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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

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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.[8–11] The metropolitan city of Delhi reports approximately 33% of the total yearly cases reported in India.[12] 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.[4] 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.[13] 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.[14] The magnitude of the DF outbreak during 2004 was relatively low, but a study by Dash et al[15] 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 DF in which DENV-3 was implicated in approximately 70% of cases and DENV-1 in approximately 30% of cases studied.[16] In 2007, despite there having been DF outbreak, circulation of DENV-3 was confirmed by detection of three DENV-3 isolates from Delhi, and two isolates of DENV-1 were reported by us.[17] No DENV-3 isolates were detected in 2008. In spite of these sporadic observations on a limited number of samples indicating active circulation of DENV-3 in this part of India, the exact trends of viral evolution in terms of genetic heterogeneity over the current decade is not clearly understood. Genotyping of DENV-3 has been performed previously with the *CprM* junction most widely used in the genotyping of Indian dengue isolates.[18,19] The D1 and D2 primer pair reported by Lanciotti et al in 1992 are capable of amplifying a 511-bp region of the *CprM* in all four types of dengue viruses.[20] The usefulness of this *CprM* junction for carrying out subtyping of dengue viruses has been established by us and other workers.[13,15,16,21,22]

The present study was designed to elucidate the nucleotide sequence diversity in the *CprM* gene junction of dengue virus and to determine the phylogeny of DENV-3 that has persistently circulated in Delhi since its re-emergence in 2003.

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, Hidden, 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[16] 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, 20 U RNase inhibitor and 50 U MultiScribe™ RT (Applied Biosystems, Foster City, CA, USA) with 2 pmols of D2 primer. The RT mix was incubated at 42°C for 30 minutes followed by 99°C for 5 minutes to inactivate MultiScribe-RT. PCR of cDNA was carried out in a 50 µL reaction volume containing 1 × RT-PCR buffer, 1.75 mM $MgCl_2$, 0.8 mM dNTPs, 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.[23,24] 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.[25] A phylogenetic tree was constructed employing the Neighbor Joining method[26] 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

Virus isolate name[a]	Date of collection (dd/mm/yr)	Age (yr)	Sex	GenBank accession no.	Pathology
India 03 04DEL03	09.10.03	30	F	AY706096	DHF (grade II)
India 03 10DEL03	26.10.03	15	M	AY706097	DF
India 03 11DEL03	26.10.03	14	F	AY706098	DF
India 03 19DEL03	30.10.03	40	M	AY706099	DF
India 05 01DEN05	28.09.05	26	F	DQ645945	DF
India 05 02DEN05	28.09.05	19	M	DQ645946	DF
India 05 03DEN05	28.09.05	25	M	DQ645947	DF
India 05 04DEN05	02.10.05	20	F	DQ645948	DHF (grade II)
India 05 05DEN05	02.10.05	21	M	DQ645949	DF
India 05 06DEN05	02.10.05	27	M	DQ645950	DF
India 05 07DEN05	02.10.05	23	M	DQ645951	DF
India 05 08DEN05	06.10.05	31	M	DQ841951	DF
India 05 09DEN05	06.10.05	17	M	DQ841952	DF
India 06 15/3/del2006	10.10.06	5	F	EU181201	DF
India 06 16/3/del2006	10.10.06	37	M	EU181202	DF
India 06 17/3/del2006	10.10.06	6	F	EU181203	DF
India 06 18/3/del2006	17.10.06	9	M	EU181204	DHF (grade III)
India 06 19/3/del2006	17.10.06	10	F	EU181205	DF
India 06 20/3/del2006	17.10.06	45	F	EU181206	DF
India 06 21/3/del2006	17.10.06	26	M	EU181207	DF
India 06 22/3/del2006	23.10.06	8	M	EU181208	DF
India 06 23/3/del2006	23.10.06	54	F	EU181209	DF
India 06 24/3/del2006	23.10.06	13	M	EU181210	DHF (grade III)
India 06 25/3/del2006	26.10.06	14	F	EU181211	DF
India 06 26/3/del2006	26.10.06	22	M	EU181212	DF
India 06 27/3/del2006	26.10.06	49	F	EU181213	DF
India 06 28/3/del2006	26.10.06	17	M	EU181214	DF
India 07 D3/1CprM/Del07	09.10.07	26	M	EU846234	DF
India 07 D3/2CprM/Del07	09.10.07	33	M	EU846235	DF
India 07 D3/3CprM/Del07	17.10.07	41	F	EU846236	DF

[a]Designated 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

Sequence name[a]	Genotype	GenBank accession no.
Philippines 56 H-87	I	M93130
India 03 strain DEL-12	III	AY770513
India 03 strain GWL-25	III	AY770511
India 03 strain GWL-60	III	AY770512
India 04 04DEL61	III	DQ323037
India 04 04DEL75	III	DQ323038
India 04 04DEL135	III	DQ323039
India 04 04DEL139	III	DQ323040
India 04 04DEL170	III	DQ323041
India 04 04DEL171	III	DQ323042
Sri Lanka 84 D1440	III	AF547229
Mozambique 85 D3Moza85	III	AY665402
Indonesia 88 den3_88	I	AY858038
Sri Lanka 89 D2803	III	AF547232
Kenya 91 251991	III	AF547239
Somalia 93 SOM079	III	AF547240
Malaysia 94 Z026	II	AB010990
Sri Lanka 94 D9397	III	AF547235
Mexico 96 BC184/97	III	AF547252
Philippines 97 PhMH-J1-97	I	AY496879
Guatemala 97 GUATE97-5	III	AB038473
Sri Lanka 98 K1	III	AF547243
Guatemala 98 GUATE98-5	III	AB038478
Nicaragua 98 7071	III	AF547262
Indonesia 98 98902890 DF DV-3	I	AB189128
Indonesia 98 den3_98	I	AY858039
Thailand 98 KPS-4-0657/207	II	AY912458
Martinique 00 H/IMTSSA/1706	III	AY099339
Puerto Rico 00 400-996	III	AF547264
Martinique 01 H/IMTSSA/2012	III	AY099340
Brazil 02 BR74886/02	III	AY679147
Venezuela 03 3 VEN03	III	AF547261
Indonesia 04 TB55i	I	AY858048
Indonesia 04 TB16	I	AY858047
Indonesia 04 PH86	I	AY858045
Indonesia 04 KJ71	I	AY858044
Indonesia 04 FW01	I	AY858040
Indonesia 04 BA51	I	AY858037
Singapore 04 D3/SG/SS710/2004	III	EU081181
Singapore 05 D3/SG/05K791DK1/2005	III	EU081182
Singapore 05 D3/SG/05K805DK1/2005	III	EU081185
Singapore 05 D3/SG/05K2418DK1/2005	III	EU081195
Singapore 05 D3/SG/05K4159DK1/2005	III	EU081217
Singapore 05 D3/SG/05K3312DK1/2005	III	EU081200
Singapore 05 D3/SG/05K3325DK1/2005	III	EU081204
Singapore 05 D3/SG/05K3927DK1/2005	III	EU081212

[a]Designated 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.[27] Although DHF has been endemic in India, four known serotypes have been implicated in various outbreaks in the past,[4,14–16,28] 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.[14,15] From 2003 onwards, DENV-3 has been found to be the predominant dengue virus circulating in Delhi,[14–16] however DENV-1 was also reported to circulating from 2006.[16,17]

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.[29] Various genomic regions of dengue viruses have been selected by researchers for molecular phylogenetic analysis in the past.[4,18,30,31] 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.[15,16,21,22] 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 hydropathy index did not show any major differences.[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.[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 and 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.[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.[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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