Comparison of Molecular Surveillance Data (2010-2023) of Dengue Virus Infection in *Aedes* Mosqutioes in Dhaka City

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Abstract: In 2010-2012 a prospected field study was conducted applying a rapid and sensitive, nested, reverse transcription polymerase chain reaction (RT-PCR) assay using oligonucleotide consensus primers for the type-specific detection of dengue viruses in field caught mosquitoes for the first time in Bangladesh. In laboratory experiments, the assay was sensitive enough to detect dengue virus from lab infected mosquitoes. Adult *Ae. aegypti* mosquitoes were caught from selected 5 dengue sensitive areas in Dhaka city and assayed by RT-PCR. Approximately 4.8% of 188 mosquito pools were positive for dengue viruses. Of the 9 RT-PCR positive *Ae.aegypti* pools (containing 9-12 mosquitoes each) 6 pools were (66.66%) positive for Den3 viruses, 2 pools positive (22.2%) for Den2 viruses and 1 pool positive for Den4 virus (11.11%). No Den1 virus positive pool had found in that study period. The predominant virus type found in mosquitoes responsible for year 2010-12 was Den3. In the current year 2023, the same experiment have done during dengue prevalent season from the month of April – September, 2023. The data showed that approximately 48% of 60 field caught mosquito pools were positive for dengue viruses. Of the 29 RT-PCR positive *Ae.aegypti* pools, 14 pools, were (48.28%) positive for Den2 viruses, 12 pools positive (41.38%) for Den3 viruses and 1 pool positive for Den4 virus (3.45%), 1 mosquito pool showed presence of both Den2 and Den3 viruses (3.45%). No Den1 virus positive pool had found in year 2023. The predominant virus type in mosquito pools in 2023 is Den2, where Den3 also co exists with Den2 in large numbers. Den4 also present in collected mosquito pools. It has been confirmed that Den2 mainly cause recent 2023 dengue outbreak in Bangladesh, Field caught *Aedes aegypti* from five stations had total MIR (minimum infection rate) of 0.72 per100 mosquitoes during year 2010-2012 but in year 2023 the total MIR 4.24 per 100 mosquitoes which is almost six times higher than before.

Keywords : RT-PCR, Den2, Den 3, Den 4, MIR

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Introduction

Dengue is a vector borne viral disease transmitted principally by a tropical mosquito vector *Aedes aegypti*[1]. It causes dengue fever and occasionally develops severe and fatal Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). Dengue is a major global health problem which is endemic in more than 100 countries , with an estimated 2.5 billion people at risk of infection[2]. Dengue is a RNA virus, belongs to family Flaviviridae, genus Flavivirus has four disease causing serotypes known as Den1, Den2, Den3 & Den4. Infection by one serotype provide lifelong immunity to that serotype but there is no cross protective immunity to other serotypes. Secondary infection with a heterologous serotype often leads to life threatening dengue DHF and DSS[3]. An outbreak of DF/DHF occurred in Bangladesh in 1999 and 2000. Dengue hemorrhagic fever had not been previously reported in Bangladesh[4]. Since then the disease has shown an annual occurrence in all major cities of the country. Now a days outbreak of dengue fever has triggered national concern in Bangladesh and Southeast Asian countries[5]. In Bangladesh's ongoing 2023 dengue epidemic season, the country has been witnessing the deadliest outbreak of dengue fever ever since the first outbreak in Bangladesh in 2005[6]. As of 17 September 2023, the Directorate General of Health Services (DGHS) has reported 167,684 hospitalizations and 822 deaths due to the *Aedes* mosquito-borne tropical disease in the 2023 outbreak year[7]. Like previous years, the outbreak started in Summer (April-May), spread and surged nationwide in the monsoons (July-August)[8]. On 3 August, the number of deaths surpassed previous years; and on 21 August, the tally of hospitalization surpassed the previous highest record of the 2019 outbreak[9]. Dhaka is the worst-hit area and the epicenter of the outbreak, with more than half of the cases being reported in the megacity[8,10]. On 25 July, hospitalizations were reported in all districts; and the tally of hospitalizations outside Dhaka city surpassed the figure of the capital on 14 August.

