Detection of Circulating Dengue Virus Serotypes of 2021 Dengue Outbreak in Dhaka, Bangladesh

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
B.Sc. in Biotechnology

Department of Mathematics and Natural Sciences
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Declaration

I hereby declare that:

1. The thesis titled, "Detection of circulating dengue virus serotypes of 2021 dengue outbreak in Dhaka, Bangladesh," that has been submitted, is my own original work while completing the Bachelor of Science (B.Sc.) degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The project titled, **"Detection of circulating dengue virus serotypes of 2021 dengue outbreak in Dhaka, Bangladesh"** submitted by Raad Rahmat (18136058) of Spring 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on January 06, 2022.

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Abstract

Dengue infection is a self-limiting febrile illness caused by four serotypes of dengue viruses (DENVs). It is one the most important, yet globally neglected, vector-borne diseases in tropical and subtropical regions of the world, where its etiological agent's mosquito vectors, female *Aedes aegypti* and *A. albopictus* are habituated. Due to rapid development of the world, in terms of urbanization and international travel, dengue has been on a staggering rise with regards to geographical distribution, frequency of outbreaks, and disease severity. During the monsoon season, many of the endemic and hyperendemic countries, including Bangladesh, are vulnerable to dengue outbreaks. While it mostly leads to silent or mild to moderate symptoms, disease severity is often increased with reemergence of a serotype of DENV following years of absence, especially in cases of heterologous secondary DENV infections. This makes identification of circulating serotypes, along with detection of the prevalent serotype(s), indispensable tools during times of potential outbreaks as it may lead to nationwide preparedness, thus, enabling the control or limitation of a potential epidemic. To address the issue, this study aimed to identify the prevalence of circulating serotypes of DENV in the recent 2021 dengue outbreak in Bangladesh. In this study, suspected dengue patients were enrolled whose blood was serologically tested for the presence of dengue NS1 antigen, and anti-Dengue IgM and IgG. Following RNA extraction with RNA Isolation Kit, presence of dengue RNA was then detected in the NS1 positive samples via real-time reverse transcriptase polymerase chain reaction (RT-PCR) using a commercial Dengue Virus Real Time PCR Detection Kit. Later on, another commercial Multiplex real-time RT-PCR Dengue differentiation kit was used on the isolated RNA positive samples for serotyping the DENVs in the samples. Accordingly, this serotyping study allowed the identification of the circulating serotypes, as well as the predominant serotype in the 2021 dengue outbreak in Bangladesh. This study found that DENV2 and DENV3 were the circulating DENV serotypes in the 2021 dengue outbreak in Dhaka, Bangladesh with DENV3 being predominant. It was also revealed that majority of the cases were secondary dengue infections.

Keywords: Dengue, Serotype, real time RT-PCR, Prevalence

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Abbreviations

ADE	Antibody dependent enhancement
ALT	Alanine aminotransferase
APTT	Activated partial thromboplastin time
AST	Aspartate aminotransferase
CBC	Complete blood count
CDC	Centers for Disease Control and Prevention
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DIC	Disseminated intravascular clotting
DSS	Dengue shock syndrome
ELISA	Enzyme-linked immunosorbent assay
IEDCR	Institute of Epidemiology, Disease Control, and Research
IFA	Immunofluorescent assay
IFN	Interferon
NS	Non-structural protein
NTR	Non-translated regions
prM	Precursor membrane protein
SD	Severe dengue
sNS1-ELISA	Serotyping NS1-Enzyme linked immunosorbent assay
TNF	Tumor necrosis factor
WHO	World Health Organization

Chapter 1: Introduction

Dengue is a self-limiting, systemic viral infection caused by dengue viruses (DENV) and is one of the major threats among vector-borne diseases in tropical and subtropical regions around the globe. Dengue is caused by one of the four serotypes of single-stranded, positive-sense RNA viruses, DENVs, DENV1, DENV2, DENV3, and DENV4, that are genetically and antigenically related to each other and belong to the family, Flaviviridae. Originating from the rainforests of Asia and Africa, DENVs slowly spread to the Eastern Mediterranean, American, and Western Pacific regions following increased international travel, arising initially from slave trading in the 15th and 16th centuries, and eventually by globalization and modernization of transportation, paving the way for the introduction of the principal mosquito vectors of dengue, the urban-adapted primary vector *Aedes aegypti* and the secondary vector *A. albopictus* from Asia and Africa into these regions (Guzman et al., 2016, Simmons et al., 2012). This was supported by the report which showed that prior to 1970, only 9 countries experienced severe dengue epidemics, with the disease now being endemic in over 100 countries with The Americas, South-East Asia, and Western Pacific regions being more seriously impacted. Asia, alone, accounts for 70% of the global dengue burden (WHO, 2021). The endemicity of dengue in Asia and Latin America was further facilitated by the meteoric rate of urbanization and population increase creating a den of vector breeding sites leading to infestation of mosquitoes in overcrowded areas (Simmons et al., 2012). As such, dengue is often hyperendemic in these regions where multiple serotypes co-circulate during times of dengue outbreak, which is primarily seen during the monsoon season where clogging of rainwater provides an ideal egg-laying habitat for mosquitoes.

The World Health Organization (WHO) reported that the number of reported dengue cases had increased 8-fold over the last two decades with 505,430 cases being reported in 2000 to 5.2 million cases in 2019. Furthermore, a model painted a more ominous picture estimating about 390 million dengue infections annually with 95 million infections manifesting clinical symptoms. Number of deaths reported to WHO had also increased staggeringly from 960 deaths in 2000 to 4,032 casualties in 2015. Bangladesh, Brazil, Cook Islands, Ecuador, India, Indonesia, Maldives, Mauritania, Mayotte (Fr), Nepal, Singapore, Sri Lanka, Sudan, Thailand, Timor-Leste, and Yemen, all reported increased number of cases in 2020 with Bangladesh, Brazil, Cook Islands,

Colombia, Fiji, Kenya, Paraguay, Peru, and Reunion island still being dealt with brutal dengue epidemics in 2021 (WHO, 2021). Another study reported that among 390 million DENV infections, 96 million are symptomatic and 2 million show severe symptoms with 21,000 deaths per year. In Asia, children in the age group of 5 to 15 years are more likely to contract dengue infection while in the American tropics, the adult population are more susceptible with the modal age of infection being 19 to 40 years (Gubler, 2011). In Africa, due to many cases being misdiagnosed as malaria, the dengue rates and age distribution are unclear, however, epideictic activity has increased in the last 40 years (Guzman et al., 2016).

Transmission occurs when a female *Aedes* mosquito becomes infected for life drawing blood from a dengue infected individual during the acute febrile and viremic phase of infection, thus, transmitting DENVs to a susceptible human during her next blood meals. The intrinsic incubation period (in humans) generally ranges from 3 to 14 days with an average of 4 to 7 days from infection to onset of symptoms (Gubler, 2014). Following the incubation period, symptoms begin to manifest in three phases, the initial febrile phase known as dengue fever (DF), the critical phase as observed in dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), and the convalescence or recovery phase (Simmons et al., 2012).

The initial febrile phase is generally characterized by fever (body temperature of ≥38.5°C), along with headache vomiting, myalgia, and muscle, eye and/or joint pain, with temporary rashes on some occasions (CDC, 2021, Simmons et al., 2012). In some instances, mild hemorrhagic manifestations in the form of petechiae and bruising at venipuncture sites and palpable liver are also observed along with instances of mild to moderate thrombocytopenia and leukopenia, and elevated hepatic aminotransferase levels. Most patients make complete recovery following 3-7 days of this period (Simmons et al., 2012).

However, in some instances, the patient may rapidly deteriorate between 4 to 7 days of illness indicated by persistent vomiting, severe abdominal pain, tender hepatomegaly, a high or increasing hematocrit level, swift decrease in the platelet count, serosal effusions, mucosal bleeding, and lethargy or restlessness (Simmons et al., 2012). These are caused by dengue non-structural-1 (NS1) antigen-mediated systemic vascular leakage where the loss of plasma results in elevated hemoconcentration, hypoproteinemia, pleural effusions, and ascites, and eventually hypotension (Beatty et al., 2015, Guzman et al., 2016, Modhiran et al., 2015, Simmons et al., 2012). If left

unmonitored and untreated, the patient may devolve and undergo shock which may be fatal. These are characteristics of DSS.

In cases of very intense and prolonged shock, massive internal bleeding or hemorrhages may be observed which may be accompanied by profuse plasma leakage. In cases involving DHF, moderate to severe thrombocytopenia, elevated partial-thromboplastin time, and reduced fibrinogen levels are often observed. Children are more likely to suffer from DSS and DHF. Critical cases may also present with liver failure, myocarditis, and encephalopathy. If fatality is averted, such increased vascular permeability is only transient and return back to normal levels within 2 to 3 days and the patient enters the convalescent stage where the symptoms subside. Secondary rashes for 1 to 2 weeks and fatigue for a few weeks are known to occur during the recovery phase as well (Simmons et al., 2012). Immunity against a specific serotype is lifelong, however, subsequent dengue infection with a different serotype of DENV has an increased likelihood of causing severe disease, including DSS and DHF (Guzman et al., 2016, Montoya et al., 2013).

Bangladesh, being a small yet overcrowded country in the tropical and subtropical region, is hyperendemic for dengue where annual outbreaks of dengue infections are often observed during the monsoon and post-monsoon period from mid-June to November or early-December. The incidence of dengue and the number of deaths caused by it vary in Bangladesh as observed by the two-decade long report by the Institute of Epidemiology, Disease Control and Research (IEDCR) under the Ministry of Health and Family Welfare of Bangladesh. For instance, in the year 2000, 5,551 dengue cases were reported with 93 casualties while 2007, 2009, 2010, and 2014 recorded very few cases of 466, 474, 409, and 375 cases, respectively with no deaths during these years. The incidence and number of fatalities, therefore, increase in clusters, not according to a specific chronological pattern. The country reported the greatest number of cases in 2019 with over 101,354 dengue cases and a record of 164 deaths (IEDCR, 2019). While such epidemics often see the co-circulation of multiple serotypes of DENVs, usually one or two of the serotypes are prevalent.

According to two different studies, in the year 2000 in Bangladesh, DENV3 was the predominant serotype while another study that was conducted between 2013 and 2016 showed altering prevalence between DENV1 and DENV2 (Akhter et al., 2019, Aziz et al., 2002, Pervin et al.,

2002). DENV2 was prevalent during the first two years of study (2013-2014) while the latter two years (2015-2016) showed predominance of DENV2 (Akhter et al., 2019). After 2002, no cases of DENV3 and DENV4 were reported by IEDCR. The former, however, reemerged in 2017 with an inevitable sharp rise in the number of cases following its reemergence. As a result, in 2018, DENV2 (41%) and DENV3 (31%) were the prevalent serotypes according to a study involving 151 dengue RNA PCR positive samples, which also reported cases of coinfection with multiple serotypes of DENVs with DENV3 being found in all multiple infection cases: 11% were DENV2 and DENV3 coinfection, DENV1 and DENV3 coinfection were observed in 5% of the cases while the remaining two showed a triple infection of DENV1, DENV2, and DENV3 (Shirin et al., 2019). DENV3 became the prevalent serotype in 2019 while the other serotypes being in circulation as well, leading to the eventual surge of dengue cases and deaths during that year (IEDCR, 2019).

Since prior dengue infection confers lifelong immunity against dengue caused by that serotype of DENV, it is likely that a significant proportion of the population are immune against DENV1 and/or DENV2 due to their persistent circulation over the last two decades. However, with the sudden reemergence of DENV3 and then DENV4, the population in Bangladesh are at a substantial risk against dengue and severe dengue (SD), as already observed with the unprecedented influx of cases in 2019, with DENV3 being predominant. The four serotypes of DENVs often have certain differences in terms of viral load and clinical manifestations which may differ geographically. For instance, in Bangladesh, DENV2 patients had a higher viral load compared to DENV1 patients which conflicted with the findings of a study conducted in Vietnam (Akhter et al., 2019). Additionally, different studies reported that DHF may be associated more frequently in patients infected with DENV2 or DENV3 with antibodies against DENV1 and that the risk factor for DSS may be increased in secondary cases of DENV2 who had prior exposure to any of the other three serotypes (Guzman et al., 1991, Sangkawibha et al., 1984). Another study found that DENV1 was associated with DHF and SD and more so compared to DENV2 and that DENV1 cases often presented with red-eyes while lower plate count and joint pain were associated with DENV2 cases more often. Furthermore, while secondary dengue caused by DENV2 was associated with increased disease severity compared to the other 3 serotypes, it was DENV1 that caused more overt symptoms during primary infections whereas, primary DENV2 and DENV3 primary cases were mostly silent (Yung et al., 2015). Higher risk of dengue following secondary infection were observed in cases of DENV1 followed by DENV2, DENV1 followed by DENV4, DENV2

followed by DENV3, and DENV4 followed by DENV3, according to another study (Aguas et al., 2019). Dengue, being a self-limiting virus, does lead to patients recovering on their own but in cases of DHF and/or DSS, physicians have to rely on symptom-based treatments due to the lack of specific anti-viral and vaccines against dengue, as such, it is very important to know prevalent and circulating DENV serotypes for nation-wide preparedness in controlling and/or limiting potential dengue outbreaks.

Serotyping of DENV can be conducted via different laboratory tests, including multiplex real-time reverse transcription polymerase chain reaction (RT-PCR), nested PCR, serotyping-NS1-enzyme linked immunosorbent assay (stNS1-ELISA), immunofluorescent assay (IFA), and different sequencing methods (Akhter et al., 2019, Aziz et al., 2002, Pervin et al., 2002, Prommool et al., 2021). To elucidate disease severity and devise possible treatment plans during the recent 2021 dengue outbreak in Dhaka, Bangladesh, this study aimed to identify the circulating serotypes of DENVs and decipher the prevalent serotype using multiplex RT-PCR.

Objectives

General Objective

The general objective of the study was to investigate the prevalence of circulating dengue virus serotypes in the recent 2021 dengue outbreak in Dhaka, Bangladesh.

Specific Objectives

1. To detect Dengue NS1 antigen among suspected dengue patients who were attending the outpatient department of BSMMU.
2. To decipher the percentage of dengue RNA positive samples among Dengue NS1 positive patients.
3. To identify the serotypes of circulating Dengue virus among the Dengue RNA positive samples in Dhaka, Bangladesh in 2021.
4. To observe the proportion of primary and secondary Dengue infections in 2021 among the Dengue RNA positive samples.

Chapter 2: Literature Review

2.1 History

Dengue is an old disease that has existed and tormented humans since ancient times (Gubler, 2006). While DENV, or more precisely DENV1, was first isolated in 1943 by Ren Kimura and Susumu Hotta during the 1943 dengue epidemic in Nagasaki, Japan, dengue cases have existed since much earlier (Scitable, n.d.). The earliest record of dengue, could be traced back to ancient China, as per the entry of a disease that was clinically compatible to dengue in the Chinese 'encyclopedia of disease symptoms and remedies' which was published during the era of Chin Dynasty (AD 265 to 420) and edited in AD 610 and 992 by the Tang Dynasty and Northern Sung Dynasty, respectively (Gubler, 2006). The first recorded dengue-like epidemic in America occurred in 1635 in Martinique and Guadeloupe while Panama experienced another outbreak in 1699 (Dick, 2012). However, the first major epidemics that were suspected to have been brought on by dengue, were documented to have ravaged three continents, Asia, Africa, and North America, in 1779 and 1780 (Gubler, 2006).

DENVs, which spilled over from non-human primates and became fully adapted to humans, were speculated to have originated from the rainforests of Asia and Africa which were natural habitats for the principal mosquito vector of DENV, *A. aegypti*, with the latter's claim to DENV origin being more substantiated according to sequence data analysis of DENVs and other flaviviruses. As such, dengue was predicted to have originated from Africa (Gubler et al., 2014, Guzman et al, 2016). However, with the clearing of forests and development of increasing number of human settlements, DENVs soon adapted to the rural environment via the peri-domestic mosquito vector, *A. albopictus*. Migration of rural dwellers to towns and cities, including in Asia, allowed the reach of DENVs to increase as well (Gubler, 2006). *A. aegypti* was introduced into the Americas in the 1600s through slave trading and soon spread globally, courtesy of the expansion of the shipping industry causing the geographical distribution of dengue to expand significantly (Guzman, 2016). This timeline coincided with the earliest report of dengue in the Americas in 1635 (Dick, 2012).

