view of DENV serotyping by RT-PCR and anti-DENV ED3 IgM/IgG.

Samples	RT-PCR serotyping*			Anti-DENV ED3 IgM/IgG serotyping	
112	DENV Positive	Single DENV Infection	32	Single DENV Infection	5
				Multiple DENV Infection	6
				Secondary DENV Infection	21
		Multiple DENV Infection	10	Single DENV Infection	2
				Multiple DENV Infection	1
				Secondary DENV Infection	7
	DENV Negative	DENV Negative	70	Single DENV Infection	11
				Multiple DENV Infection	12
				Secondary DENV Infection	47

*RT-PCR based DENV serotyping data were reproduced from our previous report[26]. In anti-DENV ED3 IgM/IgG-based DENV serotyping was done based on the ratio of IgM to IgG ≥1.5.

Figure I

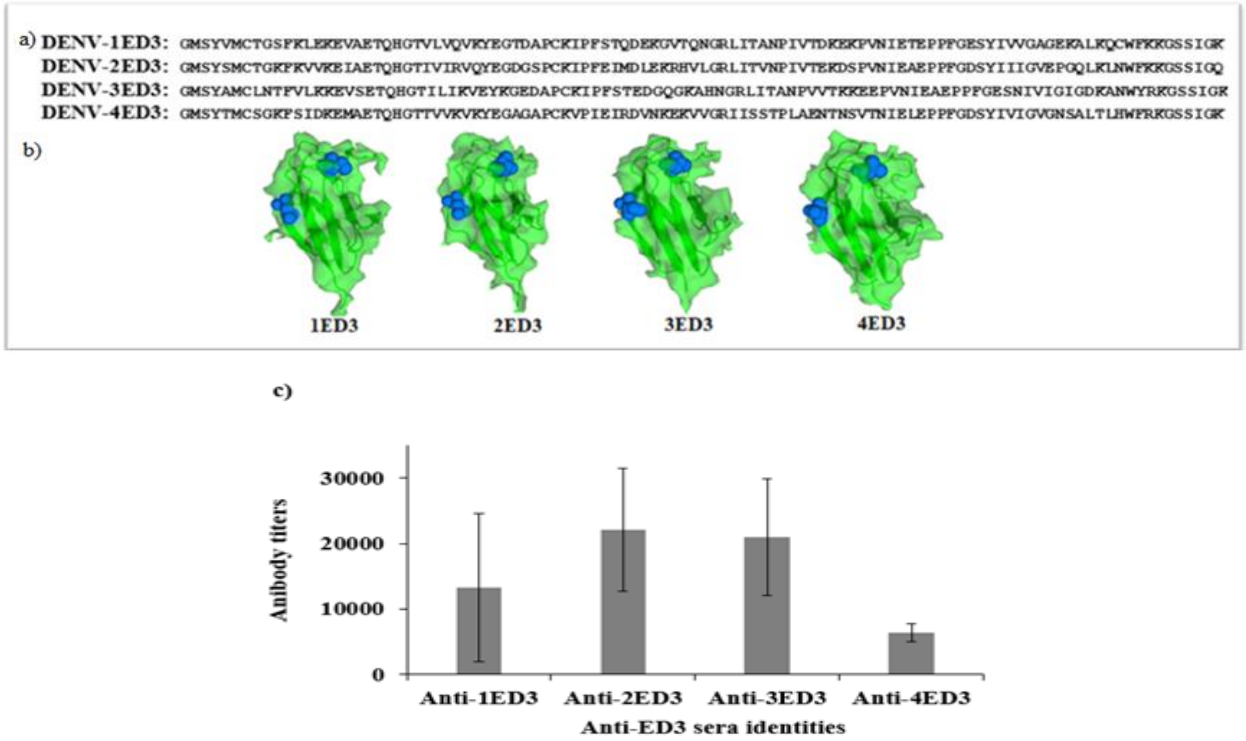


Figure II

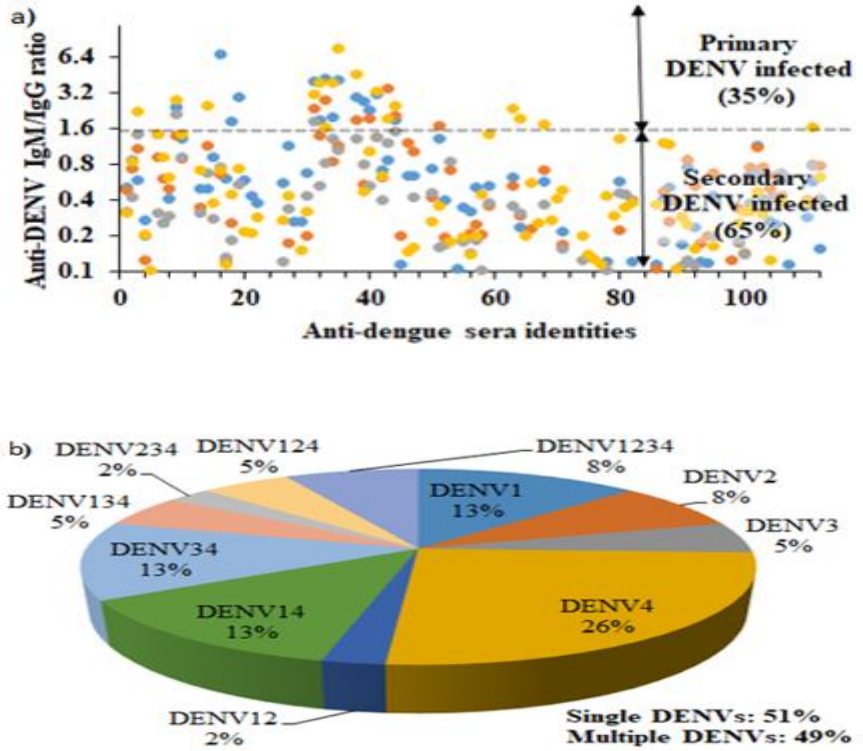
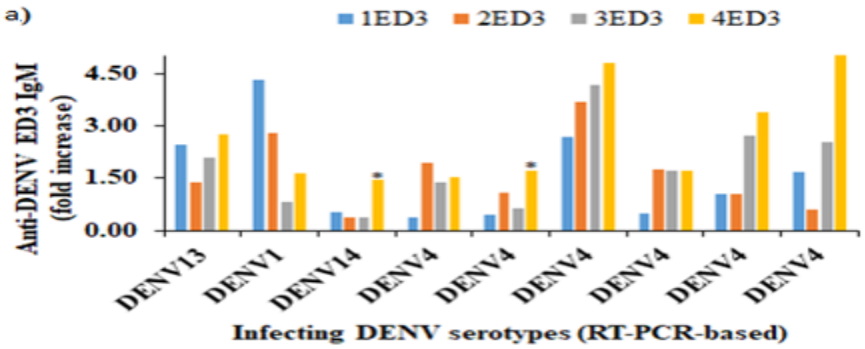