In June 2023, the Institute of Epidemiology, Disease Control and Research (IEDCR) reported that people are getting affected with the DENV-2 and DENV-3 variants this season, the two with the highest rates of infections and death[11]. In 2022 and 2021, DENV-4 and DENV-3 were found for the first time, respectively[12,13]. Although a patient does develop immunity to a certain variant after being infected with it, cross-infection with different variants raises the chance of complications and mortality. Secondary infections show changed symptoms, thus delayed hospitalizations are causing more deaths. It's notable that the death rate has more or less increased in

Bangladesh in past years[14]. With more severe cases being referred to the capital, Dhaka's hospitals struggle to control the situation.

Since no vaccine is available to prevent the disease, its control relies mainly on surveillance, vector control and case management[15].For the control and prevention of dengue fever, it is important to detect and type the virus in clinical samples and mosquitoes. Assays based on reverse transcriptase (RT-PCR) amplification of dengue viral RNA can offer a rapid , sensitive and specific approach to the typing of dengue viruses[16].

The dengue virus molecular typing method described by Lanciotti and coworkers[17] is used worldwide for diagnosis and surveillance. A two-step method with a RT-PCR followed by a nested PCR is used for serotyping of dengue viruses. RT-PCR based genotyping using type specific primers used to identify efficiently different types of dengue virus and is reported to be highly sensitive and specific[18].

In Bangladesh no significant molecular study of dengue viral RNA on field caught mosquitoes have done so far. The aim of this work is to develop a rapid, sensitive and specific molecular method (RT-PCR) for detection and typing of dengue virus in field collected mosquitoes for vector surveillance of Bangladesh in order to serve as an early warning monitoring tool for dengue outbreak.

Materials and Methods

In molecular study we first developed RT- PCR method for detection of dengue virus in lab infected mosquitoes by intra thoracically inoculating dengue patient serum . We compared ELISA and RT-PCR method in lab infected mosquitoes and established the molecular method (RT-PCR) described by Lanciotti et al. 1992 in Bangladesh for first time[19].

We applied that same molecular method in our field caught mosquitoes to detect the dengue virus. Nested multiplex RT-PCR method for typing of dengue virus in field caught mosquitoes had applied to detect the pre-dominant serotype and also the serotypes of dengue viruses that were circulating in mosquito vector of Dhaka city in 2010-2012 and in April, 2023 – September, 2023.

Collection of Dengue Vector

Our collections were carried out in five representative dengue prevalent areas in Dhaka city from July, 2010–Dec, 2012 and recent collection was carried out same those 5 areas from April, 2023–Sept, 2023. The five high risk dengue prevalent areas were-Dhaka University Campus, Shegunbagicha, Dhanmondi Residential area, Rampura and Mirpur. Most of our observation sites were independent houses, small buildings, hostels & multistoried apartment complexes. Adult indoor resting *Aedes aegypti* collections were made after sunrise and before sunset between fifteen days interval in each area during those above mentioned time period.

Molecular Detection of Dengue Viral RNA in Mosquitoes

Collection and Preparation of study sample

Capturing adult mosquitoes from field: Adult *Ae. aegypti* were captured following standard procedures between 15 days interval from September – November , 2010 , September –November, 2011, July-December 2012 and recent collections were made from April-September, 2023 during the peak dengue season from those five dengue prevalent locations in Dhaka city. Total 188 pools of *Ae. aegypti* samples were collected during the study period of 2010 to 2012 and 60 pools of *Ae. aegypti* samples were collected during this year 2023 from April to September. Collected pools were marked , processed in MEM and FBS solution and stored in cryogenic vials at -70.C for future virus isolation study.

Minimum Infection Rate (MIR): The MIR was used to compare virus infection rate *in Ae. Aegypti*, MIR= (Number of viruses detected in mosquitoes by species ÷ total number of that species tested) x100

RNA extraction

Viral RNA was extracted using a commercial reagent. A two-step method with a RT-PCR followed by a nested PCR is used for serotyping of dengue viruses. RT-PCR based genotyping using type specific primers used to identify efficiently different types of dengue virus.

Amplification of virus RNA by RT-PCR: Target sequence of the virus RNA was converted to a complementary DNA copy (c DNA) using reverse transcriptase (RT) and the dengue virus downstream consensus primer (D2), homologous to the genomic

The Journal of Eden Mohila College, 2023, Vol-3, pp. 71-83

RNA of the four serotypes, prior to enzymatic DNA amplification. Subsequently, amplification of resulting cDNA was performed using the up-stream dengue virus consensus primer (D1).

Virus typing by nested PCR

A second amplification reaction was performed with 1.0 µl of the amplified product of the first amplification reaction .The reaction mixture contained all the components described for the first amplification reaction with one exception: the primer D2 was replaced with the dengue virus type specific primersTS1, TS2, TS3 and TS4.

