USE OF NUCLEOTIDE SEQUENCING OF THE GENOMIC cDNA FRAGMENTS OF THE CAPSID/PREMEMBRANE JUNCTION REGION FOR MOLECULAR EPIDEMIOLOGY OF DENGUE TYPE 2 VIRUSES

Urvashi B Singh and Pradeep Seth

Department of Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-110029, India

Abstract. The recent emergence of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in India has been a source of concern. In the present study a quantitative comparison of 406 nucleotide long sequence from the capsid-premembrane junction region (C-PrM) of 9 dengue virus type 2 (DEN-2) isolates from Delhi with 10 DEN-2 isolates from diverse geographic areas provided sufficient information for estimating genetic relationships. The data indicated that the 1996 epidemic of DHF in Delhi was caused by genotype IV strains of DEN-2. This genotype, perhaps, displaced genotype V strains of DEN-2, which was circulating genotype in 1967. The period during which this displacement had occurred is not clear from the present study. Nonetheless, similar experience in four countries in Latin America and in Sri Lanka suggest that the introduction of new genotypes of DEN-2 displacing the circulating genotype may be associated with the appearance of DHF/DSS. More work is required to elucidate this hypothesis. Transitions at nucleotide positions 406 and 431 resulted in amino acid substitutions near (aa position 104, methionine → valine) and at the hinge region (aa position 112, valine → alanine) of C-PrM, respectively in all/most genotypes of group III and IV DEN-2 viruses analysed. Most of these virus strains have been isolated from DHF/DSS outbreaks. Significance of this observation is discussed. The data presented in this study suggest the utility of C-PrM sequence analysis for molecular epidemiology of dengue viruses.

INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) may be caused by any one of the four dengue virus serotypes (DEN 1 to 4) which are antigenically related but do not offer cross-protection. The dengue virus genome is a single stranded positive sense RNA of approximately 11Kb in length and encodes eleven distinct proteins. The gene order is capsid-premembrame/membrane-envelope-nonstructural proteins 1 to 5 (5′C-PrM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′). The first three proteins are structural proteins while the remainder are found either on the infected cell surface (NS1) or as intracellular proteins involved in virus replication.

Dengue viruses are spreading to newer geographical locations and increasing number of areas are becoming hyperendemic. The severe form of dengue fever, DHF/DSS has been attributed to several factors. Seroepidemiologic evidence suggests that DHF/DSS results from an immune enhancement produced by a secondary infection with another serotype (Halstead, 1988). Other evidence suggests that it could be related to an exceptional infection with highly virulent strains (Rosen, 1986).

Differences in virulence and/or transmission between dengue viruses have been seen

Correspondence: Dr Pradeep Seth, Department of Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-110029, India.
Tel: 91-11-6526814; Fax: 91-11-686 2663
Email:pseth@aiims.aiims.ac.in; pseth@medinst.ernet.in

in studies on sylvatic cycles in Western Africa (Robin *et al*, 1980), Vietnam (World Health Organization, 1976) and Malaysia (Rudnick, 1965). Reports from recent outbreaks of DHF in the Caribbean and South America (Rico-Hesse *et al*, 1990), Sri-Lanka (Lewis *et al*, 1993) and India (Singh *et al*, 1999) offer further indications of differences in virulence among dengue type 2 viruses.

Dengue virus variation has been studied by T_1 oligonucleotide fingerprinting of its RNA or genome sequencing, in order to classify viruses that share a common ancestry and infer their phylogeny (Rico-Hesse *et al*, 1990; Trent *et al*, 1983). Short genomic sequence analysis allows determination of genomic variability and rapid geographic classification (Rico-Hesse *et al*, 1990). Analysis of a short stretch of nucleotides from the envelope-nonstructural protein1 (E-NS1) junction revealed evolutionary relationship among dengue viruses (Rico-Hesse *et al*, 1990; Singh *et al*, 1999). Other studies used either E-protein gene region (Blok *et al*, 1989; Deubel *et al*, 1993; Lewis *et al*, 1993) or NS1 region (Blok *et al*, 1991) for phylogenetic analysis and reached similar conclusion.

In this report we demonstrate the use of nucleotide sequence of the C-PrM of DEN-2 isolates to compare them quantitatively and to study evolutionary relationships between them.

