Original Article

Diversity of Dengue Virus Serotypes in Dhaka City: From 2017 to 2021

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Abstract
This is a retrospective observational study aimed to search the alteration of circulating dengue virus (DENV) serotypes in Dhaka City for five consecutive years (2017-2021) along with disease outcome.

Methods: Routine dengue NS1 or PCR positive dengue samples from patients who visited Evercare Hospital Dhaka (ex. Apollo Hospitals Dhaka) were selected for serotype determination by serotype specific real time reverse transcriptase PCR (RT-PCR).

Results: In 2017, predominant serotype was DENV-2 (91.3%) with less than 5% of DENV-1 and DENV-3 among 161 cases. In 2018, among127 cases, DENV-2 was the predominant serotype (40.95%) followed by DENV-3 (33.07%) and DENV-1 (25.98%). In 2019, predominance of DENV-2 was totally replaced by DENV-3 (91.86%) and DENV-1 (8.14%). In 2021, only DENV-3 serotype was detected among 178 samples. Regarding serotype association with disease outcome, more severe cases (Dengue Hemorrhagic Fever/Dengue Shock Syndrome) were observed from 2019 with notable shifting of serotype dominance to DENV-3 from DENV-2 in previous years. In our cohort, the prevailing age group was 1-20 years which is analogous with many studies in Asia.

Conclusion: Dominance of DENV serotype shifted to DENV-3 in 2019 from prolonged persistence of DENV-2 and DENV-1. Continuous surveillance for circulating DENV serotype is needed for preparedness of potential outbreaks and occurrence of severe cases.

Keywords: Dengue, DENV serotype, DHF, Diversity, DENV-3.

Introduction

Dengue is a mosquito borne systemic acute viral disease, a major public health concern in urban areas of tropical and sub-tropical countries. The disease is endemic in more than 100 countries and about half of the global population is at risk of infection with this arbovirus. According to World Health Organization (WHO) estimation, about 390 million of cases are detected each year,of which average 96 million manifests clinically[1].

Dengue virus (DENV) belongs to the genus *Flavivirus*, of family *Flaviviridae,* with a single-stranded positive-sense RNA of approximately 11kb long. The DENV genomic RNA has a single open reading frame (ORF) that encodes ten proteins, consisting of three structural proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5)[2]. DENV comprises four distinct serotypes (DENV1-4) and each serotype demonstrates strain variation based on the evolution and molecular epidemiology and further classified into genetically distinct groups called genotypes[3]. Within each serotype of DENV, four to six geographically distinct genotypes have been reported. DENV-1 includes genotypes I, II, III (sylvatic), IV, V, and VI; DENV-2 includes Asian-I, Asian-II, Asian/American, American, Cosmopolitan, and sylvatic; DENV-3 includes genotypes I, II, III, IV, and V; DENV-4 includes genotypes I, II A, II B, III, and sylvatic[4-10].

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In Bangladesh, DENV-3 was isolated for the first time from a patient in 1964[11] and was found to be the main circulating serotype during the 2000 to 2002 outbreaks[12]. Thereafter, until 2012 serotype data was not available. During the years 2013-2016, DENV-1 and DENV-2 were the predominant circulating serotypes in Bangladesh[13].

Rapid and accurate serotyping of DENV is important for epidemiologic surveillance, preparedness and control of dengue outbreaks and transmission blocking strategies targeting the vector, as well as for development of vaccines and antivirals.

As predominant serotype of DENV changed over time in most of the countries, including Bangladesh, it is crucial to track and identify the circulating serotype of dengue virus at the beginning of every season for prediction of disease propagation and severity in coming season, so that it may contribute to early preparedness plan regarding dengue management. We have done a retrospective study to find out the alteration of circulating serotypes during 2017 to 2021. Here we report that the dominance of DENV-2 in 2017 and 2018 has shifted to DENV-3 in 2019 and till October, 2021, the only representing serotype was DENV-3 (100%) with no evidence of other serotypes.

