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Emergence and Continued Circulation of Dengue-2 (Genotype IV) Virus Strains in Northern India

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Dengue (DEN) is an acute mosquito borne viral disease of mankind. Off late it has become an important public health concern in Southeast Asia. Although, all the four known dengue virus serotypes (DEN-1 to 4) are reported from time to time, in the recent past, DEN-2 has emerged as the predominant type, being the causative agent of several outbreaks of dengue fever (DF) and dengue haemorrhagic fever (DHF) in India. To elucidate the true molecular epidemiology of these viruses, we have sequenced C-prM gene junction (454 nucleotides) of 11 DEN-2 viruses directly from patient serum. The C-prM gene junction was amplified initially by reverse transcription-polymerase chain reaction followed by automated DNA sequencing. These sequences provide unique information with regard to molecular epidemiology when compared to other DEN-2 sequences from diverse geographic origins. The sequence analysis revealed that most of the mutations in this region remained silent, except a few at the carboxy-terminal of the capsid. Reported phylogenetic analysis classifies DEN-2 viruses into five distinct genotypes. The Gwalior DEN-2 viruses, included in the present study were classified into genotype-IV, and were found to be most closely related to Delhi 1996 DEN-2 viruses and FJ 10/11 strains prevalent in the Fujian state of China. However, two earlier Indian isolates of DEN-2 were classified into genotype-V. The present study indicates that genotype V of DEN-2 has been replaced by genotype IV during the past decade, which continues to circulate silently in north India, and have the potential to reemerge and cause major epidemics of DF and DHF. ***J. Med. Virol. 74:314–322, 2004.***

KEY WORDS: DEN-2; C-prM; sequencing; molecular epidemiology; phylogeny

INTRODUCTION

Dengue (DEN) is the second most important mosquito borne infection after malaria causing 50 million cases of serious febrile illness resulting in approximately 24,000 deaths per annum worldwide [World Health Organization, 2002]. This is caused by four distinct but closely related serotypes of dengue viruses (DEN-1, DEN-2, DEN-3, and DEN-4) belonging to the genus *Flavivirus* of the family Flaviviridae. Initial infection with one serotype of DEN virus may lead to dengue fever (DF), which is a self limiting febrile exanthema. Dengue haemorrhagic fever (DHF) and dengue shock syndromes (DSS) are however, fatal diseases, mostly caused due to secondary infection with a heterologous DEN virus serotype or primary infection with a highly virulent homologous genotype [Halstead, 1988; Rico-Hesse et al., 1997]. Over the last two decades, the occurrence of DHF and DSS has increased throughout tropical and subtropical countries due to rapid urbanisation and greater convenience of air travel [Guzman and Kouri, 2002]. Outbreaks of DEN infections have been reported from different parts of India at regular interval [Mehendale et al., 1991; Bhattacharjee et al., 1993; Ram et al., 1998; Dar et al., 1999; Joshi et al., 2000]. DEN virus was first isolated in India in 1946 and since then all the four serotypes of DEN viruses have been isolated from various outbreaks. A major epidemic of DHF has occurred in Delhi in 1996 affecting more than 10,000 people [Dar et al., 1999]. So far, there is no effective vaccine or therapeutic measures available to combat DEN infection. Therefore, early diagnosis plays a crucial role to forecast an epidemic and to undertake effective control measures.

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The genome of DEN virion consists of a single stranded, positive sense ribonucleic acid (RNA) of approximately 10,700 nucleotides in length. It contains a single open reading frame (ORF) of approximately 10,200 nucleotides coding for a large precursor polyprotein and is flanked by two nontranslated regions (5′NTR and 3′NTR). The order of proteins encoded in the ORF is 5′-C-prM(M)-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3′. The first three are structural proteins [capsid (C), membrane (M), and envelope (E)] and the rest seven are nonstructural proteins [NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5] [Henchal and Putnak, 1990].

The genotype of the DEN virus plays an important role in the outcome of the disease. Some genotypes induce greater viraemia and are transmitted more readily, thereby having a higher potential to cause large epidemic [Rico-Hesse et al., 1997; Leitmeyer et al., 1999]. Genetic analysis is an important tool for monitoring the transmission and eventual introduction of new genotype into an endemic area. Genetic variation among DEN viruses has been studied employing RNase T1 oligonucleotide fingerprinting [Trent et al., 1990]; restriction fragment length polymorphism (RFLP) [Vorndam et al., 1994]; polymerase chain reaction-restriction endonuclease (PCR-RE) [De Paula et al., 2002]; restriction site specific-PCR (RSS-PCR) analysis [Harris et al., 1999; Miagostovich et al., 2000]. However, phylogenetic analysis based on nucleotide sequencing has been accepted universally as the most authentic method. Nucleotide sequences across different lengths of the DEN genome viz, envelope, E-NS1, C-prM, 5′ and 3′ NTRs and the complete genome have been analysed for phylogenetic analysis [Irie et al., 1989; Rico-Hesse, 1990; Deubel et al., 1993; Lewis et al., 1993; Lanciotti et al., 1997; Mangada and Igarashi, 1997; Singh et al., 1999; Singh and Seth, 2001; Twiddy et al., 2002]. Most of these studies involve sequence analysis of either cell culture or mouse brain adapted DEN isolates. However, to elucidate the exact genetic make up of DEN viruses, we have determined the nucleotide sequences of DEN viruses directly from clinical samples. In the present study, the genetic relationship among 11 DEN viruses was examined in patient sera during a DEN outbreak at Gwalior, India in 2001 [Parida et al., 2002].

