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Emergence of an independent lineage of dengue virus type 1 (DENV-1) and its co-circulation with predominant DENV-3 during the 2006 dengue fever outbreak in Delhi☆

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KEYWORDS
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CprM gene junction;
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Lineages;
Phylogenetic tree

Summary
Objectives: The sudden emergence of dengue virus type 1 (DENV-1) and its co-circulation with predominant DENV-3 was the hallmark of the 2006 dengue fever outbreak in Delhi. Viruses that circulated between 1996 and 2005 in the City have been well characterized, but the genomic diversity in 2006 strains is not known. The present study was undertaken to reveal the emerging molecular genotype(s) and evolutionary trend of the viruses responsible for the dengue fever outbreak in Delhi during 2006.
Study design: The *CprM* gene junction of the DENV isolates from the 2006 Delhi dengue fever outbreak were subjected to nucleotide sequencing. Comparative phylogenetic analysis was done using DENV-1 and DENV-3 sequences retrieved from the global database.
Results: Multiple sequence alignment revealed only substitutions, with no insertions or deletions. A dendrogram indicated emergence of a distinct lineage of DENV-1 (having similarity with the Comoros/Singapore 1993 and Delhi 1982 strains, but quite different from the Delhi 2005 lineage) and microevolution of the pre-circulating DENV-3. These findings point towards the circulation of two independent lineages of DENV-1 in Delhi during 2005 and 2006.
Conclusions: It is feared that the introduction of an independent lineage of the outbreak-associated strain of DENV-1 and its co-circulation with the deeply-rooted strain of DENV-3 in Delhi may result in yet another, possibly more severe outbreak in the near future.

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Introduction

The more frequent outbreaks of dengue than ever before in several parts of Southeast Asia and subtropical countries have led to serious public health concerns in recent years.[1,2] The situation in India during the past decade has worsened.[3–6] Dengue virus (DENV) infection generally causes a mild febrile illness, referred to as dengue fever (DF), but some may lead to severe life-threatening disease — dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). As per World Health Organization (WHO) estimates, over 50 million cases are reported worldwide, of which 500 000 may develop DHF/DSS. Rapid urbanization, the increase in human and vector populations, and expanded travel have offered added opportunities for the spread of the virus across countries and continents.[7–9]

There are four antigenically related, but genetically distinct serotypes (DENV-1 to -4), each of which contains a phylogenetically distinct cluster of viruses referred to as 'genotypes' or 'subtypes'.[10–13] These genotypes are often linked to specific geographical areas. The disease severity can be associated with a particular genotype or due to sequential infection of heterotypic virus.[14–18] Although genotyping is performed on the basis of different gene regions, *CprM* gene-based genotyping has been most widely used on Indian dengue isolates. A new set of consensus primers (D1 and D2) was first designed and developed by Lanciotti et al. in 1992,[19] which was capable of amplifying a 511 bp region of the *CprM* gene of all four types of dengue virus. Subsequently, the usefulness of this *CprM* gene region for carrying out subtyping of dengue viruses has been established by us and other researchers.[4,5,20,21]

Although several parts of India are endemic for dengue, Delhi has been worst hit during the past decade, the Delhi DF outbreaks of 2003 and 2006 being the most recent ones.[4,6] On record, all the four known serotypes of DENV have circulated in India at different times,[22] but generally one serotype dominates during a given outbreak. The first major DF outbreak in Delhi in 1996 was caused by DENV-2 genotype IV,[3] and the virus remained in circulation in and around Delhi until the end of 2002.[5] In 2003 however, we noticed DENV-3 as the leading cause of a major DF outbreak in the region,[6] and DENV-2 had been virtually replaced. This DENV-3 remained in dominant circulation until the 2006 Delhi DF outbreak, but approximately 30% of cases during 2006 were also found to be due to newly emerging DENV-1. It has been observed that a newly emerging serotype/genotype can appear suddenly and co-circulate along with the existing genotype for some time before replacing the latter in subsequent years. In view of the frequent rise and fall of the different genotype(s) of dengue viruses noticed in recent years, the present study was designed to carry out *CprM* sequence-based molecular subtyping and decipher the evolutionary trend of the two viruses (DENV-1 and DENV-3) that co-circulated during the 2006 DF outbreak in Delhi.

