Original Article

Continued Persistence of a Single Genotype of Dengue Virus Type-3 (DENV-3) in Delhi, India Since its Re-emergence Over the Last Decade

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BACKGROUND/PURPOSE: The re-emergence of an epidemic strain of dengue virus type-3 (DENV-3) in Delhi in 2003 and its persistence in subsequent years marked a changing trend in dengue virus circulation in this part of India. Its evolving phylogeny over the past decade has not been studied in detail as yet.

METHODS: Reverse transcription polymerase chain reaction and sequencing of the CprM gene junction of DENV-3 from different outbreaks since 2003 was carried out. Thirty CprM DENV-3 sequences from this study were compared with 46 other previously reported CprM DENV-3 sequences from India and other countries. Multiple sequence alignment and phylogenetic trees were constructed to determine the extent of genetic heterogeneity and trace the phylogeny of DENV-3.

RESULTS: Thirty CprM DENV-3 sequences (Accession numbers AY706096–99, DQ645945–52, EU181201–14, and EU846234–36) were submitted to GenBank. The CprM junction was found to be AT rich (approximately 53%). Nucleotide sequence alignment revealed only nucleotide substitutions. Phylogenetic analysis indicated sustained evolution of a distinct Indian lineage of DENV-3 genotype III in Delhi.

CONCLUSION: Active circulation of DENV-3 genotype III over the last decade in Delhi was evident and worrying. This genotype has been implicated in several outbreaks in South-East Asia and other parts of the world.

KEYWORDS: dengue virus, genotype, India, lineage, phylogeny

Introduction

The re-emergence of dengue virus infection is assuming epidemic proportions in various parts of the world, and at a greater frequency than previously experienced.1-5 The first recorded epidemic of dengue fever (DF), also known as dengue hemorrhagic fever (DHF), in the Asian subcontinent occurred during the 1950s. Almost 30 years later, it appeared in the Americas for the first time.6,7 Coincidentally, several outbreaks of DF were also reported in different
parts of India during the same period. The metropolitan city of Delhi reports approximately 33% of the total yearly cases reported in India. Delhi has a humid, monsoon influenced subtropical climate with daily temperatures ranging from 25–30°C, providing a conducive environment for *Aedes* mosquito breeding. The other factors that contribute to an increased population of the disease transmitting arthropod vector, *Aedes aegypti*, are rapid urbanization, water storage practices and the use of water coolers among the general population. During the post-monsoon season, the mosquito vector thrives well and transmits dengue virus amongst the human population which sometimes giving rise to a major outbreak depending on the prevalent genotype of dengue virus or its serotype.

Delhi experienced its first major DF outbreak in 1996, during which we observed complete replacement of the genotype V Dengue virus type (DENV)-2 by a more virulent genotype IV DENV-2. Vigilance over the following years confirmed a continued dominance of this DENV-2 genotype, similar to the FJ 10/11 strain in China, in Delhi and adjoining Northern India until the end of 2002. During a major DF outbreak in 2003, we suddenly observed a re-emergence and abundance of DENV-3 containing close sequence identity with a strain isolated in Guatemala in 1998. The magnitude of the DF outbreak during 2004 was relatively low, but a study by Dash et al of six isolates from the 2004 outbreak confirmed circulation of similar strains of DENV-3 in Delhi. Active circulation of this virus continued throughout 2005 and by the post-monsoon period of 2006, the whole of northern India including Delhi again witnessed a large-scale outbreak of DENV-3 which sometimes giving rise to a major outbreak depending on the prevalent genotype of dengue virus or its serotype.

**Materials and methods**

**Clinical samples**

Approval of the institutional ethical committee was obtained to carry out the present study. Acute phase serum samples were collected from suspected clinical cases of DF from different geographical locations in Delhi. Samples were collected during the post-monsoon season each year from 2003. Informed consent from all patients was obtained before collection of clinical samples.

**RNA extraction**

Viral RNA was isolated from 140 μL of serum samples or culture supernatant using a QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. Final elution was done in 50 μL of diethyl pyrocarbonate-treated water before storing at –80°C until use.