MATERIALS AND METHODS

Serum Samples

Serum samples collected from 312 febrile patients with a clinical diagnosis of DEN infection in Gwalior, India during September–November, 2001 [Parida et al., 2002] were employed in this study.

Virus

DEN virus serotype 1–4 obtained from the National Institute of Virology (NIV), Pune, India were used as reference strain in this study. All these viruses were passaged 2–3 times in C6/36 cells [Igarashi, 1978] before analysis.

RNA Isolation

Viral RNA was isolated from 140 μl DEN suspected serum samples as well as from DEN 1 to 4 infected C6/36 cell culture supernatant employing QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) following manufacturer's protocol. Finally, RNA was eluted in 50 μl of diethyl pyrocarbonate (DEPC) treated water and used as template for RT-PCR.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out following the method of Lanciotti et al. [1992] with slight modifications. Complementary DNA (cDNA) was synthesised in a 10 μl reaction volume with RT mix comprising 5×-RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 500 μM each of four deoxynucleotide triphosphate (dNTP), 10 U of recombinant RNasin® ribonuclease inhibitor, and 50 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (Promega, Madison, WI) for 1 hr at 37°C with DEN virus group specific consensus downstream primer (D2: 5′-TTGCACCAACAG-TCAATGTCTTCAGGTTC-3′) [Lanciotti et al., 1992]. The amplification of cDNA was carried out in 50 μl reaction volume with PCR mix containing 10× Magnesium free PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 1.5 mM of $MgCl_2$, 100 μM of each of four dNTP, 2.5 U of *Taq* DNA polymerase (Promega), using DEN consensus upstream primer (D1: 5′-TC-AATATGCTGAAACGCGCGAGAAACCG-3′) [Lanciotti et al., 1992] in a thermal cycler (Perkin Elmer-480). The thermal profile of the PCR reaction was—initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 2 min and extension at 72°C for 2 min, followed by a final extension step of 72°C for 10 min. The PCR products were purified from 1.2% low melting point agarose gel using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The purified PCR products were quantitated and used as template in sequencing reaction.

Sequencing Reaction

Double pass sequencing reaction was carried out employing Big dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, CA). For each sequencing reaction, approximately 25 ng of purified PCR product was mixed with a reaction cocktail containing Ampli*Taq* DNA polymerase and four dye-labelled dideoxy nucleotide terminators and 3.2 pmol of respective sense or complimentary primer (D1/D2). The reaction tubes were placed in a pre-heated (90°C) block of thermal cycler (Perkin-Elmer). Cycle sequencing parameters used were as described in the manufacturer's protocol (25 cycles of 96°C for 30 sec, 50°C for 60 sec, and 60°C for 4 min). The reaction mixture was column purified and the DNA was dried in vacuo. The dried pellet was resuspended in 10 μl of template suppression reagent (TSR), heated at 95°C for

2 min before loading on to a ABI 310 automated DNA sequencer (Perkin Elmer, Applied Biosystems).

Nucleotide and Amino Acid Sequence Analysis

The nucleotide sequences were edited and analysed by the Lasergene software package (DNASTAR Inc., Madison, WI). Multiple sequence alignments and phylogenetic analysis were done employing CLUSTALW version 1.83 [Thompson et al., 1994]. The phylogenetic tree was constructed by the Neighbour-joining method [Saitou and Nei, 1987] with bootstrap analysis of 1,000 replicates and drawn using Tree View software version 1.1.6 [Page, 1996]. A total of 18 DEN virus type-2 isolates of diverse geographical locations and global distribution were selected for the analysis.