Therefore, despite the African origin of dengue, the global spread of dengue translated to human infection, suffering, and fatality over the course of human history.

2.2 Epidemiology

2.2.1 Global epidemiology

Since branching out from the rainforests of Africa, dengue has spread globally to 129 countries (WHO, 2021). The incidence of dengue cases has also been on an astronomical rise since the 1970s and 1980s with the number of cases reported to WHO observing over an unprecedented 8-fold increase in just the last two decades (Guzman et a., 2016, WHO, 2021). While the official WHO-reported cases numbered at 5.2 million in 2019, different study models estimated a more terrifying statistic, indicating about 390 million DENV infections annually, among whom, 96 million people are likely to manifest clinical symptoms. The number of reported deaths has also seen a staggering rise from 960 in 2000 to 4,032 in 2015 (WHO, 2021). Furthermore, another study estimated that up to 3.97 billion people of the population could be at risk of dengue (Brady et al., 2012). The majority of the people that are at risk of infection live in urban regions of the tropical and subtropical countries, primarily Southeast Asia, the Pacific, and the Americas, with the former accounting up to 70% of disease burden, as depicted in Figure 2.1 (CDC, 2021, Guzman et al., 2010, WHO, 2021). Disease severity is especially dangerous for children as they are the major population among the 500,000 DHF hospitalization cases, with the fatality rate surpassing 5% in some areas (Guzman et al., 2010). While children of the age 5 to 15 years are more at risk of dengue in Asia, it is the adult population of 19 to 40 years of age that are likely to be affected in the Americas (Gubler, 2011).

Dengue is the most important arboviral disease posing a significant socioeconomic and disease burden in endemic and hyperendemic countries, like in Asia and America, where the burden of dengue is approximately 1,300 disability-adjusted life years (DALYs) per million population (Guzman et al., 2010, Murray et al., 2013). Monsoon season, in particular, sees yearly spikes in dengue cases when heavy rainfall creates water clogging in several areas which provide the ideal egg-laying habitat for the mosquito vectors, *A. aegypti* and *A. albopictus*. Coupled with the perfect breeding ground for mosquitoes and the dense urban and sometimes rural, population in endemic countries, yearly outbreaks of dengue are often observed during the monsoon and post-monsoon seasons in these areas. 2019 saw the greatest surge of dengue cases with 5.2 million people being reportedly infected. In 2020, increased number of cases in Bangladesh, Brazil, Cook Islands, Ecuador, India, Indonesia, Maldives, Mauritania, Mayotte (Fr), Nepal, Singapore, Sri Lanka,

Sudan, Thailand, Timor-Leste and Yemen were observed, with many of these countries still being affected well into 2021 (WHO, 2021).

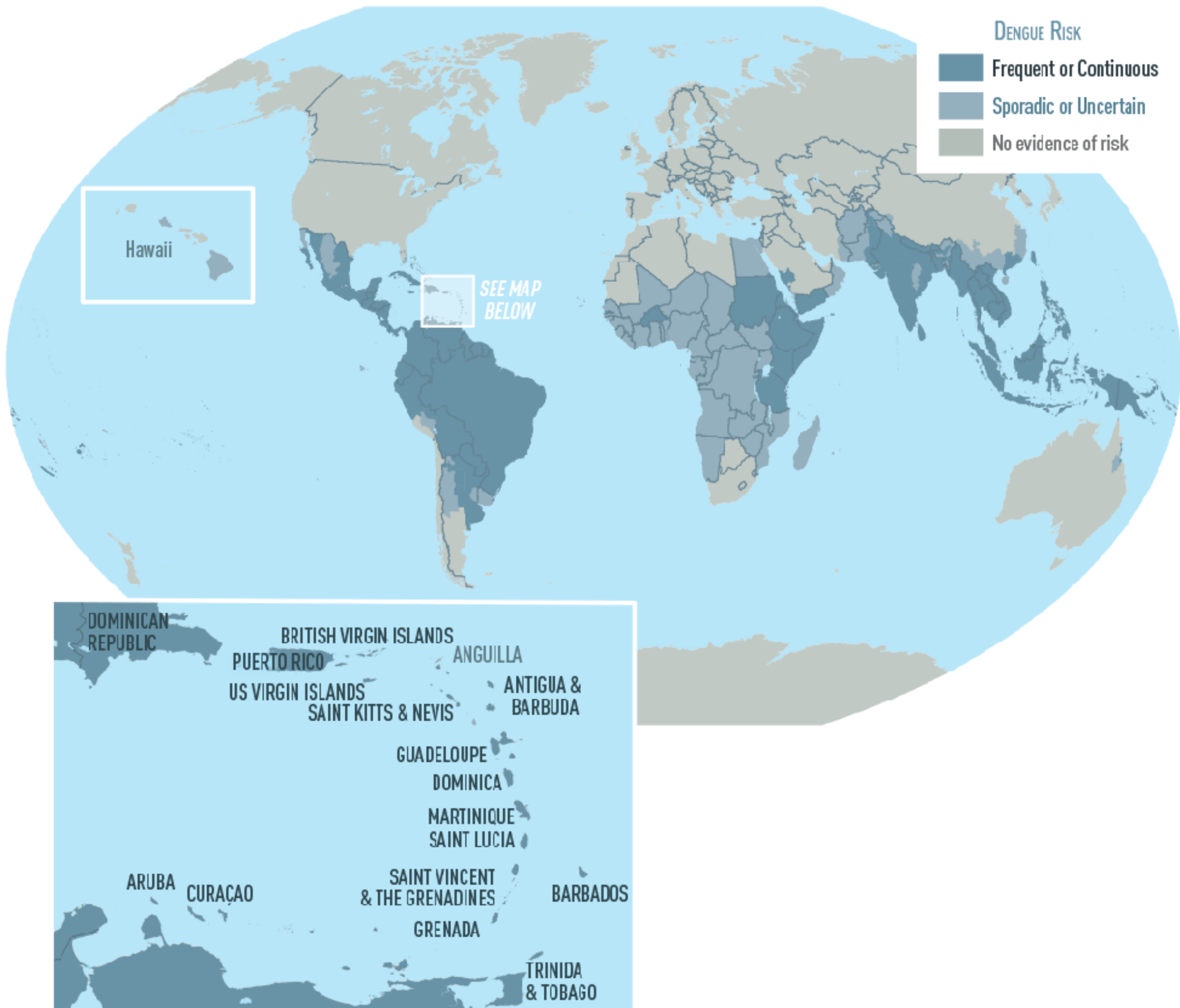


Figure 2.1: Global burden of dengue (CDC, 2021)

2.2.2 Epidemiology of Dengue in Bangladesh

Since the first reported case in Bangladesh in 1964, dengue has occurred sporadically in the country with increased incidence every few years until the first large epidemic that occurred in the year 2000 (Sharmin et al., 2015). The outbreak saw a surge in DF and DHF cases brought on by co-circulation of all four DENV serotypes, with DENV3 predominance (Shirin, 2019). Following the outbreak of 2002, DENV3 and DENV4 no longer remined in circulation in Bangladesh (Shirin et al., 2019). With DENV1 and DENV2 being the only persistent etiological agents, the number of cases remained below 7,000 with only sporadic increase in the number of cases above 1000, including a maximum of 6060 cases during 2016. However, with the re-emergence of DENV3 in 2017, there was a surge in dengue infections in 2018, while a record 101,354 cases with 164 fatalities were documented in 2019 with DENV3 being the predominant serotype (IEDCR, 2019, Shirin et al., 2019). Thus, increase in the incidence and disease severity depends on re-emergence of DENV serotypes, otherwise, sporadic outbreaks do not follow a specific pattern, other than a somewhat likely increase in reported cases every few years (IEDCR, 2019, Shirin, 2019).

Dengue cases usually occur during the monsoon and post-monsoon season from late-June to mid-December with the peak number of cases being reached in August-September. Majority of the cases recorded in 2019 was in the southern region of the country where some districts reported over 2500 cases while the capital, Dhaka, also observed increased number of dengue patients between 2000 and 2500 (Figure 2.2). With over 150,000 cases and 459 deaths from 2000-2019, dengue is, thus, responsible for a significant disease burden in Bangladesh (IEDCR, 2019).

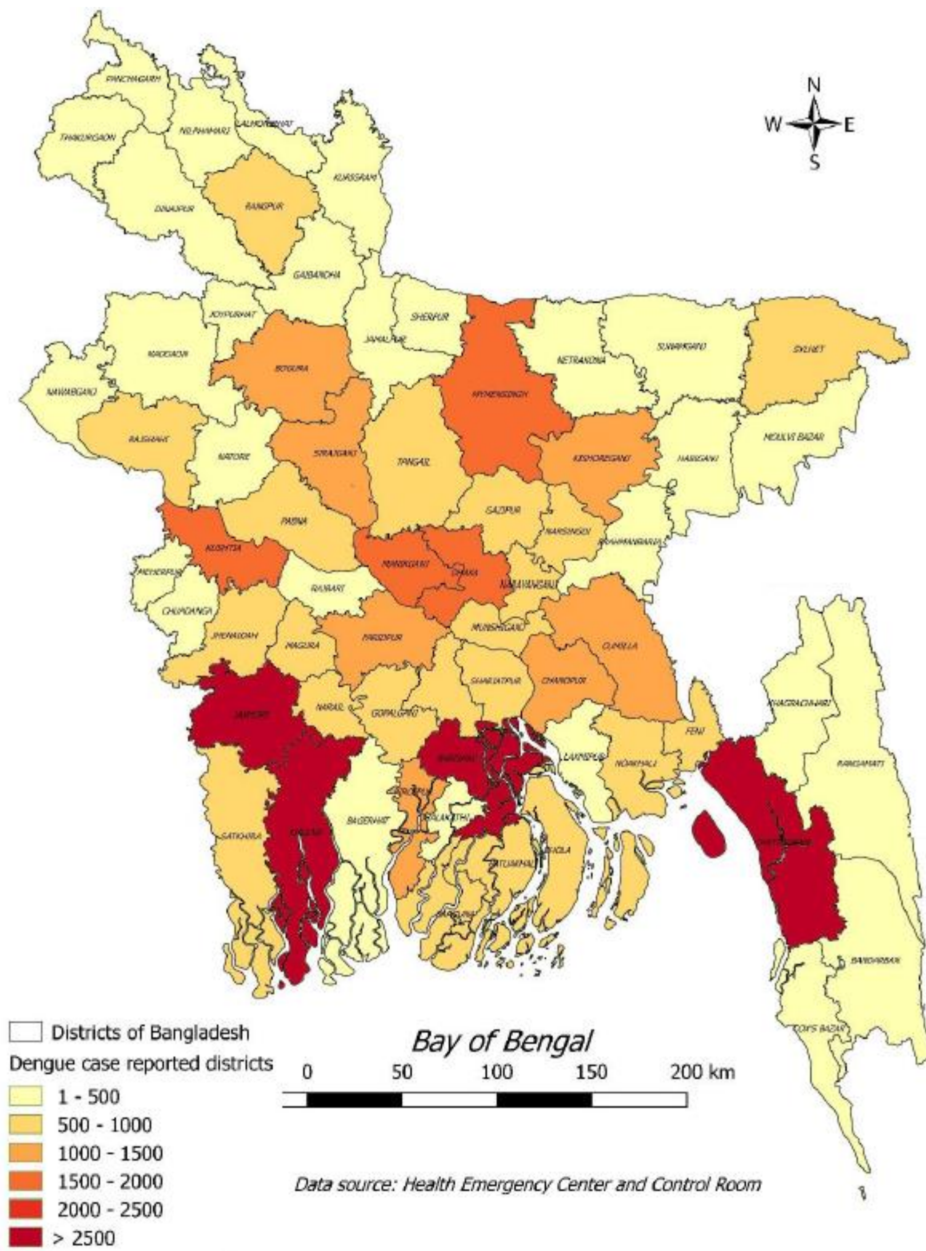


Figure 2.2: Distribution of suspected dengue cases in Bangladesh in 2019 (IEDCR, 2019)

2.3 Virologic features

DENVs are enveloped, single stranded positive-sense RNA viruses with an icosahedral symmetry and a diameter of approximately 500 Å belonging to the genus *Flavivirus* and occurs as 4 distinct antigenic serotypes (DENV1-4) (Kuhn et al., 2002, Leitmeyer et al., 1999, Simmons et al., 2012). The 10.7kb RNA genome is surrounded by a nucleocapsid and a lipid envelope bearing the envelope and coat proteins (Leitmeyer et al., 1999, Ross, 2010). The genome contains a single open reading frame (ORF) encoding a 370 kDa precursor polyprotein that is flanked by 5' and 3' non-translated regions (NTRs) which undergoes co- and post-translational proteolytic cleavage to form three structural proteins, including the capsid (C), membrane (M), and envelope (E) proteins with the E protein being 72-80% identical at the amino acid level across the four serotypes and is the target of different vaccine and immunotherapeutic studies (Brien et al., 2020, Chambers et al., 1990, Chandramouli et al., 2010, Leitmeyer et al., 1999, Wahala et al., 2010). Furthermore, 7 non-structural (NS) proteins are also formed upon cleavage, which include NS1, NS2a, cofactor for the NS3 protease (NS2b), serine protease and ATP-dependent helicase (NS3) needed for processing of virus polyprotein, NS4a, NS4b that is needed to block interferon (IFN) response, and methyltransferase and RNA-dependent RNA polymerase (NS5) (Chambers et al., 1990, Chandramouli et al., 2010, Leitmeyer et al., 1999). While all the NS proteins are needed for efficient replication, the functions of NS1, NS2a, and NS4a are not known or have scarce elucidation (Ross, 2010). The DENV structure and genome are illustrated in Figure 2.3 (Hottz et al., 2011).

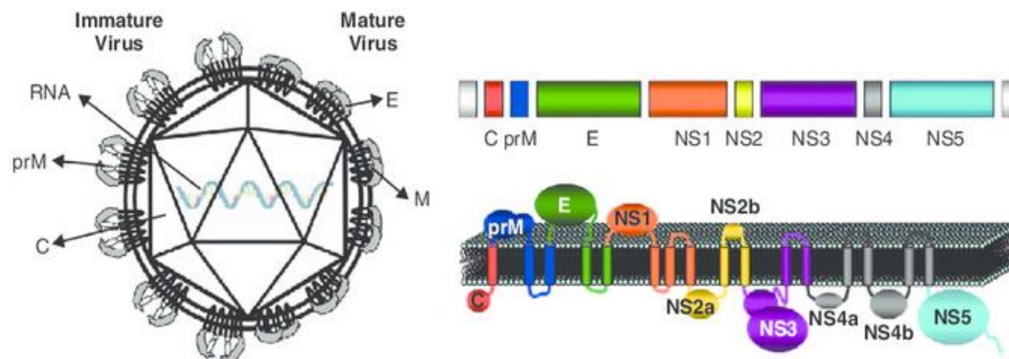
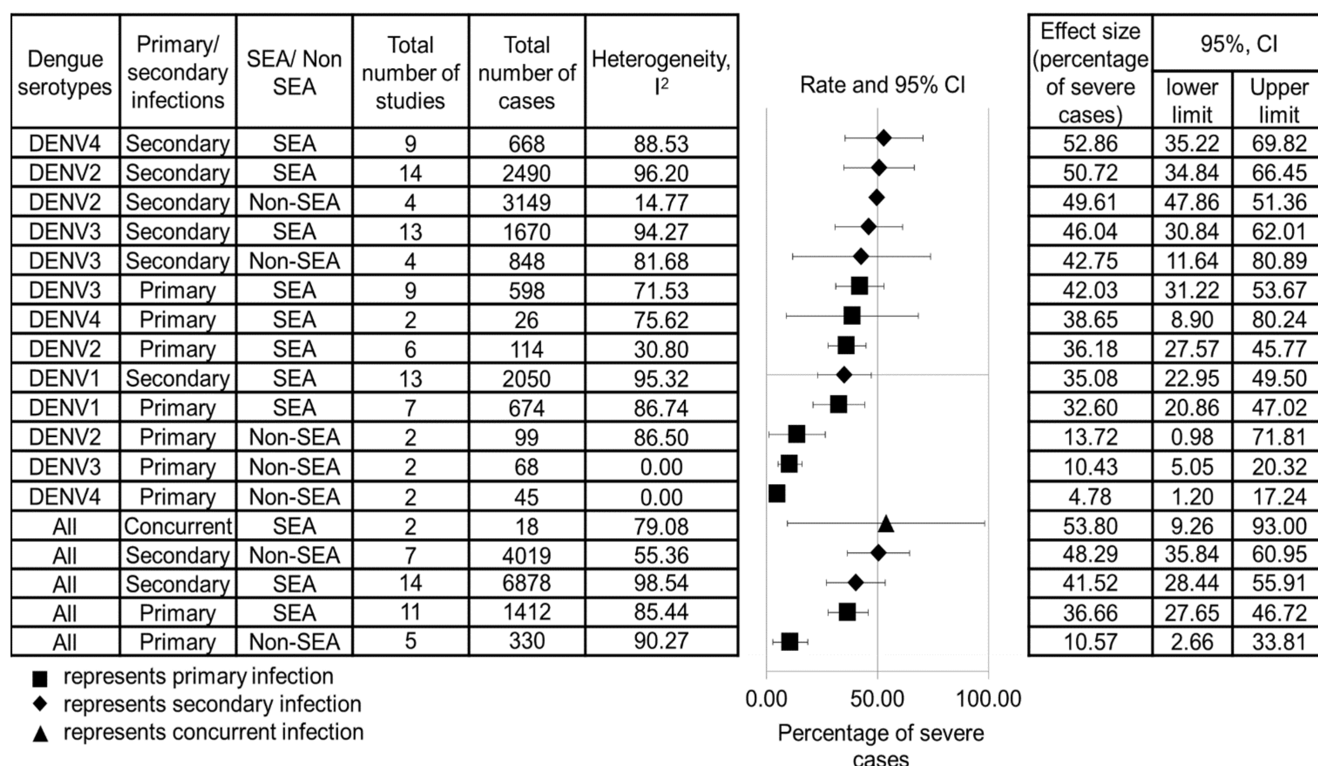