Materials And Methods
Ethical approval
Dengue serotyping study proposal was approved by the Research and Ethical Practice Committee of Evercare Hospital Dhaka (ex. Apollo Hospitals Dhaka, approval number ERC 16/2018-3). De-identified stored RNA at -80˚C was used with a different code for this research study. This study was exempt from obtaining participant's consent since only leftover specimens were used after anonymization.

Patients and clinical specimens
Patients with clinical suspicion of dengue who visited Evercare Hospital Dhaka (ex. Apollo Hospitals Dhaka) from June 2017 to August 2021 were included in this study irrespective of their age group. Subsequently for serotype determination, patients with dengue PCR Ct value >34 cycles were excluded from this study. For routine assay, 3 ml whole blood sample from adult and 0.5 ml to 1 ml from pediatric patients having clinical suspicion of dengue were collected in plain vacutainer (red top). Serum was separated and stocked at -80˚C until RNA was extracted.

RNA extraction and real time reverse transcriptase PCR
Viral RNA was extracted from 200 µl of serum following kit manufacturer's protocol (QIAampMinElute Virus Spin Kit, Qiagen, Germany for samples of 2017 to 2019 and MagMax Viral/Pathogen kit, Applied Biosystems, USA was used for samples of 2020 to 2021) and stored at -80˚C.

For samples of 2017 to 2019, CE-IVD approved commercial one step reverse transcriptase real time PCR kit from FTD (Fast Track Diagnostics, Luxembourg) was used for the detection of dengue virus. 15 µl PCR master mix containing 12.5 µl buffer, 1.5 µl primer-probe mix and 1µl enzyme was prepared for each sample, negative control and positive control and then 10 µl of the extracted RNA from samples, nuclease free water as negative and synthetic DNA as positive control was added, respectively in 0.1ml PCR tube. PCR amplification was done by Rotor Gene Q (Qiagen, Germany). According to kit manufacturer's instruction thermocycler was programmed which was as follows: 50˚C for 15 min, 94˚C for 1 min, 40 cycles of 94˚C for 8 s, 60˚C for 1min. Signal was acquired at 60˚C, and analysis was performed on the linear scale. Thresholds were set manually on each run. For target, any exponential curve crossing this threshold was considered positive. Fluorescence detected in green channel was for the amplification of dengue virus and red channel was for the internal control.

For samples of 2020 to 2021, CE-IVD approved commercial one step reverse transcriptase real time PCR kit from TRUPCR, India was used for the detection of dengue virus. 15µl PCR master mix containing 10 µl multiplex master mix, 2.65 µl dengue primer-probe mix, 2 µl internal control primer probe mix and 0.35 µl enzyme mix was prepared for each sample, negative and positive control and then 10 ul of the extracted RNA from samples, negative and positive control was added, respectively, in 0.2 ml PCR strip tube. QuantStudio 5 Dx platform (Applied Biosystems, USA) was used for PCR amplification according to kit manufacturer's instruction which was programmed as follows: 50˚C for 20 min, 94˚C for 10 min, 45 cycles of 94˚C for 15 s, 55˚C for 30 s and 72˚C for 30 s. Signal was acquired at 55˚C, and analysis was performed on the linear scale. Thresholds were set manually on each run. Fluorescence detected in FAM channel was for amplification of Dengue virus and ROX channel was for amplification of internal control.

Detection of Dengue NS1 antigen

Kits from SD Bioline, Korea were used for detection of NS1-antigen of Dengue virus. Three drops (100 μl) of serum sample were added to the well "S" and result reading was done within 15-20 minutes. Result was given after comparison with the positive control line in the device. The presence of two-color line ("C" and "T") in the result window indicates that the specimen is positive for dengue NS1 antigen, and the presence of only control line ("C") indicates negative.