RESULTS

The presence of DEN virus was confirmed in 11 acute phase patient serum samples by RT-PCR. Nucleotide sequences of C-prM gene junction (454 nucleotides) of these 11 DEN-2 viruses and four reference DEN viruses type 1 to 4 (Table I) were determined in the present study. These sequences along with sequences of eighteen other DEN-2 viruses of diverse geographical origins were retrieved from GenBank (Table II) and were aligned to the homologous gene region (nt. 162–615) of the prototype strain of DEN-2 (New Guinea C) (Fig. 1). Comparison of sequences revealed base substitution scattered throughout the entire length of the C-prM junction and there appears to be no particular region of hypervariability. However, no nucleotide deletions or insertions were detected. The nucleotide sequence homology among all the DEN-2 viruses were 87–100%. The nucleotide sequence homology among the eleven DEN-2 viruses (Gwalior strains) were 98.2–99.8% (Mean 99.03%). The highest nucleotide divergence of 1.8% was seen between GWL-177 and three other Gwalior strains viz., GWL-81, GWL-102, and GWL-90. When compared with the prototype strain (D2-NGC), these 11 strains revealed sequence homologies of 93.4–94.1% (Mean 93.67%). When the Gwalior viruses were compared with the other Indian DEN viruses (Delhi strains of 1996), the homologies between these strains were found to be 97–100% (Mean 98.28%). Total (100%) homology was found between GWL-70 and 841 strain of Delhi outbreak, 1996. When the Gwalior strains were compared with two earlier DEN isolates of India (P-23085 isolated in 1960 and sequenced in the present study) and (P9-122 isolated in 1967), there was a sequence homology of 90.5–91.6% (mean 91.05%) and 89.2–90.4% (mean 89.70%), respectively. The sequence homology of Gwalior DEN-2 isolates with other global isolates viz., C0167 THAI-96, MindanaoTP PHIL-95, N.1409 JAMA-83, and Ven-2 VENE-87 were found to be 91.9–92.5% (Mean 92.91%), 92.1–92.7% (Mean 92.35%), 91.0–91.6% (Mean 91.25%), and 88.8–89.4% (Mean 89.07%) respectively. Overall, this C-prM gene junction was found to be AU rich (53.74%). Majority of nucleotide substitution occurred at the third position of the codon and remained silent. Overall, only 14.8–18.7% nucleotide substitution resulted in amino acid substitution. Majority of mutation were transition type. The ratio of transition to transversion was 25:2.

Amino Acid Sequence Diversity

To determine the degree of relatedness of the sequences, the deduced amino acid sequences of the homologous region (151 amino acid from position 23 of capsid to 59 of prM) of DEN virus were aligned (Fig. 2). All the pair-wise comparison indicated that amino acid similarity among the Gwalior DEN-2 viruses ranged from 96.7 to 100% (mean 98.58%). These Gwalior strains revealed high amino acid sequence homology of 97–100% (Mean 98.66%) with Delhi-1996 isolates. The homology of the Gwalior isolates with the prototype DEN-2 strain (NGC) and two earlier Indian DEN-2 strains (P-23085 and P9-122) were found to be 95.4–96.7 (Mean 96.45%), 94–95.4% (Mean 95.13%), and 92.6–95.6% (Mean 94.34%) respectively. The deduced amino

TABLE I. Description of Indian Dengue Viruses Sequenced in This Study

Sl. no.	Virus ID. no.	Date of receipt of sample	Clinical status	Age (years)	Sex	Serotype	Passage history	GenBank accession no.
1	GWL-18	23-09-01	DF	13	M	DEN-2	Nil	AY324614
2	GWL-39	29-09-01	DF	12	M	DEN-2	Nil	AY324615
3	GWL-48	03-10-01	DF	50	F	DEN-2	Nil	AY324616
4	GWL-61	08-10-01	DHF	15	M	DEN-2	Nil	AY324617
5	GWL-70	10-10-01	DF	5	M	DEN-2	Nil	AY324618
6	GWL-71	10-10-01	DF	12	M	DEN-2	Nil	AY324619
7	GWL-81	11-10-01	DF	1	M	DEN-2	Nil	AY324620
8	GWL-90	13-10-01	DF	5	M	DEN-2	Nil	AY324621
9	GWL-102	14-10-01	DF	4	M	DEN-2	Nil	AY324622
10	GWL-177	22-10-01	DF	6	F	DEN-2	Nil	AY324623
11	GWL-228	30-10-01	DHF	8	F	DEN-2	Nil	AY324624
12	P23086[a]	1956	IU	IU	IU	DEN-1	SM(P-37), C6/36 (P-2)	NS
13	P23085[a]	1960	IU	IU	IU	DEN-2	SM(P-47), C6/36 (P-2)	NS
14	633798[a]	1957	IU	IU	IU	DEN-3	SM(P-30) C6/36 (P-3)	NS
15	642069[a]	1969	IU	IU	IU	DEN-4	SM(P-15) C6/36 (P-3)	NS

IU, information unknown; SM, suckling mouse; NS, not submitted.
[a]NIV reference strain.

