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LETTER

Molecular characterization of DENV-3 circulating during the post-monsoon period of 2013–14 in Delhi, India

Dear Editor,

Dengue infection is one of the emerging concerns for public health on a global scale. Over the past few years, dengue transmission has increased in the Americas, the western Pacific and southeast Asia. The magnitude, distribution, and clinical severity of dengue outbreaks have been an alarming signal in the southeast Asia region. Major outbreaks have been reported in countries in this region, including in Sri Lanka, Nepal, Bangladesh, Pakistan, and China (Koo et al., 2013; Wang et al., 2015). Recent reports have indicated an increasing trend in the number of cases in the People's Republic of China, Fiji, Malaysia, The Cook Islands, and Vanuatu, with DENV-3 affecting the Pacific Island countries after a lapse of nearly 10 years (WHO, 2015).

In the past two decades, major dengue outbreaks have been reported in Delhi, the national capital of India. All four serotypes of DENV circulate in Delhi, but only three (DENV-1, 2, and 3) have been reported as the main etiological agent in different outbreaks (Dar et al., 1999; Kukreti et al., 2008; Singh et al., 2012). The recent outbreak of dengue in 2013 was predominantly caused by DENV-2; however, DENV-1 and DENV-3 were also reported in 19% and 8% of cases, respectively (Afreen et al., 2014). In 2012, a downward shift in pre-dominance of DENV-1 and an upward shift in dominance of DENV-2 and DENV-3 were recorded (Sharma et al., 2014). This shift probably resulted in the DENV-2 outbreak in 2013. The possibility of an outbreak predominated by DENV-3 cannot now be ruled out.

The aim of the present study was to characterize the DENV-3 currently circulating in the capital. For this purpose, envelope (*E*) and non-structural 1 (*NS1*) gene regions were sequenced and analyzed. Acute phase serum samples with confirmed DENV-3 serotype were included in the study. Samples from different geographical locations of Delhi were referred to the National Centre for Disease Control for diagnosis during the post-monsoon period of 2013 and 2014.

Viral RNA was isolated from serum, using QIAmp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The *E* gene of DENV-3 was amplified using the primers, P1259A and CDC2503B described by Chao et al. (Chao et al., 2005). The cDNA was synthesized by reverse primer (CDC2503B) using GoScript™ Reverse Trancription System (Promega, Madison, USA). For cDNA amplification, GoTaq® Green Master Mix (Promega, USA) was applied, using 1 μL cDNA as a template. Initial denaturation was performed at 95 °C for 2 min, followed by 35 cycles of denaturation (95 °C for 30 s), annealing (54 °C for 50 s) and extension (72 °C for 1.5 min), with a final extension step at 72 °C for 10 min. The amplified PCR products (~1244bp) were visualized on 1% agarose gel stained with ethidium bromide.

For amplification of *NS1* gene, a new set of primer (D3NS1F, nt 2407–2434, 5′-TGAATTCGACATGGGGTGTGTCATAAAC-3′; D3NS1R, nt 3456–3478, 5′-TGCGGCCGCCGCTGAGACTAAAG-3′) was designed using the software Gene Runner (version 3.05) according to the DENV-3 reference sequence (NC_0014 75.2). Reverse transcription polymerase chain reaction (RT-PCR) was carried out in a 25 μL reaction volume using the QIAGEN OneStep RT-PCR Kit (Qiagen). The thermal profile of RT-PCR was an RT step at 50 °C for 30 min, followed by the PCR step of initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 54 °C for 50 s, extension at 72 °C for 1 min and final extension at 72 °C for 10 min. Amplified PCR products (~1072 bp) were separated in 1% agarose gel and visualized with ethidium bromide.