Figure III



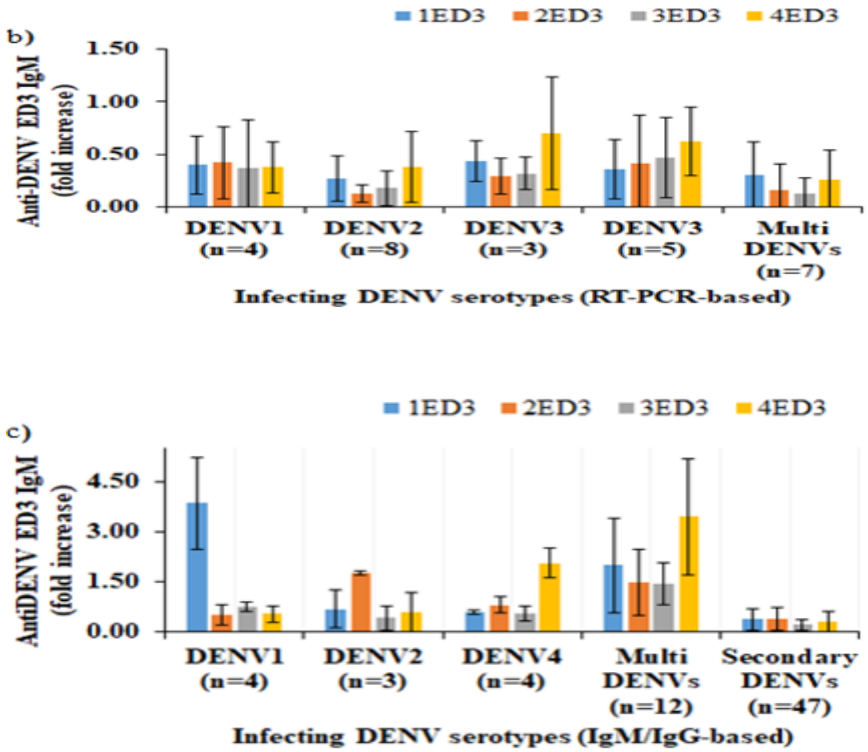
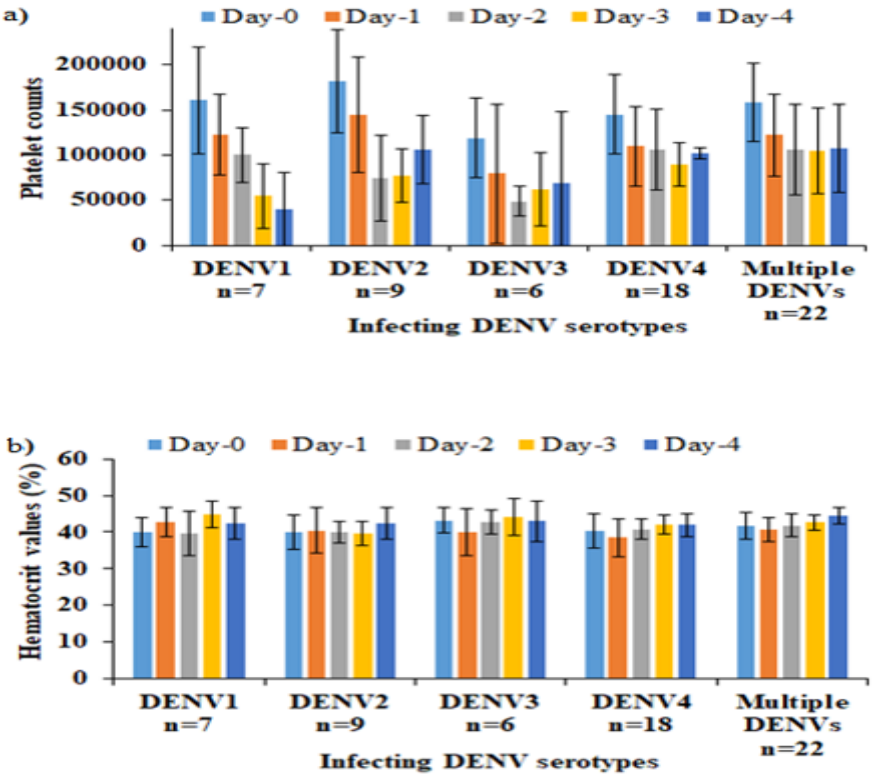

b)
1ED3
2ED3
3ED3
4ED3
Anti-DENV ED3 IgM (fold increase)
1.50
1.00
0.50
0.00
DENV1 (n=4)
DENV2 (n=8)
DENV3 (n=3)
DENV3 (n=5)
Multi DENVs (n=7)
Infecting DENV serotypes (RT-PCR-based)
c)
1ED3
2ED3
3ED3
4ED3
AntiDENV ED3 IgM (fold increase)
4.50
3.00
1.50
0.00
DENV1 (n=4)
DENV2 (n=3)
DENV4 (n=4)
Multi DENVs (n=12)
Secondary DENVs (n=47)
Infecting DENV serotypes (IgM/IgG-based)