TABLE II. Description of Global Dengue-2 Viruses Used for Comparison of Genome Sequence

Sl. no.	Virus ID. no	Year of isolation	Country of origin	Disease	Genotype	GenBank accession no.
1	New Guinea C (Prototype)	1944	New Guinea	IU	I	AF038403
2	43	1987	China	DF	I	AF204178
3	MindanaoTP	1995	Philippines	IU	I	AF177541
4	ThNH-p11	1993	Thailand	DF	II	AF022437
5	ThNH-p76	1993	Thailand	DHF	II	AF169687
6	C0167	1996	Thailand	DHF	II	AF100464
7	16681	1964	Thailand	IU	II	U87411
8	GD08	1998	China	DHF	II	AF469176
9	BR64022	1998	Brazil	DF	III	AF489932
10	N.1409	1983	Jamaica	IU	III	M20558
11	FJ-10	IU	China	IU	IV	AF276619
12	FJ11	1999	China	IU	IV	AF359579
13	841	1996	India	DHF	IV	AF047394
14	1436	1996	India	DF	IV	AF047400
15	1029	1996	India	DHF	IV	AF047397
16	1967	1967	India	DF	V	AF047402
17	Ven2	1987	Venezuela	DF	V	AF100465
18	IQT1797	1995	Peru	DF	V	AF100467

IU, information unknown.

acid sequence homology of Gwalior DEN-2 isolates with other global isolates viz., C0167 THAI-96, MindanaoTP PHIL-95, N.1409 JAMA-83 and Ven-2 VENE-87 were found to be 94–96% (Mean 95.79%), 95.4–97.4% (Mean 96.45%), 96.0–98.0% (Mean 97.13%), and 92.7–95.4% (Mean 94.4%), respectively. Compared to DEN-2 prototype strain (NGC), there were four major amino acid substitutions observed among all the Gwalior isolates at position 104 (Met → Val) & 112 (Val → Ala) of C and 52 (Lys → Asp) & 55 (Phe → Leu) of prM. When the Gwalior isolates were compared with two earlier Indian DEN-2 isolate (P-23085, P9-122), it revealed two additional amino acid substitutions at position 28 (Lys → Glu) and 31 (Thr → Val) of prM. Very few amino acid changes were observed among Gwalior DEN-2 viruses notably at position 80 of C (Val → Ile) among GWL-18, GWL-39, GWL-71 & GWL-177 and at 57 of prM (Arg → Lys) in GWL-61, GWL-81, GWL-90, GWL-102, and GWL-228.

Phylogenetic Analysis

A dendrogram was constructed by pair-wise comparison of 406 nucleotide sequences of C-prM gene junction (position 171–576 of DEN-2 genomic RNA), which revealed five different genotypic groups. The dendrogram (Fig. 3) revealed that the Gwalior isolates cluster into one group along with Delhi-1996 isolates, and FJ10 & FJ11 isolates of Fujian province of China. All these isolates belong to genotype IV of DEN-2 virus. However, two earlier Indian DEN-2 isolates of 1960 and 1967 cluster into Genotype V along with a Venezuelan isolate of 1987 and a Peruvian isolate of 1995. The prototype DEN-2 virus (NGC) grouped into a different branch (Genotype I) along with another Chinese isolate (43) and a Philippines isolate (MindanaoTP). The genotype II is represented by three Thailand isolates of 1993 and 1996, and one Chinese isolate of 1998. The genotype III was represented by a Jamaican isolate of 1983 and a Brazilian isolate of 1998.

DISCUSSION

DEN is the most important arboviral infection of mankind. It now occurs in over 100 tropical and subtropical countries with more than 2.5 billion people at risk of infection. The resurgence of DEN infections with more virulence from newer areas of India is a major public health concern. Strikingly there was a major outbreak of DEN infection in Gwalior, India during September–November 2001 [Parida et al., 2002].

So far, no suitable model/host has been identified to study DEN pathogenesis. However, most of the epidemiological studies rely on sequence analysis of DEN virus cultured either in mouse brain or in cell culture system. But direct sequencing of DEN virus from patient sera is reported to reveal the true viral RNA composition in natural host and permit to study their true correlation with pathogenesis [Leitmeyer et al., 1999]. This would avoid selection and sequencing of mutant viral RNA by isolation/cultivation in mouse brain/cell culture. We have adopted this novel approach to study the true picture of DEN virus population in human host.

Different regions of DEN genome like Envelope, E-NS1, C-prM have been utilised to study the genetic relatedness among isolates. The nucleotide sequence of C-prM gene junction was reported to yield important evolutionary information like other genomic region [Singh and Seth, 2001]. In addition, only one set of primer pair works efficiently for amplification and sequencing of this region for all the DEN virus serotypes, where as, for other region like Envelope and E-NS1 junction, four different sets of serotype specific primer pairs are required [Lanciotti et al., 1992]. This in turn leads to a more rapid and economical way of studying molecular epidemiology of DEN viruses. Selection of the C-prM gene junction for molecular epidemiology studies also include it's following features.