The PCR products of the amplified gene fragments were purified by using the QIAquick PCR purification kit (Qiagen). For sequencing, the BigDye Terminator Cycle Sequencing Kit (version 3.1; Applied Biosystems, Foster City, USA) was used, with an ABI 3130xl Genetic Analyzer (Applied Biosystems). Retrieved sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/) and accession numbers were acquired (Table 1). A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was carried out to confirm the identity of the strains. For sequence comparison with previously reported Indian and other geographically diverse DENV-3 isolates (Supplementary Table S1), multiple sequence

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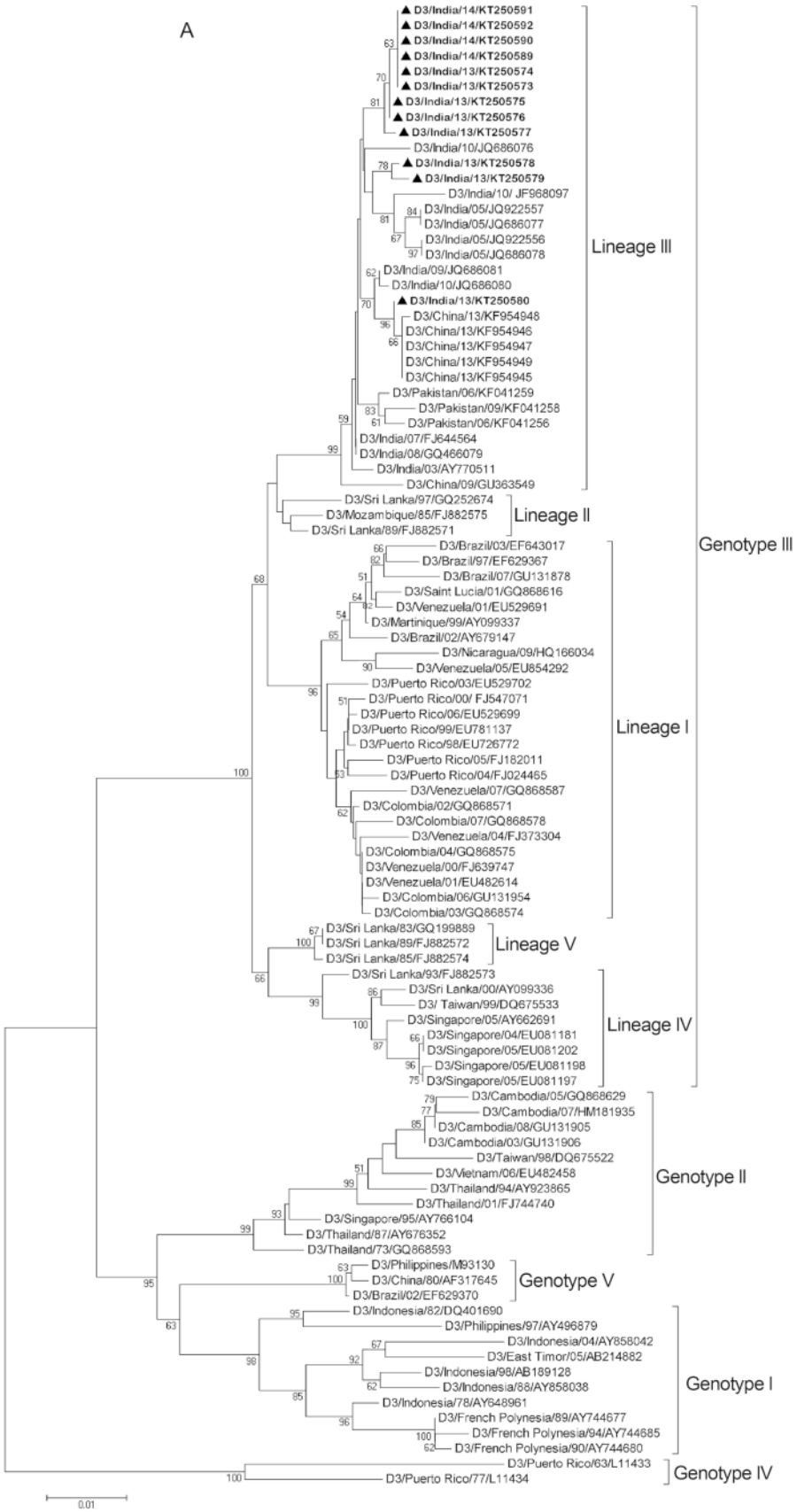
Table 1. Details of DENV-3 isolates from India sequenced in the study.