**cDNA synthesis and amplification of CprM junction**

Reverse transcription polymerase chain reaction (RT-PCR) was carried out as described previously using the CprM gene-specific primers, D1 and D2, which amplified a 511-bp amplicon as reported by Lanciotti et al.20 Briefly, cDNA was synthesized in a 10 μL reaction volume comprising 5 × RT buffer, 10 mM dNTPs, 100 mM DTT, 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 2 pmols of D1 primer, on a ABI 9700 PCR thermal cycler. The thermal profile consisted of an initial
denaturation step at 95°C for 10 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and a final extension at 72°C for 10 minutes. The PCR products were gel purified from 1.2% (w/v) agarose gels using the Accu-Prep Gel Extraction Kit (Bioneer, USA). Positive samples were selected for automated nucleotide sequencing.

Automated nucleotide sequencing
Thirty amplified products from RT-PCR positive samples, from 2003–07, were subjected to automated nucleotide sequencing. Sequencing was carried out using Big dye-terminator cycle sequencing ready reaction kit (Applied Biosystems). For each sequencing reaction approximately 25 ng of purified PCR product was mixed with 3.2 pM of respective primer (D1 or D2) and a reaction mixture containing AmpliTaq DNA polymerase and four dye-labeled di-deoxy nucleotide terminators. The reaction was placed onto a pre-heated thermal cycler. Cycle sequencing parameters consisted of 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The reaction mixture was purified by precipitation with 3 M Sodium acetate (pH 4.6) and 75% isopropanol and the product vacuum dried. The DNA pellet was re-suspended in 10 μL of template suppression reagent, heated at 95°C for 2 minutes and immediately chilled on ice, then mixed and briefly centrifuged before loading onto an ABI 310 automated capillary DNA sequencer (Applied Biosystems).

Database submission
Thirty DENV-3 CprM sequences were determined in this study, submitted to GenBank at www.ncbi.nlm.nih.gov, and accession numbers acquired. Prior to this, BLAST searches were carried out to confirm the virus type. See Table 1 for a description of these viruses. An intermediate region of 425 bp was selected for multiple sequence alignment.

Sequences comparison
Thirty seven previously reported DENV-3 CprM sequences from different geographical locations (South-East Asia, Africa, Americas) and nine sequences from India were retrieved from the National Center for Biotechnology Information database (Table 2) and selected for comparison with the 30 DENV-3 sequences from this study. Sequences are designated by the country of isolation and the last two digits from the year of isolation, and identified by virus identity.

Multiple sequence alignment
Nucleotide sequences from sense and anti-sense strands were aligned using Sequence Navigator software. Multiple sequence alignment was performed using BioEdit software version 7 employing the Clustal W multiple alignment option. These nucleotide sequences were translated into amino acid sequences for comparison with other Indian and globally submitted sequences. The Lasergene 5 software package (DNASTAR Inc, Madison, WI, USA) was used to examine the percent identity and diversity among sequences.

Phylogenetic tree
Phylogenetic analysis was carried out using MEGA version 3.1. A phylogenetic tree was constructed employing the Neighbor Joining method with bootstrap analysis of 1000 replicates. The tree was rooted using DENV-1, DENV-2 and DENV-4 CprM sequences (GenBank accession numbers NC_001477, NC_001474, and NC_002640, respectively).

Results
A total of 30 DENV-3 isolates from 2003–07 (4 from 2003, 9 from 2005, 14 from 2006 and 3 from 2007) were confirmed by RT-PCR, partial nucleotide sequencing and Blast analysis in this study. CprM sequences of these 30 DENV-3 isolates from Delhi were submitted to GenBank and accession numbers obtained (Table 1). An intermediate region of 425 bp from these sequences were aligned (nt 186–610) with nine previously reported Indian sequences and 37 previously reported sequences globally. Sequence alignment revealed only nucleotide substitutions, with no insertions or deletions apparent. This region was found to be AT rich with the AT composition of the 30 DENV-3 sequences in this found to vary from 52.07–54.15%. The majority of mutations observed in the Indian DENV-3 CprM sequences were transitions that were mostly synonymous in nature. All the Indian sequences, except for one from Gwalior isolated in 2003 (GWL-60), exhibited a mean sequence identity of 98%
Table 1. Indian DENV-3 isolates sequenced in this study

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²Designated by country of isolation and the last two digits of the year of isolation, followed by virus ID. DF = dengue fever; DHF = dengue hemorrhagic fever.