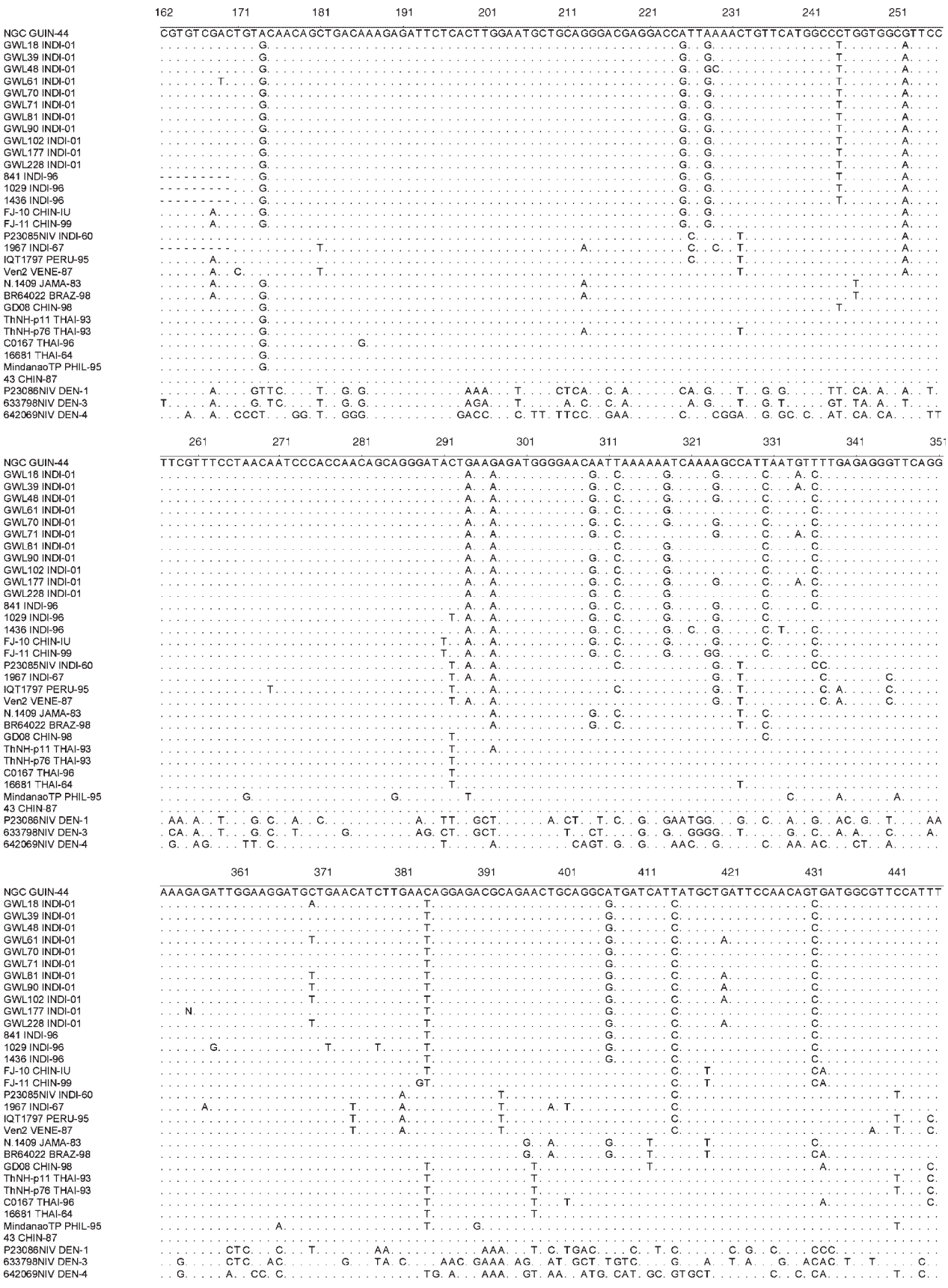

Fig. 1. Multiple sequence alignment of C-prM gene junction [nucleotide 162–615 corresponding to the prototype DEN-2 virus (NGC)]. Dot (.) indicates nucleotide similarities with DEN-2 (NGC). Dash (-) and gap ( ) indicates sequence not available. Each strain is abbreviated with strain designation followed by first four letters of country of origin and last two digits of the year of isolation.