Serotype specific real-time reverse transcriptase PCR

For dengue serotype identification we used commercial Genesig one step reverse transcriptase real time PCR kit from Primer design, UK. Four Dengue subtype specific primer and probe mixes are provided in a single tube and detected through the four different channels as described in the kit contents. The primer and probe mixes provided exploit the TaqMan® principle. Briefly, 5 ul RNA was taken in 0.2 ml PCR tube and then added to 10 ul Oasig master mix, 1 ul dengue primer probe mix and 4 ul nuclease free water. Reverse transcription was done in Rotor Gene Q and QuantStudio 5 Dx platform at 55°C for 10 minutes followed by enzyme activation at 95°C for 2 minutes and finally 50 cycles of denaturation at 95°C for 10 seconds and annealing and extension together at 60°C for 60 seconds. Then different dengue serotypes were detected in different channels according to the kit manufacturer's instruction.

Results And Discussion

Serotype diversity and shifting of dominance

Dengue outbreaks occurring in many countries of the world, including Bangladesh, is considered a major public health concern. As Bangladesh is surrounded by dengue-endemic neighbors like India and Myanmar, there is always a risk of virus importation and transmission from neighboring countries. Over the past several decades, the rising trend of dengue incidence is due to increased travel, climate change, population growth, urbanization, and poor implementation of effective control measures. Frequent changes of serotypes were observed in almost every year, so, monitoring of DENV serotypes in early season of every year is important for proper management of mass population. In this study, we have detected the prevailing dengue serotype of five consecutive years (2017-2021) in Dhaka city to track the diversity of circulating serotypes [Table-1].

Table 1: Serotype distribution in five consecutive years (2017-2021)

Years	Serotype done	Serotype positive	DENV1 N (%)	DENV2 N (%)	DENV3 N (%)	DENV4 N (%)
2017	181	161	7 (4.35)	147 (91.30)	7 (4.35)	0
2018	167	127	33 (25.98)	52 (40.95)	42 (33.07)	0
2019	116	86	07 (8.14)	0	79 (91.86)	0
2020	30	1	0	0	1 (100)	0
2021	221	178	0	0	178 (100)	0

Samples for serotype determination were selected on routine NS1 or PCR positivity from suspected patients visiting our hospital. Routine PCR positive samples with Ct value ≤30 was preferred, but up to 34 Ct was considered for serotyping PCR as serotyping PCR often showed negative result when routine PCR Ct value >34.

In 2017, total 181 dengue positive cases were selected for serotyping and among them serotyping of 161 were successful. Serotyping PCR of 20 samples were unsuccessful probably due to low viral amount. Out of 161 we found 7 (4.35%) DENV-1,147 (91.3%) DENV-2, and 7 (4.35%) DENV-3.

The predominance of DENV-2 was also found in Dhaka city during the year 2013-2016 [13]. Dominance of dengue serotype in Dhaka city has been shifted to DENV-2 from DENV-3 found in first dengue epidemic in 2000-2002[14-16]. Dominance of DENV-2 serotype was reported in Pakistan in 2017[17]. However, in India DENV-1 was predominant in 2017[18].

In 2018, we could serotype 127 samples and out of those we found 33 (25.98%) DENV-1, 52 (40.95%) DENV-2, and 42 (33.07%) DENV-3. This result shows that in 2018, percentage of DENV-2 decreased but was still dominant over other serotypes and DENV-1 and DENV-3 increased significantly. A similar study in 2018 was reported from IEDCR of Bangladesh where DENV-2, DENV-3 and DENV-1 was found 41%, 31% and 9%, respectively[19].

In 2019, 86 samples were serotype positive from 116 selected samples. The result shows that predominance of DENV-2 is totally replaced by DENV-3 (91.86%) and

other serotypes were only few. A study by Riad et al conducted on Bangladeshi population showed that DENV-3 was the dominating serotype in 2019, which originated an extreme and unprecedented surge in the number of infections. About 100,000 cases were reported in 2019 which is more than double the number of combined cases in the previous 19 years[20,21]. Huge number of cases were documented in 2019, which may be due to mass awareness program by Government regarding disease severity caused by reemergence of DENV-3 after a prolong presence of DENV-2 (2003-2018). Other possibilities of this outbreak may be, the expanding transmission capability of the emerging serotype from humans to mosquitoes, and vice versa, vector competence, and other environmental factors. A study in Myanmar showed over the entire 3-year period (2017-2019), the most prevalent serotype in Myanmar was DENV-3 (46.2%) followed by DENV-1 (30.1%) & DENV-4 (19.8%)[22].