MATERIALS AND METHODS

Virus isolates and cell line

The DHF epidemic in Delhi began in mid August and lasted until mid November 1996. We collected eight isolates from patients presenting at different time during the epidemic (Table 1). Two isolates, 838/96 and 841/96, were from two children who had DF and DHF respectively and were admitted to our hospital during the initial part of the epidemic. Three isolates, 979/96, 980/96 and 1029/96 were from patients who were infected during the peak of the epidemic and three isolates 1432/96, 1436/96 and 1451/96 were from patients who reported towards the culmination of the epidemic. These viruses were isolated in *Aedes albopictus* cell line, C6/36, by standard technique (Henchal *et al*, 1983).

RNA extraction

Viral RNA was extracted by the method of Chomczynski and Sacchi (1987) with minor

Table 1
Dengue-2 virus isolates from 1996 and 1967 Delhi epidemics used for genome sequence analysis.

Virus isolate ID #	Date of receipt of sample	Age/Sex	Diagnosis	Genbank accession #	Passage history
838/96	16/09/96	7/F	DF	AF 047393	C6/36[a] 2[b]
841/96	16/09/96	7/M	DHF	AF 047394	C6/36[a] 2[b]
979/96	26/09/96	16/F	DF	AF 047395	C6/36[a] 2[b]
980/96	26/09/96	16/F	DF	AF 047396	C6/36[a] 2[b]
1029/96	30/09/96	9/M	DHF	AF 047397	C6/36[a] 2[b]
1432/96	24/10/96	20/F	DHF	AF 047399	C6/36[a] 2[b]
1436/96	24/10/96	20/F	DF	AF 047400	C6/36[a] 2[b]
1451/96	31/10/96	58/M	DHF	AF 047401	C6/36[a] 2[b]
1967	1967		DF	AF 047402	SM ?, C6/36[a] 2[b]

[a]C6/36: *Aedes albopictus* cell line; SM: suckling mouse brain.
[b]# of passages in the cell line; ? passage history not known.

modifications as described earlier (Singh *et al*, 1999). Briefly, virus infected cells were lysed with guanidine-isothiocyanate buffer and the cellular RNA was precipitated out and finally dissolved in DEPC (Diethylpyrocarbonate) treated water containing RNAsin.

Amplification of dengue virus RNA

A stretch of 511 bp of C-PrM (*134 - 644*) was amplified using forward primer D1 (*134-161*) 5′-TCAATATGCTGAAACGCGCGAG AAACCG-3′ and reverse primer D2 (*616-644*) 5′-TTGCACCAACAGTCAATGTCTT CAGGTTC-3′. These primers were described earlier by Lanciotti *et al* (1992) for detection and typing of all the four dengue viruses by PCR from clinical samples. Viral RNA was first reverse transcribed to c-DNA using reverse primer D2 and MoMLV reverse transciptase at 42ºC for 1 hour, followed by inactivation of RT at 99ºC for 5 minutes. Thirty cycle PCR amplification was performed in the same tube using the GeneAmp RNA PCR Kit (Perkin Elmer, USA) according to the manufacturer's instructions with following amplification profile: initial denaturation at 95ºC for two minutes followed by cyclic amplification consisting of denaturation (95ºC for 30 seconds), annealing (58ºC for 2 minutes) and extension (72ºC for 2 minutes). In addition, a final extension of 7 minutes at 72ºC was given at the end of 30 cycles. Positive displacement pipettes and cotton plugged disposable tips were used to avoid carryover contamination. Each test included a positive control, RNA extracted from uninfected C6/36 cells as negative sample control and a minimum of three negative controls containing distilled water in place of sample. The amplified product was electrophoresed into 1.2% agarose gel with molecular weight marker. The gel was stained with ethidium bromide and visualized in an ultraviolet transilluminator (UVP, USA).

DNA sequencing

The amplified products (amplicons) were purified by Wizard PCR Preps Purification system (Promega Corpn, USA) and subjected to sequencing on ABI 373 sequencer with the help of ABI prism, Dye terminator cycle sequencing kit (Perkin Elmer, Switzerland) as per manufacturer's recommendations. Both forward (D1) and reverse (D2) primers were employed to sequence both strands of the amplified product.