Applying Nested RT-PCR Method for Detection of dengue viral RNA in field-caught mosquito specimens

Nested RT-PCR method was followed for simultaneous detection and typing of dengue virus in field caught mosquitoes. The correctly sized DNA product of 511 bp was obtained after first amplification with consensus primers D1 and D2. Each DNA product was then correctly typed by second round of amplification with the type specific primers. The expected sizes of second amplification products were 482 bp (Den1), 119 bp (Den2), 290 bp (Den3) and 389 bp (Den4).

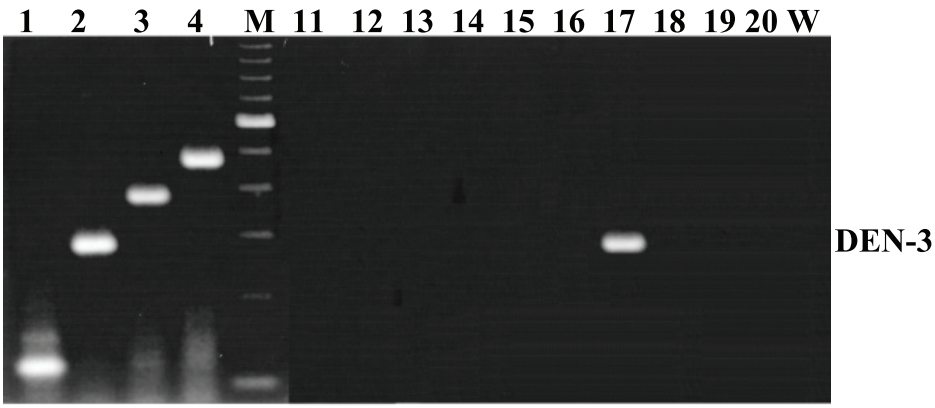

Fig 1. Detection and typing of dengue virus by nested RT-PCR from field caught mosquito pools (2010-2012). Lane1- Den2, Lane2-Den3 , Lane-3 Den4 & Lane-4 Den1 prototype, M-100bp ladder (lowest band shows 100 bp), Lane(6-15) represent field caught mosquito sample(11-20) from different location , where in lane 12 sample 17 shows Den3 DNA band. Lane 16 represent the negative control (W-water)

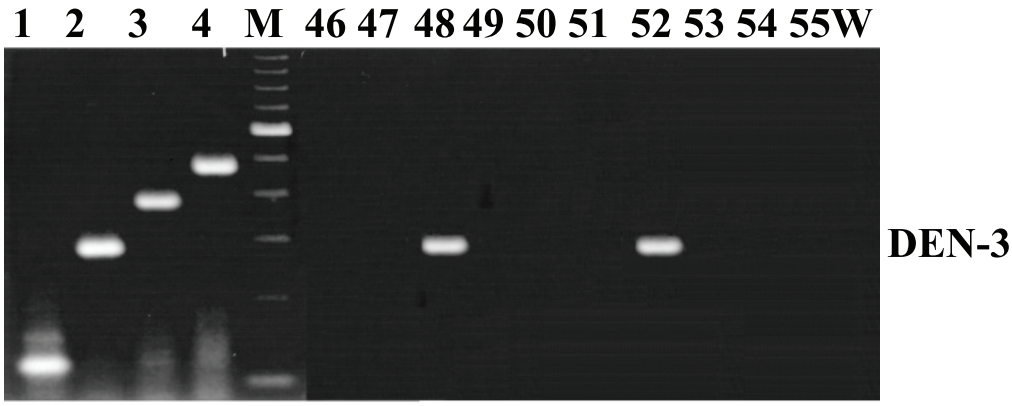

Fig 2. Detection and typing of dengue virus by nested RT-PCR from field caught mosquito pools(2010-2012). Lane1-Den2, Lane 2-Den3, Lane-3Den4 & Lane-4 Den1 prototype, M-100bp ladder (lowest band shows 100 bp), Lane(6-15) field caught mosquitoes from different location, represent sample 46-55 where in lane 8 and 12 sample 48 and 52 show Den-3 DNA band respectively. Lane 16 represent the negative control (W)

