Genetic characterization of dengue virus from patients presenting multi-serotypic infections in the Northern West Bengal, India

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Abstract
Northern West Bengal, popularly known as North Bengal, is a dengue-endemic area, which has been severely affected by Dengue in the past few years resulting in massive hospitalizations and deaths. Genetic characterization of the circulating endemic dengue virus (DENV) serotypes is of paramount importance for the epidemiological understanding of the infection and subsequent vaccine development. The present study was conceived to characterize circulating dengue serotypes and to undertake phylogenetic study. EDTA blood samples of all \( N = 83 \) NS1-positive cases of patients with acute febrile illness referred to different health care facilities were collected and processed for RNA isolation followed by the complementary DNA (cDNA) preparation. Serotype determination of dengue infection was done using conventional PCR by targeting the viral C-prM region. Phylogenetic tree was constructed by implementing the Maximum likelihood method. Out of 83 blood samples 17 were detected to be positive for the presence of dengue viral RNA. DENV3 was found to be the predominant serotype in the single-infection cases; however, we have detected multi-serotypic co-infections throughout the study. Joint pain was found to be the most valuable symptom for the prognosis of dengue. Sequence analyses suggested that both DENV1- and DENV3-circulating genotypes are in the genotype III group and remain closely related to the Indian clade. To the best of our knowledge, this is the first report on the genetic characterization of circulating DENVs in North Bengal, which may contribute to the study of dengue epidemic and pathogenesis.

Keywords C-prM · DENV3 · Dengue outbreak · Multi-serotypic · North Bengal

Introduction
Dengue viral disease, although considered a neglected tropical disease, has become a major public health concern worldwide during the last few decades. It is reported in 100 countries each year and globally 3.6 billion people are at risk of dengue fever (DF) and dengue hemorrhagic fever (DHF) [1]. It is one of the most common mosquito-borne acute systemic viral diseases, caused by the dengue virus (DENV), a positive-sense, single-stranded RNA virus of the genus Flavivirus (Family Flaviviridae) [2]. Near about 400 million dengue cases and 500,000 hospitalizations with 24,000 deaths (mainly in children) occur annually throughout the world due to dengue [2–4]. According to molecular and epidemiological studies, DENV has four genetically different serotypes (those having up to 70% homology among each other) which are further classified into various genotypes [5–10]. The mosquito vectors of the DENV are dependent on the environmental conditions of tropical and sub-tropical countries of the world. The risk of dengue outbreak by the vector is believed to be caused by temperature suitability. DENV infection is transmitted from non-human primates to humans or human to human, generally, through two mosquito species, Aedes aegypti and A. albopictus. The peri-urban areas have been linked to greater risk, particularly
in highly connected areas, suggesting that human movement between population centers is an important facilitator of dengue spread.

The Indian subcontinent has suitable weather for dengue virus transmission by the mosquito vectors for the cause of several epidemic outbreaks every year. All the serotypes of DENV circulate and cause several epidemic outbreaks throughout India from 1963 to date every year [11–14]. Each year more than 1 lakh infections and 200 to 400 deaths take place in India because of Dengue [15]. The most recent, seriously affected dengue outbreak was in 2017, with 188,401 infections and 325 deaths [15]. Being a dengue-endemic region, West Bengal, especially Kolkata (erstwhile Calcutta) also faces severe dengue outbreaks and death every year since 1963 [16–22]. Most recently in 2017, the total number of infections and death was 37,746 and 46 due to dengue in West Bengal [15]. The burden of dengue has now become an important public health concern not only in the Kolkata region but also in rural West Bengal. Incidence of dengue is on the increase and is spreading to geographical regions not previously reported like the northern part of West Bengal, popularly known as North Bengal [23, 24]. However, there are a lack of detailed molecular studies on the overall prevalence of the dengue serotypes circulating in endemic zones, like the North Bengal region. This is also reported that circulating DENV is rapidly changing their genomes by accumulating mutations randomly as the RNA genome is prone to mutational changes [25]. These genomic changes may result in the emergence of new serotypes/genotypes of DENV with augmented pathogenicity and antigenic properties, as occurred in the newly discovered fifth serotype (DENV5), the nomenclature, however is yet to be approved by ICTV [26]. So, it is very much important to characterize the circulating DENV in North Bengal and their changes in the genetic material in this geographical region.

