

Sequencing and Phylogeny of Dengue virus serotype 1 circulating in Lucknow, India

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Abstract

Background: In India majority of the dengue outbreaks are attributed to DENV-2 and DENV-3. However, DENV-1 is increasingly being implicated as a major serotype during recent outbreaks in India, including the Delhi outbreak in 2006. In Lucknow, we found a similar trend. We sequenced our strains to determine if similar strain is circulating here. In the present study, we sequenced C-prM gene junction of 5 DENV-1 isolated from Lucknow, India. For comparison, we retrieved 23 other Indian and 31 global reference sequences from NCBI database, making sure that Indian and global isolates from all decades are available for comparative analysis.

Methods and Findings: 298 samples from clinically suspected cases were tested by IgM MAC ELISA, Viral culture and RT PCR, Real time PCR. 66 cases came out to be positive by various methods. Five serum samples found positive for DENV-1 RNA were further sequenced for analysis. On sequence analysis, we observed that the Lucknow DENV-1 sequences showed sequence similarity of 96.1%-97.8% (mean 96.95%) with the 1980 and 1993 sequences from Thailand and Comoros and 95.9%-97.85 (mean 96.85%) with the 1993 sequence from Singapore. On comparison of our sequences with the other Indian sequences, mean sequence divergence of 2.1%, 4.15% and 5.05% were observed with Gwalior 2002, Delhi 2006 and Vellore 1962-1964 respectively.

Conclusion: DENV-1 isolates from this study clustered as a distinct subclade and signifies that a new lineage of DENV-1 has evolved.

Keywords: Dengue, phylogeny, Lucknow, India



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Introduction

Dengue is the most important arthropod-transmitted viral disease that affects humans in the world today. Each year, an estimated 50 million cases of dengue occur, with 40% of the world's population at risk [1]. In the past few decades,

there has been an expansion of the geographical distribution of both the mosquito vector and the viruses, resulting in increased epidemic activity. Dengue viruses belong to the family Flaviviridae, genus Flavivirus, with four antigenically related but distinct serotypes, DENV-1, DENV-2, DENV-3 and DENV-4. Dengue virus has a single stranded positive-sense

RNA genome of approximately 10,700 bases in length [2]. In the present study, we carried out a molecular epidemiological study of dengue virus that has been circulating in Lucknow using sequence comparison and phylogenetic analysis. Molecular characterizations of these viruses are necessary to identify the molecular subtype/genotype and to determine the introduction of any new lineages. Moreover it also helps in to gain insight into degree of genetic variability, rates and pattern of evolution [2]. Dengue virus genome has three major structural genes, the capsid, pre-membrane and envelope genes along with seven non-structural genes [3]. Different regions of dengue genome have been selected for molecular phylogenetic analysis in the past, but many studies, have reported the C-prM gene junction as a powerful tool in genotyping. The C-prM gene junction employs a single pair of primers for amplification and sequencing of any of the four serotypes of dengue virus [4, 5].

India is endemic for dengue and has witnessed several dengue outbreaks in the past. All the four known serotypes have been implicated in these outbreaks, but the major outbreaks have been caused by DENV-2. From 2003 onwards, there has been a shift in the cause of these outbreaks from DENV-2 to DENV-3, which has been found to be the predominant dengue virus circulating to predominant dengue virus serotype circulating in Northern India [6,7,8].

Majority of the dengue outbreaks are attributed to DENV-2 and DENV-3 viruses, but DENV-1 has not been recorded in major dengue outbreaks. However, DENV-1 was isolated from different parts of India at regular intervals, since its first isolation from southern India (Vellore) in 1956. It is increasingly being implicated as a major serotype during recent outbreaks in India, including the Delhi outbreak in 2006 [8, 9, 10]. The lack of sequence analysis affects the effective monitoring of circulating genotypes in India. Therefore, we planned to sequence and analyze a few of the DENV-1 strains that were isolated.

