Unusual Emergence of Guate98-like Molecular Subtype of DEN-3 during 2003 Dengue Outbreak in Delhi

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Abstract

With a view to identifying the molecular subtype of the circulating dengue virus responsible for a major outbreak of dengue fever (DF) / dengue haemorrhagic fever (DHF) in and around Delhi during the post-monsoon period in 2003, 32 serum samples were collected from clinically suspected cases. These were subjected to reverse transcription/polymerase chain reaction (RT/PCR) for amplification of 511 bp C-PreM gene region of the dengue virus. Seven specimens, yielding a satisfactory quantum of viral RNA, were subsequently processed for automated nucleotide sequencing. Five of the seven analysed isolates showed close DNA sequence homology with Guate96-98 strains of DEN-3 virus, whereas two turned out to be genotype IV of DEN-2. Earlier, DEN-2 (genotype IV) had been identified as the etiological agent during a major DF/DHF outbreak in Delhi in 1996 and also in 2000. Though DEN-2 continues to prevail, DEN-3, having a close sequence homology with Guate96-98 strains, seems to have entered India for the first time in late 2003, resulting in a major DF/DHF outbreak. How the Guate96-98 strain of DEN-3 entered India remains to be linked epidemiologically.

Keywords: Dengue outbreak, molecular typing, CpreM gene, DEN-3, Delhi.

Introduction

Dengue fever/dengue haemorrhagic fever (DF/DHF) is caused by one or more of the four antigenically-related dengue virus serotypes DEN-1 to DEN-4. It is widespread in tropical and subtropical countries in the world and is a serious cause of morbidity and mortality, threatening about one third of the total human population[1-3]. Many outbreaks and epidemics of DF/DHF have been reported in different parts of India during the past four decades[4-8]. In Delhi alone, a number of outbreaks of dengue virus infection were recorded in 1967, 1970, 1982, 1988 and 1990[9-13]. Again in 1996, a major DHF outbreak, resulting in 10,252 cases with 432 deaths, occurred in and around Delhi. DEN-2 genotype IV was the predominant etiological agent[14,15].

Delhi and its adjoining areas were again struck by a major outbreak of DF/DHF between September and December 2003. The present study was undertaken to unveil the predominant molecular subtype of the dengue virus involved in this outbreak.

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Materials and methods

Clinical specimens

A total of 32 serum samples from clinically suspected acute cases of DF/DHF were collected from different hospitals in Delhi between September and December 2003. Most of the serum samples were collected within the first five days of the clinical onset. Samples were transported to the laboratory within six hours of collection and stored at –70 °C until processed. Thirty-two sera are considered adequate to establish the predominant molecular subtype of dengue virus.

Virus RNA isolation

Thirty-two serum samples were subjected to dengue viral RNA isolation. 140µl sera sample was processed for RNA isolation using QIAamp viral RNA Kit (QIAGEN, Germany) using standard kit protocol. Finally, viral RNA was eluted in 30µl nuclease-free water.

Reverse transcription/polymerase chain reaction (RT/PCR)

RT-PCR was carried out using previously reported D1 and D2 primers that were M13 tailed for the convenience of nucleotide sequencing. This primer set is capable of amplifying all the four types of dengue viruses (DEN-1 to DEN-4).

Primer D1 (with M13F tail)

(5'-TGTAAAACGACGGCCAGTTCAATATGCTGAAACGCGCGAGAAACCG-3')
(M13 forward primer sequence underlined)

Primer D2 (with M13R tail)

(5'-CAGGAAACAGCTATGACCTTGCACCAACAGTCAATGTCTTCAGGTTC-3')
(M13 reverse primer sequence underlined)

Complementary DNA (cDNA) synthesis and gene amplification of 511bp CpreM gene region of the dengue virus was performed using one step GeneAmp RNA Gold RT PCR Kit (Applied Biosystems, USA) with D1 and D2 primers for detection and typing of all the four types of dengue virus[16]. Briefly, 50µl reaction mix containing final concentration of 1X of 5X RT buffer, 1.5mM $MgCl_2$, 200µM dNTPs, 5mM dithiotheratol and 10pmol of D1 and D2 primers, 10U RNAase inhibitor, 15U of MuLV MultiScribe reverse transcriptase, 2.5U of Amplitaq Gold DNA polymerase and 5 µl of extracted viral RNA. RT was performed at 42 °C for 20 minutes on GeneAmp 9700 PCR System. Then pre-hold at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 minute and final extension for 72 °C for 10 minutes and hold at 4 °C. Appropriate positive and negative controls were used in RT/PCR. PCR products were electrophoresed on 1.5% agarose gel along with 100bp DNA ladder marker (MBI Fermentas, USA) and were visualized on gel documentation system (Biometra, Germany).