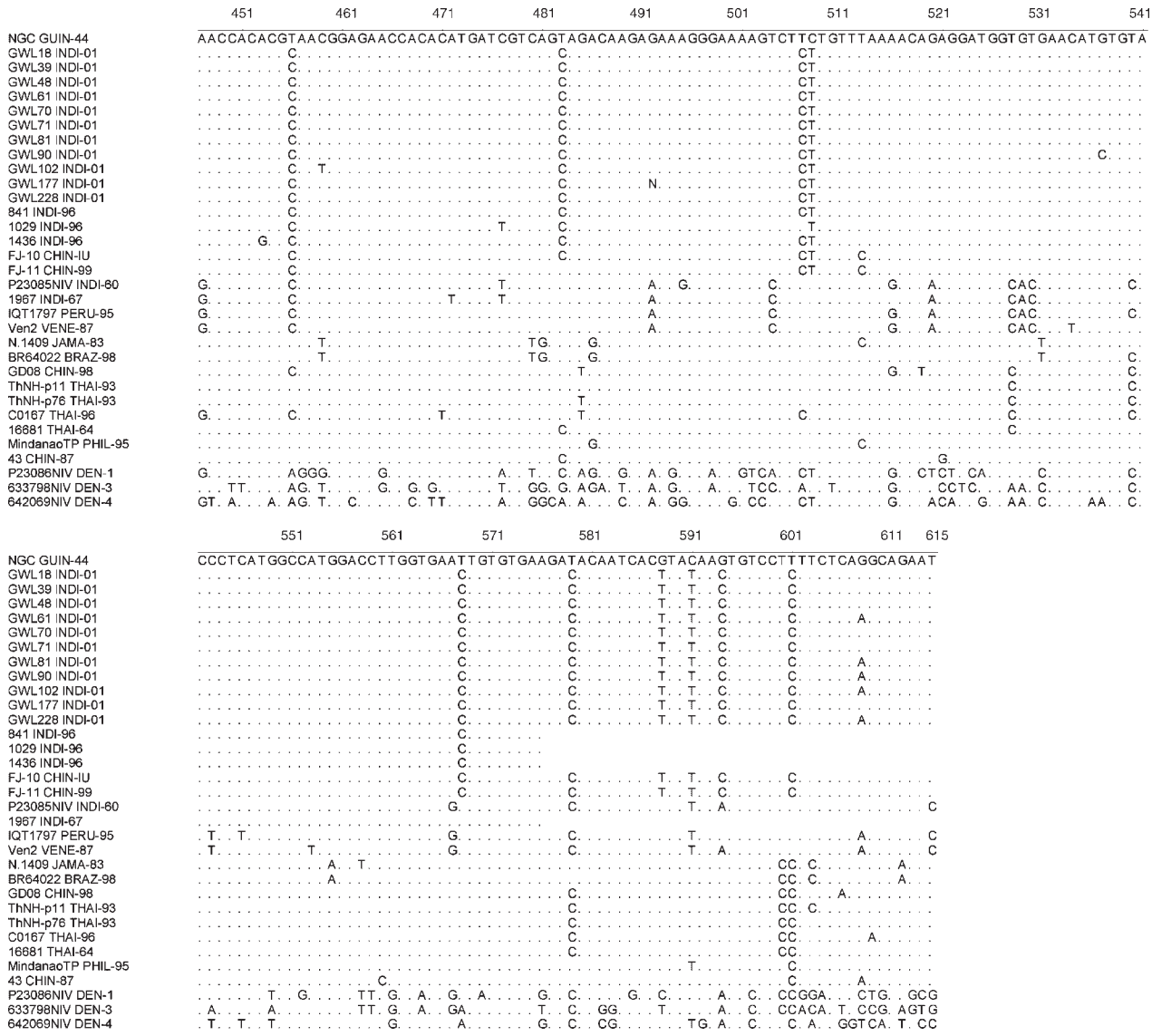

Fig. 1. (*Continued*)

1. The majority of mutation in this C-prM region occurs at the third position of the codon resulting in silent mutation, i.e., do not lead to a change in amino acid sequence of the corresponding polypeptide.
2. There is a uniform rate of random substitution of nucleotide in this C-prM junction. There is no hyper variable region that might affect the expression of important viral epitopes.
3. This region is AU rich (around 54%), which tend to be more subjected to random mutation compared to GC rich region.

With the above points in mind, we have sequenced the C-prM junction to study the genetic relatedness among Den-2 isolates in the present study. The nucleotide sequence analysis of the C-prM junction revealed that there is no significant difference between the Gwalior isolates recovered at different stages (i.e., early, middle, and late stage) of the outbreak. This is due to the fact that the outbreak was contained within a period of 60 days, there by hardly giving any time for the virus to evolve during this outbreak. There is also no significant diagnostic sequence difference between the viruses recovered from patients showing different clinical severity (i.e., DF and DHF). This is due to the absence of any known virulence associated marker in the C-prM gene junction.