Sample no.	Isolate name	Year	Genotype	Accession number	
				E gene	*NS1* gene
1	1/D3/Del/2013	2013	III	KT250573	KT250581
2	2/D3/Del/2013	2013	III	KT250574	KT250582
3	3/D3/Del/2013	2013	III	KT250575	KT250583
4	4/D3/Del/2013	2013	III	KT250576	KT250584
5	5/D3/Del/2013	2013	III	KT250577	KT250585
6	6/D3/Del/2013	2013	III	KT250578	KT250586
7	7/D3/Del/2013	2013	III	KT250579	KT250587
8	8/D3/Del/2013	2013	III	KT250580	KT250588
9	1/D3/Del/2014	2014	III	KT250589	KT250593
10	2/D3/Del/2014	2014	III	KT250590	KT250594
11	3/D3/Del/2014	2014	III	KT250591	KT250595
12	4/D3/Del/2014	2014	III	KT250592	KT250596

alignment was performed using the Clustal W multiple alignment tool available in BioEdit (version 7.0.5.3). To represent the three-dimensional structure of the proteins, Cn3D (version 4.3.1) was used (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml). Phylogenetic analysis was performed using MEGA (version 5.0). A Tamura Nei model of nucleotide substitution with a gamma distribution of between-site rate variation was employed to construct the neighbor-joining tree with 1000 bootstrap replicates. To calculate nucleotide diversity per site (Pi), Tajima's D-test statistics, and nucleotide-based statistics (Ks, Kst, Ks*, Kst*, Z, Z* and Snn), we used DnaSP (version 5.10). F statistics (Fst) and the N statistics (Nst) were also calculated by DnaSP (version 5.10) to estimate diversity and gene flow (Nm) among the phylogenetic lineages.

In our study, total 12 samples (eight from 2013 and four from 2014) were sequenced for both the *E* and *NS1* gene regions. The 981 bp (from nucleotides 1418–2398) gene region of *E* was selected for multiple sequence alignment. All the 12 isolates sequenced revealed 0.00423 nucleotide diversity per site (Pi). These isolates showed sequence identity of 98.36%–99.67% with other Indian isolates. Sequence comparison with the prototype strain H87 (M93130) revealed a few amino acid substitutions, which were also present in other previously reported Indian isolates (Supplementary Figure S1). In the phylogenetic analysis, all Indian and global isolates could be classified as five different genotypes of DENV-3 (Figure 1A). The analysis revealed clustering of all Indian isolates within the same group (genotype III) with recent isolates from China (GU363549, KF954945-KF954948) and Pakistan (KF041256, KF041258 and KF041259). Extensive analysis of the phylogenetic tree revealed the presence of the five lineages of genotype III. Lineage I isolates formed a separate clade in closeness to lineage II, and was represented by the isolates from the Americas, including Brazil, Saint Lucia, Venezuela, Puerto Rico, Colombia, and Martinique. Lineage II was represented by the isolates from Sri Lanka (GQ252674 and FJ882571) and Mozambique (FJ882575), and was found in close proximity to lineage III. All the Indian isolates were categorized under lineage III. Isolates from Sri Lanka, China's Taiwan, and Singapore clustered as lineage IV, while lineage V was formed only by the three older isolates from Sri Lanka (FJ882572, FJ882574, and GQ199889). The nucleotide diversity per site (Pi) in all genotype III sequences was recorded as 0.02345. Genetic differentiation between the five lineages was analyzed by Ks = 9.80970, Kst = 0.57351, Ks* = 2.22754, Kst* = 0.25983, Z = 428.15909, Z* = 5.73874, and Snn = 1.0. The statistical significance of nucleotide-based statistics was tested by the permutation test with 1000 replication value ($P < 0.001$). Nst and Fst recorded for the lineages were 0.67884 and 0.67537; $\chi^2 = 284$, $P = 0.0013$. Estimated gene flow (Nm) was 0.12. Tajima's D value (–1.02015, $P > 0.10$) showed a neutral evolutionary trend for all the DENV-3 isolates.