with Guatemala 98, and 98.45% with Martinique 01 and Puerto Rico 00 sequences. GWL-60 was divergent from other Indian sequences and demonstrated sequence identity of 98.6–99.3% with the Sri Lanka 98, Martinique 00, Guatemala 97, Mexico 96 and Nicaragua 98 sequences. When the GWL-60 sequence was compared with other Indian sequences, seven nucleotide substitutions were observed. Four T>C transitions at nucleotide positions 203, 256, 271 and 289; two C>T transitions at positions 206 and 295; and a single G>A transition at position 265 could be seen. None of these nucleotide substitutions gave rise to any amino acid changes. A year by year analysis of Indian DENV-3 sequences revealed that Guatemala 98, Martinique 01 and Puerto Rico 00 exhibited a mean sequence identity of 98.25% with the 2003 sequences, 97.75% with 2004 sequences, 98.6% with 2005 sequences, 97.5% with 2006 sequences and 97.75% with 2007 sequences. All Indian sequences showed a sequence divergence of 0.2–3.4% amongst themselves, with a mean year-wise divergence of 1.20–1.65%. In the 2003 and 2004 Indian sequences a “T” was present at nucleotide position 358. This was replaced by a “C” residue in all Indian
### Table 2. Indian and global DENV-3 reference sequences used for comparison in this study

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*aDesignated by country of isolation and the last two digits of year of isolation, followed by virus ID.*
sequences isolated after 2005. The transition at this position did not produce any amino acid changes in the deduced amino acid alignment. Nucleotide changes were also observed in virus sequences from each year, with 2003 and 2004 sequences exhibiting a lower number of mutations than sequences from 2005–07. Although the 2006 sequences, 27/3/del2006 and 28/3/del2006 contained two G>C transversions (nucleotide positions 281 and 554), one A>C transversion (nucleotide position 555) and 1 A>G transition (nucleotide position 564); only 2 G>C transversions gave rise to two amino acid changes, alanine to proline and aspartate to proline at amino acid positions 63 and 154, respectively. The isolate D3/1CprM/Del07 contained a single A>C transversion at nucleotide position 197, resulting in an amino acid change at position 35 where lysine was replaced by glutamine. However, in sequences D3/2CprM/Del07 and D3/3CprM/Del07, two nucleotide substitutions, A>G and C>T at nucleotide positions 277 and 307, did not result in any amino acid changes.

A phylogenetic tree was constructed using pair-wise comparison of a 425 nt region from the CprM junction (nt 186–610) of virus isolates sequenced in this study and from other countries including Asia, Africa and Americas (Figure). The phylogenetic tree demonstrated that all DENV-3 isolates were clustered in three distinct genotypes with all Indian DENV-3 isolates from 2003–07 classified as genotype III yet divergent as a distinct Indian lineage. This lineage shows a close relationship to the Guatemala 98, Puerto Rico 00 and Martinique 01 isolates. The 2003 Indian isolate, GWL-60, tended to cluster with viruses from Sri Lanka, Martinique, Nicaragua, Mexico and Guatemala isolated from 1996–2000. Genotype III consisted of viruses from various regions of the world such as the Americas, Africa and South-East Asia. DENV-3 strains from Africa (Mozambique 85, Kenya 91 and Somalia 93) clustered together forming a separate group within genotype III. Similarly, strains from Singapore isolated in 2004 and 2005 could also be grouped together with the Brazil 02, Venezuela 03 and Sri Lanka 84 isolates. Other viruses amongst this genotype did not show geographical clustering.

The oldest virus strain, H-87, isolated from the Philippines in 1956 was classified as genotype I clustering with viruses from Indonesia and Philippines isolated from 1988–2004. Genotype II consisted of two viruses from Thailand and Malaysia isolated in 1998 and 1994, respectively. Due to the unavailability of the DENV-3 genotype IV CprM sequences we could not include these while constructing the phylogenetic tree.

**Discussion**

Outbreaks of dengue and dengue hemorrhagic fever have been reported from almost all tropical and sub-tropical regions of the world. This is probably due to the significant increase in human and mosquito populations courtesy of rapid urbanization and increased frequency of long-distance travel by humans. Although DHF has been endemic in India, four known serotypes have been implicated in various outbreaks in the past, with most major and severe outbreaks of DF/DHF having been caused by DENV-2. In 2003, there was a noticeable shift in the cause of these outbreaks from DENV-2 to DENV-3. From 2003 onwards, DENV-3 has been found to be the predominant dengue virus circulating in Delhi, however DENV-1 was also reported to circulating from 2006.