Most of the nucleotide changes in this region occurred at third position of the codon and remained silent. However, compared to prototype DEN-2 strain (NGC), there were few amino acid changes among Gwalior Dengue-2 isolates. All the three Cysteine residues at position 34, 45, and 53 of prM remained conserved among all the DEN-2 isolates owing it's role in maintaining the conformation of premembrane. Most of the amino acid changes were conserved. However, some

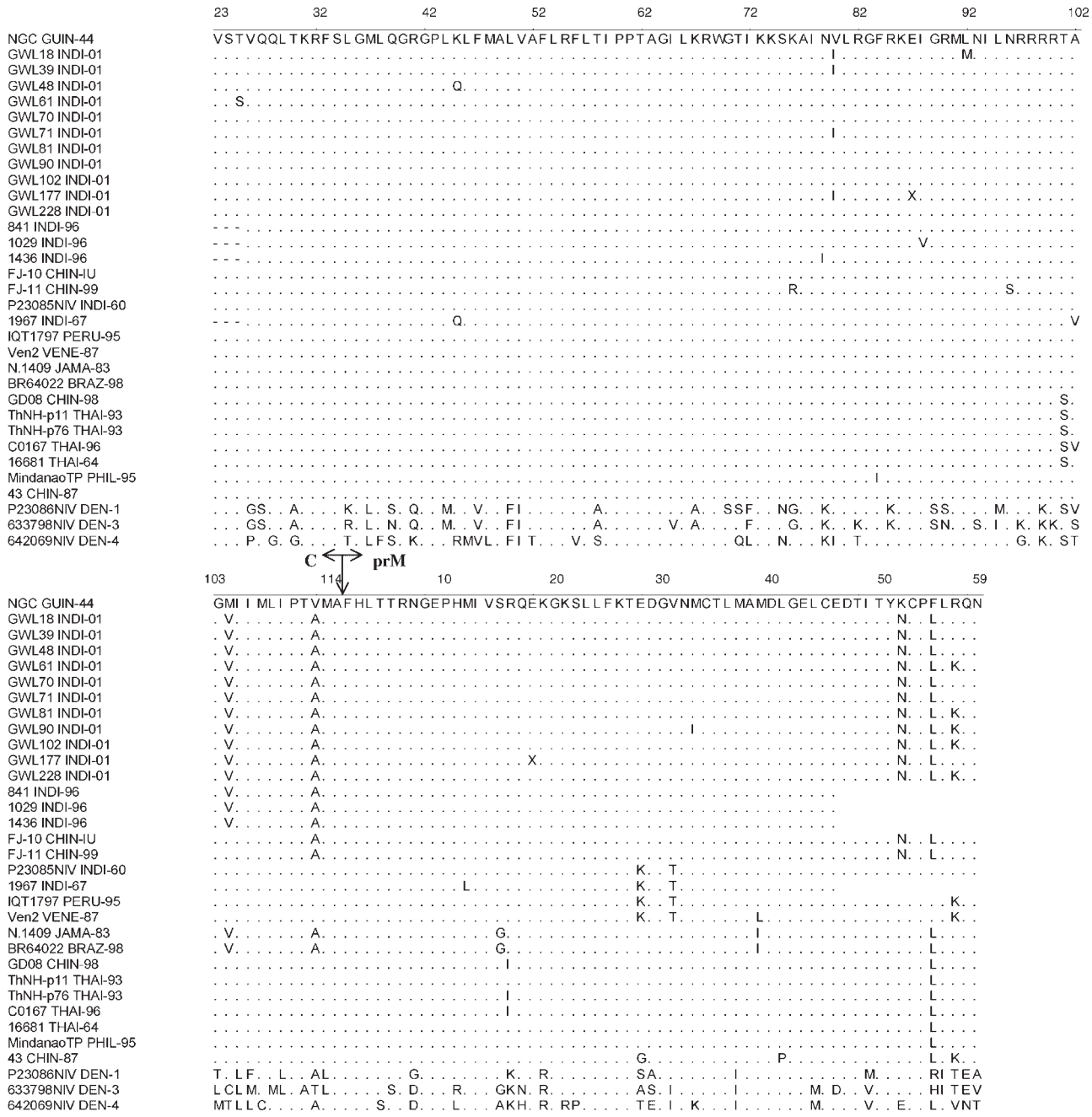

Fig. 2. Multiple sequence alignment of deduced amino acid (aa) corresponding to the aa 23 of Capsid to aa 59 of prM of prototype DEN-2 virus (NGC). Dot (.) indicates aa similarities with DEN-2 (NGC). Dash (-) and gap ( ) indicates sequence not available. Each strain is abbreviated with strain designation followed by first four letters of country of origin and last two digits of the year of isolation.