Figure IV


a)
Day-0
Day-1
Day-2
Day-3
Day-4
Platelet counts
200000
150000
100000
50000
0
DENV1 n=7
DENV2 n=9
DENV3 n=6
DENV4 n=18
Multiple DENVs n=22
Infecting DENV serotypes
b)
Day-0
Day-1
Day-2
Day-3
Day-4
Hematocrit values (%)
60
50
40
30
20
10
0
DENV1 n=7
DENV2 n=9
DENV3 n=6
DENV4 n=18
Multiple DENVs n=22
Infecting DENV serotypes

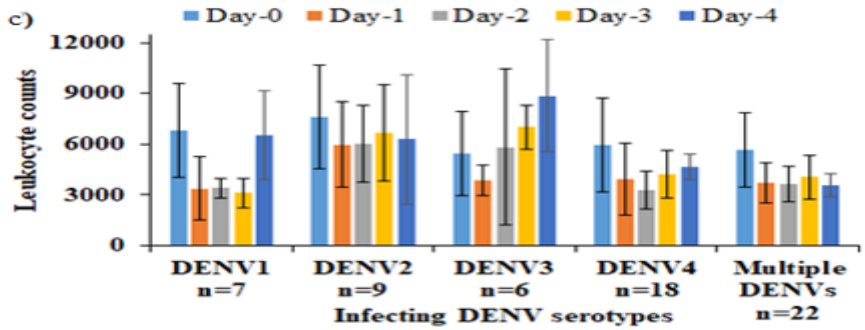
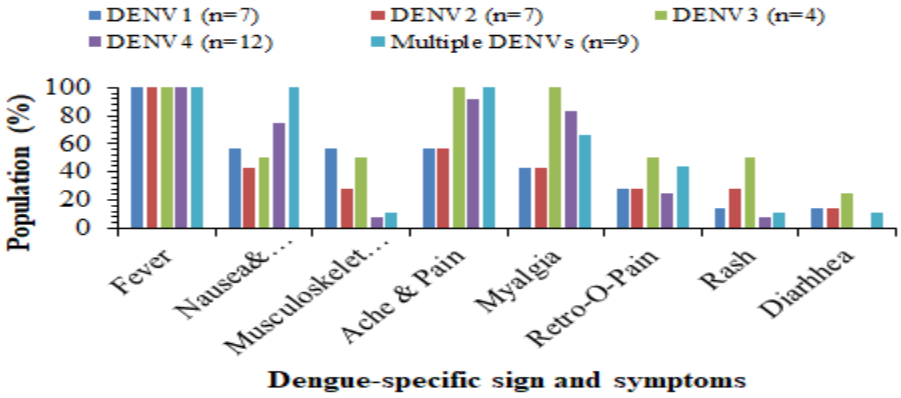


Figure V



ACKNOWLEDGEMENT:

MMI thanks Professor Yutaka Kuroda of Tokyo University of Agriculture and Technology for providing ED3 variants and the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B), for providing the Swiss albino mice. MMI thanks Research and Publication Cell of the University of Chittagong, Bangladesh for funding.

Competing Interests:

No conflict of interest.

Ethics approval:

This study has been conducted under ethical approval from the Ethical Review Board of Chittagong Medical College Hospital (CMC/PG/2018/580), and ethical approval from Chittagong University [AERB-FBSCU-20221031-(1)] with written consent of the participants. All patients' data were totally anonymous and kept confidential.