In this study we carried out a molecular epidemiological study of DENV-3 that has been circulating in Delhi since its re-emergence in 2003, using sequence comparison and phylogenetic analysis. Genotypic identification has proven to be a useful tool for determining the origin and spread of epidemics and in correlating virulence of strains. Various genomic regions of dengue viruses have been selected by researchers for molecular phylogenetic analysis in the past. Although the dengue virus genome has three major structural genes, known as the capsid, pre-membrane and envelope genes, along with seven non-structural genes, many studies have reported the CprM junction as a powerful target in genotyping. The CprM junction was found to harbor epidemiologically important sequence information with a single pair of primers able to be used for amplification and sequencing of any of the four dengue virus serotypes. For phylogenetic analysis of our sequences, we retrieved previously reported sequences of the same region from the National Centre for Biotechnology Information GenBank database.

Analysis of Indian DENV-3 sequences revealed features that are characteristic of the CprM junction. It was found that most of the mutations in our sequences were
Figure. Phylogenetic tree of DENV-3. The tree was generated based on a 425 bp region of the CprM gene junction. Each isolate was designated by country of isolation and the last two digits of year of isolation, followed by the 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 Indian sequences used for comparison in bold italics.
silent and that the region examined was AT rich, hence more prone to mutations. The hydrophilic amino acids were mostly found on the surface of the protein and thought to be involved in immunological interactions. A number of amino acid changes were observed in the 2006 and 2007 sequences where non-polar alanine was replaced by polar proline and positively charged lysine replaced by uncharged glutamine. These changes might not be very significant in terms of antigenicity as the hydrophathy index did not show any major differences.\textsuperscript{32} However, another amino acid change from negatively charged aspartate to non-polar proline also occurred in these isolates. The implications of unique changes in recent Indian DENV-3 warrant further studies to understand their virulence and epidemic potential. The 2006 and 2007 Delhi isolates exhibiting amino acid changes were isolated from DF patients and no specific amino acid substitution could be identified with a severe form of disease. It is noteworthy to mention that the four Indian isolates from this study causing DHF in patients (Table 1) did not exhibit any common nucleotide or amino acid substitutions but when compared with the amino acid sequences of DENV-3 causing DF they showed identical results. No significant correlation was seen between the CprM sequences and differences in clinical severity, confirming the absence of any known virulence marker in this region.

The phylogenetic tree classified all 76 DENV-3 sequences into their respective subtypes/genotypes as designated by Lanciotti et al.\textsuperscript{18} All 30 Indian isolates belonged to genotype III along with other geographically diverse strains. However, they clustered as a distinct Indian lineage, thereby suggesting independent evolution of these viruses. Indian isolates from this lineage were found to be closely related to a Central American isolate, Guatemala 98 (Guate 98-5), suggesting this virus to be the progenitor for this lineage. A single Indian isolate, India 03 GWL60, was classified into another group suggesting alternative origin from the other Indian strains. This study confirms previous reports that circulating Indian DENV-3 strains are related to strains that caused dengue outbreaks in Guatemala and Martinique in the 1990s.\textsuperscript{33}

The oldest DENV-3 strain, Philippines 56, was classified as genotype I along with other strains from the Philippines and Indonesia isolated over two decades, suggesting the persistence of this genotype in the area for a long time.

It is evident that genotype III of DENV-3 circulates throughout the world, whereas other genotypes are localized in particular geographic regions. This indicates a higher potential of genotype III to spread and dominate in geographically diverse regions of the world. This genotype has also been implicated in major dengue epidemics in several parts of Asia, Africa and the Americas and has the potential to cause an international dengue pandemic.\textsuperscript{34}

The findings of this study indicate independent evolution of DENV-3 leading to emergence of a distinct Indian lineage within genotype III since 2003. Since similar strains of DENV-3 genotype III have been reported to cause major outbreaks in Asia and other parts of the world, continued surveillance is warranted to monitor the incursion and spread of this virus so that timely and effective control strategies can be instituted before the onset of the next outbreak.

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