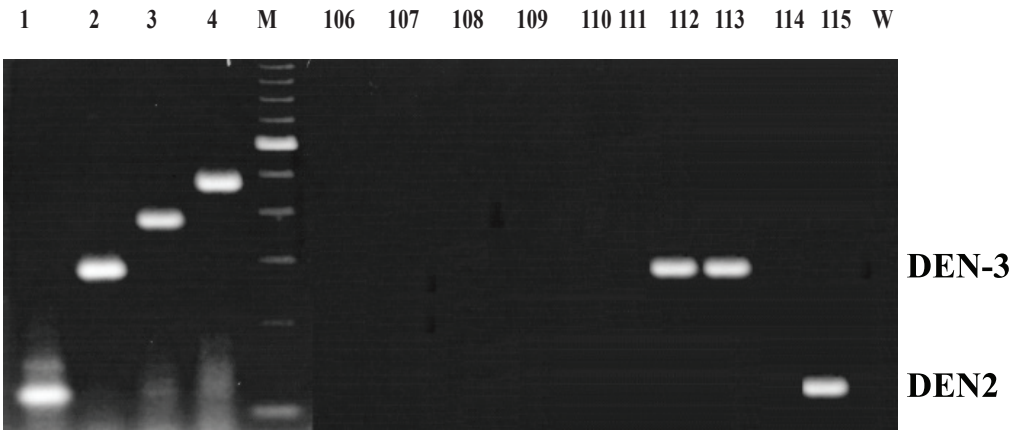

Fig 3. Detection and typing of dengue virus by nested RT-PCR from field caught mosquito pools (2023). Lane1-Den2, Lane 2-Den3, Lane-3Den4 & Lane-4 Den1 prototype, M-100bp ladder (lowest band shows 100 bp), Lane(6-15) field caught mosquitoes from different location, represent sample 106-115 where in lane 12&13 sample 112and 113 show Den3 DNA band respectively and in lane 15, PCR product of sample 115 show Den2 DNA band. Lane 16 represent the negative control (W-water)

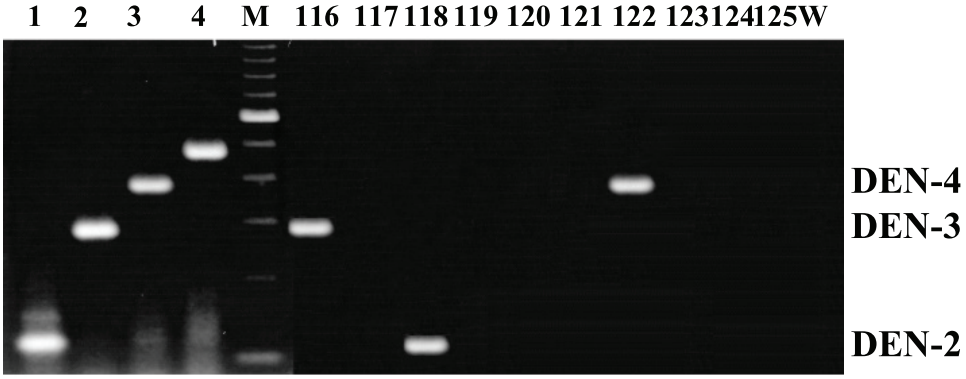

Fig- 4. Detection and typing of dengue virus by nested RT-PCR from field caught mosquito pools (2023). Lane1-Den2, Lane 2-Den3 , Lane-3 Den4 & Lane-4 Den1 prototype, M- 100bp ladder (lowest band shows 100 bp), Lane(6-15) field caught mosquitoes from different location, represent sample 116-125 where in lane 6 sample 116 show Den3, in lane8 sample118 show Den2 and in lane 12 sample 122 show Den4 DNA bands respectively. Lane 16 represent the negative control (W-Water)

Detection of Predominant dengue virus serotype in field caught *Ae.aegypti* mosquito pools (2010-2012)

Of the 9 RT-PCR dengue positive *Ae.aegypti* pools (containing 9-12 mosquitoes each) 6 pools were (66.66%) positive for Den3 viruses, 2 pools positive (22.2%) for Den2 viruses and 1 pool positive for Den4 virus(11.11%). No dengue 1 virus positive pool had found in that study time. The predominant virus type found in mosquitoe responsible for year 2010-12 was Den3.