The phylogenetic analysis based on the 823 bp gene region (nucleotides 2498–3320) of *NS1* also revealed a similar segregation of sequences, as shown by the tree based on the *E* gene region (Figure 1B). A few sequences were removed from the analysis because of unavailability of sequence for the *NS1* gene region. All sequenced isolates had sequence identity of 96.35%–100% to other Indian isolates from previous years. Recent isolates from China (KF954945–KF954948) also showed close identity (99.02%–99.63%) to these isolates. Sequence identity of 99.27%–99.78% to Pakistan isolates (KF041256, KF041258, and KF041259) was observed.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D3/India/10/JQ686076
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D3/India/10/ JF968097
D3/India/05/JQ922557
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D3/India/09/JQ686081
D3/India/10/JQ686080
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D3/China/13/KF954948
D3/China/13/KF954946
D3/China/13/KF954947
D3/China/13/KF954949
D3/China/13/KF954945
D3/Pakistan/06/KF041259
D3/Pakistan/09/KF041258
D3/Pakistan/06/KF041256
D3/India/07/FJ644564
D3/India/08/GQ466079
D3/India/03/AY770511
D3/China/09/GU363549
Lineage III
D3/Sri Lanka/97/GQ252674
D3/Mozambique/85/FJ882575
D3/Sri Lanka/89/FJ882571
Lineage II
D3/Brazil/03/EF643017
D3/Brazil/97/EF629367
D3/Brazil/07/GU131878
D3/Saint Lucia/01/GQ868616
D3/Venezuela/01/EU529691
D3/Martinique/99/AY099337
D3/Brazil/02/AY679147
D3/Nicaragua/09/HQ166034
D3/Venezuela/05/EU854292
D3/Puerto Rico/03/EU529702
D3/Puerto Rico/00/ FJ547071
D3/Puerto Rico/06/EU529699
D3/Puerto Rico/99/EU781137
D3/Puerto Rico/98/EU726772
D3/Puerto Rico/05/FJ182011
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D3/Venezuela/07/GQ868587
D3/Colombia/02/GQ868571
D3/Colombia/07/GQ868578
D3/Venezuela/04/FJ373304
D3/Colombia/04/GQ868575
D3/Venezuela/00/FJ639747
D3/Venezuela/01/EU482614
D3/Colombia/06/GU131954
D3/Colombia/03/GQ868574
Lineage I
D3/Sri Lanka/83/GQ199889
D3/Sri Lanka/89/FJ882572
D3/Sri Lanka/85/FJ882574
Lineage V
D3/Sri Lanka/93/FJ882573
D3/Sri Lanka/00/AY099336
D3/ Taiwan/99/DQ675533
D3/Singapore/05/AY662691
D3/Singapore/04/EU081181
D3/Singapore/05/EU081202
D3/Singapore/05/EU081198
D3/Singapore/05/EU081197
Lineage IV
Genotype III
D3/Cambodia/05/GQ868629
D3/Cambodia/07/HM181935
D3/Cambodia/08/GU131905
D3/Cambodia/03/GU131906
D3/Taiwan/98/DQ675522
D3/Vietnam/06/EU482458
D3/Thailand/94/AY923865
D3/Thailand/01/FJ744740
D3/Singapore/95/AY766104
D3/Thailand/87/AY676352
D3/Thailand/73/GQ868593
Genotype II
D3/Philippines/M93130
D3/China/80/AF317645
D3/Brazil/02/EF629370
Genotype V
D3/Indonesia/82/DQ401690
D3/Philippines/97/AY496879
D3/Indonesia/04/AY858042
D3/East Timor/05/AB214882
D3/Indonesia/98/AB189128
D3/Indonesia/88/AY858038
D3/Indonesia/78/AY648961
D3/French Polynesia/89/AY744677
D3/French Polynesia/94/AY744685
D3/French Polynesia/90/AY744680
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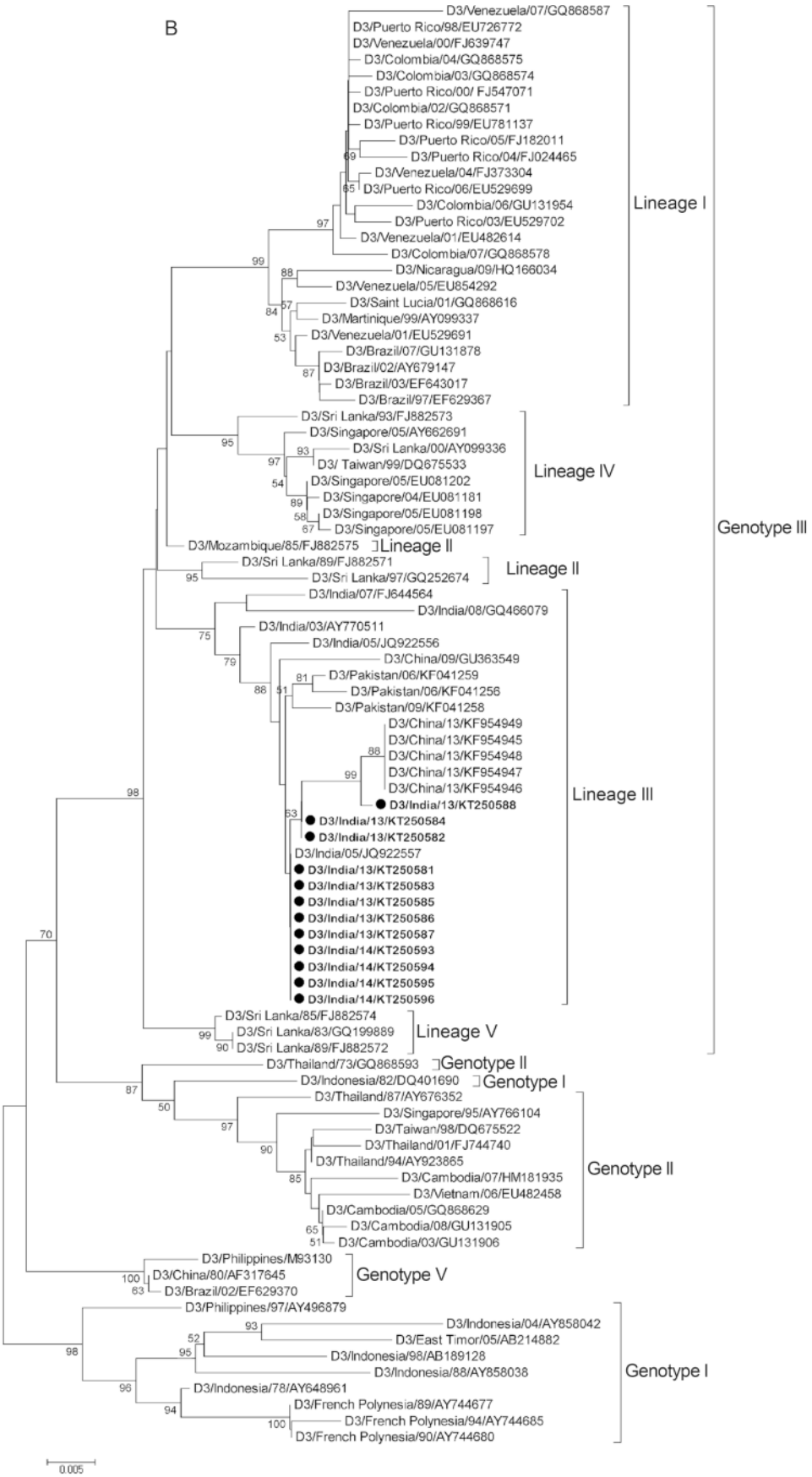
D3/Puerto Rico/77/L11434
Genotype IV
0.01