Figure 2.3: The structure and genome of DENV (Hottz et al., 2011)

2.4 Serotypes of Dengue virus

DENVs have been classified into four serotypes, DENV1-4, whose genomic sequences are about 65-70% similar with the difference resulting in ≥30% divergence in amino acid sequence causing different patterns of protein folding (Chong and Khan, 2019, Lisova et al., 2014, Modis et al., 2005, Soo et al., 2016). These differences arise due to the combined effects of multiple serotypes co-circulating in a specific region, rapid mutation rate of RNA viruses, and increased human activities that promote spread of DENVs. This could eventually lead to the emergence of a novel DENV serotype that may differ in critical neutralizing epitopes (Monath, 1994). These differences prevent cross-serotype immunity between the DENV serotypes. As such, despite a prior dengue infection in an individual conferring lifelong immunity, this defense is specific towards the infecting DENV serotype. Therefore, secondary dengue infections caused by a different serotype of DENV are common occurrences in hyperendemic regions. While the risk of DF and DHF has been associated with all the four serotypes, disease severity is significantly greater in cases of heterologous secondary DENV infections (Halstead et al., 1970, Wichmann et al., 2004).

The increased disease severity associated with secondary dengue infection by a different serotype can be attributed to a phenomenon known as antibody dependent enhancement (ADE). Dengue usually leads to synthesis of serotype-specific antibodies which confer lifelong immunity against the specific infecting DENV serotype and short-lasting immunity against other serotypes as well. However, after some time, the antibodies become unable to neutralize the different virus serotype but, instead, forms immunocomplexes that have a high affinity towards Fcγ receptors on the surfaces of macrophages and other cells. This enhances the entry of DENV into cells during secondary heterologous dengue infection and subsequently raises viral replication as well. Due to this phenomenon, increased damage, hence, more prominent disease symptoms are often observed in these cases (Figure 2.4) (Soo et al., 2016).



Dengue serotypes	Primary/ secondary infections	SEA/ Non SEA	Total number of studies	Total number of cases	Heterogeneity, I^2	Effect size (percentage of severe cases)	95%, CI lower limit	95%, CI Upper limit
DENV4	Secondary	SEA	9	668	88.53	52.86	35.22	69.82
DENV2	Secondary	SEA	14	2490	96.20	50.72	34.84	66.45
DENV2	Secondary	Non-SEA	4	3149	14.77	49.61	47.86	51.36
DENV3	Secondary	SEA	13	1670	94.27	46.04	30.84	62.01
DENV3	Secondary	Non-SEA	4	848	81.68	42.75	11.64	80.89
DENV3	Primary	SEA	9	598	71.53	42.03	31.22	53.67
DENV4	Primary	SEA	2	26	75.62	38.65	8.90	80.24
DENV2	Primary	SEA	6	114	30.80	36.18	27.57	45.77
DENV1	Secondary	SEA	13	2050	95.32	35.08	22.95	49.50
DENV1	Primary	SEA	7	674	86.74	32.60	20.86	47.02
DENV2	Primary	Non-SEA	2	99	86.50	13.72	0.98	71.81
DENV3	Primary	Non-SEA	2	68	0.00	10.43	5.05	20.32
DENV4	Primary	Non-SEA	2	45	0.00	4.78	1.20	17.24
All	Concurrent	SEA	2	18	79.08	53.80	9.26	93.00
All	Secondary	Non-SEA	7	4019	55.36	48.29	35.84	60.95
All	Secondary	SEA	14	6878	98.54	41.52	28.44	55.91
All	Primary	SEA	11	1412	85.44	36.66	27.65	46.72
All	Primary	Non-SEA	5	330	90.27	10.57	2.66	33.81

Figure 2.4: Pooled effect size of the relationship between dengue serotypes and percentage of severe cases (Soo et al., 2016)

The different serotypes of DENV often differentiates in terms of viral load and clinical manifestations. In Bangladesh, it was observed that DENV2 patients had a higher viral load compared to DENV1 patients which conflicted with the findings of a study conducted in Vietnam (Akhter et al., 2019). Different studies reported that DHF may be associated more frequently in patients with secondary dengue caused by DENV2 or DENV3 following a past exposure to DENV1 and that the risk factor for DSS may be increased in patients with DENV2 who had antibodies against any of the other three serotypes (Guzman et al., 1991, Sangkawibha et al., 1984). Another study found that DENV1 was more associated with DHF and SD compared to DENV2 and that DENV1 cases often presented with red-eyes while lower platelet count and joint pain were more often observed in patients infected with DENV2. Furthermore, while DENV2 led to increased disease severity in secondary cases compared to the other 3 serotypes, it was DENV1 that led to more overt symptoms during primary infections. On the other hand, primary DENV2 and DENV3 cases were mostly silent (Yung et al., 2015). Additionally, it was found that certain

sequence of DENV infections led to increased risk of symptom manifestation- DENV1 followed by DENV2, DENV1 followed by DENV4, DENV2 followed by DENV3, and DENV4 followed by DENV3 (Aguas et al., 2019).

Serotyping of DENV can be conducted via different laboratory tests, including multiplex real-time RT-PCR, conventional nested PCR, stNS1-ELISA, IFA, and different sequencing methods (Akhter et al., 2019, Aziz et al., 2002, Pervin et al., 2002, Prommool et al., 2021).

PCR remains the mainstay DENV serotyping method with multiplex real-time RT-PCR being more widely used compared to conventional nested RT-PCR due to its high sensitivity, specificity, and rapidity. While nested RT-PCR is more robust, utilizes basic thermal cycler and regent, and is cheaper than real-time RT-PCR, it's sensitivity is lower, it is more time consuming than real-time RT-PCR, and the result interpretation from gel electrophoresis analysis are open to subjective errors. However, both PCR techniques show high specificity during the identification of DENV serotypes. Dot-blot hybridization test can also be utilized, but they are not widely used in recent times as they not very sensitive. Therefore, PCR, more specifically, real-time RT-PCR, is the more popular diagnostic method. On the other hand, primer and probe selection are of crucial importance in real-time RT-PCR as mismatching will open ways to PCR failure (de Oliveira Poersch et al., 2005, Guzman and Kouri, 1996, Harris et al., 1998, Lanciotti et al., 1992).

Modified stNS1-ELISA is based on the serologic detection of serotype-specific dengue NS1 antigen. The assay uses four different serotype-specific antibody pairs to not only detect the presence of DENV and diagnose dengue but also allows the identification of the DENV serotype in the patient serum. While the sensitivity of this method is slightly less than that of real-time RT-PCR, it is suitable for resource limited laboratories as it is simpler, less technologically-intensive, and cheaper to use since serum samples can be directly assayed without pre-processing, thus, eliminating the need for RNA-extraction and reverse transcription. Furthermore, the window for diagnosis is elongated since NS1 remains in patients sera longer than DENV RNA. Therefore, it is emerging as an alternative technique to real-time RT-PCR (Prommool et al., 2021).

IFA uses serotype-specific monoclonal antibodies for simple, economical, reliable, and rapid detection of DENV and identify the serotypes. However, the major problem with detection and serotyping via monoclonal antibodies is that false negative results may be obtained in cell cultures with low viral concentrations (Guzman and Kouri, 1996).

Serotyping DENVs via sequencing is also an available but less utilized method due to the huge cost associated with sequencing technologies. A non-hyper variable region, such as region of *C-prM* gene junction, may be chosen as the sequence does not vary much compared to other RNA viruses and the mutations that occur are usually silent. While sequence diversity between the serotypes and relative conservation within a specific serotype could be utilized to accurately identify the DENV serotypes, the rapid mutation rate of RNA viruses which is capable of making vaccine development difficult and complicated, and the high cost disparity between current sequencing techniques and the widely employed real-time RT-PCR make sequencing a less popular diagnostic method for serotyping DENVs (Chong and Khan, 2019, Fatima et al., 2011).

2.5 Transmission and Viral life cycle

DENVs are primarily spread to humans via bites from infected mosquito vectors, *A. aegyptus* and *A. albopictus* (CDC, 2019). Although the viruses initially emerged from sylvatic cycles involving non-human primates and *Aedes* mosquitoes, they are now fully adapted to humans. The female mosquitoes become infected during feeding on an infected person's blood during the acute febrile and viremic phase (4-12 days) of dengue (even if the individual is asymptomatic). Human to mosquito transmission occurs for up to 2 days before the onset of symptoms and 2 days following resolution of fever. Once inside the mosquito, the DENV makes it way to midgut cells of the mosquitoes before being disseminated into various secondary tissues, including the salivary glands during the extrinsic incubation period of 10-12 days when the ambient temperature is 25-28°C. However, this period does vary, for instance, between 5-12 days depending on temperature fluctuations, virus genotype, and initial viral concentration (Guzman et al., 2016, WHO, 2021).

Since the mosquito gets infective for life, it can continue to spread DENVs to susceptible humans throughout its lifespan of approximately 1 weeks in general, or more than 2 weeks in some instances. The intrinsic incubation period from infection via mosquito bites to onset of symptoms ranges from 3-14 days (Figure 2.4) (Guzman et al., 2016). Despite vertical transmission rate being low, DENVs can spread from pregnant mother to fetus causing babies to suffer from pre-term birth, low birthweight, and fetal distress. Furthermore, only one documented case of DENV being spread via breast milk has been reported while the spread of dengue through blood transfusion, organ transplant, needle stick injury are rare occurrences (CDC, 2019).

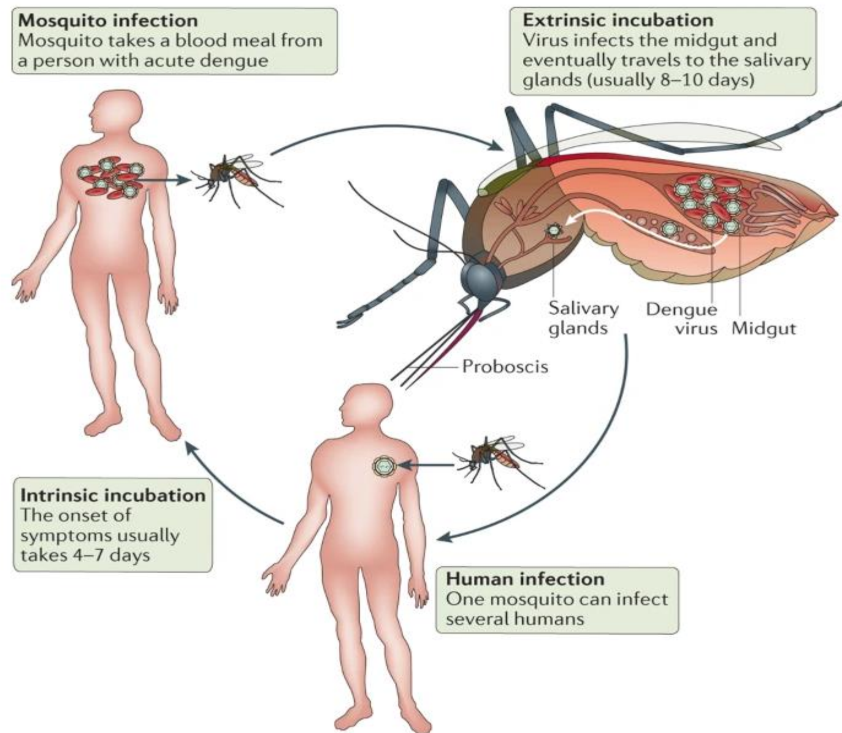


Figure 2.5: Transmission of DENV (Guzman et al., 2016)

DENVs can infect a wide variety of cell types, including epithelial cells, endothelial cells, hepatocytes, muscle cells, dendritic cells, monocytes, bone marrow cells, and mast cells, however, the identity of the cellular receptors involved in viral attachment and entry into cells are unknown (Guzman et al., 2016). Different studies indicated that heparan sulfate, dendritic cell-specific ICAM3-grabbing non-integrin, macrophage mannose receptor 1, heat shock protein 70 (HSP70) and HSP90 could be candidate receptors, but are still not confirmed (Chen et al., 1997, Guzman et al., 2016). Upon attachment of mature DENV particles to the host cell receptor via its E protein, entry occurs through receptor mediated endocytosis. Once inside the cells, alterations in pH allow the E protein to be rearranged, which helps the viral and endosomal membranes to fuse together, thus, enabling the nucleocapsid to be released in the cytoplasm of cells and be disassembled to release the capped viral genomic RNA (Guzman et al., 2016).

The RNA is translated into a long precursor polyprotein that is co- and post-translationally cleaved by NS2b or NS3 viral protease and host proteases into individual viral proteins. The NS proteins get localized to the endoplasmic reticulum (ER)-derived vesicle packets which serve as the site of

replication and get transcribed. The NS1 protein formed is initially associated with the ER due to the addition of high mannose carbohydrate (CHO) moieties while another set of NS1 proteins becomes anchored to glycosyl-phosphatidylinositol (GPI). Both sets of NS1 proteins are then transported to the cell surface via an unknown pathway where it associates with lipids, for instance, cholesterol while some NS1 that was bound directly to cell surface glycosaminoglycans (GAGs) can also be secreted (Guzman et al., 2016).

The precursor M protein (prM) and the E protein become imbedded into the ER membrane and enclose the premature viral particles during its entry into the ER lumen. During its trafficking via the secretory pathway, the low pH of the trans-Golgi networks allows the prM and E protein to be rearranged to enable the prM protein to be cleaved by furin protease to form the mature M protein. The mature viral particles then get released from the cells, while the ones with prM still intact remain non-infective (Figure 2.5) (Guzman et al., 2016).