Materials and methods

Clinical samples

One hundred and seventy-nine acute phase serum samples from clinically suspected cases of dengue were collected from different geographical locations of Delhi during the peak phase (October) of the 2006 Delhi DF outbreak. Informed consent from the patients was obtained after explaining the nature and possible consequences of the study. Approval of the Institute's ethics committee was also given to carry out the study. Samples were transported to the laboratory with the due precautions recommended for handling RNA viruses.

cDNA synthesis and amplification of target *CprM* gene junction

Viral RNA was extracted from 140 μl of serum samples using the QIAamp Ultrasens virus kit (Qiagen, Germany) following the manufacturer's protocol. The final elution was done in 50 μl of diethyl pyrocarbonate (DEPC)-treated water before storing at −70 °C until use. The reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out as previously described by us[6] using *CprM* gene-specific primers (D1 and D2) that amplified the 511 bp amplicon reported by Lanciotti et al. in 1992.[19] cDNA was synthesized in a 10 μl reaction volume with a reverse transcriptase (RT) mix comprising 5× RT buffer, 10 mM dNTPs, 100 mM DTT, 20 U RNase inhibitor, and 50 U of MultiScribe RT (ABI, USA) with reverse primer (D2: 5′-TTGCACCAACAGTCAATGTCTTCAGGTTC-3′). The RT mix was incubated at 42 °C for 20 min followed by 99 °C for 5 min for inactivation of MultiScribe RT. Amplification of the cDNA was carried out in a 50 μl reaction volume with PCR mix containing 1× RT-PCR buffer, 1.75 mM $MgCl_2$, 0.8 mM dNTPs, 2.5 U AmpliTaq Gold DNA polymerase (ABI, USA) using forward primer (D1: 5′-TCAATATGCTGAAACGCGCGAGAAACCG-3′), in a PCR thermal cycler (ABI 9700). Thermal profile included initial denaturation at 95 °C for 10 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The PCR products were gel-purified from 1.2% agarose gel using Accu-Prep gel extraction kit (Bioneer, USA).

Automated nucleotide sequencing

Purified amplicons were subjected to automated nucleotide sequencing with both forward and reverse primers separately. Sequencing was carried-out using the Big Dye terminator kit (ABI, USA). Approximately 25 ng of purified PCR product was mixed with 3.2 pM respective primer (D1/D2) and a reaction mixture containing AmpliTaq DNA polymerase and four dye-labeled dideoxynucleotide terminators. The reaction mixture was placed onto a preheated thermal cycler. Cycle sequencing parameters included 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. The reaction mixture was purified by precipitation with 3 M sodium acetate (pH 4.6) and 75% isopropanol, and the extension product was vacuum dried. The DNA pellet was resuspended in 10 μl of template suppression reagent (TSR), heated at 95 °C for 2 min and immediately chilled on ice, mixed and after brief spin, finally loaded onto the ABI 310 genetic analyzer (ABI, USA).

Sequence alignments and phylogenetic analysis

CprM gene sequences obtained in the present study were submitted to GenBank at www.ncbi.nlm.nih.gov (accession

Table 1 Details of patients and DENV-3 and DENV-1 isolates sequenced in this study