REFERENCES

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496(7446):504–7. doi:10.1038/nature12060.
2. World Health Organization. Dengue and Severe Dengue Key Facts. World Health Organization. 2021;
3. Fu J, Tan BH, Yap EH, Chan YC, Tan YH. Full-length cDNA sequence of dengue type 1 virus (Singapore strain S275/90). Virology. 1992;188(2):953-958
4. Holmes EC, Twiddy SS. The origin, emergence and evolutionary genetics of dengue virus. Infection Genetics and

Evolution. 2003;3(1): 19–28. doi: 10.1016/s1567-1348(03)00004-2.

5. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis. 2000;181(1):2-9. doi: 10.1086/315215.

6. Yoon I-K, Nisaluk A, Kalayanarooj S, Thaisomboonsuk B, Bhoomiboonchoo P, et al. Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 2010. Int J Infect Dis. 2012;16(1):E141. doi:https://doi.org/10.1016/j.ijid.2012.05.319

7. Balmaseda A, Hammond SN, Pérez L, Tellez Y, Saborío SI, Mercado JC, et al. Serotype-specific differences in clinical manifestations of dengue. Am J Trop Med Hyg. 2006;74(3):449-456.

8. Corwin AL, Larasati RP, Bangs MJ, Wuryadi S, Arjoso S, Sukri N, et al. Epidemic dengue transmission in southern Sumatra, Indonesia. Trans R Soc Trop Med Hyg. 2001;95(3)257-265

9. Pereira Passos MN, Jabor Garcia Santos LM, Reis Pereira MR, GuimarãesCasali C, De Paula MenezesDrumond Fortes B, Ortiz Valencia LI, et al. Clinical differences observed in patients with dengue caused by different serotypes in the epidemic of 2001/2002, occurred in Rio de Janeiro. Rev Soc Bras Med Trop. 2004;37(4):293-295. doi:https://doi.org/10.1590/S0037-8682200400040000

10. Khurram M, Qayyum W, Hassan SJ ul, Mumtaz S, Bushra HT, Umar M. Dengue hemorrhagic fever: Comparison of patients with primary and secondary infections. J Infect Public Health. 2014;7(6):489-495.

11. Senaratne UTN, Senaratne UTN, Murugananthan K, Murugananthan K, Sirisena PDNN, Carr JM, et al. Dengue virus co-infections with multiple serotypes do not result in a different clinical outcome compared to mono-infections. Epidemiol Infect. 2020;148:e119

12. Stanaway JD, Shepard DS, Undurraga E A, Halasa Y A, Coffeng LE, Brady OJ, et al. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. Lancet Infect Dis. 2016;16(6):712-723.

13. Shirin T, Muraduzzaman AKM, Alam AN, Sultana S, Siddiqua M, Khan MH, et al. Largest dengue outbreak of the decade with high fatality may be due to reemergence of DEN-3 serotype in Dhaka, Bangladesh, necessitating immediate public health attention. New Microbes and New Infections. 2019;16(29)100511.doi: 10.1016/j.nmni.2019.01.007

14. Reddy MN, Dungdung R, Valliyott L, Pilankatta R. Occurrence of concurrent infections with multiple serotypes of dengue viruses during 2013–2015 in northern Kerala, India. PeerJ . 2017;5:e2970. doi:10.7717/peerj.2970.

15. Singh A, Bisht P, Bhattacharya S, Guchhait P. Role of Platelet Cytokines in Dengue Virus Infection. Frontiers in Cellular and Infection Microbiology. 2020;10:561366. doi.org/10.3389/fcimb.2020.561366

16. Soo KM, Khalid B, Ching SM, Chee HY. Meta-analysis of dengue severity during infection by different dengue virus serotypes

in primary and secondary infections. PLoS One. 2016;11(5):e0154760.

17. Tazeen A, Afreen N, Abdullah M, Deeba F, Haider SH, Kazim SN, et al. Occurrence of co-infection with dengue viruses during 2014 in New Delhi, India. Epidemiol Infect. 2017;145(1)67-77.doi.org/10.1017/S0950268816001990

18. Zhang J, Shu Y, Shan X, Li D, Ma D, Li T, et al. Co-circulation of three dengue virus serotypes led to a severe dengue outbreak in Xishuangbanna, a border area of China, Myanmar, and Laos, in 2019. Int J Infect Dis. 2021;107:15-17

19. Chanama S, Anantapreecha S, A-Nuegoonpipat A, Sa-Gnasang A, Kurane I, Sawanpanyalert P. Analysis of specific IgM responses in secondary dengue virus infections: Levels and positive rates in comparison with primary infections. J ClinVirol. 2004;31(3):185-189. doi: 10.1016/j.jcv.2004.03.005.