Phylogenetic analysis

The sequences thus obtained were aligned by Eyeball Sequencing Editor (ESEE) programme version 3.0s (Cabot, 1995). The phylogenetic analysis including estimation of DNA distances by Kimura two-parameter method, bootstrapping and generation of Neighbour-Joining tree were performed by Molecular Evolutionary Genetics Analysis (MEGA) package version 1.01 (Kumar *et al*, 1993). Sequences were compared with those of DEN-2 strains from different geographic areas that have been previously published (Table 2).

RESULTS

A short nucleotide sequence of cDNA of C-PrM encompassing nucleotides 171-576 (406bp) was selected for comparison among DEN-2 isolates to derive evolutionary information for epidemiologic interpretation on the basis of following criteria:

a) Zuckerkandl and Pauling (1965) suggested that the area of genome selected for phylogenetic study should show differences in base sequences in allelic stretches of DNA that do not lead to differences in amino acid sequence in the corresponding polypeptide chains. In other words, the base sequence of the codon may be changed but its translated product, that is amino acid, may remain the same, so that if the majority of mutations should occur in the third position of the codon they would be, therefore, mostly silent.

b) Eck (1963) proposed that middle letter of the triplet of each codon is recognized by transfer RNAs only as "purine" or "pyrimidine". Therefore, any base substitution of the

Table 2
Global dengue 2 viruses used for genome sequence analysis.

Virus Isolate ID#	Code (genotype)	Genbank accession No.	Country	Year
NGC (Prototype)	D_2NG (I)	AF 038403	New Guinea	1944
ThNH-P36/93	THNH36 (II)	AF 022441	Thailand	1993
ThNH-P16/93	THNH16 (II)	AF 022440	Thailand	1993
ThNH-P14/93	THNH14 (II)	AF 022439	Thailand	1993
ARAC-8110827	D2-JAM (III)	M 15075	Jamaica	1982
FLD2CPM1	M1(III)	X 51708	Malaysia	1990
FLD2CPM2	M2(III)	X 51709	Malaysia	1990
FLD2CPM3	M3(III)	X 51710	Malaysia	1990
DEN2CPMP	VIETNAM(III)	Z 74048	Vietnam	1996
DEN2CPG	CHINA(III)	X 65239	China	1994

middle letter, which is recognized by the transfer RNA, would effect the synthesis of the polypeptide chain. The process of natural selection generally takes care of such substitutions. On the other hand, the base substitution not recognized by the transfer RNA will be random and will provide no selective advantage. Therefore, such a genome region showing a uniform rate of random substitution should be selected for phylogeny.

c) The area of genome should have no hypervariable regions that may be affected by immune selection of epitopes, hence obscuring long term evolutionary trends. In addition, high nucleotide variation may probably mask random mutational events that give clues to evolutionary relationships.

d) Benzer (1961) pointed out that a genetic region rich in AT pairs will tend to be more subjected to substitution than that rich in GC pairs since AT base pairs are held together much less strongly than GC base pairs. Thus random mutations in the AT rich regions are likely to influence the evolution.

e) Blok *et al* (1992) reported that each protein of flaviviruses (including dengue viruses) evolved at a different rate so much so that there is an almost four fold variation among the proteins. As compared to highly conserved NS5 region which shows very little variation C-PrM and E-NS1 regions changed 2-3 times and 1.5 times more rapidly, respectively.

We noticed that most of the mutational changes occurred in the third position of the codon and mostly remained silent in the region of C-PrM junction chosen for sequence comparison. It showed random mutations in uniform manner with no hypervariable region. The E nucleotide gene sequence (Blok *et al*, 1989; Chu *et al*, 1989), on the other hand, shows variation in a non-uniform manner and has regions of "hot spots" or intervals of high nucleotide variations that might be affected by immune selection of epitopes, thus masking the random mutation events. Further, contrary to the observations made by Rico-Hesse (1990), we found a significant uniform variation ranging from 6-12% in this region across DEN-2 strains isolated from different parts of the world, including an earlier strain of DEN-2 from India (Balaya *et al*, 1969). We also noticed that the C-PrM region selected for sequence comparison was rich in AT pairs (AT ratio varied from 52.3% to 57.1% among 22 DEN-2 strains examined).