The objective of this study was to identify the prevalent DENV serotype circulating in the North Bengal region. To the best of our knowledge, this is the first report on the characterization of DENV genotypes using the molecular technique in the study region. We have also tried to characterize the partial sequences of circulating DENV and delineate their phylogenetic relationships with that of the sequences from India as well as from different parts of the world.

Materials and methods

Study case definition

The study area was based on the northern region of West Bengal, popularly known as North Bengal. Dengue-infected patients reporting from Siliguri town and the Darjeeling, Jalpaiguri, and Alipurduar districts of North Bengal were mainly focused on in the study. In total, 83 patients having positive results in the NS1 (Non-structural protein 1) ELISA test were enrolled, and clinical and demographic information were recorded in a framed questionnaire. Blood samples and data collection were done only after taking written consent from the patients or the patient’s party. The study was approved by the Institutional Human Ethical committee (Ref. No.-Zoo/3059/2021), as per the Helsinki Declaration of 2013. The description and characteristics of NS1 and IgM-positive status of the subject patients are presented in Supplementary Table 1.

Viral RNA extraction and cDNA preparation

Viral RNA was extracted from 250 µl of whole blood using Trizol LS reagent (Thermo Fisher Scientific Inc., USA) following the manufacturer’s instructions. Extracted RNA was converted into complementary DNA (cDNA) using the SuperScript IV Reverse Transcriptase enzyme (Thermo Fisher Scientific Inc., USA). Reverse transcription-polymerase chain reaction (RT-PCR) was done in a 20 µl reaction mixture containing 10 µl extracted RNA, 0.1 µM reverse primer SBD2 [27], 1X RT buffer (Thermo Fisher Scientific Inc., USA), 10 mM dNTP mix (Sigma-Aldrich, USA), RNase Inhibitor (Thermo Fisher Scientific Inc., USA), and Hi-grade RNase-free water (GCC-Biotech, India). The RT-PCR cycling program involved single cycle heating at 90 °C for 10 min, reverse transcription reaction at 42 °C for 60 min, and enzyme inactivation at 72 °C for 15 min. The prepared cDNA was stored at − 20 °C for Dengue detection and serotype identification by polymerase chain reaction (PCR).

PCR amplification and serotype identification

The first round of PCR was done using the universal common primers (forward-SBD1 and reverse-SBD2) for all DENV serotypes, as described by Lanciotti et al. [27], targeting 511 base pair (bp) of C-prM gene junction. The amplification reaction was conducted using 5 µl of cDNA samples, 0.4 µM of each primer, 2.5 mM Magnesium chloride (MgCl₂), 1X PCR buffer (with dye) (Promega, USA), 200 µM of each dNTPs (Sigma-Aldrich, USA), 1.5 U of Taq polymerase enzyme (Sigma-Aldrich, USA), and Hi-grade RNase-free water (GCC-Biotech, India) to 25 µl final reaction mixture. The PCR was done in a Thermal cycler (Eppendorf, Germany) for 35 cycles using the following program: 30 s at 94 °C, 1 min at 60 °C, 1 min at 72 °C, with a final extension of 10 min at 72 °C. The amplified PCR product of 511 bp was gel electrophoresed and visualized on 1% Agarose gel, pre-stained with ethidium bromide.

The amplified products from the first round of PCRs were subjected to the second round of PCRs (nested) for
determining the DENV serotypes using serotype-specific primers, SBDG1-DENV1, SBDG2-DENV2, SBDG3-DENV3, and SBDG4-DENV4 [27]. Nested PCR was done in 25 µl reaction mixture with 1000 times diluted PCR products, 0.4 µM of SBD1, 0.4 µM each of the serotype-specific primers, 2.5 mM MgCl₂, 1X PCR buffer (Promega, USA), 200 µM of each dNTPs (Sigma-Aldrich, USA), 1.5 U of Taq polymerase (Sigma-Aldrich, USA), and Hi-grade RNase-free water (GCC-Biotech, India). The PCR was run according to the same program in the thermal cycler (Eppendorf, Germany). The amplified PCR products of different serotypes were electrophoresed in 1% Agarose and visualized by UV light after ethidium bromide staining.