Patient ID	Age/sex	Clinical diagnosis[a, b]	ID of isolated virus	GenBank accession No.	Serotype
D-201	33/M	DF	01den06	EF080812	DENV-3
D-203	25/M	DF	02den06	EF080813	DENV-3
D-81	40/F	DF	03/3/del2006	EF126994	DENV-3
D-82	19/F	DF	04/3/del2006	EF126995	DENV-3
D-212	10/M	DF	05/3/del2006	EF126996	DENV-3
D-166	29/F	DHF (grade II)	06/3/del2006	EF126997	DENV-3
D-179	15/F	DF	INDI06DEN07	EF546768	DENV-3
D-224	30/M	DF	INDI06DEN08	EF546769	DENV-3
D-178	5/M	DF	INDI06DEN09	EF546770	DENV-3
D-232	23/M	DF	INDI06DEN10	EF546771	DENV-3
D-217	15/M	DF	INDI06DEN11	EF546772	DENV-3
D-168	50/F	DHF (grade II)	INDI06DEN12	EF546773	DENV-3
D-119	25/M	DF	INDI06DEN13	EF546774	DENV-3
D-85	22/F	DF	INDI06DEN14	EF546775	DENV-3
D-162	16/M	DHF (grade II)	01den06	EF080814	DENV-1
D-74	18/M	DF	02den06	EF080815	DENV-1
D-181	31/M	DF	03/1/del2006	EF126998	DENV-1
D-161	15/F	DHF (grade III)	04/1/del2006	EF126999	DENV-1
D-77	27/F	DF	05/1/del2006	EF127000	DENV-1
D-78	34/F	DF	06/1/del2006	EF127001	DENV-1

[a] All cases belonged to Delhi.
[b] The majority of the cases presented with clinical symptoms such as headache (80%), myalgia (74%), rash (53%), vomiting (39%), epistaxis (15%), melena (4%), and fever ranging from 39 to 40 °C.

numbers are given in Table 1). A BLAST search was carried out to confirm the identity of strains. For comparison, we retrieved 50 DENV-1 and 43 DENV-3 *CprM* sequences belonging to diverse geographical locations from the global database, as shown in the phylogenetic trees in Figures 1 and 2, respectively. The DNAStar software package was used to examine the percent identity and diversity among sequences. Phylogenetic analysis was done using MEGA version 3.1,[23] and the evolutionary tree was constructed using the neighbor-joining method[24] with a bootstrap value of 1000 replicates.

Results

RT-PCR and nucleotide sequencing revealed that 20 samples were positive for the presence of dengue virus-specific nucleic acid. The BLAST search of these 20 *CprM* gene sequences confirmed 14 cases as DENV-3 and six as DENV-1. While DENV-3 sequences were closely related to the Delhi strains of 2003—05 and Guatemala strains isolated in 1998, the DENV-1 sequences were found to be similar to the Comoros/Singapore 1993 strains and the Delhi 1982 strain. Multiple sequence alignment of the 14 DENV-3 and six DENV-1 sequences of this study indicated substitutions that spanned the whole sequence, and no insertions or deletions were identified.

For comparative phylogenetic analysis of DENV-1, we selected a *CprM* gene interval of 354 nucleotides (nucleotides 208 to 561) of DENV-1 from the previously reported global sequences. On sequence analysis it was observed that the 2006 Delhi DENV-1 *CprM* sequences showed sequence similarity of 98.6—97.7% (mean 98.15%) and 97.2—97.7% (mean 97.45%), respectively, with the 1993 sequences from Singapore and Comoros. On comparison with other Indian sequences from Delhi sampled in 1982 and 1970 (closely related to outbreak-associated strains from Myanmar), we observed sequence similarity of 97.7—98% (mean 97.75%) and 95.8—96.3% (mean 96.05%), respectively. Three nucleotide changes (all transitions) were found in common in the 2006 Delhi DENV-1 sequences, 1993 Singapore/Comoros sequences, and 1982 Delhi sequences: T→C transitions were observed at nucleotide positions 208 and 532, and a G→A transition at nucleotide position 550. However, in 2005 DENV-1 sequences from Delhi, three changes were in common with strains from South America (Brazil, Argentina, and Paraguay) *viz*. T→C at nucleotide position 244, C→T at position 253, and A→G at position 388. Two nucleotide changes (C→T at position 469 and T→G at position 520) were found exclusively in 2006 DENV-1 sequences from Delhi. Sequence divergence of 3.1% and 4.8% was observed when 2006 Delhi sequences were compared with 2005 Delhi sequences and 1962—64 Vellore (South India) sequences, respectively. The DENV-1 Delhi sequences of 2006 of this study also showed a sequence divergence of 0.8% among themselves. Comparison with reference sequences revealed that only transition mutations occurred in 2006 Indian (Delhi) DENV-1 sequences, which were mostly synonymous in nature. Non-synonymous mutations were observed amongst 2006 Delhi isolates 01den06 and 03/1/del2006, where isoleucine was replaced by threonine (amino acid position 59), and in 02den06 and 04/1/del2006 where leucine was replaced by serine (amino acid position 155).