Methods

Clinical samples

Serum samples from 298 clinically suspected cases of pediatric age group were collected from Department of pediatrics, Chhatrapati Shahuji Maharaj Medical University, Lucknow in year 2005-2008. Informed consent from their parents was obtained before collection of clinical samples. Approval of the ethical committee of both institutions was obtained to carry out the present study. All samples were tested by various methods (IgM MAC ELISA, Viral culture and RT PCR, Real time PCR). Five serum samples found positive for DENV-1 RNA were included in this study.

Virus

India was used as reference strain to India was used as a reference strain Dengue virus serotype 1 (P-23086) obtained from the National Institute of Virology (NIV), Pune, India was used as reference strain in this study. This is the prototype Indian DENV-1 virus isolated from Vellore, India in 1956.

Extraction of viral RNA Reverse transcription-Polymerase chain reaction (RT-PCR)

Viral RNA was extracted from 140 µl of serum samples and P-23086 infected C6/36 supernatant by using QIAamp viral RNA mini kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Finally, RNA was eluted in 50 µl of nuclease free water and stored at - 80°C until use.

cDNA synthesis and amplification of C-prM junction

A one-step single tube serotype-specific multiplex PCR was performed with RNA from control dengue viruses and viral isolates using a multiplex RT-PCR protocol. The amplification was carried out in a 50 µl total reaction volume with Access quick RT-PCR kit according to the manufacturer's protocol, along with five primers viz., forward D1 and four serotype specific reverse primers (Ts1, Ts2, Ts3 and Ts4) as described by Saxena et al [11].

Briefly, five primers targeting the capsid gene (C-prM) were included in the assay, resulting in different size RT-PCR products of the dengue serotypes (DENV- 1,482 bp, DENV-2,119 bp, DENV-3,290 bp, DENV-4,389 bp).

The thermal profile includes first reverse transcription at 48°C for 45 second, than initial denaturation at 95°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 72°C for 1 minute, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The PCR amplified products were analyzed on 2% agarose gel using Accu-Prep gel extraction kit (BIO RAD, UK).

Sequencing reaction

DNA sequencing was performed on gel purified PCR products using dye-terminator method of ABI (Applied Biosystems, Foster City, CA). The nucleotide sequence was resolved by a 377 automated DNA sequencer (Applied Biosystems).

Sequence alignment and phylogenetic analysis

The C-prM gene regions sequences of DENV in this study were submitted to GenBank at <http://www.ncbi.nlm.nih.gov> and accession numbers acquired (**Table 1**); prior to which

BLAST search was carried-out to confirm the virus type. Previously reported DENV sequences of different gene regions of dengue virus genome from India and also from different geographical locations across the globe were retrieved from NCBI database and selected for comparison with DENV sequences from this study. Sequences from diverse geographic locations, such as those from Asia, Africa, America and Europe were selected for comparison. Sequence analysis was carried out using various Bioinformatics softwares. BLAST was carried out for the consensus sequence of all the gene regions of all the study cases. The result displayed BLAST hits of our sequence with the sequences having similar local region from the database in the next window with the scores of each hit. BLAST hits and scores of our query sequences with the sequences from the database confirmed that the region amplified by PCR was of desired gene region Dengue virus. Multiple sequence alignment was performed using BioEdit software employing Clustal W Multiple Alignment. Phylogenetic analysis was carried-out using MEGA version 3.1. Phylogenetic tree was constructed employing Neighbor Joining method with bootstrap analysis of 1000 replicates.

Statistical Analysis

The relationship between the frequencies of the clinical parameter and complication was analyzed using Pearson correlation co-efficient and linear regression analysis. Statistical analysis was conducted using SPSS software.