Sequencing and sequence submission

After amplification of 511-bp fragment of C-prM junction region, PCR products were purified from agarose gel using Quick Gel extraction and PCR purification combo kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer’s protocol. Serotype-specific PCR products of respective sizes were also purified from gel for sequencing. The purified PCR products were sequenced by Sanger dideoxy sequencing from AgriGenome Labs, Kerala, India. Each PCR product was sequenced at least twice from both ends. Raw sequences were digitally curated in BioEdit 7.0.5.3 software [28] and unique sequences were submitted to National Center for Biotechnology Information (NCBI) GenBank (Accession numbers: MW302125.1, MT949456.1, MW389650.1, MW389651.1, MW487378.1, and MW487379.1).

Sequence alignment and phylogenetic analyses

Submitted DENV sequences obtained from positive samples were subjected to NCBI nrBlast searches and were then aligned in BioEdit 7.0.5.3 to check sequence ambiguity and prepare the full-length sequence [28]. For the phylogenetic analysis of DENV sequences, a few Indian and non-Indian partial sequences of the C-prM region of both DENV1 and DENV3 were selected and downloaded from the GenBank global database (NCBI, National Institutes of Health, Bethesda, MD, USA). For the DENV1 serotype, 11 Indian and 19 non-Indian and for the DENV3 serotype, 10 Indian and 14 non-Indian sequences were included in the study. We have also used other Indian serotypic sequences as outgroups in the phylogenetic tree generation. All the sequences along with our submitted sequences (MW487378 and MT949456) separately for both DENV1 and DENV3 were aligned in MEGA software (version 10) using ClustalW [29, 30]. A phylogenetic tree was constructed in MEGA software (version 10) using the Maximum likelihood and Kimura 2-parameter model with 100 bootstrap replicates [29, 30].

Statistical analysis

For statistical analysis, patients were categorized into different groups depending on sex, age (< 30 years or above), and PCR positivity. The inclusion criterion for statistical analysis was only positive for the NS1 test. All the data were analyzed using IBM SPSS Statistics for Windows, version 21. The categorical variables were presented as frequencies and percentages. Relative risk factor identification was done by binary logistic regression analysis, based on the dependent variables, like joint pain, vomiting, and rash, and independent variables, like age, sex, and PCR positivity. The Odds ratio (with 95% confidence interval) determinants with p values less than or equal to 0.20 were selected for representation.

Results

Detection and serotype determination of DENV by PCR amplification

All samples were subjected to PCR amplification using SBD1 and SBD2 as primers and DENV cDNAs as templates. Seventeen (17) samples scored positive showing 511-bp fragments in ethidium bromide-pre-stained agarose gel (Supplementary Fig. 1). Positive samples were further subjected to serotype detection using serotype-specific primers [27], followed by agarose gel electrophoresis analysis. Few samples were scored positive for single-serotypic DENV infection among the RT-PCR-positive samples. DENV3-specific amplification was observed in 5 samples. However, 12 samples showed multi-serotypic DENV infections. Among the multi-serotypic patient samples, four (33.33%) were positive for both DENV2 and 3, two (16.66%) for DENV1, 2, and 3; one (8.33%) for DENV2, 3, and 4; and five (41.66%) were positive for all the 4 serotypes. Samples with a single infection with DENV3 showed a band of 290 bp in 1% Agarose gel (Supplementary Fig. 2). PCR products from multi-serotypic infections from a single patient’s blood are presented in Supplementary Fig. 3 showing bands of 482 bp (DENV1), 119 bp (DENV2), 290 bp (DENV3), and 392 bp (DENV4).