A phylogenetic tree generated on the basis of the sequences of the present study and previously reported DENV-1 *CprM* sequences revealed clustering of isolates from different countries in three distinct genotypes (genotypes I, II, and III) (Figure 1). However, it is noteworthy that all Indian DENV-1 sequences (from this study and those reported by

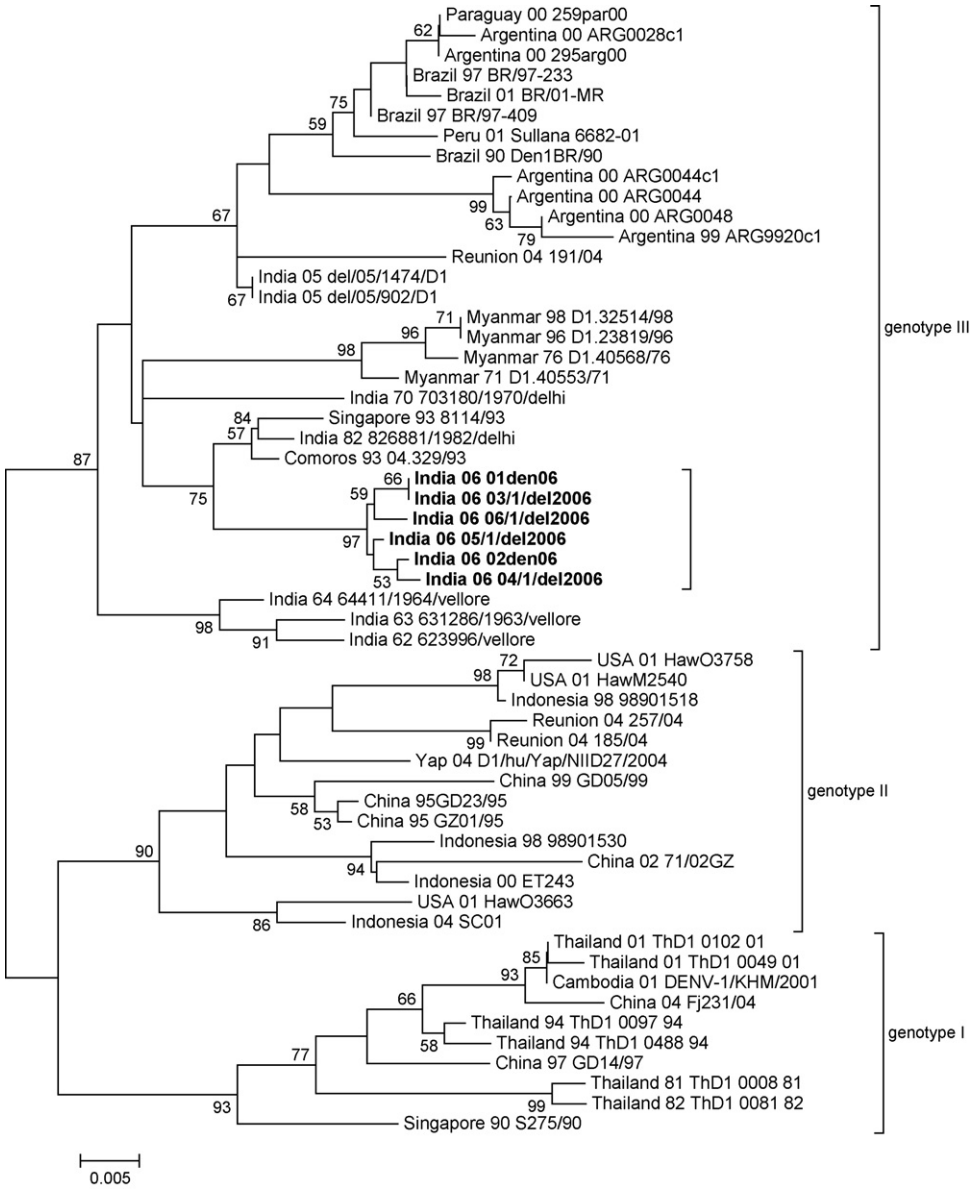

Figure 1 DENV-1 phylogenetic tree. The tree was generated by the neighbor-joining method based on 354 bp nucleotide sequences of *CprM* gene region. Each strain is denoted by country of isolation and last two digits of year of isolation, followed by virus ID. Bootstrap support values (>50%) are shown for major nodes on the tree. All horizontal branch lengths are drawn to scale. DENV-1 sequences of the present study are denoted in bold.

other workers) belonging to different outbreaks, clustered in genotype III in close proximity with the viruses from South America, Myanmar, Africa, and Singapore, indicating circulation of DENV-1 genotype III in India. Although, within genotype III, six DENV-1 isolates of the present study clustered as a distinct subclade, these were found to be closely related to 1993 isolates sampled from Comoros and Singapore and 1982 isolate sampled from Delhi, India. However, based on the supportive bootstrap value (Figure 1), it is evident that a new lineage of DENV-1 in Delhi has evolved recently. It was observed that these six DENV-1 strains of 2006 were also related to 1970 Delhi strain and outbreak-associated Myanmar strains of 1971, 1976, 1996, and 1998. Indian isolates of 2005 sampled in Delhi (del/05/1474/D1, del/05/902/D1) clustered with DENV-1 strains from the Americas, exhibiting close proximity with American lineage. Segregation of Delhi 2005 and 2006 isolates into two different groups in the phylogenetic tree (Figure 1) revealed that there was independent existence of two distinct lineages of DENV-1 in this part of the country. Three DENV-1 strains that circulated in the southern part of India during 1962—64 (631286/1963/Vellore, 623996/Vellore, and 64411/1964/vellore) clustered separately (third lineage) from other Indian strains. Three distinct lineages of Indian DENV-1 genotype III (1962—64 Vellore lineage and Delhi 2005 and 2006 lineages) are evident from the phylogenetic tree (Figure 1). However, whether the existence of Vellore (South India) lineage still exists cannot be confirmed because of the non-availability of south Indian sequences of recent years. Genotype II was more cosmopolitan, with viruses from Hawaii, Indonesia, China, Yap State,