Table 1. RT-PCR positive *Ae. Aegypti* mosquito pools and (%) of dengue virus serotypes detected in Dhaka city (2010-2012)

No of pools assayed	Total no. of mosquitoes	No. of positive pools	No. of positive pools and dengue virus serotypes(%)			
			DEN1	DEN2	DEN3	DEN4
188	1251	9(4.8%)	0	2(22.22%)	6(66.66%)	1(11.11%)

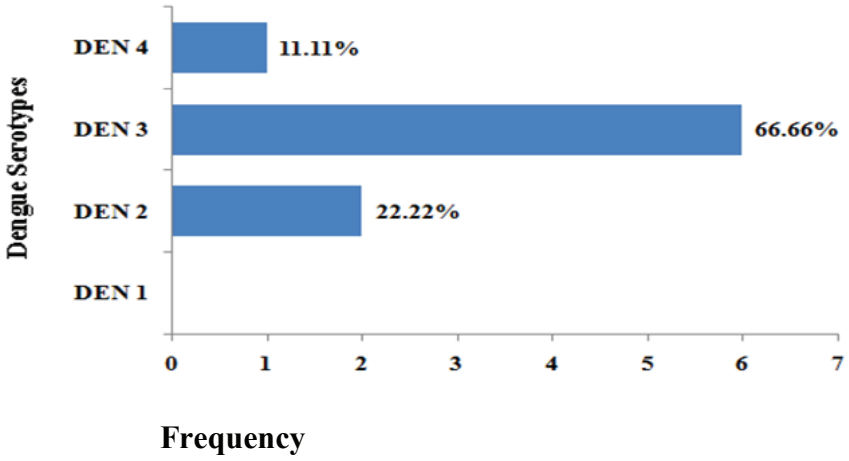

Fig 5. Percent of dengue virus serotypes in field caught *Ae. aegypti* mosquito specimens (2010-2012)

Detection of Predominant dengue virus serotype in field caught *Ae.aegypti* mosquito pools (2023, April-September)

Of the 29 RT-PCR dengue positive mosquito pools14 pools were (48.28%) positive for Den2 viruses, 12 pools positive (41.38%) for Den3 viruses and 1 pools positive for Den4 virus (3.45%),1 mosquito pool showed presence of both dengue Den2 and Den3 viruses (3.45%). The predominant virus type in year 2023 is Den2, where Den3 also co-exists with Den2 in large number in mosquito pools.

Table 2. RT-PCR positive *Ae. Aegypti* mosquito pools and (%) of dengue virus serotypes detected in Dhaka city during 2023 dengue outbreak (April - September 2023)

No of pools assayed	Total no. of mosquitos	No. of positive pools	No. of positive pools and dengue virus serotypes(%)				
			DEN1	DEN2	DEN3	DEN2+DEN3	DEN4
60	684	29 (48%)	0 (0.00%)	14(48.28%)	12(41.38%)	1(3.45%)	1(3.45%)

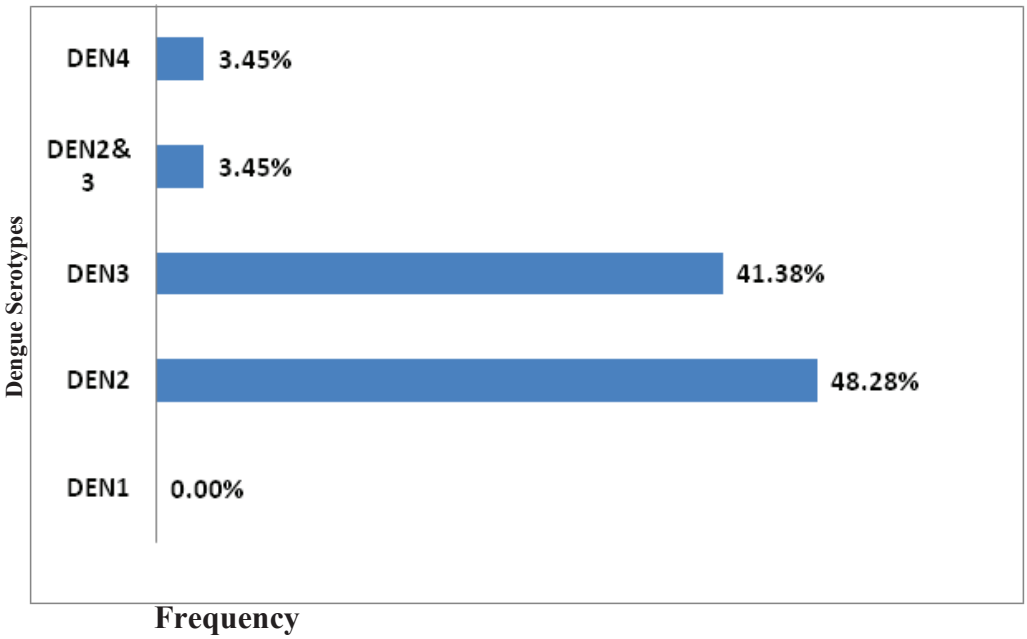

Fig 6. Percent of dengue virus serotypes in field caught *Ae. aegypti* mosquito specimens (April-September, 2023)