Finally, another advantage of using C-PrM region for sequence comparison and phylogenetic analysis is that only one set of primer pair is required for gene amplification of a 511 bp region and its subsequent se-

quencing for all the 4 dengue virus serotypes (Lanciotti *et al*, 1992). On the other hand, 4 different sets of primer pairs are required for amplifying 240 bp region of E-NS1 from each of the 4 dengue virus serotypes (Deubel *et al*, 1993; Chungue *et al*, 1993, 1995).

Nucleic acid sequences of cDNA of C-PrM of all viruses listed in Table 1 were aligned to DEN-2 prototype strain New Guinea C (NGC). In addition, sequences of the same region of some selected DEN-2 isolates from other geographical regions (listed in Table 2) have been included for comparison. The sequence data of 1996 epidemic strains show a similarity of 96% to 100% (mean 99%, data not shown). These isolates were from patients with varying disease severity (DF/DHF) who got infected in the beginning, the peak and towards the end of the epidemic. No significant difference was observed at the genome level to correlate with the severity of disease in these patients or to suggest any evolution during the epidemic period. The majority of mutations occurred at the third position of the codon and was mostly silent (data not shown). However, transitions at positions 406 and 431 resulted in amino acid substitutions in all the isolates of 1996 from Delhi (Table 3). When compared to 1967 strain of DEN-2 these strains showed a similarity of 88% to 90% (mean 90%, data not shown).

A comparison with NGC revealed a similarity of 91-94% (mean 93.4%) and with Jamaican strain a similarity of 89-93% (mean 91.6%) was seen (data not shown). A similarity ranging from 88% to 90% was observed on comparison with Malaysian strains M1, M2 and M3 isolated from patients with DHF, DSS and DF respectively (Samuel *et al*, 1990). Similarly, a similarity of 89-92% was seen on comparison with DEN-2 strains from Thailand (ThNH-p14/93, ThNH-p16/93 and ThNH-p36/93), Vietnam (DEN2CPMP) and China (DEN2CPG).

The predicted amino acid sequence of the C-PrM encompassing aa 26-112 of the capsid region and aa 113-160 of the pre-membrane region of the viruses listed in Tables 1 and 2 revealed that the proteins were highly conserved in majority of the strains analysed and had a similarity of 94% to 100% (Fig 1). The majority of amino acid substitutions in the capsid region were common to 1996 DEN-2 isolates. Significant among these were the changes at amino acid positions 104 (methionine → valine) and 112 (valine → alanine) which were near and at the C-PrM junction, respectively (Fig 1, Table 3).

Phylogenetic relationship of 1996 Delhi epidemic strains with other global strains was determined by Neighbour-Joining method (Fig 2). The trees thus generated closely resembled that described earlier using E-NS1 junction sequence (Singh *et al*, 1999). All the strains of 1996 Delhi epidemic form a cluster (genotypeIV) which is distinct from genotype I represented by prototype strain NGC, genotype II which includes 1993 Thailand epidemic strains of DEN-2, genotype III which is represented by Jamaican strain (ARAC-8110827), Vietnamese strain (DEN2CPMP), Chinese strain (DEN2CPG) and Malaysian strains M1(FLD2CPM1), M2 (FLD2CPM2) and M3 (FLD2CPM3) and genotype V which is constituted by an isolate from an epidemic of DF in Delhi in 1967. The relationships shown in Fig 2 were independent of the type of phylogenetic analysis algorithms used for the generation of phylogenetic trees (maximum parsimony, UPGMA) (data not shown).

DISCUSSION

Dengue virus infection is an important emerging disease. It is presently unknown whether specific viral genotypes are responsible for more severe forms of the disease or for any particular world-wide epidemic. Nonetheless, precise identification of a virus genotype is a necessary step for defining the source of epidemic virus for a better understanding of dengue transmission, introduction into newer areas and virus evolution. The global transmission of dengue viruses has been followed by comparing short nucleotide sequences of E gene, NS1 gene or E-NS1 junction