Results

Out of 298 clinically suspected cases of dengue fever total 66 cases came out to be positive by various methods (IgM MAC ELISA, Viral culture and RT PCR, Real time PCR). Virus culture

showed positive CPE in 53 samples which was further confirmed by RT PCR. Multiplex PCR from CPE positive cultures revealed two serotypes of which 29 were of DENV-1 type and 24 were of DENV-3 type. Sequencing and phylogeny of 5 DENV-1 viruses was done.

Nucleotide sequence and amino acid alignment analysis of DEN-1

For nucleotide sequence analysis of DENV-1, we have selected a 368bp (nt 100-467) region of the C-prM gene. Details of these sequences are described in **Table 1**. These sequences were compared and aligned with twenty three previously reported Indian sequences and thirty one sequences reported globally (**Figure 2**). On sequence analysis, we observed that the Lucknow DENV-1 sequences showed sequence similarity of 96.1%-97.8% (mean 96.95%) with the 1980 and 1993 sequences from Thailand and Comoros and 95.9%-97.85 (mean 96.85%) with the 1993 sequence from Singapore. On comparison of our sequences with the other Indian sequences, mean sequence divergence of 2.1%, 4.15% and 5.05% were observed in Gwalior 2002, Delhi 2006 and Vellore 1962-1964, respectively. The Lucknow DENV-1 sequences of this study also showed a sequence divergence of 1% among themselves. Comparison with the reference sequences revealed only transition mutations which were mostly synonymous in nature. Five T-C transitions were at nucleotide position 115, 151, 160, 275 and 413, A-T transition was at nucleotide position 137 and A-G transition was at position 295. (**Figure 3**)

Phylogeny of the DEN-1

A phylogenetic tree was constructed using pairwise comparison from the DENV-1 C-prM gene junction of virus isolates sequenced in this study with the 23 sequences from India

Table 1. Details of Indian DENV-1 strains sequenced in this study.

Virus isolate name	Date of collection	Age	Sex	GeneBank accession no.	Pathology
DEN/01/UP/INDIA_2008	2008	5 months	M	JF290425	DF
DEN/03/UP/INDIA_2006	2006	3 months	F	JF290427	DF
DEN/04/UP/INDIA_2008	2008	2 months	F	JF290428	DF
DEN/05/UP/INDIA_2008	2008	18 days	M	JF501211	DF
DEN/06/UP/INDIA_2008	2008	3 months	M	JF501212	DF