In 2020, we received very limited number of dengue samples probably due to COVID-19 pandemic situation and only a single case of DENV-3 was found among 24 dengue NS1 positive cases and 6 routine PCR positive samples where Ct values were >32. This large number serotype negativity (29 among 30) may be the cause of low viral load. Similar situation was found in Guangzhou, China in 2020 where they reported few dengue cases (only two local cases) attributable to the effect of COVID-19 pandemic[23]. A discordant picture was seen in Singapore where dengue fever surge was observed with predominance of DENV-3 in 2020. They narrated the cause of this surge was due to focus on COVID-19 pandemic and lack of preventive measures to confine the spread of new and predominant DENV-3 serotype against which the population was not immune[24].

In 2021, we did serotype of 178 dengue positive samples and surprisingly all are found to be DENV-3 serotype (100%).

Bangladesh Council of Scientific and Industrial Research (BCSIR) also found only DENV-3 serotype in Dhaka city in 2021, although, with only small (20) number of samples[25].

Gender distribution and age prevalence
The present study is showing male predominance, with 60.04% male and 39.96% female. Studies in Singapore, Myanmar and India showed similar male predomi-

nance[22,26,27]. However, this difference may not indicate more susceptibility of male to dengue infection, rather it is associated with the socio-economic status of the region studied. Females in these areas are less privileged and may not get equal opportunity to have treatment for fever. On the contrary, males, often being the only earning person to maintain family cost, get more attention from other members of the family to seek medical care. Reports from South America showed either equal proportions of male and female dengue cases or a greater proportion of female cases[28,29]. In three consecutive years (2017, 2018 & 2019), 1-10 years age group was the dominating age group whereas in 2021, it was 11-20 [Table-2]. Thus, dengue infection occurred mainly in children and adolescence where maximum positive cases (40.51%) were among 1-20 years age group. Children are particularly vulnerable to the disease because their immune systems are weaker than adults and they tend to play outside where there is less protection against the mosquitos. Schools are a hotbed of dengue because many have open windows and lack mosquito repellents. Children and adolescents under 15 years of age are the most affected group in Asia, the hyperendemic area for dengue fever (DF) and dengue hemorrhagic fever (DHF)/ dengue shock syndrome (DSS), which corresponds with our findings[30]. The age distribution is different in America where these syndromes occur in all age groups, although most fatalities during epidemics occur in children[31].

Table 2: Age and gender distribution of study population

Age (Years)	2017 (n=161)		2018 (n=127)		2019 (n=86)		2020 (n=01)		2021 (n=178)	
	Male (%)	Female (%)	Male (%)	Female (%)	Male (%)	Female (%)	Male (%)	Female (%)	Male (%)	Female (%)
<1	2 (1.2)	1 (0.6)	10 (7.9)	5 (3.9)	2 (2.3)	2 (2.3)	0	0	1 (0.6)	2 (1.1)
1 - 10	19 (11.8)	14 (8.7)	18 (14.2)	12 (9.4)	18 (20.9)	15 (17.4)	0	0	12 (6.7)	10 (5.6)
11-20	15 (9.3)	5 (3.1)	13 (10.2)	6 (4.7)	11 (12.8)	11 (12.8)	0	0	33 (18.5)	12 (6.7)
21-30	12 (7.5)	15 (9.3)	6 (4.7)	7 (5.5)	3 (3.5)	4 (4.6)	0	0	24 (13.5)	8 (4.5)
31-40	22 (13.7)	10 (6.2)	12 (9.4)	8 (6.3)	4 (4.6)	3 (3.5)	0	0	24 (13.5)	16 (9)
41-50	13 (8.1)	10 (6.2)	10 (7.9)	7 (5.5)	3 (3.5)	1 (1.2)	1 (100)	0	11 (6.2)	7 (3.9)
51-60	5 (3.1)	6 (3.7)	6 (4.7)	2 (1.6)	6 (7)	2 (2.3)	0	0	4 (2.2)	5 (2.8)
61-70	4 (2.5)	4 (2.5)	2 (1.6)	1 (0.8)	0	1 (1.2)	0	0	1 (0.6)	3 (1.7)
>70	3 (1.9)	1 (0.6)	1 (0.8)	1 (0.8)	0	0	0	0	1 (0.6)	4 (2.2)
Total	95	66	78	49	47	39	1	0	111	67