20. BandaruAK ,Vanumu CS, Early predictors to differentiate primary from secondary dengue infection in children. Med J Dr DY Patil Univ. 2016;9(5):587-593

21. Rafique I, Saqib MAN, Munir MA, Qureshi H, Taseer I ul H, Iqbal R, et al. Asymptomatic dengue infection in adults of major cities of Pakistan. Asian Pac J Trop Med. 2017; 10(10):1002-1006

22. St. John AL, Rathore APS. Adaptive immune responses to primary and secondary dengue virus infections. Nature Reviews Immunology. 2019;(4):218-230.

23. Lai SC, Huang YY, Wey JJ, Tsai MH, Chen YL, Shu PY, et al. Development of Novel Dengue NS1 Multiplex Lateral Flow Immunoassay to Differentiate Serotypes in Serum of Acute Phase Patients and Infected Mosquitoes. Front Immunol. 2022; 13:852452.doi: 10.3389/fimmu.2022.852452.

24. Falconar AKI, De Plata E, Romero-Vivas CME. Altered enzyme-linked immunosorbent assay immunoglobulin M (IgM)/IgG optical density ratios can correctly classify all primary or secondary dengue virus infections 1 day after the onset of symptoms, when all of the viruses can be isolated. Clin Vaccine Immunol. 2006;13(9)1044-1051

25. Cucunawangsih, Lugito NPH, Kurniawan A. Immunoglobulin G (IgG) to IgM ratio in secondary adult dengue infection using samples from early days of symptoms onset. BMC Infect Dis. 2015;15:276.doi: 10.1186/s12879-015-1022-9.

26. Roy MG, Uddin K, Islam D, Singh A, Islam MM. All four dengue virus serotypes co-circulate in concurrent dengue infections in a single dengue session in Chittagong, Bangladesh. Bioresearch Commun. 2022;8(1):1042-1048.doi:https://doi.org/10.3329/brc.v8i1.57042.

27. Pitcher TJ, Sarathy V V, Matsui K, Gromowski GD, Huang CYH, Barrett ADT. Functional analysis of dengue virus (DENV) type 2 envelope protein domain 3 type-specific and DENV complex-reactive critical epitope residues. J Gen Virol. 2015;96(2):288–293. doi: 10.1099/vir.0.070813-0.

28. Kulkarni MR, Islam MM, Numoto N, Elahi M, Mahib MR, Ito N, et al. Structural and biophysical analysis of sero-specific immune responses using epitope grafted Dengue ED3 mutants. BiochimBiophys