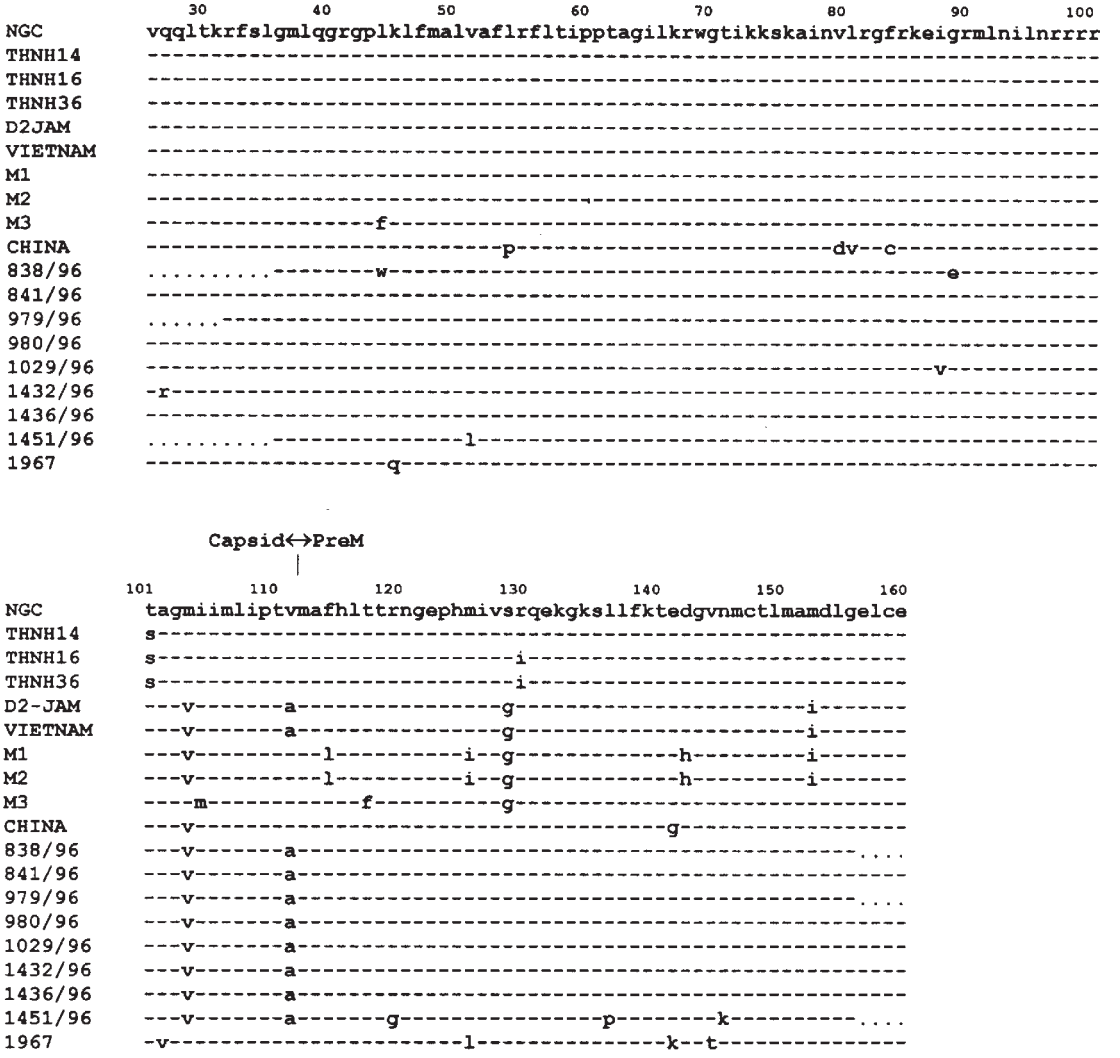

Fig 1–Predicted amino acid sequence alignment of C-PrM region (aa 26 → 160) of 9 DEN-2 isolates from Delhi (see Table 1 for details) with that of prototype DEN-2 strain, New Guinea C (NGC, genotype I). Corresponding nucleotide sequences have been deposited with the Genbank (see Table 1 for accession #). Amino acid sequences of representative strains belonging to other genotypes (see Table 2 for details), obtained from Genbank, were included for comparison. Dashes (-) indicate identities and dots indicate information not available. Amino acid positions are numbered according to Hahn *et al* (30) with prototype DEN-2 strain NGC as reference. Single letter amino acid abbreviations: a=alanine; c=cysteine; d=aspartic acid; e=glutamic acid; f= phenylalanine; g=glycine; h=histidine; i=isoleucine; k=lycine; l=leucine; m=methionine; p=proline; q= glutamine; r=arginine; s=serine; t=threonine; v=valine; w=tryptophane; y=tyrosine.

regions by several workers (Blok *et al*, 1989, 1991; Deubel *et al*, 1993; Lewis *et al*, 1993; Rico Hesse *et al*, 1990, 1997; Singh *et al*, 1999).