Patient’s characteristics

The study cohort (n = 83) presented a higher proportion of males (~ 61.5%), compared to females (~ 38.5%). Most NS1-positive patients were < 30 years old (60.2%), with a mean age of ~ 27 years. There was an average duration of fever of ~ 3.4 days among the participants. Out of 83 patients, 73.5% showed fever ≥ 3 days, but 43.4%, 14.5%, and 12% of patients showed joint pain, rash, and
vomiting tendency, respectively (Table 1). The detailed description of symptoms found in the 17 PCR-positive patients, with detected DENV serotypes is represented in Table 2. The frequency distribution of different characteristics of PCR-positive patients (n = 17) was done, where most patients were < 30 years old (64.7%), with a mean age of ~27 years. Among the PCR-positive cases, 76.5% showed fever ≥ 3 days, but 58.8%, 17.6%, and 11.8% of patients showed joint pain, rash, and vomiting tendencies, respectively (Table 1). Most infections of dengue were found to be multi-serotypic (70.6%), compared to single-serotypic (29.4%). As any DHF or DSS patients were not found in the study group, all patients were cured without any severity and death. There was no significant change found between the patients infected with single and multiple serotypes, as the low positivity and serotype determination. It was observed that most of the adults in the age range 21–60 years showed multi-serotypic infection compared to single-serotypic infection. The percentage of having joint pain in patients infected with multiple serotypes of DENV was greater than the patients infected with a single serotype.

Relative risk analysis

Female or male patients did not show any significant risk despite being dependent on other symptoms, like vomiting, rash, and joint pain. Suffering from joint pain is significantly risky for patients > 30 years old, with an Odds ratio (OR) of 3.269 (p = 0.011). PCR positivity was also dependent on all the symptoms but at various levels. Patients having joint pain showed more risk than those without joint pain of being PCR positive (OR 2.198, p = 0.155). Table 3 represents the binary logistic regression analysis to estimate the risk factors from different symptoms relative to sex, age, and PCR positivity.

| Table 1 Study sample characteristics | Frequency [N (%)] | Frequency, [N (%)] |
|-------------------------------------|-------------------|-------------------|
| **Variable**                        | **N=83**          | **N=17**          |
| Sex                                 |                   |                   |
| Male                                | 51 (61.5)         | 11 (64.7)         |
| Female                              | 32 (38.5)         | 06 (35.3)         |
| Age                                 |                   |                   |
| ≥ 30 years                          | 33 (39.8)         | 06 (35.3)         |
| < 30 years                          | 50 (60.2)         | 11 (64.7)         |
| Fever                               |                   |                   |
| < 3 days                            | 22 (26.5)         | 4 (23.5)          |
| ≥ 3 days                            | 61 (73.5)         | 13 (76.5)         |
| Rash                                |                   |                   |
| Yes                                 | 12 (14.5)         | 03 (17.6)         |
| No                                  | 71 (85.5)         | 14 (82.4)         |
| Vomiting                            |                   |                   |
| Yes                                 | 10 (12.0)         | 02 (11.8)         |
| No                                  | 73 (88.0)         | 15 (88.2)         |
| Joint pain                          |                   |                   |
| Yes                                 | 36 (43.4)         | 10 (58.8)         |
| No                                  | 47 (56.6)         | 07 (41.2)         |
| PCR positivity                      |                   |                   |
| Yes                                 | 17 (20.5)         | NA                |
| No                                  | 66 (79.5)         | NA                |
| Infection type                      |                   |                   |
| Single serotype                     | NA                | 05(29.4)          |
| Multiple serotype                   | NA                | 12 (70.6)         |
| Duration of fever (days), Mean (95% CI) | 3.39 (3.10–3.67) | 2.88 (2.57–3.19) |
| Age (years), Mean (95% CI)          | 27.29 (23.66–30.92) | 27.47 (18.96–35.98) |