Acta.2015;1854(10):1438–1443.doi:10.1016/j.bbapap.2015.07.004.
29. Elahi M, Islam MM, Noguchi K, Yohda M, Kuroda Y. High resolution crystal structure of dengue-3 envelope protein domain III suggests possible molecular mechanisms for serospecific antibody recognition. Proteins.2013;81(6):1090–1095. doi.org/10.1002/prot.24237
30. Islam MM, Miura S, Hasan MN, Rahman N, Kuroda Y. Anti-Dengue ED3 Long-Term Immune Response With T-Cell Memory Generated Using Solubility Controlling Peptide Tags. Front Immunol. 2020;11:333.doi: 10.3389/fimmu.2020.00333.
31. Kuno G, Gómez I, Gubler DJ. An ELISA procedure for the diagnosis of dengue infections. J Virol Methods. 1991;33(1–2):101–113.
32. Crill WD, Hughes HR, Delorey MJ, Chang GJ. Humoral immune responses of dengue fever patients using epitope-specific serotype-2 virus-like particle antigens. PLoS One [Internet]. 2009;4(4):e4991.
33. Lardo S, Utami Y, Yohan B, Tarigan SMMU, Santoso WD, Nainggolan L, et al. Concurrent infections of dengue viruses serotype 2 and 3 in patient with severe dengue from Jakarta, Indonesia. Asian Pac J Trop Med . 2016;9(2):134–140.
34. Rahman M, Rahim R, Hasan A, Murad AS, Biswas M. Co-circulation of Three Dengue Virus Serotypes in 2017 in Dhaka city : First report from Bangladesh. Bioresearch Communications. 2019;5(1):637-641.
35. Pervin M, Tabassum S, Sil BK, Islam MN. Isolation and serotyping of dengue viruses by mosquito inoculation and cell culture technique: An experience in Bangladesh. Dengue Bulletin. 2003;27:81-90.
36. Elahi M, Islam MM, Noguchi K, Yohda M, Toh H, Kuroda Y. Computational prediction and experimental characterization of a “size switch type repacking” during the evolution of dengue envelope protein domain III (ED3). BiochimBiophysActa. 2013;1844(3):585–592.
37. Bhatnagar J, Blau DM, Shieh WJ, Paddock CD, Drew C, Liu L, et al. Molecular detection and typing of dengue viruses from archived tissues of fatal cases by RT-PCR and sequencing: Diagnostic and epidemiologic implications. Am J Trop Med Hyg. 2012;86(2):335-340. doi: 10.4269/ajtmh.2012.11-0346
38. Anwar A, Wan G, Chua KB, August JT, Too HP. Evaluation of Pre-Analytical Variables in the Quantification of Dengue Virus by Real-Time Polymerase Chain Reaction. J Mol Diagnostics.2009;11(6):537–542.
39. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med. 2006;27:126–139. . doi.org/10.1016/j.mam.2005.12.003
40. Koo C, Kaur S, Teh ZY, Xu H, Nasir A, Lai YL, Khan E, Ng LC ,Hapuarachchi HC. Genetic variability in probe binding regions explains false negative results of a molecular assay for the detection of dengue Virus. Vector-Borne Zoonotic Dis. 2016;16(7):489–495. doi.org/10.1089/vbz.2015.1899.
41. AzeredoELd, Monteiro RQ, Pinto LMO. Thrombocytopenia in Dengue: Interrelationship between Virus and the

Imbalance between Coagulation and Fibrinolysis and Inflammatory Mediators. Mediators Inflamm. 2015;3(313842). doi.org/10.1155/2015/313842

42. Stanaway JD, Shepard DS, Undurraga EA, Halasa YA, Coffeng LE, Brady OJ, et al. The global burden of dengue: an analysis from the Global Burden of Disease Study 2013. Lancet Infect Dis. 2016;16(6):712-723.

43. Raafat N, Blacksell SD, Maude R. A review of dengue diagnostics and implications for surveillance and control. Transations R Soc Trop Med Hyg . 2019;113(11):653–660. doi.org/10.1093/trstmh/trz068.

44. Nayak V, Dessau M, Kucera K, Anthony K, Ledizet M, Modis Y. Crystal structure of dengue virus type 1 envelope protein in the postfusion conformation and its implications for membrane fusion. J Virol. 2009;83(9):4338–44. doi.org/10.1128/JVI.02574-08

45. Rouvinski A, Guardado-Calvo P, Barba-Spaeth G, Duquerroy S, Vaney M, Kikuti CM, Sanchez MEN, Dejnirattisai W, Wongwiwat W, Haouz A, Girard-Blanc C, Petres S, Shepard WE, Despres P, Arenzana-Seisdedos F, Dussart P, Mongkolsapaya J. Screation GR, Rey FA.Recognition Determinants of Broadly Neutralizing Human Antibodies Against Dengue Viruses. Nature. 2015;520(7545):109-113.doi: 10.1038/nature14130.