Gene sequencing and phylogenetic analysis

PCR amplicons were purified using Centricon-100 columns (Millipore, USA) and subjected to automated dideoxy chain

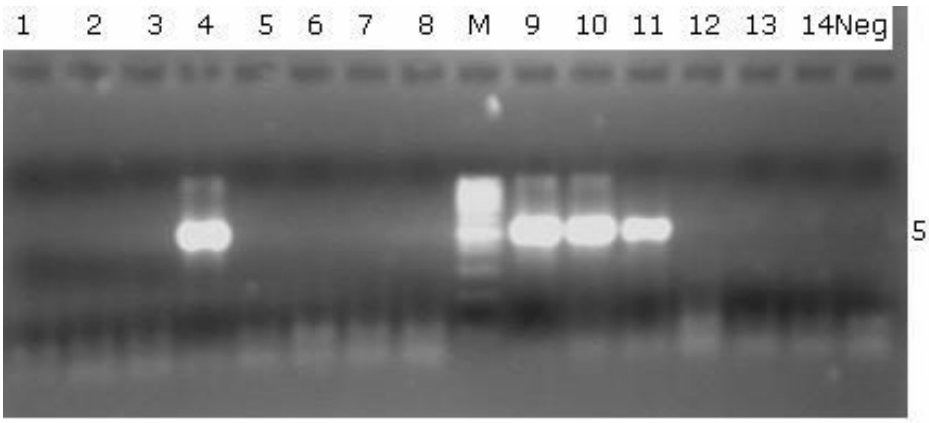
termination nucleotide cycle-sequencing using commercial ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit with Amplitaq DNA Polymerase FS, following the manufacturer's protocol and run on ABI PRISM™ 310 Genetic Analyser (Applied Biosystems, USA). Nucleotide sequences were edited and aligned using Sequence Navigator Software. Subsequently, blast search (http://www.ncbi.nlm.nih.gov/blast) and phylogenetic analysis using DNA Star software, were done to reveal the dengue virus molecular subtype.

Result and discussion

During the study, a majority of the subject patients had clinical symptoms of DF and only sporadic cases presented symptoms of DHF/DSS {DF: 24 (75%), DHF: 7 (21.9%) and DSS: 1 (3.1%)}. The samples belonged to all age groups ranging from 5 to 50 years. The male-female ratio was 18:14. The mean platelet count was 77,120, which ranged from 18,000 to 250,000. Most of the samples collected within five days from the onset of the fever were selected for RT-PCR testing.

Out of the 32 serum samples subjected to RT-PCR, only seven yielded amplification of 511bp C-PreM gene region of dengue virus. Lane 1-14 in the Figure included clinical samples, 100bp DNA ladder marker in lane M and negative control in lane Neg. Automated nucleotide sequencing of these seven RT/PCR products revealed two groups of sequences, the first group had five almost identical sequences, while the second group had two similar sequences. All samples were subjected to blast search, which revealed that five isolates had a very close sequence homology (=98%) with Guate98 AB038478 strain of dengue type 3 (DEN-3), while two turned out to be DEN-2 and showed =99% homology with Delhi96 AF047394 strain Delhi 2000 strains (Table). These Cpre M gene sequences were submitted in the Gene Bank wide accession AY 706094-AY706099.

Figure. ***PCR products visualization on 1.5% agarose gel***



Gel picture showing 511bp CpreM gene amplification of dengue virus. 1-14: dengue suspected clinical samples, M: 100bp DNA Ladder marker and Neg: negative control

Table. *Clinical and molecular analysis data of seven samples, yielding RT-PCR positivity for 511bp CpreM gene of dengue virus*

S. No.	Sample ID*	Serum collection	Clinical state	Age/Sex	Platelet count	RT-PCR 511bp	Assigned genotype on blast search
1	04DEL03	22.09.03	DF	30/F	89,000	+ve	DEN-3
2	06DEL03	24.09.03	DF	20/M	79,000	+ve	DEN-2
3	10DEL03	07.10.03	DHF	15/M	39,000	+ve	DEN-3
4	11DEL03	08.10.03	DF	14/F	NA	+ve	DEN-3
5	16DEL03	01.10.03	DF	34/F	18,000	+ve	DEN-2
6	19DEL03	04.10.03	DHF	40/M	38,000	+ve	DEN-3
7	26DEL03	13.10.03	DHF	16/M	36,000	+ve	DEN-3