CI Confidence interval
Sequence analysis and phylogenetic tree construction

Multiple sequence alignment of endemic isolates of DENV1 and DENV3 showed few key mutations when compared with other reported sequences of DENV from different regions of the world. These mutations may lead to a change in protein structures, as some amino acid substitutions were found (Table 4 and 5). The multiple sequence alignment of different DENV sequences isolated from this study region and as well as other sequences identified few mutations. Two representative alignments, presenting the DENV1 and DENV3 sequences from one North Bengal (NB), two Indian, and three other countries showed the mutations (Supplementary Fig. 4 and 5). The nucleotide sequences used in this study were similar to both DENV1 and DENV3, respectively, as found in NCBI Megablast searches. The phylogenetic tree of DENV1 serotypes showed that the sequence of North Bengal DENV1 (genotype III) clustered together with all the Indian sequence taxa (Fig. 1). The other genotypes of DENV1 and DENV3 were also observed to be clustered with sequences from the same region or other countries.

### Table 2 Description of symptoms with characteristics of PCR-positive samples

| Patient number | Sex | Age | Symptoms | NS1 test | IgM/IgG | DENV Serotype detected |
|----------------|-----|-----|----------|----------|---------|------------------------|
| Patient 5      | M   | 5   | Fever from 2 days | Positive | Negative | DENV-3                |
| Patient 6      | F   | 20  | Fever from 4 days, joint pain | Positive | Negative | DENV-3                |
| Patient 18     | M   | 9   | Fever from 3 days, vomiting 5 times, Rash | Positive | Negative | DENV-3                |
| Patient 19     | M   | 14  | Fever from 2 days, joint pain | Positive | Negative | DENV-3                |
| Patient 22     | M   | 25  | Fever from 3 days | Positive | Negative | DENV-2, 3, 4          |
| Patient 23     | M   | 14  | Fever from 3 days | Positive | Negative | DENV-2, 3             |
| Patient 32     | F   | 24  | Fever from 2 days, joint pain | Positive | Negative | DENV-2, 3             |
| Patient 33     | M   | 22  | Fever from 3 days | Positive | Negative | DENV-2, 3             |
| Patient 35     | F   | 29  | Fever from 3 days, joint pain, Vomiting, Rash | Positive | Negative | DENV-1, 2, 3          |
| Patient 39     | F   | 38  | Fever from 3 days, joint pain | Positive | Negative | DENV-1, 2, 3, 4       |
| Patient 40     | F   | 60  | Fever from 3 days, joint pain, Rash | Positive | Negative | DENV-1, 2, 3, 4       |
| Patient 42     | M   | 18  | Fever from 3 days, body pain | Positive | Negative | DENV-1, 2, 3, 4       |
| Patient 45     | M   | 13  | Fever 3 days | Positive | Negative | DENV-3                |
| Patient 49     | F   | 48  | Fever from 3 days | Positive | Negative | DENV-2, 3             |
| Patient 51     | M   | 60  | Fever from 2 days, joint pain | Positive | Negative | DENV-1, 2, 3, 4       |
| Patient 55     | M   | 38  | Fever from 3 days, Joint pain | Positive | Negative | DENV-1, 2, 3, 4       |
| Patient 69     | M   | 30  | Fever from 4 days, Joint Pain | Positive | Negative | DENV-1, 2, 3, 4       |

### Table 3 Relative risk estimation with Odds ratio in binary logistic regression analysis

| Odds ratio | Lower bound–upper bound | \( P \) value depicted |
|------------|-------------------------|------------------------|
|            | Joint pain | Vomiting | Rash |
| Sex        |            |          |      |
| Female:Male | 1.261     | 1.704    | 1.164 |
| Age group  |            |          |      |
| 30+:<30    | 0.517–3.071 | 0.452–6.426 | 0.336–4.037 |
|            | 0.610     | 0.432    | 0.811 |
| 30+:<30    | 3.269     | 1.607    | 1.630 |
|            | 1.307–8.178 | 0.427–6.054 | 0.477–5.569 |
|            | 0.011*    | 0.483    | 0.436 |
| PCR        |            |          |      |
| Positive:Negative | 2.198 | 0.967    | 1.357 |
|            | 0.743–6.503 | 0.186–5.035 | 0.324–5.679 |
|            | 0.155*    | 0.968    | 0.676 |