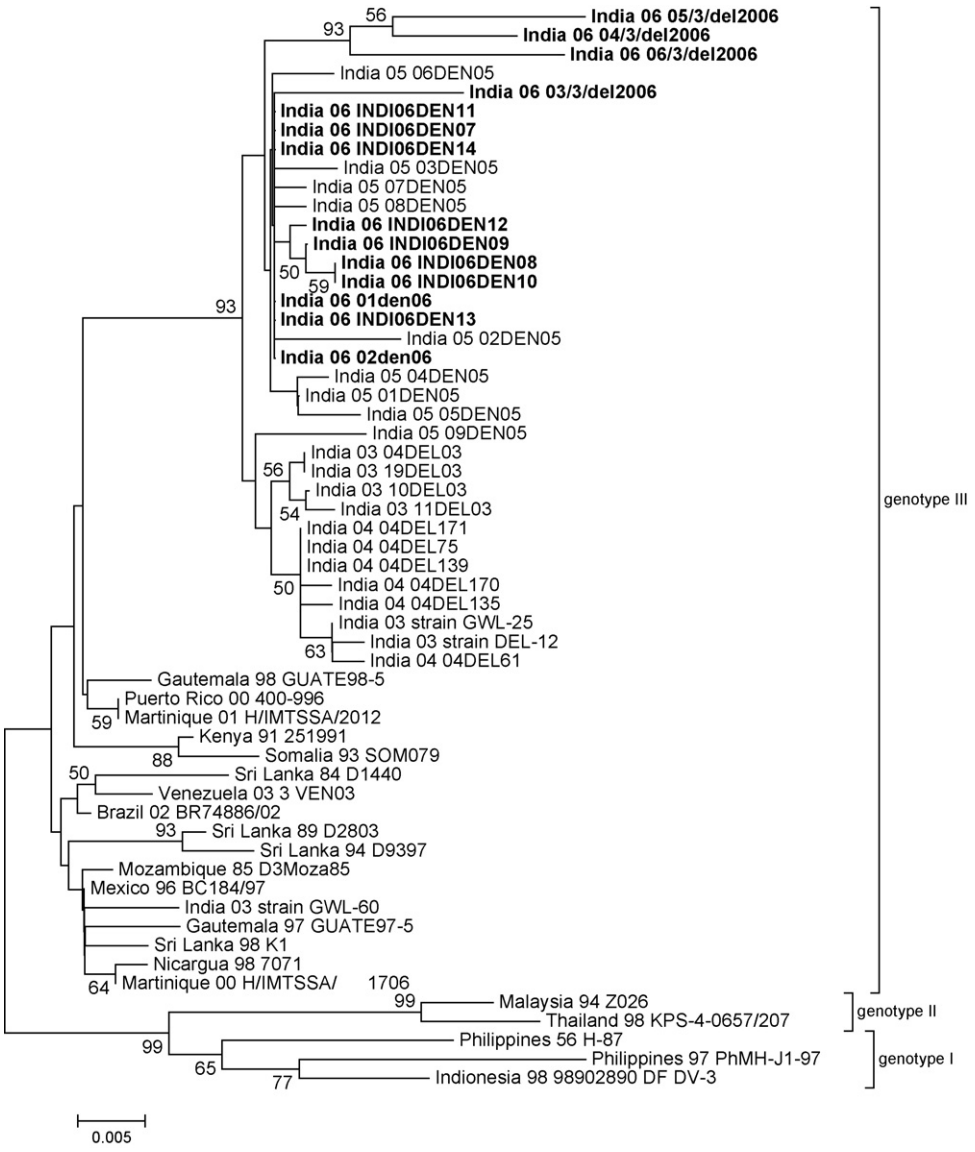

Figure 2 DENV-3 phylogenetic tree. The tree was generated by the neighbor-joining method based on 434 bp nucleotide sequences of *CprM* gene region. Each strain is denoted by country of isolation and last two digits of year of isolation, followed by virus ID. Bootstrap support values (>50%) are shown for major nodes on the tree. All horizontal branch lengths are drawn to scale. DENV-3 sequences of the present study are denoted in bold.

and Reunion Island, but genotype I mainly consisted of viruses from Southeast Asia, particularly Thailand and China.

Depending on the availability of global DENV-3 sequences for comparison in the database, a 433 bp region (nucleotides 180—612) of the DENV-3 *CprM* gene junction was selected for phylogenetic analysis. When DENV-3 *CprM* sequences were analyzed, it was observed that 2006 Delhi sequences were closely related to sequences from Guatemala (1998) and presented a nucleotide identity of 95.9—98.2% (mean 97.05%). On comparison of Delhi 2006 sequences with other Indian sequences from years 2003, 2004, and 2005, mean sequence divergence of 2.85%, 2.15%, and 1.6%, respectively, were observed. Common amino acid mutations observed in 2006 DENV-3 sequences were isoleucine→valine (amino acid position 88) and aspartate→asparagine (amino acid position 121) in 04/3/del2006 and 06/3/del2006; isoleucine→phenylalanine (amino acid position 127), glycine→glutamate (amino acid position 122), and arginine→lysine (amino acid position 55) in 04/3/del2006 and 05/3/del2006; and valine→glycine (amino acid position 128) in INDI06DEN08, INDI06DEN10, and INDI06DEN12.