Out of 32 samples subjected to RT-PCR for 511bp Cpre M gene of dengue virus, only 7 turned positive, while 25 did not show any amplification.
All five DEN-3 strains had =98% nucleotide sequence homology with Guate98 strains.
Both DEN-2 strains had =99% nucleotide sequence homology with already circulating Delhi96 and Delhi2000 strains.
NA: Information not available

Several studies have shown that dengue virus infection has been endemic in different parts of India, as documented for over four decades[17,18], and almost all the four known serotypes of dengue virus (DEN-1 to DEN-4) have been reported. The metropolitan city of Delhi witnessed several outbreaks of DF/DHF in 1967, 1970, 1982, 1988,1996 and 2000. DEN-1 and DEN-3 viruses were associated with the 1970 epidemic, DEN-1 and DEN-2 with the 1988 epidemic, while genotype IV of DEN-2 was responsible for the major DHF outbreak in 1996[15]. Our previous findings revealed genotype IV of DEN-2 as the predominant type circulating from 1996 onwards, based on RT-PCR and C-PreM gene sequencing, although cases of DEN-1 were also detected[19-21].

During the present study, the majority of the patients had clinical symptoms of DF and only sporadic cases presented with symptoms of DHF or DSS. The samples referred to our laboratory for molecular characterization were accompanied by IgM serology results. When cross-checked, we found that five RT/PCR-positive samples were IgM-negative, while two RT/PCR-positive samples were dengue IgM-positive. Previous studies had also shown that most, but not all, RT/PCR-positive samples had negative IgM serology. The reason for this is attributed to the fact that the virus had not been recovered from most of the DF/DHF patients beyond the 5th day of the onset of

the symptoms, while detectable levels of dengue-specific IgM antibodies appear after the 4th or the 5th day. Our findings re-established that the ideal time for the collection of samples for molecular test was between days 1-5.

The nucleotide sequence alignment and blast search of the seven RT/PCR-positive samples in the present study, when compared with those of earlier ones, revealed that only ~29% (2/7) belonged to the already prevalent genotype IV of DEN-2; whereas the majority ~71% (5/7) showed close genomic homology (=98%) with GUATE98 AB038478 strain of dengue type 3 (DEN-3) which caused a widespread dengue outbreak in Guatemala in the late Nineties[22]. A similar strain is also reported to have been reintroduced in Marlinique (French West Indies)[23] and in Rio de Janerio, Brazil (unpublished data vide Gene Bank Accession No. AY679147). The first reported evidence of DEN-3 in Delhi was in 1970, based on serotyping, but no genomic data of the 1970 strain of DEN-3 is available. It is difficult to ascertain, after a long gap of 33 years, whether the old 1970 strain of DEN-3 had re-emerged during the current outbreak; or Guate98 DEN-3 strain (prevalent in South American countries) had been introduced for the first time in India.

The changing epidemiology of different subtypes of dengue virus and their co-existence and/or replacement of one type by the other is well documented[24]. During the present outbreak, we found DEN-3 as the predominant type, but it did not seem to completely replace the previously circulating DEN-2. Prior to 1977, co-existence of DEN-2 and DEN-3 in the Americas had also been reported[25]. The first epidemic of DEN-3 in Jamaica and Puerto Rico was witnessed in 1963, which was followed by another epidemic of DEN-3 in Colombia and Puerto Rico in the mid-1970s, and in the Pacific islands in early 1980s[26]. In 1994, a new strain of DEN-3 was introduced in the Americas, causing a major epidemic of DF/DHF in Nicaragua and an outbreak of DF in Panama[27]. But this DEN-3 was genetically different from the DEN-3 strains which circulated in the Americas earlier. Interestingly, in 1994, this DEN-3 genotype was reported to have a close identity with those strains which caused DHF epidemic in some of the South-East Asian countries around the same period[28]. This DEN-3 strain subsequently spread from Asia to Central America and Mexico in 1995 and caused major epidemics. A classic example of the replacement of one type by the other is evident from the fact that, in 1971, DEN-2 was introduced into the Pacific areas followed by a new strain of DEN-1 in 1975 and DEN-4 by 1979, and in early 1980s by yet another new strain of DEN-3[29]. These reports support our current findings that, although DEN-2 (genotype IV) has been predominant in northern India over the past few years, Guate96-98-like DEN-3 strain dominated during the major outbreak of DF in Delhi in 2003.

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