Odds ratio values are highlighted in bold
Italics emphasizes the \( P \) values

\*\( P \) value < 0.20

### Table 4 Mutations found in the DENV1 sequence isolated from the study region

| Nucleotide position in ON123654 | Codons in the sequences and Amino acid (in short abbreviation) | North Bengal (MW487378) | Other sequences from India | Other country sequences |
|---------------------------------|---------------------------------------------------------------|-------------------------|---------------------------|------------------------|
| 230–232                        | ATG-Met or TTG-Leu                                            | CTG or TTG-Leu           |                           |                        |
| 302–304                        | AGC-Ser or GGC-Gly                                            | GGC or GGT or GGA-Gly    |                           |                        |
| 362–364                        | AGC-Ser or AAC-Asp                                            | AAC or AAT-Asp           |                           |                        |
| 410–412                        | CTC-Leu or CTT-Leu                                            | CTT-Leu or TTC-Phe       |                           |                        |

ON123654 is the GeneBank Accession number of North Indian isolate of DENV1
sequences from other countries were outlier groups depending on the similarities. However, the DENV1 (III), isolated from South America, showed closeness with all the Indian sequences than with North America or other viral sequences [31]. Here, DENV2, DENV3, and DENV4 sequences from Indian isolates were used as outgroups in the phylogenetic tree. The phylogenetic tree of DENV3 serotypes showed a close resemblance of the North Bengal sequence with Indian DENV3 (genotype III) (Fig. 2). Three sequences of DENV1, DENV2, and DENV4 serotypes were used as outgroups in the phylogenetic tree construction.

### Discussion

The burden of dengue is one of the most important health issues in West Bengal every year. The unchecked growth and development of dengue vectors have made West Bengal, especially the North Bengal region a dengue-prone region in eastern India [32]. In the present study, most of the subjects were <30 years old with a mean age of ~27 years, therefore adult patients outnumbered the pediatric age group, which appears contradictory to the observation, made by several previous researchers [12, 14, 20, 33–35]. However, frequency related to gender with preponderance among males is found to be similar here [14, 33, 35]. The higher

| Nucleotide position in MN253125 | Codons in the sequences and Amino acid (in short abbreviation) |
|----------------------------------|---------------------------------------------------------------|
|                                  | North Bengal (MT949456) | Other sequences from India | Other country sequences |
| 286–288                          | GTT-Val                  | GTT-Val                      | GTC-Val or ATC-Ile   |
| 382–384                          | AAA-Lys                  | AAA-Lys                      | AGA-Arg or CAA-Gln  |
| 415–417                          | ATA-Ile                  | ATA-Ile or GTA-Val           | ATG-Met or ATA-Ile  |
| 427–429                          | GCA-Ala                  | GCA-Ala or ACA-Thr           | ACA-Thr             |
| 598–600                          | CAC-His                  | CAC-His                      | CTC-Leu or TTC-Phe  |

MN253125 is the GeneBank Accession number of North Indian isolate of DENV3

![Phylogenetic tree of studied and global database DENV1 sequences by Maximum likelihood method (100 bootstrap replicates).](image)

The sequences of Indian DENV2, 3, and 4 serotypes were included as the out-group. The references denoted with blue-colored text were the Indian isolates of DENV1. The DENV1 sequence from the present study is denoted in a black-bordered square. The bootstrap values were represented as decimal numbers.
proportion of male victims is evident in this study as compared to females among the affected patients.

So far clinical features are concerned; fever and joint pain were found to be most common among NS-positive patients. Female patients especially those above the age of 30 years were found to be having joint pain more as compared to males. This observation is similar to the report by Halsey et al. [36]. Also, acute phase symptoms were more commonly observed in PCR-positive patients in the present study. This observation is similar to the observation made by Verma et al. [37]. The data relating to acute phase symptoms presented in NS1-positive patients related to PCR positivity were not statistically significant due to higher p values, which might be related to the small sample size. A cohort-based study is essential to understand the role of different clinical parameters in the infection severity.

Among the dengue RNA-positive cases, fever > 3 days was the most important diagnostic symptom, followed by joint pain, rash, and vomiting, which may be related to viral load. Less PCR positivity was found in the present study, it may be due to comparatively lower viral load in the respective patient’s blood.