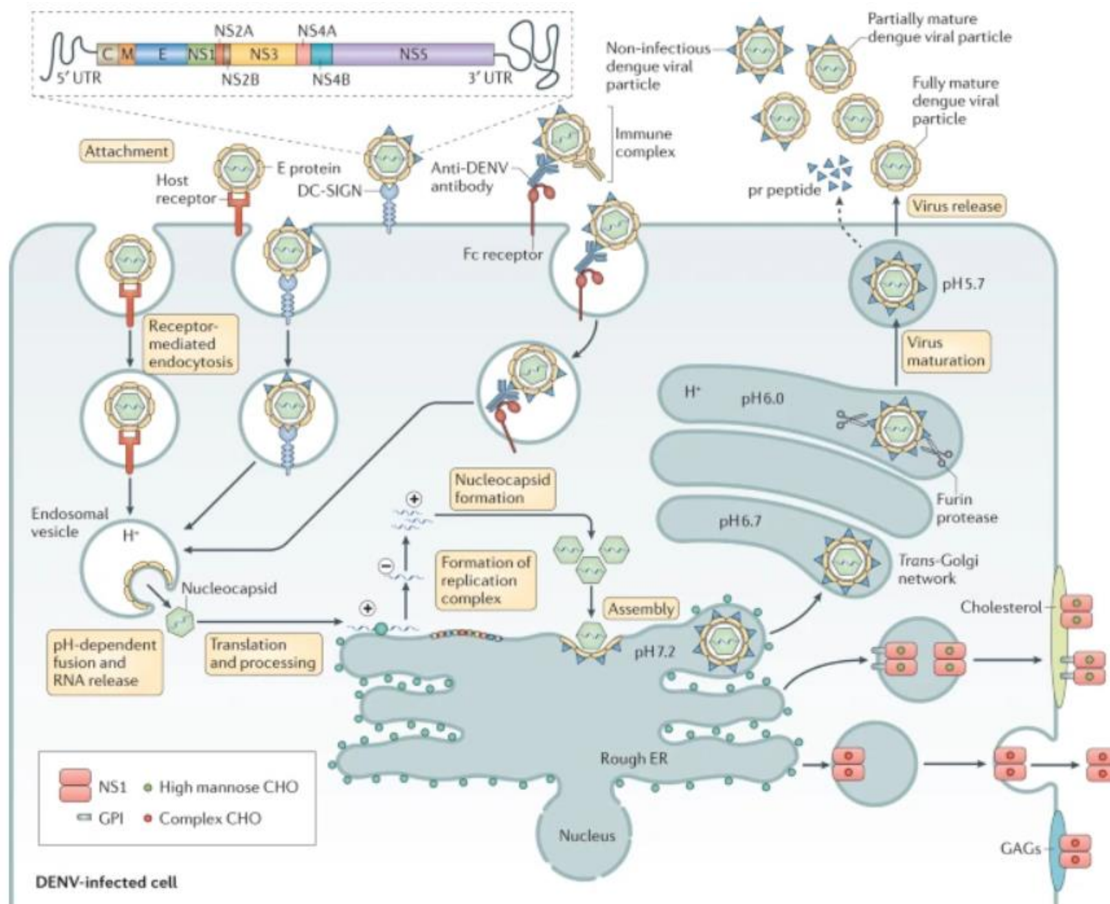


Figure 2.6: DENV life cycle (Guzman et al., 2016)

2.6 Pathophysiology

DENV is introduced into the body during mosquito feeding which results in its inoculation into the dermis, epidermis, and blood. This causes the macrophages, dendritic cells, and Langerhans cells to become infected in the skin and upon migration, spreading DENV to the lymph nodes, subsequently triggering the recruitment of monocytes and macrophages to become infected as well. This results in the dissemination of DENV throughout the lymphatic system due to the large number and variety of cells that can be infected, particularly blood-derived monocytes, myeloid dendritic cells and splenic and liver macrophages, which belong to the mononuclear lineage (Johnston et al., 2000, Marovitch et al., 2001).

Dengue symptoms vary from inapparent to mild to severe and may even lead to a range of physiological abnormalities that can affect multiple systems, such as the liver, blood coagulation, complement, hematopoiesis, and the vascular systems with disease severity usually increasing in cases of heterologous dengue infections due to the phenomenon of ADE (Guzman et al., 2016). DHF and DSS are associated with increased vascular and microvascular permeability brought on by NS1-induced endothelial barrier dysfunction that causes vascular leakage and TLR4 activation resulting in the production of inflammatory cytokines that may increase vascular permeability as well (Beatty et al., 2015, Guzman et al., 2016, Modhiran et al., 2015). Dengue may also lead to the transient suppression of bone marrow mediated by IFN-α production and increased platelet destruction at the peripheries during the febrile and early convalescent stages. These lead to thrombocytopenia where the platelet count may drop to as low as 5,000 per ml (Binder et al., 1997, Guzman et al., 2016). DHF and DSS also impair coagulation system homeopathy that disrupts the regulation of clot formation via increased activated partial thromboplastin time (APTT) and reduced levels of fibrinogen (Guzman et al., 2016). The binding of NS1 to thrombin to form NS1-thrombin complexes and recombinant NS1 (rNS)1-induced prothrombin activation increased APTT, while the release of anticoagulating heparan sulfate or chondroitin sulfate from the glycocalyx by NS1 also adds to the altered homeostasis. Furthermore, increase of procoagulant markers, coupled with reduced levels of anticoagulants may also lead to disseminated intravascular clotting (DIC) (Guzman et al., 2016, Lin et al., 2012). Albeit coagulopathy is usually minor and short in duration for most patients, it could lead to major bleeding caused by erythrocyte extravasation in the gastrointestinal tract, or in case of children with severe shocks, such altered

coagulation homeostasis is worsened by prolonged hypotension and tissue hypoxia (Guzman et al., 2016).

The complement system, which was activated to control DENV infection, contributes to DENV pathogenesis due to the latter's interaction with the coagulation system. Complement activation could be induced in secondary dengue infection via the classical pathway by circulating immune complexes or via an NS1 mediated alternate pathway in primary dengue infections, which is observed especially in children. DHF and DSS are also often correlated with liver enlargement and dysfunction which may be caused by edema brought on by increased vascular permeability or infection of hepatocytes which leads to an inflammatory response (Guzman et al., 2016). Furthermore, changes in liver enzyme levels, for instance, increase in blood aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as, transaminase, and serum bilirubin, alkaline phosphatase and gamma-glutamyl transpeptidases levels, are seen (Guzman et al., 2016, Nguyen et al., 1997). DENV infection of the hepatocytes results in their apoptosis which are then engulfed by Kupffer cells to form Councilman bodies, which is a characteristic feature of dengue and yellow fever (Figure 2.6) (Couvelard et al, 1999, Guzman et al., 2016, Martina et al., 2020).

Although the disease severity may vary due to prior dengue infections and sequence of the infecting DENV serotype, the clinical manifestations and systemic dissemination of DENV are similar for all serotypes.

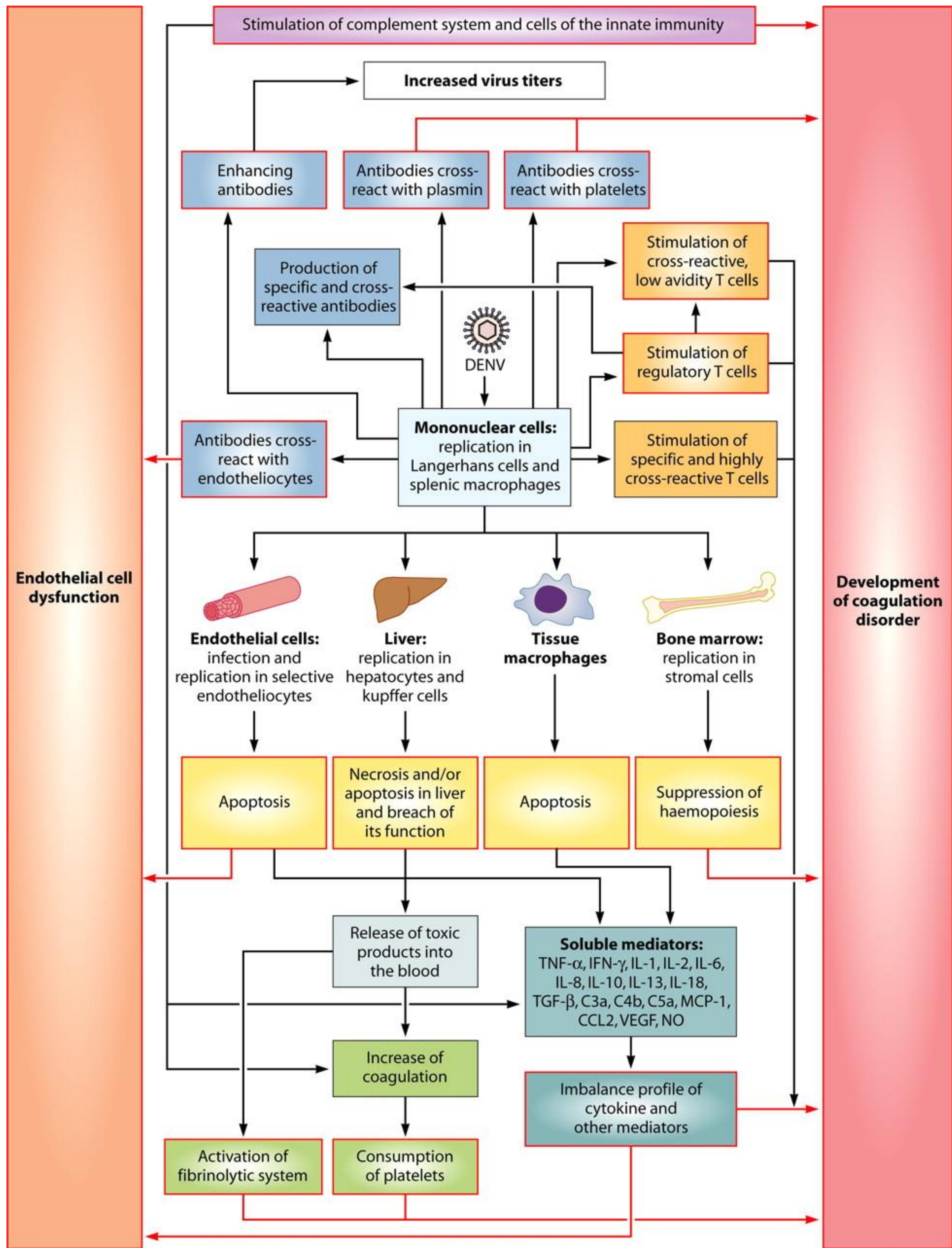


Figure 2.7: Pathophysiology of dengue virus infection (Martina et al., 2020)

2.7 Signs, Symptoms, and Diagnosis

Dengue is a short but dynamic illness where the clinical manifestation can dramatically change or worsen despite the short duration of the disease (usually less than a week in 90% of cases) (Guzman et al., 2016). The incubation period in humans generally last on an average of 4-7 days (Simmons et al., 2012). The common symptoms of dengue include fever, headache, retroorbital pain, myalgia, bone and back pain, arthralgia, nausea, vomiting, and rashes, among others (Figure 2.7) (CDC, 2021). Symptoms usually arise in three phases: the initial febrile phase as observed in most patients who exhibit clinical manifestations, the critical phase, which is characteristic in DHF and DSS, and the convalescence or recovery phase (Guzman et al., 2016, Simmons et al., 2012).

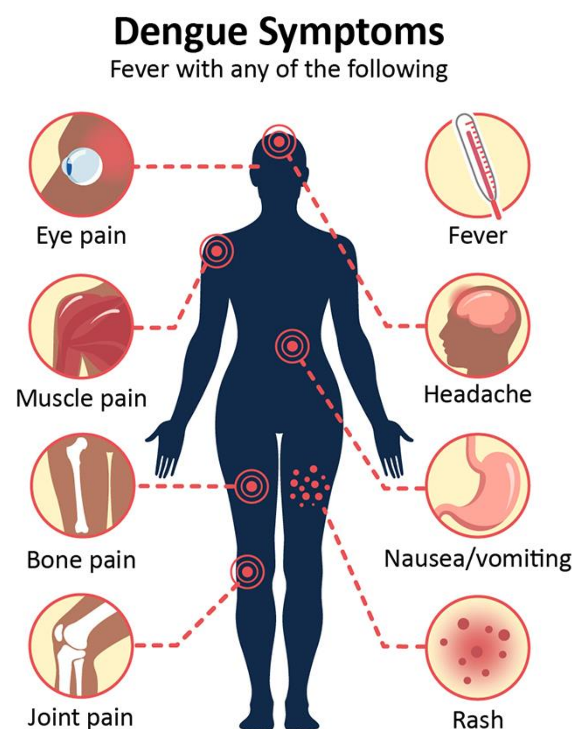


Figure 2.8: Common symptoms of dengue virus infection (CDC, 2021)

The initial febrile phase is generally characterized by fever with a body temperature of ≥38.5°C. Headache, myalgia, vomiting, and muscle, eye and/or joint pain, and temporary rashes are also sometimes observed during this phase (CDC, 2021, Simmons et al., 2012). Hemorrhages, for instance, petechiae and bruising at venipuncture sites and palpable liver are also known to occur infrequently during the febrile phase. Furthermore, mild-to-moderate thrombocytopenia and leukopenia, and elevated hepatic aminotransferase levels sometimes accompany fever as well. Majority of the patients recover within 3-7 days of this period without undergoing a critical stage (Simmons et al., 2012).

However, conditions of some patients may become critical between 4 to 7 days of illness presenting with persistent vomiting, severe abdominal pain, tender hepatomegaly, a high or increasing hematocrit level, thrombocytopenia, serosal effusions, mucosal bleeding, and lethargy or restlessness (Simmons et al., 2012). These are characteristic features of DSS which brought on by dengue NS1 antigen-mediated systemic vascular leakage. The resulting loss of plasma may also lead to elevated hemoconcentration, hypoproteinemia, pleural effusions, ascites, and eventually hypotension (Beatty et al., 2015, Guzman et al., 2016, Modhiran et al., 2015, Simmons et al., 2012). Untreated patients may devolve rapidly, undergoing shocks which may be fatal.

DHF is another critical manifestation of dengue arising from very intense and prolonged shock and characterized by massive internal bleeding or hemorrhages which may be accompanied by profuse plasma leakage. Moderate to severe thrombocytopenia, elevated partial-thromboplastin time and reduced fibrinogen levels are often observed as well. Children have thinner vascular walls and as such, are more likely to suffer from DSS and DHF. Complications arise when patients also exhibit liver failure, myocarditis, and encephalopathy. If fatality is averted with proper supportive care, increased vascular permeability return back to normal levels within 2 to 3 days and the patient enters the convalescent stage where the symptoms subside with exceptions of secondary rashes and fatigue, which are known to occur during the recovery phase but are not medically concerning (Simmons et al., 2012).

Accurate and early diagnosis of dengue is essential in patient management and usually involves detection of the virus, viral RNA, antigens or antibodies, or a combination of these techniques. Following the onset of illness, virus can be detected in the serum, plasma, circulating blood cells, and other tissues for 4-5 days and thus, virus isolation, nucleic acid, or antigen detection can be

employed to diagnose dengue. However, serology is the primary method following post-acute stages of illness (WHO, 2009). Serologic tests usually involve the use of commercial kits for the detection of dengue NS1, IgM, and IgG via enzyme-linked immunosorbent assay (ELISA) and less-sensitive rapid test assays. Furthermore, molecular techniques, including RT-PCR and real-time RT-PCR allow faster, more sensitive, and specific detection, while multiplex real-time RT-PCR helps to determine the DENV serotype. Virus isolation and identification, albeit being highly specific, has a relatively low sensitivity while being more expensive and resource and time-intensive, as such, it is employed less often (Guzman et al., 2016). Different laboratory methods that can be used to diagnose dengue are summarized in Table 2.1 and illustrated in Figure 2.8 (Simmons et al., 2012, WHO, 2009).

Biomarkers for severe dengue may include high level of viraemia and NS1 protein, the level of microparticles that are produced as a consequence of apoptotic cell death and cellular activation, the level of some immune-response mediators, such as IL-1 receptor-like 1 (IL1RL1; also known as ST2), tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), and some biochemical alterations. These, however, has not yet been approved for routine practice. Other common signs of severe dengue are low/decreasing white blood cell and platelet count, high/increasing hematocrit level, fluid accumulation detected by X-ray or ultrasonography, prolonged bleeding time, increased APTT, elevated levels of liver enzymes activated complement with high levels of C3a and C5a, fibrin split products, low levels of fibrinogen, pleural effusions that can be detected by chest X-ray, and gallbladder wall thickening and ascites that can be picked up by abdominal sonograms. Therefore, close monitoring of these parameters, along with indications of shock, should be carefully monitored in dengue patients (Guzman et al., 2016).