"DF"denotes Dengue Fever

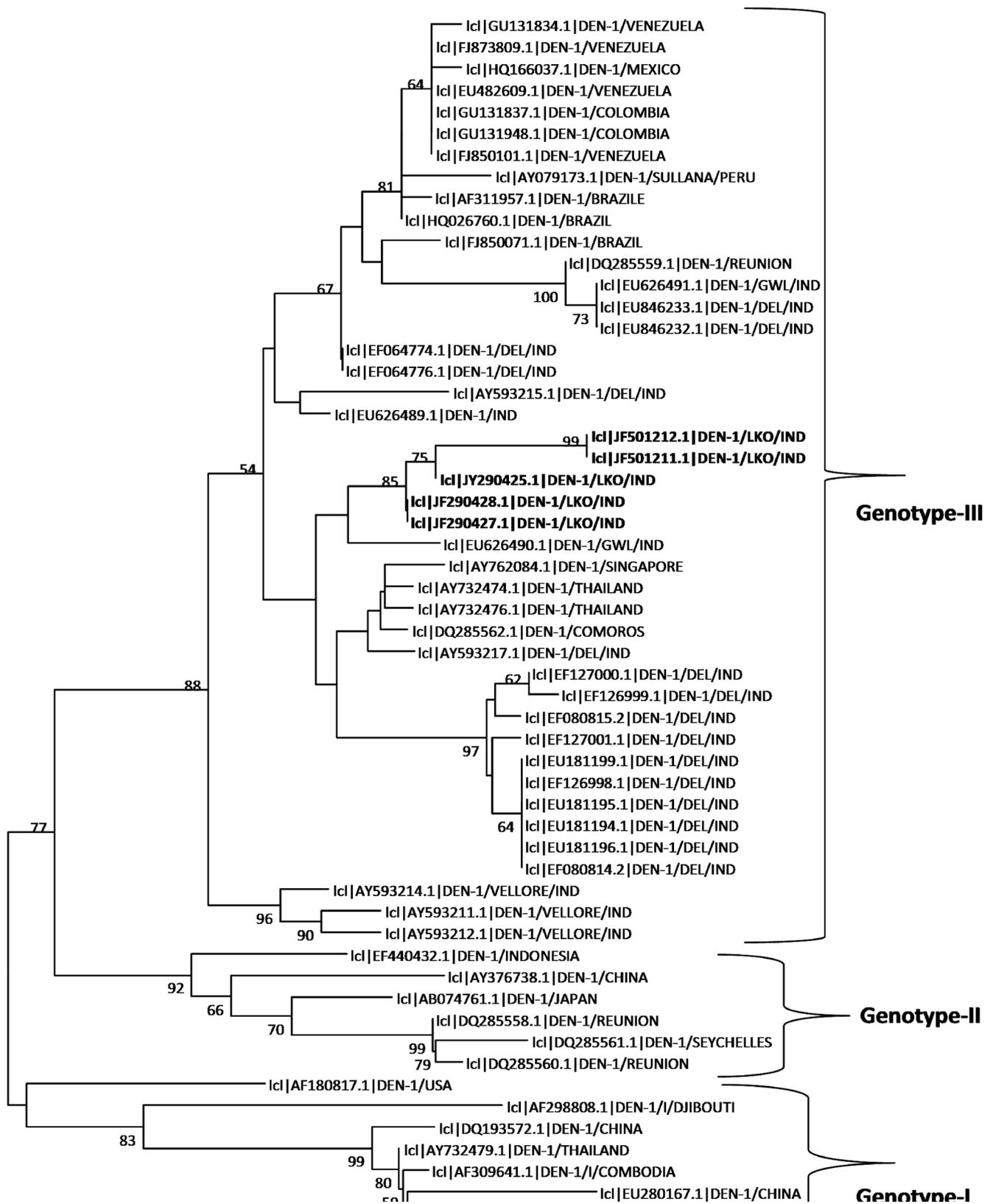


Figure 1. Phylogenetic tree of DENV-1. The tree was generated based on 404 bp region of the CprM gene junction. Strains in the trees are shown by their GeneBank accession number, serotype, place and or country of origin. Lucknow strains are shown highlighted in bold.