In the present study eight strains of DEN-2 isolated from 1996 Delhi epidemic of DHF and one from 1967 Delhi epidemic of DF were compared to some of the global DEN-2 isolates in the region of C-PrM junction. A genotype was defined as a group of dengue viruses having no more than 6% sequence difference within the chosen interval (Rico-

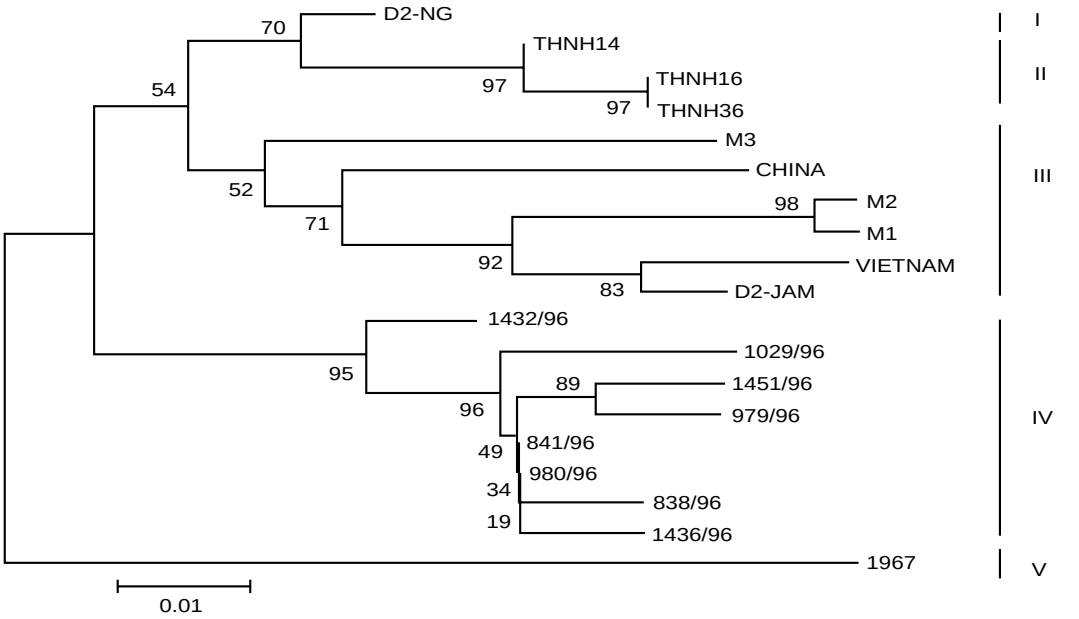

Fig 2–Phylogenetic analysis of c-DNA sequences of C-PrM region of 10 DEN-2 isolates from DF/DHF epidemics in Delhi (see Table 1). Nucleotide sequences have been deposited with the Genbank (see Table 1 for accession #). The analysis is based on 357 alignable positions (positions in alignment containing one or more gaps were excluded from analysis) in the C-PrM region. Sequences of DEN-2 isolates from other parts of the world (obtained from Genbank) were added for comparison (see Table 2). The numbers at the branch points indicate the percentage bootstrap values in 500 bootstrap replications. Genotypes of DEN-2 viruses are indicated in Roman numerical I to V.

Hesse, 1990). This lower arbitrary value for the definition of a genotype was chosen, as the rate of variation of dengue viruses in nature seems to be lower than that seen in other RNA viruses. A 0-4% difference among isolates collected at different time points shows that this epidemic was caused by a single genotype of DEN-2. This was expected as this epidemic began in mid August 1996 and lasted until mid-November 1996, which hardly gave any time to the virus to show genetic variability. In addition, a difference of 10-12% from the 1967 Delhi epidemic strain indicates that the genotype of the current DEN-2 strains was different from the earlier isolate. Similarly, a difference ranging from 6% to 11% from other global isolates suggests that these isolates were of different genotype. These observations are strengthened by a recent report from our laboratory on sequence analysis of the E-NS1 junction region of these viruses revealing a 0-3% difference within the 1996 DEN-2 isolates and 8-12% difference with some selected DEN-2 isolates from other parts of the world (Singh *et al*, 1999).