Table 2.1: Summary of different dengue diagnostic methods (WHO, 2009)

Diagnostic methods	Diagnosis of acute infection	Time to results	Specimen	Time of collection after onset of symptoms	Facilities	Cost
Viral isolation and serotype identification	Confirmed	1–2 weeks	Whole blood, serum, tissues	1–5 days	Mosquito or cell culture facilities, BSL-2/BSL-3[a] laboratory, fluorescence microscope or molecular biology equipment	$$$
Nucleic acid detection	Confirmed	1 or 2 days	Tissues, whole blood, serum, plasma	1–5 days	BSL-2 laboratory, equipment for molecular biology	$$$
Antigen detection	Not yet determined	1 day	Serum	1–6 days	ELISA facilities	$$
	Confirmed	>1 day	Tissue for immuno-chemistry	NA	Facilities for histology	$$$
IgM ELISA	Probable	1–2 days	Serum, plasma, whole blood	After 5 days	ELISA facilities	$
IgM rapid test		30 minutes			No additional supplies	
IgG (paired sera) by ELISA, HI or neutralization test	Confirmed	7 days or more	Serum, plasma, whole blood	Acute sera, 1–5 days; convalescent after 15 days	ELISA facilities BSL-2 laboratory for neutralization assay	$

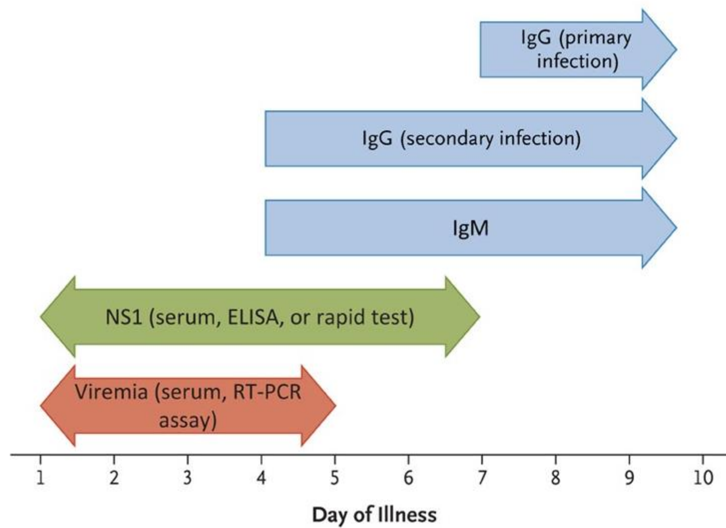


Figure 2.9: Laboratory diagnosis of dengue (Simmons et al., 2012)

2.8 Treatment, Management, and Prevention

At present, there are no effective targeted antiviral agents or globally licensed vaccines against dengue, compelling the reliance on supportive treatments based on symptoms (Simmons et al., 2012). While the clinical spectrum of the disease is wide and ranges from very mild to severe manifestations, timely decisions regarding hospitalizations, and treatment and management at primary and secondary care facilities are critical determinants in patient prognosis. WHO-recommended treatment plan involves making an overall assessment of the patient through patient history and physical examination, followed by a confirmatory laboratory tests, complete blood count (CBC) test, hematocrit test, and other tests elucidating liver function, glucose, serum electrolytes, urea and creatinine, bicarbonate or lactate, cardiac enzymes, ECG, and urine specific gravity to diagnose dengue, determine the severity, and disease phase. This will help make management decisions regarding home, in-hospital, or emergency treatment of the patient, and patient categorization into Group A, B, and C, respectively.

Group A patient who can tolerate adequate volumes of fluid and do not exhibit warning signs of severe disease (for instance, lymphocytopenia, thrombocytopenia, or elevating hematocrit) should be given oral rehydration therapy to make up for fluid loss due to fever and vomiting, paracetamol with 6 hours or more dosing intervals for high fever, and constant monitoring of symptoms urine output, bleeding, and temperature pattern, as well as blood cell count and hematocrit levels to determine the requirement of hospitalization. Group B patients exhibit warning signs, have other co-existing conditions (such as pregnancy, infancy, old age, diabetes mellitus, renal failure, chronic hemolytic diseases), or other extenuating social circumstances (for example, living alone or very far from a healthcare facility) and thus, require in-hospital therapy. Treatment for group B patients involve oral or intravenous fluid therapy with isotonic solutions, like 0.9% saline solution, Ringer's lactate, or Hartmann's solution at different intervals depending on a number of parameters: hematocrit level, vital signs, urine output, peripheral perfusion, blood glucose, and other ones that correlate with organ function. Furthermore, constant monitoring is also critical for group B patients. Patients that require urgent treatment usually exhibit severe symptoms correlated with severe DSS, DHF, or severe organ impairment are categorized as group C patients. The treatment involves judicious intravenous fluid resuscitation using just the adequate volume of crystalloid solution or colloid solution at a hospital with intensive care facilities. Regular

monitoring of the patient to determine the required volume of fluid therapy and/or blood transfusions, freshly-packed red cells, oxygen supplementation in case of severe bleeding are critical. Complications, such as, fluid overload, hyperglycemia, hypoglycemia, electrolyte and acid/base imbalance, Hyponatremia, hypokalemia, hyperkalemia, serum calcium imbalances and metabolic acidosis, co-infections, and nosocomial infections should also be checked and treated appropriately (WHO, 2009).

Vector management and control help reduce or prevent the transmission of DENV. Some of these measures involve preventing access to water-clogged areas that serve as egg-laying habitats for the mosquito vectors, for instance, by using nets, to keep them from laying eggs, or destroying the habitats altogether by emptying and cleaning them. Other methods could involve using different insecticides (for instance, larvicides like temephos or methoprene, or adulticides like cold aerosols or thermal fogs) or other biological control agents (such as the fish, *Poecilia reticulata* or predatory copepod species) that target different developmental stages of the mosquitoes. By the employment of one or a combination of these approaches, transmission of DENV via mosquito vectors could be controlled (WHO, 2009). Furthermore, by wearing long-sleeved clothes (especially during day-time), using window screens, coils, vaporizers, and by actively campaigning to alert the at-risk communities and enlightening them regarding the control and prevention measures, transmission of DENV can be substantially reduced (WHO, 2009, 2012). New approaches that target vectors are also being developed, including the release of genetically modified male mosquitoes that sterilize the wild-type female population or by introducing strains of the obligate intracellular bacterium wolbachia into *A. aegypti* via its embryos (Simmons et al., 2012).

While one vaccine, Dengvaxia® (CYD-TDV), that was developed by Sanofi Pasteur, is licensed in 20 countries, it is yet to be prequalified by WHO. It is a live recombinant tetravalent dengue vaccine that is administered in 3 doses on a 0/6/12 month schedule. Additionally, five other dengue vaccines are currently in clinical development with two (developed by NIH/Butantan and Takeda), are already at phase III trials (WHO, 2012). However, with no licensed vaccines or antiviral agents available in Bangladesh, symptomatic treatment, and vector management and control remain the mainstay treatment, management, and preventative methods in the country (Shirin et al., 2018).

Chapter 3: Materials and Methods

3.1 Patient recruitment

All the patients in this study were recruited from the Department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU) where they gave blood samples during testing for dengue infection following informed written consent (Appendix I).

Inclusion criteria of the study was to collect blood samples from the patients, irrespective of age and sex who had fever for five days of disease onset with the body temperature being a minimum of 100°F (or just below 38°C) during sample collection, thus, meeting the requirements of the WHO's definition of DF and DHF (WHO, 2021).

Exclusion criteria included febrile illness for more than five days with an already determined source of infection, cases with chronic diseases, for instance, tuberculosis, bronchial asthma, congenital heart diseases, and renal failure, or patients with a bleeding tendency that had been persistent from birth.

Thus, following collection of informed written consent from the patients or the patients' guardians (if the patient was <18 years of age), patient's history was collected according to a predesigned questionnaire (Appendix II) detailing the aforementioned criteria.

3.2 Duration of study

From patient recruitment to subsequent testing and analysis, the total duration of the study was 7 months (June 01, 2021 to December 30, 2021).

3.3 Sample size

A total of 524 dengue suspected febrile patients were initially recruited in the study, of whom 150 tested positive for dengue NS1 in their serum in routine laboratory testing at the Department of Virology, BSMMU. Among them, 120 patients consented to participate in the study, from whom, 90 NS1 positive samples with complete patient history, including dengue IgM and IgG status were selected for further study.

3.4 Sample collection, Labeling, and Storage

5 mL of venous blood was collected from consenting patients and was taken in appropriately labeled Ethylene diamine tetraacetic acid (EDTA) tubes via aseptic techniques. Afterwards, each sample's serum was separated, properly labeled, and preserved at -20°C until subsequent testing.

3.5 Report collection

Patient reports from routine dengue NS1 antigen immunochromatography tests carried out at the Department of Virology, BSMMU were collected. Among the NS1 positive patients, the 90 individuals who had full patient history, including reports depicting dengue IgM and IgG status, and consented to participate in the study, were selected for further study.

3.6 Laboratory procedure

In this study, suspected dengue patients were enrolled whose blood was serologically tested for the presence of dengue NS1 antigen. Following RNA extraction with GeneProof PathogenFree RNA Isolation Kit, presence of dengue RNA was then detected in the NS1 positive samples via real-time reverse transcriptase polymerase chain reaction (RT-PCR) using VISASURE Dengue Virus Real Time PCR Detection Kit. Multiplex real-time RT-PCR with FTD Dengue differentiation kit was later carried out with the RNA positive samples for serotyping the DENVs in the samples.

3.6.1 Viral RNA extraction

NS1 positive serum samples were subjected to total RNA extraction procedure using Gene Proof Pathogen Free RNA Isolation Kit (GeneProof, Brno, Czech Republic).

Procedure:

1. 600 µL of Lysis Buffer RAV1, containing carrier RNA, was added to 150 µL of sample and thoroughly mixed before the mixture was incubated at 70°C for 5 minutes.
2. 600 µl of 96-100% ethanol was added to the clear lysis solution and mixed via vortexing for 10-15 seconds.
3. RNA virus column was placed in a collection tube (2 ml) and was loaded with 700 µl of the lysed sample before being centrifuged at 8,000 x g for 1 minute. The residual lysis solution was also loaded onto the column and centrifuged at 8,000 x g for 1 minute. The RNA virus column was then transferred into a new collection tube while the previous one was discarded.
4. 500 µl of Wash Buffer RAW was pipetted into the column and centrifuged for 1 minute at 8,000 x g before the collection tube was discarded and the column transferred into a new one.
5. Afterwards, 600 µl of Wash Buffer RAV3 was pipetted into the column and centrifuged for 1 minute at 8,000 x g before the collection tube was discarded and the column transferred into a new one.
6. 200 µl of Buffer RAV3 was then added to the column and centrifuged for 2-5 minutes at 11,000 x g before the collection tube was discarded and the column transferred into a new one.
7. Centrifugation at 11,000 x g was then carried out for 1 minute.
8. The RNA virus column was then placed into a new microcentrifuge (Eppendorf/1.5 ml) tube where 50 µl of RE (elution) buffer (which was preheated to 70°C) was added and the mixture incubated for 1-2 minutes at room temperature. Centrifugation at 11,000 x g was then carried out for 1 minute.

3.6.2 Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of dengue virus RNA

DENV RNA in extracted RNA samples from the NS1 positive samples was detected via real time RT-PCR using VISASURE Dengue Virus Real Time PCR Detection Kits (CerTest Biotec SL., Zaragoza, Spain). The detection system employed a one-step real time RT-PCR where reverse transcription by reverse transcriptase and subsequent amplification of the 3' non-coding region using specific primers and fluorescent labeled probes occurred in the same reaction well.

Procedure:

1. The reaction mixture was prepared by adding 15 µl of rehydration buffer into each well of the required number of wells of the 8-well strips where each well already contained a stabilized mixture of enzymes (reverse transcriptase and DNA polymerase), primers and probes labeled with FAM and VIC (for sample probes and internal control probes, respectively), buffer, dNTPs, stabilizers, and internal control (IC).
2. Afterwards, 5 µl of RNA samples, dengue virus positive control and negative control was added in respective tubes creating a reaction volume of 20 µl and the wells closed with the provided caps.
3. The plate was then centrifuged and then loaded onto the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Massachusetts, United States) thermal cycler for 45 cycles of amplification whose cycle parameters were set as:

Table 3.1: Real-time RT-PCR cycle parameters

Cycles	Step	Time	Temperature
1	Reverse transcription	15 minutes	45°C
1	Initial denaturation	2 minutes	95°C
45	Denaturation	10 seconds	95°C
	Annealing/Extension (Data collection*)	50 seconds	60°C

4. Fluorogenic data were collected during the extension step through FAM (dengue virus) and VIC (IC) channels with the passive reference option for ROX being set as "none."

Result interpretation for detection of DENV RNA:

According to manufacturer's instructions, a sample with cycle threshold (CT) value less than 40, with or without an amplification signal for IC, was considered positive for dengue virus RNA while a sample was deemed negative for DENV if there was an absence of amplification signal in the detection system, but IC was positive. The run was invalid if both detection system and IC amplification signals were absent or if either the negative control showed positive result or the positive control showed negative result.

3.6.3 Multiplex real-time RT-PCR for detection and differentiation of dengue virus serotypes

The RNA samples that yielded positive results for presence of DENV RNA was used to detect the presence of specific DENV serotypes and distinguish between them via multiplex real-time RT-PCR using FTD Dengue differentiation kit (Fast-track Diagnostics, Sliema, Malta). The FTD kit enabled the performance of an *in vitro* test that allowed for qualitative detection of specific nucleic acids of different DENV serotypes using different primer/probe mix for DENV1, DENV2, DENV3, and DENV4.

Procedure:

1. The reagents, including the primers and probes (DD PP), positive (PC) and negative controls (NC), and the 2X RT-PCR buffer were thawed while the 25X RT-PCR enzyme was placed in the freezer or cooling block during the duration of the experiment.
2. The required amount of 2X RT-PCR buffer was pipetted into a 1.5 ml tube.
3. The required amount of DD PP was then added to the 2X RT-PCR buffer.
4. The PCR master mix was then created by pipetting the required amount of 2X RT-PCR buffer to DD PP and 2X RT-PCR buffer mixture, and 25X RT-PCR enzyme into a 1.5mL tube in sequential order before mixing via a vortex and spinning it down.

Table 3.2: Amount of required reagents

Number of reactions		1	9	32	64
FTD-44-32/64	Buffer	12.5 µl	112.5 µl	400 µl	800 µl
	PPmix	1.5 µl	13.5 µl	48 µl	96 µl
	Enzyme	1 µl	9 µl	32 µl	64 µl
	Total	15 µl	135 µl	480 µl	960 µl

5. 15µL of the master mix was later added into their respective wells of a 96-well plate, followed by addition of 10µL of extracted RNA samples, positive control, or negative control and mixed briefly. The plate was subsequently sealed using ABI optical adhesive film, mixed with a vortex, and briefly centrifuged.
6. The plate was loaded onto the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Massachusetts, United States) thermal cycler for 40 cycles of amplification whose cycle parameters was set as:

Table 3.3: Multiplex real-time RT-PCR cycle parameters

Cycles	Step	Time	Temperature
1	Reverse transcription	15 minutes	50°C
1	Initial denaturation	1 minute	94°C
45	Denaturation	8 seconds	94°C
	Annealing/Extension (Data collection*)	1 minute	60°C

7. Fluorogenic data were collected during the extension step with the passive reference option for ROX was set as “none.”

Result interpretation for determination of DENV serotype:

For result interpretation of FTD dengue detection, according to manufacturer's instructions, the detection programming was set such that probes that were labeled with dyes that emitted fluorescent signals that was detected in the FAM (520nm), JOE (550nm), CY5 (670nm), or ROX (610nm) channel upon amplification of the DENV nucleic acid was interpreted as detection of DENV1, DENV2, DENV3, and DENV4, respectively. For a run to be valid and result interpreted as positive, the negative control needed to be below the threshold, the positive control needed to produce a positive amplification trace with Ct value below 33.

3.7 Statistical analysis

The quantitative data was categorized into different groups according to necessity following standard classifications and analyzed accordingly. Statistical analysis, sorting, and editing were conducted using the IBM SPSS software, version 28.0.0.0 (SPSS Inc., Chicago, IL, USA).