A phylogenetic tree constructed on the basis of DENV-3 sequences from different parts of the world segregated into three genotypes (Figure 2). Fourteen DENV-3 strains of the present study clustered within genotype III of DENV-3 along with the previously reported Indian and global sequences from the Caribbean, Americas, Africa, and Sri Lanka. Our 2006 Delhi DENV-3 sequences were closely related to Guatemala strain (GUATE98-5). Genotype II comprised viruses from Malaysia and Thailand isolated in 1994 and 1998, respectively. Three DENV-3 strains (Philippines 56 H87, Indonesia 98902890 DF DV-3, and Philippines PhMH-J1-97) clustered

together to form genotype I. Due to non-availability of the DENV-3 genotype IV *CprM* global sequences, we could not include these while constructing the phylogenetic tree.

Discussion

India is endemic for dengue having witnessed several DF/DHF outbreaks in the last decade.[3,4,6,25] The last major DF outbreak in India occurred between September and October 2006 involving more than 12 000 cases and nearly 184 deaths, of which approximately 3366 cases and 65 deaths were reported from Delhi alone.[26] Molecular characterization of the viruses implicated in this outbreak was deemed necessary with the view to: (1) identify molecular subtype/genotype of the dengue virus, (2) determine the introduction of new or evolving lineages, if any, and (3) elucidate the changing pattern of molecular epidemiology of dengue viruses in this part of India.

Particular genotypes are associated with disease severity and changes in the prevalent type may also lead to increased severity of the outbreak. In our study, an interesting pattern of emergence and circulation of dengue viruses in Delhi has come to light. From 1996, DENV-2 genotype IV was circulating in Delhi[3] when DENV-3 in co-circulation with DENV-2 emerged in 2003,[6] replacing DENV-2 in subsequent years. During the present study on the 2006 Delhi DF outbreak samples, we observed emergence and co-circulation of DENV-1 with predominance of DENV-3, thereby increasing the risk of severity of the disease in a population already exposed to a previous serotype. Such study of epidemiological dynamics in populations in which more than one DENV serotype co-circulate simultaneously is of special significance.[27] If the trend observed in our present study continues, the probability of dominant emergence of DENV-1 (suppressing the pre-existing DENV-3) in coming years cannot be ruled out. Emergence of a new virus serotype is mostly associated with severe disease outbreaks, thereby making the situation more alarming.

CprM gene-based RT-PCR using the specific primer set evaluated by Lanciotti et al. in 1992 and Harris et al. in 1998[19,28] has the potential to identify all four dengue virus serotypes. In addition, studies suggest that *CprM* gene sequences also produce epidemiologically valuable information. The usefulness of the *CprM* gene junction for molecular epidemiology of DENV-2 and DENV-3 strains has been established time and again by us and other researchers.[4,5,20,21] We have studied the sequence of these dengue viruses directly from the patient sera as has also been supported by other researchers.[5,16]

Analysis of translated amino acid sequences revealed changes in our DENV-3 isolates, INDI06DEN08, INDI06DEN10, and INDI06DEN12 where non-polar valine was replaced by non-polar glycine, hence no significant change. In addition, in amino acid changes in 04/3/del2006, 06/3/del2006 (isoleucine→valine and aspartate→asparagine) and 04/3/del2006, 05/3/del2006 (isoleucine→phenylalanine and arginine→lysine), no significant changes were observed in hydropathy index.[29] However, in DENV-1 2006 Delhi isolates 01den06 and 03/1/del2006, where polar isoleucine was replaced by non-polar threonine, and again in 02den06 and 04/1/del2006, where polar leucine was replaced by non-polar serine, the implications in terms of disease severity could not be established.