Comparison of Minimum Infection Rate between year 2010- 2012 and 2023

Table 3. Comparison of Minimum infection rates (MIR) between year 2012 and 2023 *Ae. Aegypti* species caught in different dengue prevalent areas in Dhaka city.

Stations in Dhaka	No of mosquitoes assayed by RT-PCR	No of dengue viruses detected	MIR	No of mosquitoes assayed by RT-PCR	No of dengue viruses detected	MIR
	Year2010-2012			April-September 2023		
Rampura	246	2	0. 81	144	8	5.55
Dhanmondi	246	4	1.22	140	12	8.57
Mirpur	269	2	0 .74	170	6	3.5
Shegunbagicha	231	0	0. 43	120	2	1.67
D.U Campus	259	1	0. 39	110	1	1
All stations	1251	9	**0. 72**	684	29	**4.24**

Infection rates for *Aedes aegypti* dengue prevalent areas were expressed as MIR (Minimum Infection rate) and compared (table- 3). *Ae. aegypti* caught from five indoor stations had MIR ranging from 0. 39-1.22 per 100 mosquitoes with an **MIR of 0.72** for the combined stations. Dhanmondi residential area showed the highest (1.22) MIR and Dhaka University Campus showed the lowest (0.39) MIR (2010-2012).

In Recent year, April-September, 2023 MIR ranging from 1-8.57 per 100 mosquitoes with an **MIR of 4.24** for the combined stations. Dhanmondi residential area showed the highest (8.57) MIR and Dhaka University Campus showed the lowest (1.00) MIR.

Discussion

In early outbreaks (2000–2002), Den3 was the prevalent serotype, albeit all four serotypes were circulating. Dengue serotypes from clinical patient serum was isolated by Mohammed A.I , et al. 2003 and they concluded that Den-3 was then circulating in Bangladesh which may entered from neighboring countries. These findings were in agreement with the presence of Den2, Den3 & Den4 in Dhaka city .

Our findings in 2010- 2012 also indicated Den3 as the predominant serotype in mosquito population and multiple virus serotypes also were co-circulating in mosquitoes as well as patients in Dhaka city.

Later, Muraduzzaman et al. revealed that Den1 and Den2 were the prevalent circulating serotypes between 2013 and 2016[20]. However, Den3 emerged after a hiatus in 2017 with the prevalent serotype of Den2. Since the largest outbreak in 2019, Den3 has been the most prevalent circulating serotype while this year 2023, Den2 has become the predominant serotype.

Our recent finding from April-September, 2023 also indicate that Den2 is the predominant serotype in mosquito population, Den3 also circulating in remarkable percentages and virus type Den4 also present in mosquito population in Dhaka city in year 2023.

Three genotypes (I, II, II) of Den3 and the cosmopolitan genotype of Den2 are currently circulating in Bangladesh. Arguably, the emergence of the Den4 serotype, which has been missing for more than 20 years, could pose a significant public health threat to Bangladesh because of secondary infection.

Evidence of more than one viral serotypes should be taken as a warning that outbreaks of DHF might occur in the future. However, we found 2010-2012 survey that the overall rate of dengue virus infection per 100 *Aedes aegypti* females during the study period (MIR) was as high as 0.72, that gave us an impression that a low grade infection and transmission was than present in Dhaka city. In the year 2023 mosquito collection from field and molecular typing confirmed that multiple serotypes co- circulating in Dhaka city and the overall rate of dengue virus infection per 100 *A. aegypti* females (MIR) is 4.24, which is almost six times higher than earlier study. This condition make a dengue out break in 2023. Following the years of Den3 predominating which may result in higher numbers of severe cases as a result of second infection with heterologous serotype of Den2 has been identified as the predominant.

So it may have been established in our study that RT-PCR based surveillance of dengue viral infection in *Aedes* mosquitoes and the large scale study for identification of serotypes of dengue virus by RT-PCR in field caught mosquitoes in Bangladesh could serve as an early warning monitoring system of dengue outbreak.

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