3.8 Workflow:

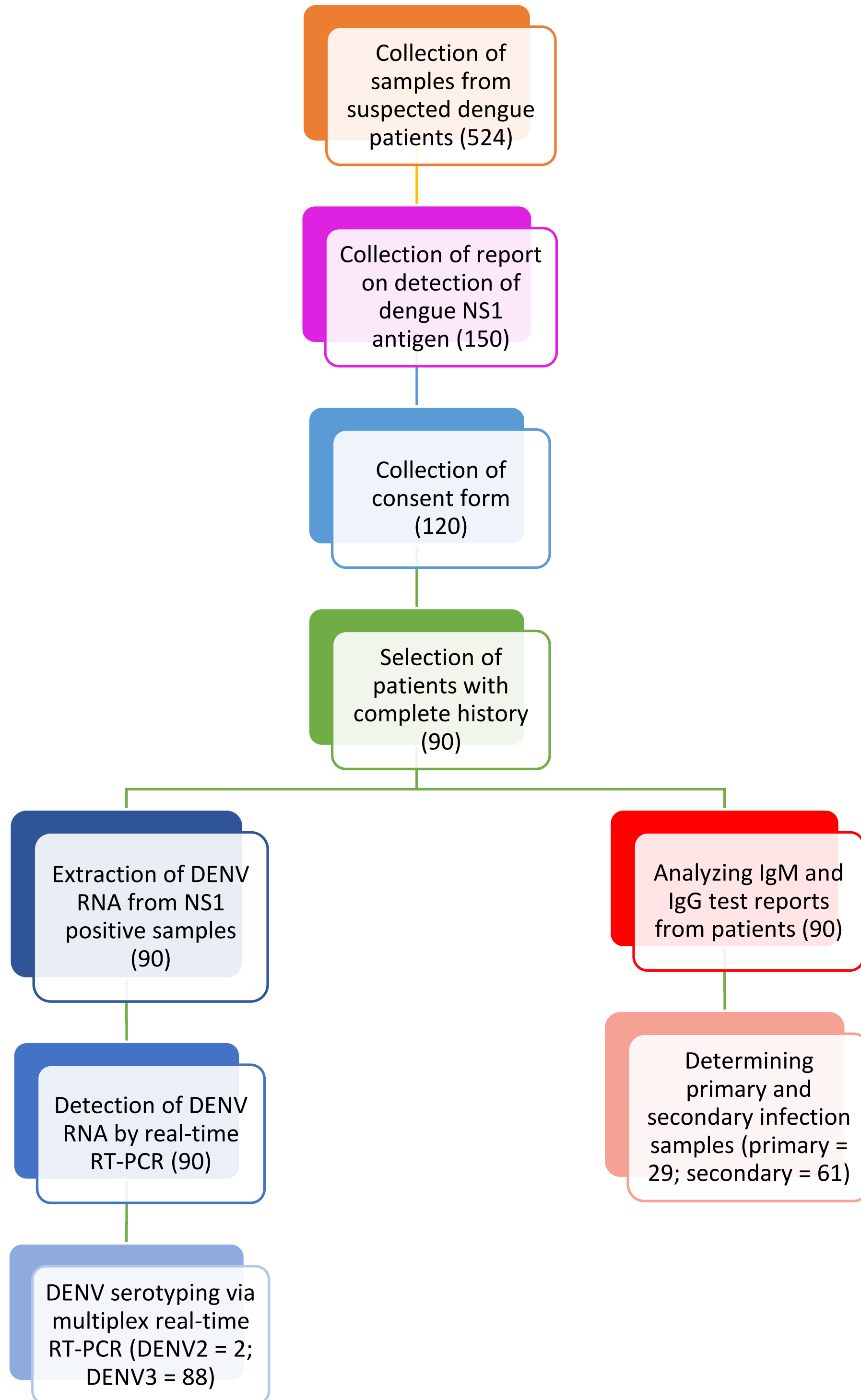


Figure 3.1: Workflow of the study

Chapter 4: Results

4.1 Sociodemographic features of the study population

Table 4.1 summarizes the sociodemographic features of the study population. The mean age of the patients was 32.78 ± 15.32 years (range: 03-76 years), with 35 among the 90 patients (38.9%) belonging to the 21-30 years age group, thus, constituting the maximum number of patients among the age groups. The other age groups, 0-10, 11-20, 31-40, 41-50, 51-60, and >60, consisted of 6 (6.7%), 11 (12.2%), 13 (14.4%), 12 (13.3%), 8 (8.9%), and 5 (5.6%) patients, respectively. This has been illustrated in Figure 4.1, graphically. Among the study population, 33 (36.7%) were female and the remaining 57 (63.3%) were male. According to religious beliefs, Muslims made up majority of the population with 77 (85.6%) patients, while 12 (13.3%) were of the Hindu faith and the remaining 1 patient (1.1%) was a Buddhist. 61 (67.8%) individuals were married and the remaining 29 (32.2%) were unmarried. Additionally, 79 (87.8%) of the participants belonged to the middle socioeconomic status while 6 (6.7%) and 5 (5.6%) responded to have belonged to the lower and upper socioeconomic class, respectively. 43 (47.8) of the respondents received higher education with 22 (24.4%), 12 (13.3%), 5 (5.6%), 5 (5.6%), and 3 (3.3%) receiving HSC, SSC, secondary, primary, and no education, accordingly. While 73 (81.8%) answered that they had adequate nutrition and 13 (14.4%) having high nutritional status, 4 (4.4%) patients had poor nutritional intake.

Table 4.1 Sociodemographic features of the study population

Sociodemographic features		Frequency	Percentage
Age	0-10	6	6.7%
	11-20	11	12.2%
	21-30	35	38.9%
	31-40	13	14.4%
	41-50	12	13.3%
	51-60	8	8.9%
	>60	5	5.6%
Sex	Female	33	36.7%
	Male	57	63.3%
Religion	Muslim	77	85.6%
	Hindu	12	13.3%
	Buddhist	1	1.1%
Marital status	Married	61	67.8%
	Unmarried	29	32.2%
Socioeconomic status	Lower	6	6.7%
	Middle	79	87.8%
	Upper	5	5.6%
Education	No education	3	3.3%
	Primary	5	5.6%
	Secondary	5	5.6%
	SSC	12	13.3%
	HSC	22	24.4%
	Higher	43	47.8%
Nutritional status	Poor	4	4.4%
	Average	73	81.1%
	High	13	14.4%

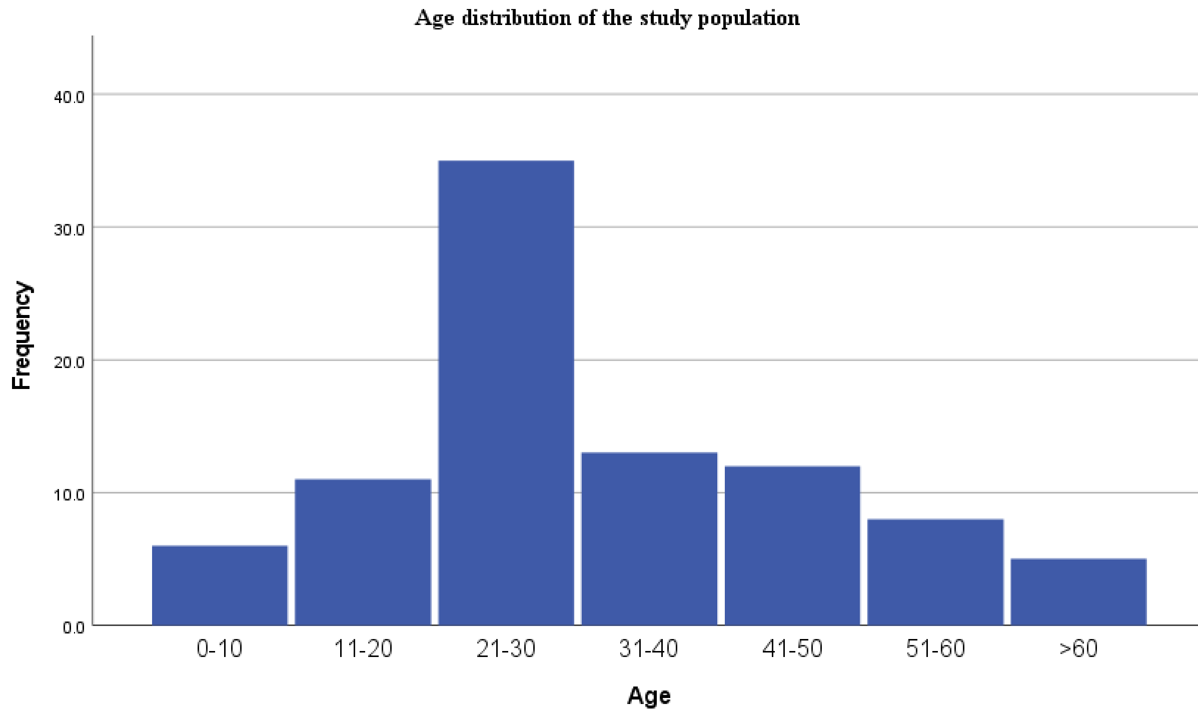


Figure 4.1: Age distribution of the study population

4.2 Clinical and laboratory profiles of the study population

As shown in Table 4.2 and Figure 4.2, all 90 patients (100%) in the study population had fever. The mean days of fever among the patients was 3.11 ± 0.409 days. Additionally, among the common symptoms of dengue, 79 (87.8%), 43 (47.8%), 47 (52.2%), 31 (34.4%), 18 (20.0%), 28 (31.1%), 35 (38.9%), 38 (42.2%), and 11 (12.2%) of the patients suffered from headache, retroorbital pain, myalgia, arthralgia, back pain, anorexia, nausea, vomiting, and abdominal pain.

Table 4.2: Clinical profile of the study population

Symptoms	Number of patients	Percentage (%)
Fever	90	100
Headache	79	87.8
Retroorbital pain	43	47.8
Myalgia	47	52.2
Arthralgia	31	34.4
Back pain	18	20.0
Anorexia	28	31.1
Nausea	35	38.9
Vomiting	38	42.2
Abdominal pain	11	12.2

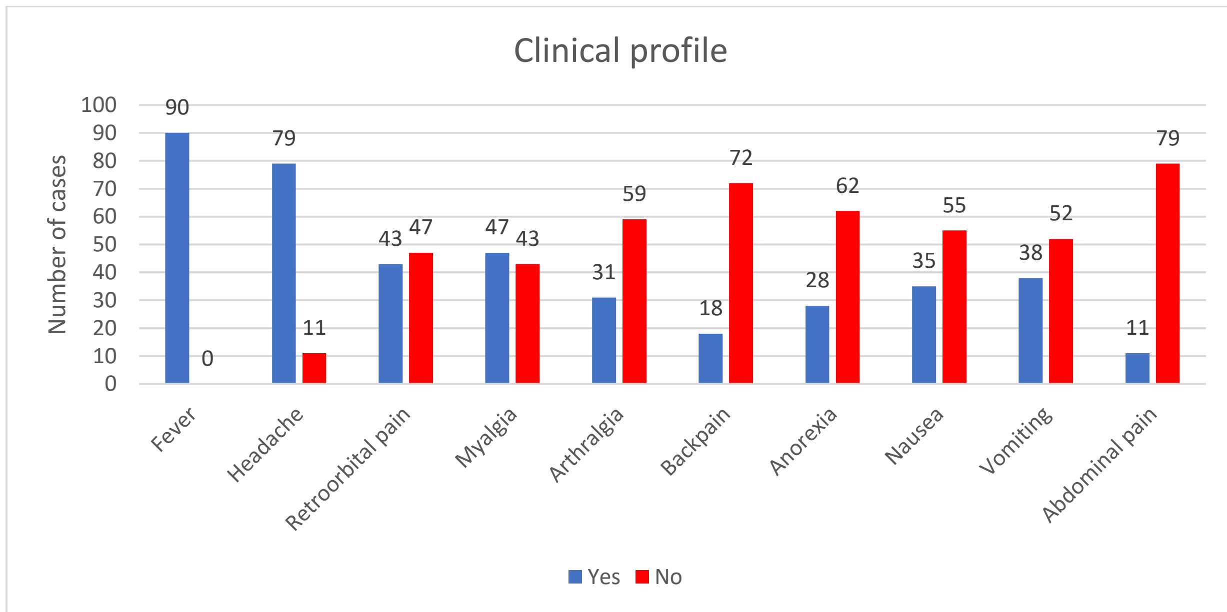


Figure 4.2: Clinical profile of the study population

According to Table 4.3, the mean haemoglobin level of the patients was 12.88 ± 1.25 g/dl while the average platelet count was $188.10 \times 10^9 \pm 43.35 \times 10^9$/L, and ALT, leukocyte count, lymphocyte level, neutrophil level, and hematocrit (HCT) level were 40.43 ± 4.05 U/L, $4.22 \times 10^9 \pm 1.81 \times 10^9$/L, 29.92 ± 12.77%, 63.38 ± 13.34%, and 38.79 ± 5.01%, respectively.

Table 4.3: Laboratory profile of the study population

	Minimum	Maximum	Mean
Haemoglobin (g/dl)	10.20	15.90	12.88 ± 1.25
Platelet (/L)	120×10^9	285×10^9	$188.10 \times 10^9 \pm 43.35 \times 10^9$
ALT (U/L)	34.00	50.00	40.43 ± 4.05
Leukocyte (/L)	1.20×10^9	9.00×10^9	$4.22 \times 10^9 \pm 1.81 \times 10^9$
Lymphocyte (%)	12.60	56.00	29.92 ± 12.77
Neutrophil (%)	32.00	83.00	63.38 ± 13.34
HCT (%)	10.00	46.00	38.79 ± 5.01

4.3 NS1 positive patients among the study population

Among 524 dengue-suspected febrile patients, 150 (28.62%) tested positive for dengue NS1 antigen (Figure 4.3) and among the 120 NS1 positive patients who consented to participate in the study, 90 patients with complete history, including dengue IgM and IgG status were selected for further testing.

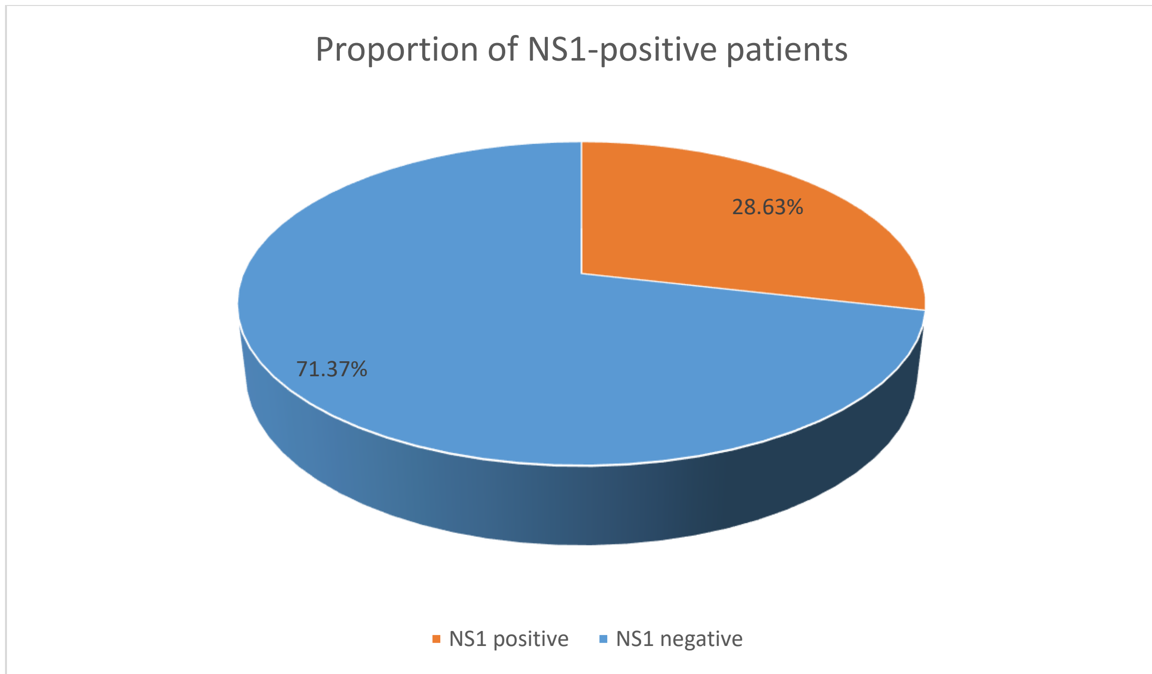


Figure 4.3: Proportion of NS1 positive patients among the dengue suspected patients

4.4 Detection and Serotyping of DENV among NS1-positive samples

Real-time RT-PCR detected the presence of DENV RNA in all 90 NS1-positive samples (100%). Following multiplex real-time RT-PCR, it was revealed that 88 (97.8%) samples were positive for DENV3, while 2 (2.2%) were DENV2 positive (Figure 4.4). DENV1 and DENV4 were not detected in any of the patients in the study population. Only DENV3 was detected in all 60 samples from June 2021 to September 2021 while in October-November 2021, 28 samples (93.3%) were DENV3 positive, and 2 (6.7%) samples tested positive for DENV2 (Figure 4.5). The PCR amplification plots of DENV2 and DENV3 have been provided in Appendix III and IV, respectively.