Five different genotypes of DENV-1 and four genotypes of DENV-3 have been proposed.[13,25,30] On analysis of *CprM* sequences of DENV-1 (354 bp) and DENV-3 (433 bp) of our study, we observed the presence of three genotypes each in DENV-1 and DENV-3. Phylogenetic analysis revealed that 2006 DENV-3 strains of this study were closely related to the strains that circulated in Delhi between 2003 and 2005, which were similar to strains from Guatemala.[6,31] In the case of DENV-1, we observed clustering of sequences below the genotype level that correlated with the geographical origin and/or time of isolation, which appears to represent distinct lineages. Interestingly, the data retrieved from NCBI GenBank revealed the existence of a different lineage of DENV-1 in South India (Vellore) during 1962–64 (accession numbers **AY593212** and **AY593211**) and another lineage in Delhi during 2005 (accession numbers **EF064776** and **EF064774**). However, all the six DENV-1 strains of 2006 in the present study may have evolved from Delhi 1982 or Singapore/Comoros 1993 strains.

The dendrogram suggests that Delhi had at least two different lineages of DENV-1 emerging independently during two consecutive years (2005 and 2006), 2005 strains clustering with American lineage, while 2006 strains forming a distinct lineage, but clustering with the Indian (1982), African (Comoros), and Southeast Asian (Singapore) strains. Based on E gene sequences, Domingo et al.[32] also reported the presence of two new lineages of Indian DENV-1 strains belonging to years 2001–2005. Given the geographical or temporal association among sequences belonging to the same lineage, a classification based on lineages facilitates surveillance and the tracking-down of the origin of dengue virus isolates.[32]

The inadequate availability of Indian sequence data for the past years from homologous gene region has been a limiting factor that prevents researchers from interpreting information on phylogeny of these viruses. In our study, DENV-1 strain (S275/90) sampled in Singapore in 1990 grouped in genotype I. However, this strain was classified as genotype III on the basis of full-length E gene sequences,[25] where it clustered with strains from the Americas, Africa, and Southeast Asia. Domingo et al.[32] found this strain to be a product of a recombination event with parents from different lineages. This strain is a recombinant between American and Asian lineage. Such recombinations may be revealed at times when phylogenetic trees are drawn on the basis of different genes, thereby explaining the reason for ambiguity in results.

The dendrogram and increase in divergence of DENV-3 sequences each year (Figure 2) suggests that these viruses have undergone microevolution, as is evident from the temporal structure of the distinct clade of these viruses, and that they have been constantly evolving each year since its emergence in 2003, although the stochastic or selective pressure on this virus has not been studied. A DENV-3-associated outbreak was last reported in India in 1994.[33] However, DENV-3 was reported to be the etiologic agent of the first major DHF outbreak in neighboring Bangladesh in 2001[34] and was also implicated in outbreaks in Sri Lanka in the recent past.[35,36] Furthermore, there was no indication of either serotype confirming association of any particular variant with serious dengue disease, as the viruses isolated from DHF patients fell at different locations on the phylogenetic tree. No significant correlation was seen between

the *CprM* sequences and the differential clinical severity, confirming the absence of any known virulence marker in this region.

It is possible that the emergence of an outbreak-associated strain/lineage of DENV-1 along with the pre-existing DENV-3 in Delhi may result in another major DF/DHF outbreak in the near future. The changing epidemiology of dengue virus infection in Delhi is a cause of concern, more so because the emergence of a newer genotype and/or lineage may hamper timely forecasting of the probable serotype/genotype of the virus. Findings of any such study will be useful if an early warning signal on the emerging genotypes (particularly DHF-causing strains) is conveyed to the public health authorities before or during the early phase of the outbreak, so that the recommended control measures are implemented stringently and in time.

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Conflict of interest: No conflict of interest to declare.

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