The dengue infections in West Bengal were mainly by DENV1, 2, and 4 serotypes between 2008 and 2010 [20]. But a massive dengue outbreak was reported in Kolkata by DENV3 serotype with increased numbers of DHF and DSS cases in 2012 [20]. However, previously DENV3 infection was also reported in 2005 in the Kolkata region [18]. The re-emergence of circulating DENV3 may be due to the accumulation of mutations in the genome and the selection pressure. It has been reported that there might be a co-circulation of all 4 dengue serotypes, however, with a preponderance of DENV2 in West Bengal [33]. The present study identified that several patients were affected by dual, triple, or all 4 serotypes of DENV with several combinations, thus indicating the circulation of all 4 serotypes throughout the study region (Table 2). We also found that, in the cases of single-serotypic infection, DENV3 was most prevalent, though DENV3 was found in both single and multiple infection cases. Previously few studies also reported similar reports involving co-infections with multiple serotypes [33, 38, 39]. The high frequency of multi-serotypic infection may be due to the feeding behavior of the mosquito vector, Aedes aegypti, which feeds multiple times during a single gonotrophic cycle [40]. Co-infection with several serotypes in a single patient is also caused by “super-infection,” when bitten by a mosquito carrying one DENV serotype, closely followed by a bite from another mosquito carrying a second serotype [41]. Additionally, travel history, along with logistic development, may also contribute to the infection by multiple dengue serotypes in a single person. Circulation of more than one serotype and virulent genotypes are responsible for major outbreaks with high mortality in any

Fig. 2 Phylogenetic relationships among the endemic DENV3 serotype and other sequences by the Maximum likelihood method of Mega X (100 bootstrap replicates). The Tree was based on the partial capsid and pre-membrane junction region of all the strains of DENV3 serotypes. Each sequence is abbreviated by its isolation region or country followed by its serotype, genotype, and GenBank accession number within the bracket. The sequences of Indian DENV1, 2, and 4 serotypes were included as the out-group. The references denoted with blue-colored text were the Indian isolates of DENV3. DENV3 sequence from the present study is denoted in a black-bordered square. The bootstrap values were represented as decimal numbers...
geographical region. Co-infections with multiple serotypes were also reported in Kerala [39, 42], Karnataka [43] and Telangana [44], and Tamil Nadu [45]. In the year of 2009, the co-infection of DENV was reported in Delhi with a frequency of 19% [39]. But several studies then reported the frequency of co-infections in different states of India, which ranged from 43 to 62% [43, 45, 46], and more interestingly 100% in Kerala [39], which suggests a huge increment frequency of co-infection. One previous study suggested that co-infection did not affect the disease severity [41]. But co-infection was found to be showing a severe spectrum of disease in DENV-infected patients in another study [47]. Our data showed that all 4 DENV serotypes circulate within West Bengal, which could result in secondary infections that occur during times when antibodies might offer protection instead of an enhancement. To the best of the authors' knowledge, this is the first study from North Bengal, which observed the circulation of all four serotypes with different combinations of co-infections.

DENV C-prM partial region were used for the detection and serotyping of circulating DENV serotypes among dengue patients. This region of the genome has epidemiological importance and the DENV serotypes differ substantially in this flanking region so a phylogenetic study was performed using the same sequences of that region. DENV C protein helps in the recruitment of the viral RNA during assembly and the release of the genome; whereas, prM is important for the formation and maturation of the viral particle inside the host cell. Any significant change in this region of DENV may exert a great impact on the disease manifestation and severity. Nucleotide substitutions, identified in this our study, may have a role in the protein synthesis, modification, and virus assembly in the host cell. The significance and implications of the mutations in the regions of C-prM require validation through bioinformatics studies.