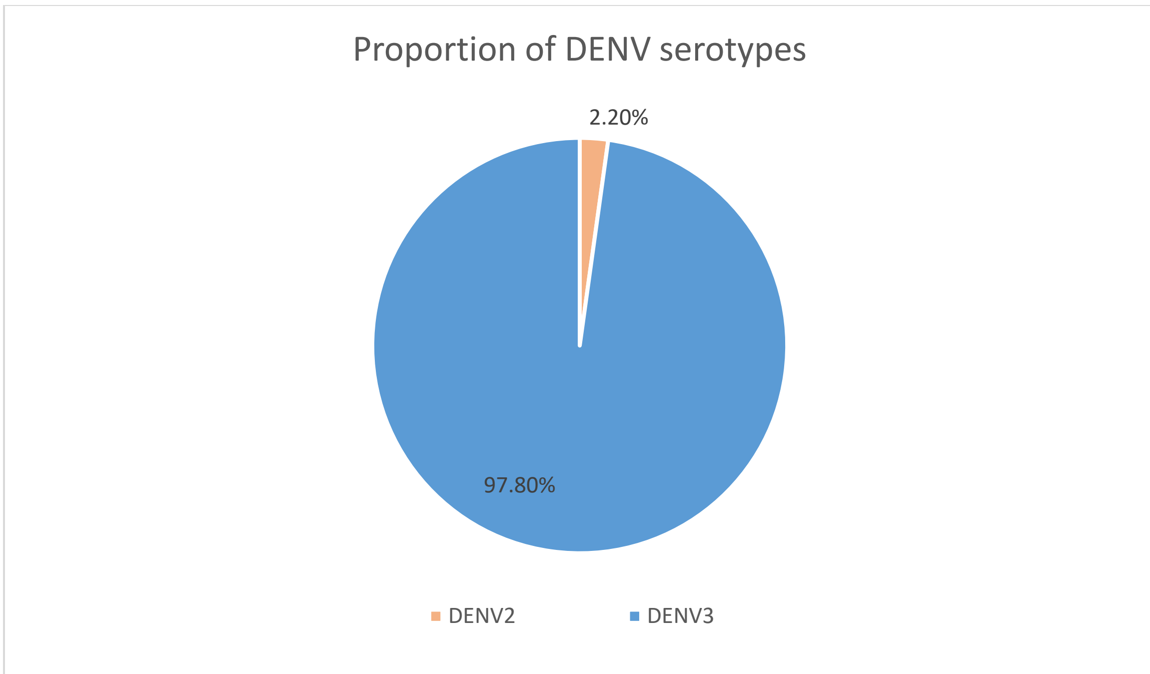


Figure 4.4: Proportion of DENV serotypes among dengue patients from June to November, 2021

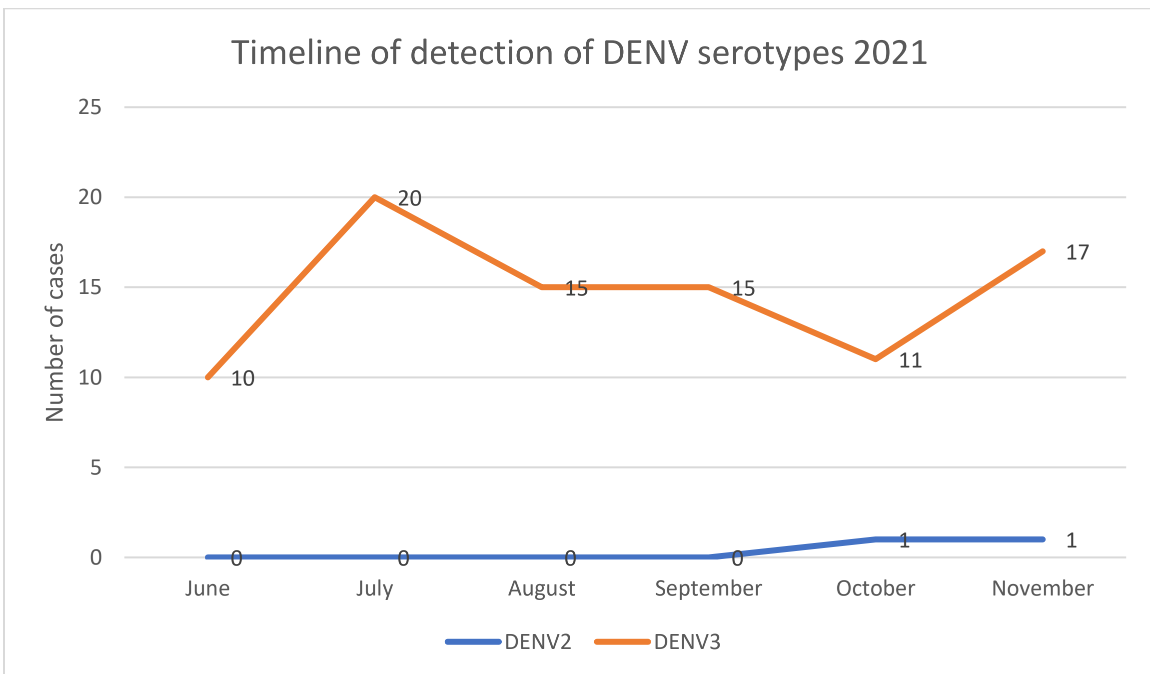


Figure 4.5: Monthly serotype findings from June to November, 2021

4.5 Type of infection and its prevalence in DENV serotypes, and patient age and sex

As illustrated in Figure 4.6, it was revealed that 61 (67.80%) of the dengue cases were secondary infections while the remaining 29 (32.20%) cases were primary DENV infections. According to Table 4.4, both cases of DENV2 were secondary infections while 59 (67.05%) of the DENV3 cases were secondary dengue as well and the rest of the DENV3 cases [29 (32.95%)] were primary infections. The result, however, was not statistically significant (Chi-square, *P*>0.05).

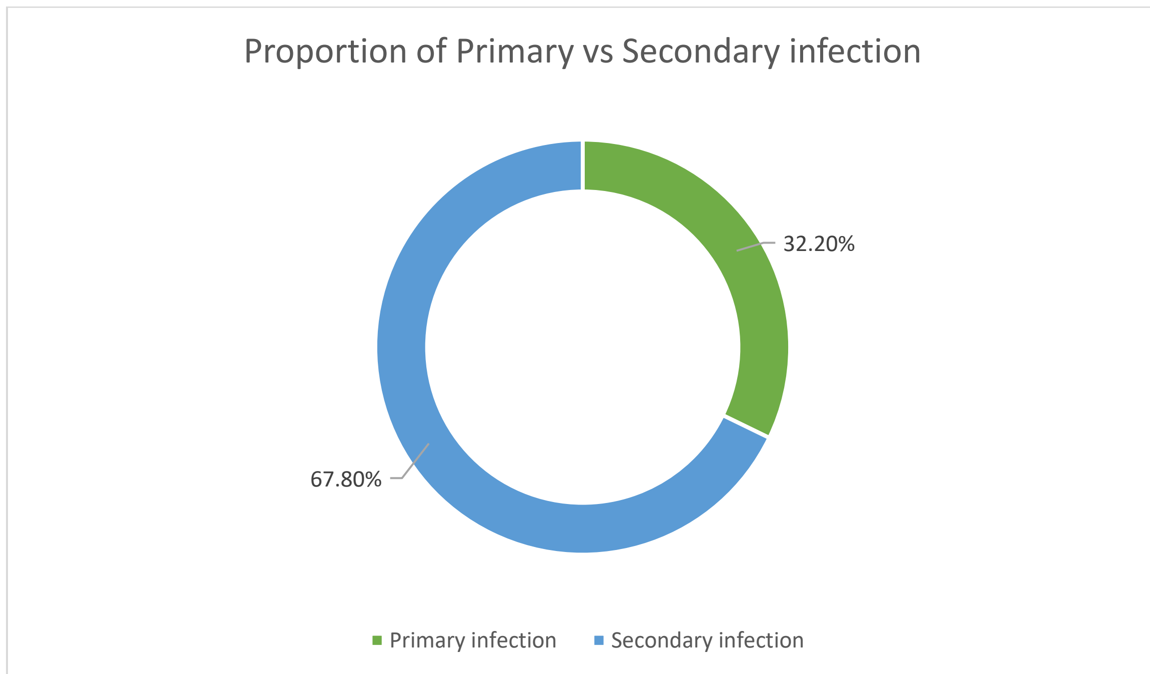


Figure 4.6: Proportion of primary vs secondary Dengue infections among the Dengue RNA positive patients in the year 2021

Table 4.4: Distribution of DENV serotypes among dengue infection in the year 2021

		Infection type		
		Primary	Secondary	Total
Serotype	DENV2	0	2	2
	DENV3	29	59	88
Total		29	61	90

Crosstabulation of infection type against age (Table 4.5) showed that 80% of study patients aged 60 or above suffered from secondary infections. Albeit occurring in lower proportions, and in line with the proportion of the secondary infections among all the patient population, secondary infection was the predominant infection type across all age groups. The percentage of secondary infections among the 0-10, 11-20, 21-30, 31-40, 41-50, and 51-60 age groups were 66.67%, 81.82%, 71.43%, 61.54%, 50.00%, and 62.50%. The relationship between type of infection and age groups, however, was not statistically significant (Chi-square, $P>0.05$).

Table 4.5: Age wise type distribution of dengue infection in the year 2021

		Infection type		
		Primary	Secondary	Total
Age groups	0-10	2	4	6
	11-20	2	9	11
	21-30	10	25	35
	31-40	5	8	13
	41-50	6	6	12
	51-60	3	5	8
	>60	1	4	5
Total		29	61	90

Table 4.6 shows that among the 33 females, 13 (39.39%) suffered from primary DENV infections while 20 (60.61%) experienced secondary infections. In case of the 57 males of the study population, primary and secondary dengue infections were observed in 16 (28.07%) and 41 (71.93%) patients, respectively. The result was not significant (Chi-square, $P<0.05$).

Table 4.6: Gender wise type distribution of dengue infection in the year 2021

		Infection type		
		Primary	Secondary	Total
Sex	Female	13	20	33
	Male	16	41	57
Total		29	61	90

4.6: Prevalence of DENV serotypes in different age groups and genders

Crosstabulation of DENV serotypes against different age groups (Table 4.7) showed that both DENV2 cases involved patient in the 21-30 years age group. DENV3 infected all age groups, primarily the 21-30 years group with 33 cases while affecting 6, 11, 13, 12, 8, and 5 patients among the patients in the 0-10, 11-20, 31-40, 41-50, and >60 years age group, respectively. However, no significant association was found between DENV serotypes and age groups (Chi-square, *P*>0.05). Furthermore, Table 4.8 depicted that among the 33 females, 1 suffered from DENV2 infection and the remaining 32 females were infected with DENV3. In case of the 57 males of the study population, DENV2 and DENV3 infections were observed in 1 and 56 patients, respectively. The results were not significant (Chi-square, *P*<0.05).

Table 4.7: Age wise distribution of DENV serotypes in the year 2021

		Serotype		
		DENV2	DENV3	Total
Age groups	0-10	0	6	6
	11-20	0	11	11
	21-30	2	33	35
	31-40	0	13	13
	41-50	0	12	12
	51-60	0	8	8
	>60	0	5	5
Total		2	88	90

Table 4.8: Gender wise distribution of DENV serotypes in the year 2021

		Serotype		
		DENV2	DENV3	Total
Sex	Female	1	32	33
	Male	1	56	57
Total		2	88	90

Chapter 5: Discussion

The identification of circulating DENV serotypes is of paramount importance with regards to nationwide preparedness in order to limit and manage potential dengue outbreaks with possible mass casualties caused by the reemergence of specific DENV serotypes after years of absence from circulation. This study, therefore, aimed to determine the prevalence of circulating DENV serotypes in Dhaka, Bangladesh in 2021. In this study, suspected dengue patients were recruited, and the reports of their dengue NS1 antigen status were collected and analyzed. After obtaining informed written consent and patient history from willing participants, 90 dengue NS1 positive patients with complete patient history, including IgM and IgG test report results, were selected for the detection of the presence of DENV RNA using real-time RT-PCR before subsequently serotyping the DENVs via multiplex real-time RT-PCR.

The findings revealed that 28.9% of febrile patients were dengue NS1 positive and presence of DENV RNA was detected in all the patients' serum samples. Males are likely to be more infected by DENV as observed in this study as 63.3% of the population were male while 36.7% were female. Similar findings were observed by Tahmina et al. (2019) where 28.02% of their study population was NS1 positive and among them, 58.1% were male and 41.9% were female. This could be attributed to male citizens venturing outdoors more often than females due to work, prayers in mosque, lunching out, among other activities. Furthermore, it is the young adult age group (21-30 years) that are also likely to be more at risk of dengue since they accounted for majority (38.9%) of the study population. Young adults are generally known to remain outdoors during the day time because of education, work, social gatherings, and so on which could explain them being exposed to mosquito bites more than the other age groups and contracting dengue. If the more active working population was considered between the ages of 21 and 40, over half of the study population (53.3%) belonged to these age groups (21-30 and 31-40). All the patients suffered from fever and majority of them experienced headache while about half of the patients endured myalgia and retroorbital pain. On the other hand, only 11 patients experienced abdominal pain. Since patient history was collected within 2-4 days of symptom onset, the laboratory findings did not reflect any parameter of noteworthy interest.

Real-time RT-PCR detected the presence of DENV RNA in all 90 NS1 positive samples (100%). Multiplex real-time RT-PCR for serotyping revealed that 88 (97.8%) samples were positive for DENV3, while 2 (2.2%) were DENV2 positive. DENV1 and DENV4 were not detected in any of the patients in the study population with the former being absent from circulation for the first time in the last decade (IEDCR, 2021, Tahmina et al., 2019). The dwindling proportion of DENV1 and DENV2 and absence of DENV4 are further substantiated by the findings of IEDCR whose study showed that DENV4 has not been in circulation for years while, DENV1's prevalence has decreased from 50% in 2015 to 3% in 2019 and 0% in 2021. Similarly, DENV2 has decreased in prevalence from 76% in 2016 to 1% in 2019. Furthermore, both DENV2 cases were detected in October and November and prior to this study and as of September 22, 2021, IEDCR had not reported any cases of DENV2 infections in 2021 (IEDCR, 2021). This could be accounted to DENV1 and DENV2 being in constant circulation since the last two decades and as such, leading to the development of immunity by majority of the population against them while DENV3 had been absent from circulation since 2002 and since its reemergence in 2017, its prevalence has been increasing and has been the predominant serotype in the last three years as its absence from circulation prevented immunity from being developed by the Bangladeshi people (Shirin et al., 2019). The two DENV2 cases were observed in the 21-30 years age group while the remaining 33 patients in that age group were DENV3 cases. The two DENV2 infected both male and female and in accordance with the proportions of the two sexes in the study, 63.6% of the DENV3 cases involved males and the remaining 36.4% affected females. Therefore, there is not much variance in the infectivity of the two serotypes in terms of age and sex.

Patient history showed that majority (67.8%) of the patients had secondary dengue infections. Marginally different results were obtained by a study conducted by Titir (2021) et al. in 2019 where IgG was found in 60.5% of the samples. The slight variation could be attributed to the passage of time resulting in more dengue infections, especially during the 2019 outbreak in Bangladesh. Since DENV2 had been in circulation for the last few decades, it explains both DENV2 infections in this study being secondary infections. Furthermore, 67.0% of DENV3 cases were secondary infections while the remaining 33.0% were primary infections. Since its reemergence in 2017, DENV3 has been increasing in prevalence, including being the predominant serotype the last three years and as such, a high proportion of DENV3 secondary infections were obtained in this study. Similarly, majority of the age groups (other than the 41-50 years where

primary and secondary infections were equal in number) included patients who suffered from secondary dengue, especially in respondents aged 60 and above and between the ages of 11-20, which included 80.0% and 81.2% secondary dengue cases, respectively. Accordingly, similar proportions of secondary dengue were obtained for both sexes with females having 60.6% secondary infections and the number being slightly higher at 71.9% for males. Therefore, it can be inferred that dengue secondary infections do not vary much with regards to age and sex.