Further analysis of the data shows that in this region the transitions were predominant with very few transversions. Most of the transitions were in the third position of the codon and were mostly silent. However, transitions at nucleotide positions 406 (A→G) and 431 (T→C) resulted in amino acid substitutions methionine → valine (at amino acid position 104) and valine → alanine (at amino acid position 112) respectively, near and at the junction of the capsid and pre-membrane region. Substitution of methionine to valine (aa 104) has also been observed among DEN-2 strains isolated from Jamaica, Vietnam, Malaysia and China. Methionine, which is a

polar amino acid having a hydropathic index of 1.9, is substituted near the junction of capsid and pre-membrane protein by a non-polar amino acid valine with bulky side chain and hydropathic index of 4.2. This substitution is likely to make this region of capsid more hydrophobic and thus promoting interaction with the immune response as well as with other proteins. On the other hand, replacement of non-polar amino acid valine with another non-polar amino acid alanine at the C-PrM junction may not have significant effect on the hydrophobicity of the protein in that region. Incidentally, all these strains, except D2-JAM, were isolated during epidemics of DHF in Vietnam, Malaysia, China and India. D2-JAM was isolated from a patient with DF in Jamaica (Rico-Hesse *et al*, 1997). Apart from these substitutions no significant difference was observed among the strains isolated from patients with varying degree of severity of the disease at the genome level.

The phylogenetic analysis of the sequence data generated Neighbour-Joining trees (Fig 2). It can be seen that while DEN-2 isolates from 1967 Delhi epidemic belonged to the genotype V and probably represented the circulating genotype during that period, the 1996 Delhi epidemic strains clustered together as genotype IV. A similar comparison in the E-NS1 region of these isolates showed clustering with other genotype IV strains from Somalia, Seychelles and Torres Straits (Singh *et al*, 1999). It appears that the earlier genotype V strains were replaced by genotype IV strains in and around Delhi. In Sri Lanka also, earlier DEN-2 isolates of 1968-1969, which clustered with genotype I and probably represented the circulating genotype during that period, were replaced by genotype IV strains during early 1990s after a period of 24 years (Lewis *et al*, 1993). Probable origin of these strains is in the South Pacific Islands, where these viruses existed since late 1970s and early 1980s (Lewis *et al*, 1993). The period during which this displacement of earlier genotypes of DEN2 strains with genotype IV strains would have taken place in this part of the subcontinent is not clear at present.

Similar approach with the E-NS1 region sequence data has previously been shown to reflect the geographic origin of DEN-2 strains found in other parts of the world. For example, in Latin American countries, the native American genotype has been replaced by the Southeast Asian genotype (Rico-Hesse *et al*, 1997). Interestingly, introduction of new genotypes of DEN-2 displacing the circulating genotype has been associated with the appearance of DHF/DSS in at least four countries of Latin America (Rico-Hesse *et al*, 1997), Sri Lanka (Lewis *et al*, 1993) and India (Singh *et al*, 1999). More work is required to give credence to this hypothesis.

In terms of clinical presentation of the disease (DF *vs* DHF/DSS), there was no segregation of virus strains in the phylogenetic tree. However, this should not be taken to exclude the possibility of significant genetic diversity between DEN strains causing DF and those associated with more severe form of the disease since only a small gene segment of C-PrM region was selected for analysis. Perhaps sequence of complete genome of the virus may throw some light on this aspect.

To conclude, the present investigation clearly demonstrates the advantage of using C-PrM region for studying the evolution of dengue viruses over E-NS1 region. Although it yields similar information, unlike E-NS1 region here only one set of primer pair is required for gene amplification of a 511 bp region for virus typing and its subsequent sequencing for all the four dengue virus serotypes. Thus it is less time consuming, less labor intensive and less expensive than using E-NS1 region.

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