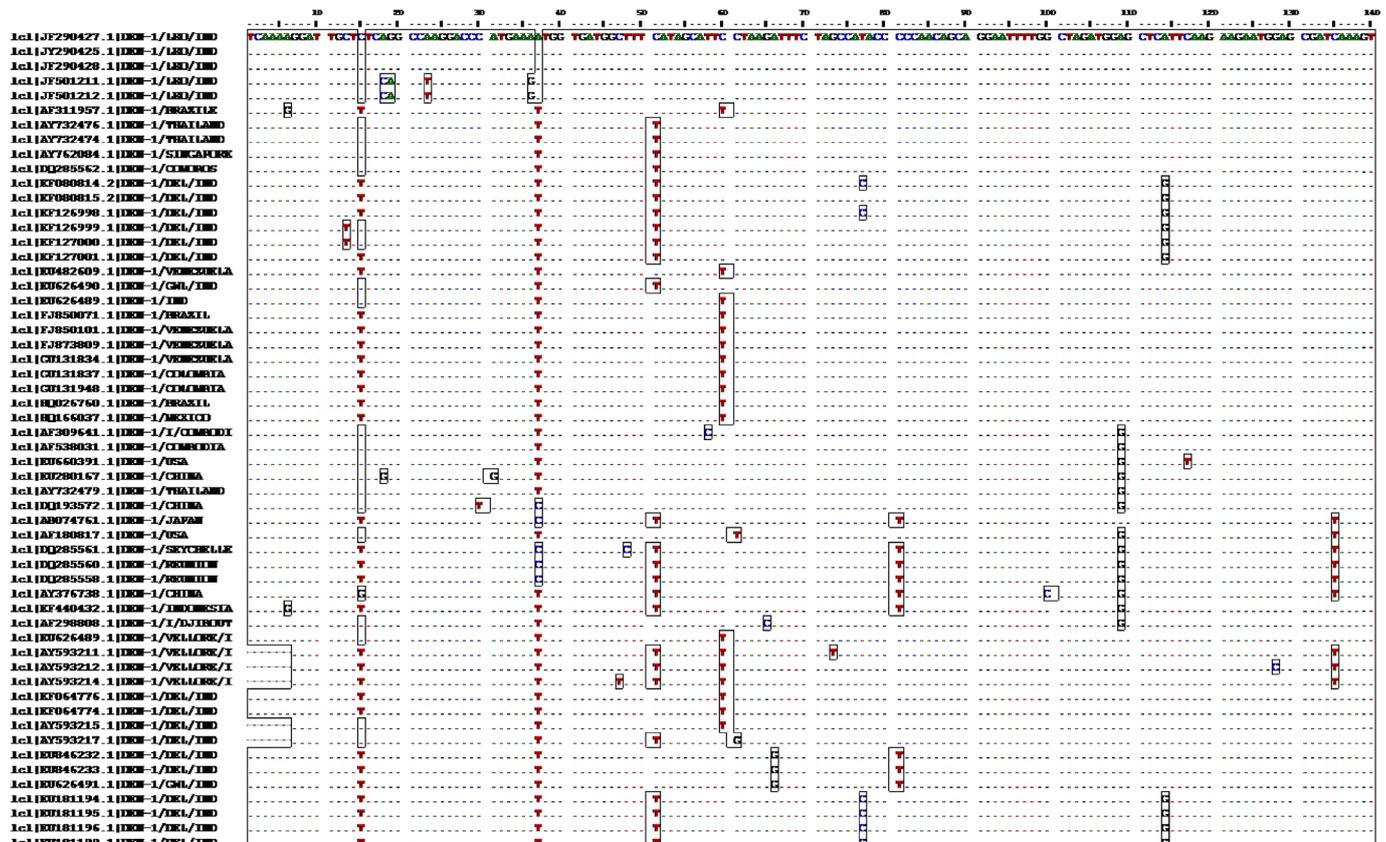


Figure 3. Nucleotide (nt) alignment of CprM gene junction sequences of all Indian and global DEN-1 showing changes in comparison to the consensus sequence. The Indian sequences that are sequenced in this study are the first five strains in the figure. Dot (.) indicates nt homology with the consensus.

and 31 from different parts of the world, revealed clustering of isolates in three distinct genotypes (genotype I, II and III) (Figure 1). But all the Indian DENV-1 sequences of this study and those reported by others from different outbreaks, clustered in genotype III along with viruses from Mexico, Colombia, Brazil and Venezuela. All the five DENV-1 isolates from this study clustered as a distinct subclade.

Discussion

Uttar Pradesh is the largest state constituting majority of northern India. Lucknow is the capital of this state with a population near 5 million. Several epidemics of febrile illness associated with hemorrhagic manifestation have occurred in this region in past [12]. First major epidemic occurred in year 1968 after the rains at Kanpur, which is an adjoining city to Lucknow [13]. Again in 1969, a similar epidemic occurred in Kanpur, covering cases of DENV-4 and DENV-2 viruses. In 1996 again an epidemic of DF/DHF occurred in Lucknow,

Kanpur and their adjoining areas [14]. In 2003, there was an outbreak of Dengue in Lucknow and surrounding areas of Uttar Pradesh, India [15]. In 2004, again in the post-monsoon season, there was resurgence of dengue infection in this region [12]. The virus from this region has never been sequenced in the past. Therefore we planned to sequence the virus in order to investigate the genetic distribution of this serotype circulating in this part of country. Strain typing is a critical tool for molecular epidemiological analysis and can provide important information about the spread of dengue viruses.