The phylogenetic tree of DENV1 revealed that the DENV1 strain isolated from the present study falls into genotype III, which has very close proximity with the other DENV1 strains reported from different parts of India like Delhi, Tamil Nadu, Arunachal Pradesh, Kerala, and Hyderabad (Supplementary Table 2). The strain isolated from South America also shows close relation with the Indian clade of III genotypes. It may ascribe to the travel history of infected patients from South America to Asian countries (especially India). However, one new genotype V (African and American) of DENV1 emerged in India in 2013, which showed proximity to the Indian genotype III strains [48]. The out-group in the tree (DENV2, DENV3, and DENV4) remained in a separate and discrete clade with a substantial difference in the sequences with all the DENV strains (Fig. 1). The DENV3 strain isolated and sequenced in the present study was identified as genotype III, which is largely distributed throughout the world. The North Bengal strain showed very close proximity with the other DENV3 III strains of different regions of India like Rajasthan, Pune, Tamil Nadu, Mumbai, Hyderabad, and Delhi. The other genotypes of DENV3, genotypes I and II were originally different from the Indian III genotype and localized in particular geographical regions. Genotype III had evolved more frequently by circulating all over the world so detection of the genetic changes have special importance in epidemiological study and vaccine development. The out-group has distributed distinctly from all the DENV3 strains in the tree (Fig. 2).

Our finding is a strong indication that the dengue outbreaks are caused by different serotypes and genotypes, which are circulating all over India and evolving each year. Multi-serotypic co-infections of DENV in the northern part of West Bengal suggests its possible significant role in the pathogenesis of severe dengue cases. Co-circulation and its relativeness to different hematological parameters during dengue infection should be evaluated further for a better understanding of the role of co-infections. There are several queries on the outcome of the infection severity due to viral replication in one single host co-infected with multiple serotypes. The possibility of mutual interference among different dengue serotypes during replication within the same host also needs to be investigated in detail. Year-wise surveillance of infecting strains of DENV and their changes, using the molecular technique for the emergence of new subtypes, is thus important in public health. As there are a few limitations of this study, like less sample number, we have studied the dengue isolates from the North Bengal region only during one epidemic year. However, it is the first and foremost molecular characterization study in these dengue-endemic regions of northern West Bengal.

**Limitations**

The major limitation of the present study was the sample size, which needs to be large and categorized into different groups, like primary infection, secondary infection, DHF, and DSS. A detailed correlation study is needed for understanding the disease manifestation during different types of infections. An epidemiological study involving dengue cases for several continuous years is an urgent need to investigate DENV evolution and epidemiological changes of circulating DENV serotypes, targeting different important genes of the genome, e.g., envelope protein and non-structural proteins. Correlation between single serotypic and multi-serotypic DENV infection with that of disease pathophysiology and severity of disease is also one of the important perspectives. Determination of viral load in the patient’s blood is similarly an important parameter for the disease manifestation and modulation.
**Conclusion**

In this study, we have presented genetic epidemiological evidence of both single- and multi-serotypic infections of dengue among the patients of the North Bengal region. The most prevalent serotype circulating in this region seems to be DENV3 both in single- and multiple-serotypic infections. The isolated Dengue strains of DENV1 (III) and DENV3 (III) are evolutionarily close to other isolates identified in different states of India. The sequence information about the key viral genes of the circulating strains may help in the study of genetic relatedness of circulating isolates/serotypes and the study of disease severity vis-a-vis secondary infections. Therefore, better knowledge on co-infection of DENV may help us to understand the expanding global dengue epidemic.

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**Author contribution** SB and BKG conceptualized the work and designed the experiments and SKR collected samples, performed the experiments, and analyzed the data. SKR prepared the draft manuscript. Data interpretation, analyses of the research outcomes, critical revision, and approval of the manuscript for publication were done by SB and BKG. All authors read and approved the final manuscript. BKG acted as the clinical expert and played a role in the clinical implication of the data collected.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

**Declarations**

**Competing interests** The authors declare that there are no relevant financial or non-financial competing interests to report.

**Ethical approval** All the experiments involving human blood samples were performed according to ethical standard precautions and protocols, approved by the Institutional Human Ethical Committee of the University of North Bengal (Ref. No.-Zoo/3059/2021) as per the Helsinki Declaration of 2013.

**Informed consent** Informed consent was obtained from all the individual participants included in this study.

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