Disease severity and serotype
We compared diseases severity (classical/severe dengue) in admitted patients in our hospital each year during the span of 2017 to 2021 and clinically more severe cases (34.41%) were found in 2019 followed by 31.19%, 26.32%, 18.55%, 11.22% in 2021, 2020, 2018, 2017

respectively. Dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS), dengue with severe thrombocytopenia were considered as severe dengue. Most affected age group was 1-10 years with male predominance [Table-4]. We also correlated disease severity with different serotypes and a greater number of severe cases (40.7%) were observed in 2019 compared to 13.7%, 8.7%, 5% in 2021, 2018, 2017 respectively. We ignored the single case of 2020 [Table-3]. Although clinically diagnosed cases were huge in 2019 but serotype was done with 116 cases, out of which 86 were serotype positive. Among these 86, clinically severe cases were 35 and non-severe cases were 51. All 35 severe cases were DENV-3 and among non-severe 51 cases, 44 (86.3%) were DENV-3 and 7 cases (13.7%) were DENV-1. In 2019, most of the non-severe cases were routinely tested by NS1 only. As it was a retrospective study and huge number of samples were requested for routine dengue PCR, we didn't include NS1 positive samples for serotyping. For this reason, higher percentage of DENV-3 and severity data may be influenced by lower number of serotyped non-severe cases. From 2017 the reemergence of DENV-3 was noted and seen to replace the dominance of DENV-2 and in 2019, DENV-3 was the flourishing serotype without any evidence of DENV-2. As during 2013-2016 Bangladesh population was exposed by DENV-1 and DENV-2 serotype mainly, reemergence of DENV-3 in 2017 and onwards, caused serotype specific cross reactivity that may be related with disease severity and fatality. Moreover, DENV-3 has so far been reported to be the most pathogenic serotype; DENV-1 and DENV-2 have been noticed to be nearly similar in their pathogenic traits, whereas DENV-4 is probably the least pathogenic serotype.[32]

Table 3: Severity correlation with different serotypes (2017-2021)

Year	Classical dengue fever				Severe dengue fever				Severity percentage**
	DENV1 (%)	DENV2 (%)	DENV3 (%)	Total	DENV1 (%)	DENV2 (%)	DENV3 (%)	Total	
2017(n=161)	6 (3.9)	143(93.5)	4(2.6)	153	0	7(87.5)	1(12.5)	8	5%
2018 (n=127)	32(27.6)	46 (39.7)	38 (32.7)	116	1 (9.1)	6 (54.5)	4 (36.4)	11	8.7%
2019 (n=86)	7 (13.7)	0	44 (86.3)	51	0	0	35(100)	35	40.7%
2020 (n=1)	0	0	0	0	0	0	1(100)	1	100%
2021(n=178)*	0	0	82(100)	82	0	0	13(100)	13	13.7%

Conclusion
Identification of circulating DENV serotype at the beginning of every season is important as changing serotype is allied with disease intensity and fatality. We observed serotype shift of DENV-3 from prolonged persistence of DENV-2 and DENV-1 which resulted in more severe outcome of infection particularly in children and adolescents in recent years. As there is no specific treatment for dengue, continuous surveillance and early preparedness plan regarding dengue management is required to prevent and control dengue related mortality and morbidity in each year. It is a single centered study in Dhaka and there is a lack of diversity of population. A large-scale multi-centric study of serotype prevalence along with circulating genotype determination is recommended for further evaluation.

Author Contributions
All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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