This study, thus, identifies DENV3 as the predominant DENV serotype in the 2021 dengue outbreak in Dhaka, Bangladesh with DENV2 being in co-circulation. Further extensive studies to confirm the absence of DENV1 from circulation in Bangladesh must be conducted.

Chapter 6: Conclusion and Recommendations

This study concludes that DENV2 and DENV3 are co-circulating in the 2021 dengue outbreak in Dhaka, Bangladesh with DENV3 being the predominant serotype. It further confirms the absence of DENV1 and DENV4 with the former being absent from circulation for the first time in the last decade and therefore, more studies should be carried out to confirm its absence from circulation. If DENV1 remains absent from circulation for several years, vigilance need to be maintained with regards to circulating DENV serotypes in Bangladesh and its neighboring countries to prevent unexpected reemergence of DENV1 or DENV4 as such an unforeseen circumstance without nationwide preparedness could lead to a massive outbreak and mass casualties as observed with the 2019 dengue outbreak caused by the reemergence of DENV3 in Bangladesh. Disease severity will be of more concern given that majority of the cases identified in this study were secondary infections, and in case of secondary dengue caused by a heterologous DENV serotype, severe symptoms or fatalities may also result. Therefore, studies to ascertain the circulating DENV serotypes must be carried out annually in Bangladesh.

Limitations of the study

Financial and time constraints led to limitations in sample size. Additionally, follow-ups regarding laboratory profiles to elucidate disease severity or other correlated features were not performed.

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Appendix I

Consent form:

Title of the study: "Detection of circulating dengue virus serotypes of 2021 dengue outbreak in Dhaka, Bangladesh."

Purpose of the study:

Dengue is a viral disease and is one of the leading causes of mortality and morbidity in Bangladesh. The country is usually hit with dengue outbreaks during the monsoon seasons every annually and since reemergence of a dengue virus serotype (Dengue virus has 4 serotypes) that has been absent from circulation for years may result in devastating symptoms and even death, especially, in cases of secondary dengue infections caused by a different serotype, identifying the circulating dengue virus serotype is crucial. Clinical features of dengue fever vary from fever, severe headache, eye and body pain, and rash to internal and external bleeding, shock, even death. This will help with nationwide preparedness to help in dengue management. In this study, 3 ml serum from 5 ml NS1 antigen positive blood will be preserved for further study.

Procedure, risks and hazards:

With all aseptic precaution blood sample will be collected from you by experienced phlebotomist. We assure you that the whole procedure is completely health hazardless.

Potential benefits:

During the study period you don't have to bear any expenses, neither will you be provided with money.

Confidentiality:

The hard and soft copy of all documents will be kept secret in a locker which will be accessible only by the chief investigator. You will be given an identification number thus you can claim your documents further.

Self-participation:

The decision of taking part in the study will be totally yours. You have every right to withdraw yourself from study participation at any time. There will be no violation of your rights after signing the consent form.

Questions:

If you have any question then you can ask, we will try to answer. During the study period you can contact the investigator for any further questions.

Giving consent:

I am satisfied about participation and purpose of the study after consulting with the researcher. I know that the decision will be only mine to take part in the study or to withdraw myself from the study. I have read the above facts/ all are read in front of me. I totally agree to take part in the particular study.

……………………………………..............................

Signature of the interviewer with Date

……………………………………..............................

Signature/Thumb print of the participant with Date

…………..………………………………………………….

Signature/Thumb print of witness with Date

সম্মতিপত্র:

গবেষণার শিরোনামঃ "Detection of circulating dengue virus serotypes of 2021 dengue outbreak in Dhaka, Bangladesh."

গবেষণার উদ্দেশ্যঃ

ডেঙ্গু রোগ একটি ভাইরাসজনিত জ্বর, যা বর্তমানে আমাদের দেশে মারাত্মক আকার ধারণ করেছে। যেকোনো বয়সের মানুষ এ জ্বরে আক্রান্ত হতে পারে। ডেঙ্গু রোগ হলে জ্বর, প্রচণ্ড মাথাব্যথা, চোখব্যথা, শরীরব্যথা, র‍্যাশ ছাড়াও রোগটি মারাত্মক আকার ধারণ করলে শরীরের ভিতরে ও বাইরে বিভিন্ন স্থানে রক্তক্ষরণসহ রোগীর মৃত্যু পর্যন্ত হতে পারে। ডেঙ্গু ভাইরাসের ৪ ধরনের সেরোটাইপ ও প্রতিটি সেরোটাইপ এর কয়েক ধরনের জেনোটাইপ রয়েছে। জেনোটাইপ সনাক্তকরণ এর মাধ্যমে রোগটির তীব্রতা, বিস্তৃতি ও প্রতিরোধ এর কার্যকর উপায় সম্পর্কে ধারণা করা সম্ভব। এ লক্ষ্যে, বঙ্গবন্ধু শেখ মুজিব মেডিকেল বিশ্ববিদ্যালয় এর ভাইরলজি বিভাগ একটি গবেষণার উদ্যোগ নিয়েছে। যা ভাইরাস এর বর্তমান সেরোটাইপ এ সহায়ক হবে। এই গবেষণার জন্য ৫ মি.লি. রক্ত (NS1 এন্টিজেন পজিটিভ) হতে ৩ মি.লি. সেরাম গবেষণার কাজে ব্যবহারের জন্য সংরক্ষন করা হবে।

গবেষণা ঝুকি

গবেষণার জন্য প্রয়োজনীয় রক্তের নমুনা সম্পূর্ণ জীবাণুমুক্তভাবে অভিজ্ঞ লোক দ্বারা সংগ্রহ করা হবে। এ গবেষণার সম্পৃক্ততার জন্য আপনার কোন ক্ষতি হবে না।

গবেষনায় অংশগ্রহণের সুবিধাদিঃ

এই গবেষণায় অংশগ্রহনের জন্যআপনার কোন টাকা খরচ হবে নাএবং আপনাকে কোন টাকা দেয়া হবে না।

গোপনীয়তাঃ

এই গবেষণা চলাকালীন আপনার এবং আপনার যাবতীয় তথ্য যত্নের সাথে গোপন রাখা হবে। আপনার আইডি নম্বর সম্বলিত সব ধরনের কাগজপত্র আপনার নাম ও ঠিকানাসহ গোপনীয়তার সাথে সংরক্ষণ করা হবে।

স্বেচ্ছামূলক অংশগ্রহণঃ

এই গবেষণায় আপনার অংশগ্রহণ সম্পূর্ণ ঐচ্ছিক। আপনি গবেষণায় অংশগ্রহণে অস্বীকৃতি জানাতে পারেন অথবা গবেষণা চলাকালীন যে কোন সময় গবেষণা থেকে নিজেকে প্রত্যাহার করে নিতে পারবেন। এই ফর্মে স্বাক্ষর করলে আপনার আইনগত কোন অধিকার খর্ব হবে না।

প্রশ্নাবলীঃ

যদি আপনার কোন প্রশ্ন থাকে তবে দয়া করে জিজ্ঞাসা করুন, আমরা তার উত্তর প্রদান করতে যথাসাধ্য চেষ্টা করবো। যদি ভবিষ্যতে আপনার অতিরিক্ত কোন প্রশ্ন থাকে তাহলে গবেষণারত চিকিৎসক, ভাইরোলোজী বিভাগ, বঙ্গবন্ধু শেখ মুজিব মেডিকেল বিশ্ববিদ্যালয়ে যোগাযোগ করতে পারবেন।

সম্মতির স্বীকারোক্তিঃ

"আমি উক্ত গবেষণায় নিয়োজিত চিকিৎসকের সাথে এই গবেষণা নিয়ে আলোচনায় সন্তুষ্টি প্রকাশ করছি। আমি বুঝেছি যে, এই গবেষণায় আমার অংশগ্রহণ সম্পূর্ণভাবে ঐচ্ছিক এবং আমি যে কোন সময় কোন বাধ্যবাধকতা ছাড়াই এই গবেষণা কার্যক্রম থেকে নিজেকে বিরত রাখতে বা প্রত্যাহার করে নিতে পারব। আমি উপরোক্ত শর্তগুলো পড়েছি/ আমার সামনে পঠিত হয়েছে এবং আমি স্বেচ্ছায় এই গবেষণায় অংশগ্রহণ করতে সম্মতি জানাচ্ছি"।

……………………………………………………………………

সাক্ষাতকার গ্রহণকারীর স্বাক্ষর ও তারিখ

……………………………………………………………………

অংশগ্রহণকারীর স্বাক্ষর/টিপসহি ও তারিখ

……………………………………………………………………

স্বাক্ষীর স্বাক্ষর ও তারিখ

Appendix II

Questionnaire

I.D No:…………… Date:……………………

1. Name: Contact No:
2. Age:
3. Present Address:
4. Residential area: i) Urban ii) Rural
5. Socioeconomic status: i) Upper class ii) Middle class iii) Lower class
6. Housing: i) Pacca house ii) kaccha house iii) Apartment
7. Religion: i) Muslim ii) Hindu iii) Christian iv) Buddhist
8. Occupational status of patient:
9. Educational status: i) No education ii) Primary iii) Secondary v) SSC vi) HSC vii) Above
10. Nutritional status: i) High ii) Average iii) Poor
11. Marital status: i) Married ii) Unmarried iii) Widow iv) Separated
12. Use of mosquito net: i) Yes ii) No
13. Hygienic status around the house: i) Present ii) Absent
14. Onset of symptoms:
15. Clinical features:
    - Fever with duration:
    - Headache
    - Retroorbital pain
    - Myalgia
    - Arthralgia
    - Back pain
    - Anorexia
    - Nausea
    - Vomiting
    - Abdominal pain
16. Any past history of dengue infections:

17. Laboratory findings:
    - Leukocytes
    - Neutrophil
    - Lymphocytes
    - HB%
    - HCT
    - Platelet
    - ALT
18. Dengue NS1:
19. Dengue IgG:
20. Dengue IgM:
21. Dengue serotype:

Signature:

Appendix III

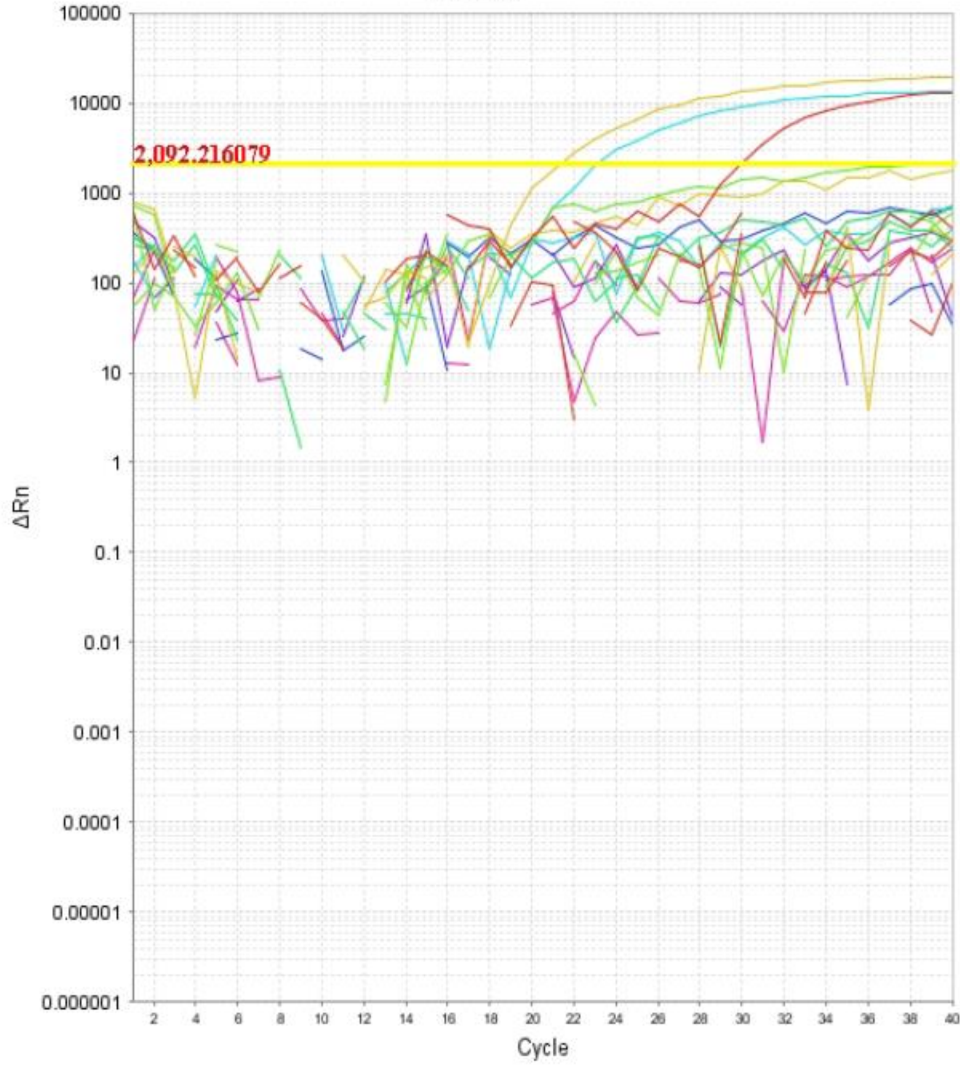
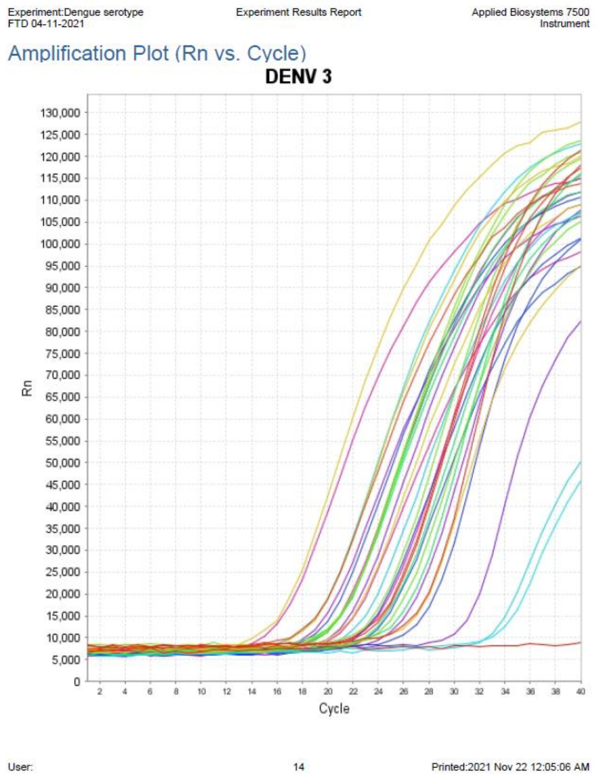


Figure A.III: DENV2 Amplification plot (logarithmic)

Appendix IV



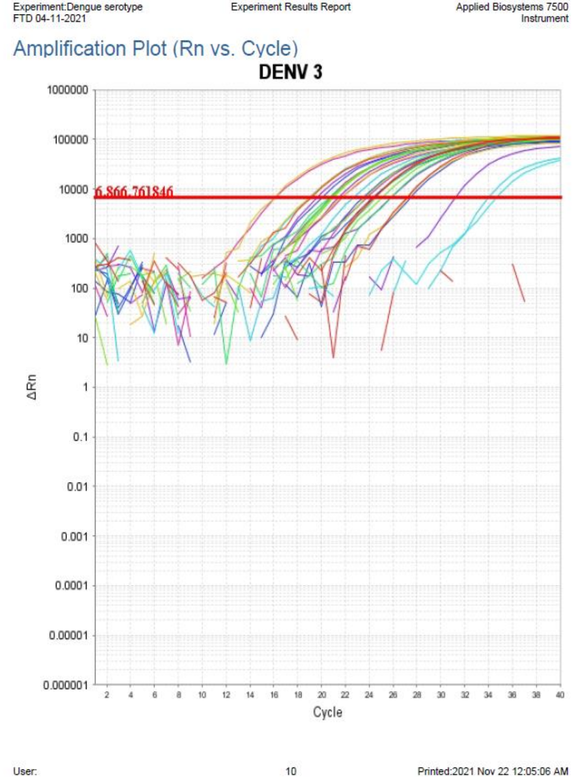


Figure A.IV: DENV3 Amplification plot (logarithmic plot: right; linear plot: left)