Based on pairwise comparison of C-prM gene junction of virus isolates sequenced in this study all the five DENV-1 isolates from this study clustered as a distinct subclade (based on sequence similarity of $\geq 95\%$) within genotype III and signifies that a new lineage of DENV-1 in Lucknow has evolved. These strains are very close to Gwalior (City in Madhya Pradesh, adjoining state to Uttar Pradesh) strains of India with mean sequence divergence of 2.1%. Though Delhi is just 300 KM away from Lucknow, the virus shows a sequence divergence

of 4.15%. Majority of Indian DENV-1 genotype III viruses recovered in past were phylogenetically distinct, the viruses recovered in 2001-02, 2004 and 2006-07 from northern India belong to separate lineages. This type of distinct lineage pattern has not been observed in Indian DENV-2 and DENV-3 viruses [16]. Domingo *et al*, and Kukreti *et al*, has reported earlier the emergence of two different lineages of DENV-1 in India during 2005-2006 and designated them as India-1 (close to American strains) and India-2 (related to Singapore 1993 isolate) [9, 17]. Since our strains are also close to Singapore 1993 isolates (mean sequence similarity of 96.85%) therefore we can classify them as India-2 strains. However, strains from South India (Vellore) recovered during 1962-1964 also formed a distinct lineage and were designated as India-3. These strains do not cluster with any other Indian and global isolates [9]. Recently, a study by Kukreti *et al*, has reported the emergence of another independent lineage India-4, that consists of viruses from Delhi and Gwalior [18]. Other Indian DENV-1 isolated in 1956, 1970, 1982 and 2004 could not be designated in a separate lineage.

Genotype I and II has to Genotype I and II have never been found in India. Genotype I consists of viruses circulating in other Asian countries including Thailand, Cambodia and China, but the presence of an African isolate from Djibouti in this group, signifies the introduction of these Asian viruses in Djibouti or vice-versa. Genotype II consists of viruses from Indonesia, China, Japan and Reunion islands. None of the Indian strains including ours could be classified as genotype I or II. Our study shows that both DENV-1 and DENV-3 are co-circulating in this region of India. Dar L *et al* have also reported similar findings from Delhi 2003 outbreak. They reported that all the 4 dengue virus serotypes have been circulating simultaneously with no particular type predominating. This finding suggests that dengue is now becoming endemic in this region of the country.

Conclusion

In the present study we found DENV-1 as a major emerging serotype in this part of country. DENV-1 isolates of this study clustered as a distinct subclade and signifies that a new lineage of DENV-1 has evolved in Lucknow. This emergence of DENV-1 in a population already exposed to DENV-2 and 3 serotypes might be the cause of increased severity of the disease. The emergence of a new serotype is associated with severe disease outbreaks, hence making the situation more alarming. This type of epidemiological study in a population where more than one dengue virus serotype co-circulates simultaneously is of special significance (19). The effect of co-circulation of serotypes in a population on disease severity needs to be further studied. Detailed and continuous epidemiological surveillance is warranted to monitor the incursion and spread of dengue viruses, which will help to undertake effective control and management strategies at the earliest.

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Competing interests

The authors declare that they have no competing interests.

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Supporting information

- File 1: Table describing name, accession numbers, year of isolation and country of isolation of the DEN-1 strains enrolled in this study.
- File 2: Figure showing a phylogenetic tree analysis of DEN-1 strains isolated in Lucknow
- File 3: Figure showing nucleotide (nt) alignment of C-prM gene junction sequences of all Indian and global DEN-1 showing changes in comparison to the consensus sequence

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