non-conservative changes were seen among Gwalior strains notably at position 104 of C (Met → Val). The polar amino acid Methionine at the carboxyl termini of capsid is substituted by non polar Valine. This will make this region more hydrophobic and assist in better interaction with other proteins involved in the immune response. Another strongly basic amino acid Lysine at position 52 of prM is replaced by a polar amino acid Asparagine. Other amino acid substitutions involving Valine → Alanine at position 112 of capsid and Phenyl alanine → Leucine at position 55 of prM may not have much significance, since these involves only interchange of hydrophobic aminoacid. Compared to earlier Indian DEN-2 isolate of 1960 and 1967, two additional amino acid changes were observed among Gwalior isolates in the premembrane. Strongly basic Lysine is substituted by strongly acidic Glutamic acid at position 28 and polar Threonine is substituted by hydrophobic Valine at position 31 of prM. These two substitutions were noted among all DEN-2 global

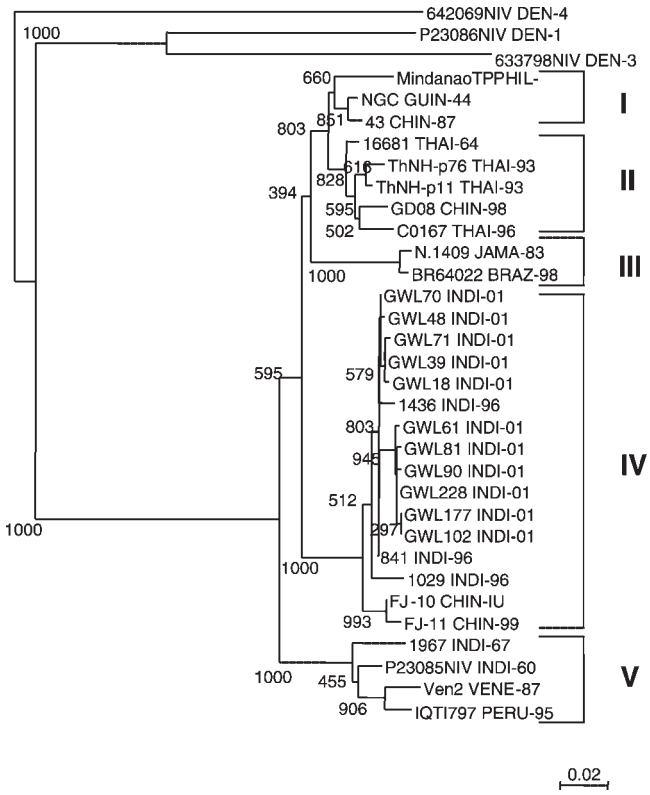

Fig. 3. Phylogenetic tree among dengue viruses generated by neighbour-joining method. Bootstrap values are indicated at the major branches. Representative strains of DEN-1, DEN-3, and DEN-4 were used to root the tree. Each strain is abbreviated with strain designation followed by first four letters of country of origin and last two digits of the year of isolation. Bootstrap values are indicated at the branch points.

isolates analysed in this study except in one each from Peru and Venezuela.

When the sequence of the Gwalior DEN isolates were compared with other global DEN-2 viruses, it was found that the Gwalior isolates were related most closely to Delhi-1996 isolates followed by Chinese-1999 isolates. Genotype classification of DEN virus is based on less than 6% nucleotide divergence within a selected genomic region. This comparative lower value for genotype classification is based on the hypothesis that the rate of variation among DEN virus is comparatively less owing to the fact of it's lifecycle involving both human and mosquitoes [Rico-Hesse, 1990]. Phylogenetic analysis revealed that Gwalior dengue-2 viruses belong to Genotype-IV. Involvement of genotype IV of DEN-2 virus has also been reported earlier from the Delhi epidemic-1996 [Singh et al., 1999]. However, two earlier Indian DEN-2 isolates of 1960 and 1967 clustered into Genotype V along with two South American isolates. This indicates that the dengue-2 virus circulating during 1960s in India belong to genotype-V. Later, this genotype was replaced by genotype IV, as evident from the isolates of the Delhi-1996 outbreak. Further studies incorporating the isolates of the intervening period (1967–1996) are warranted to exactly pinpoint the period of genotype replacement. The current outbreak by the genotype IV of DEN-2 virus at Gwalior may be attributed to the fact of close proximity (300 km) and frequent travel between Delhi and Gwalior. This indicates that the genotype IV is the dominant genotype of dengue-2 viruses involved in the major outbreaks in northern India. These viruses appear to have been circulating silently and possess high potential to reemerge at anytime. This also correlates with the findings of Rico-Hesse et al. [1997] that the emergence of new genotype in an area is almost invariably associated with increased incidence of DHF.

This study suggests that the Gwalior DEN epidemic is attributed to reemergence of Genotype IV of Dengue-2 viruses. Reemergence and spread of DEN in newer areas is a major source of concern and must be monitored carefully to identify the source of infection and to undertake effective control measures.

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