B
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D3/Venezuela/01/EU482614
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D3/Brazil/07/GU131878
D3/Brazil/02/AY679147
D3/Brazil/03/EF643017
D3/Brazil/97/EF629367
Lineage I
D3/Sri Lanka/93/FJ882573
D3/Singapore/05/AY662691
D3/Sri Lanka/00/AY099336
D3/ Taiwan/99/DQ675533
D3/Singapore/05/EU081202
D3/Singapore/04/EU081181
D3/Singapore/05/EU081198
D3/Singapore/05/EU081197
Lineage IV
Genotype III
D3/Mozambique/85/FJ882575
Lineage II
D3/Sri Lanka/89/FJ882571
D3/Sri Lanka/97/GQ252674
Lineage II
D3/India/07/FJ644564
D3/India/08/GQ466079
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D3/India/14/KT250593
D3/India/14/KT250594
D3/India/14/KT250595
D3/India/14/KT250596
Lineage III
D3/Sri Lanka/85/FJ882574
D3/Sri Lanka/83/GQ199889
D3/Sri Lanka/89/FJ882572
Lineage V
D3/Thailand/73/GQ868593
Genotype II
D3/Indonesia/82/DQ401690
Genotype I
D3/Thailand/87/AY676352
D3/Singapore/95/AY766104
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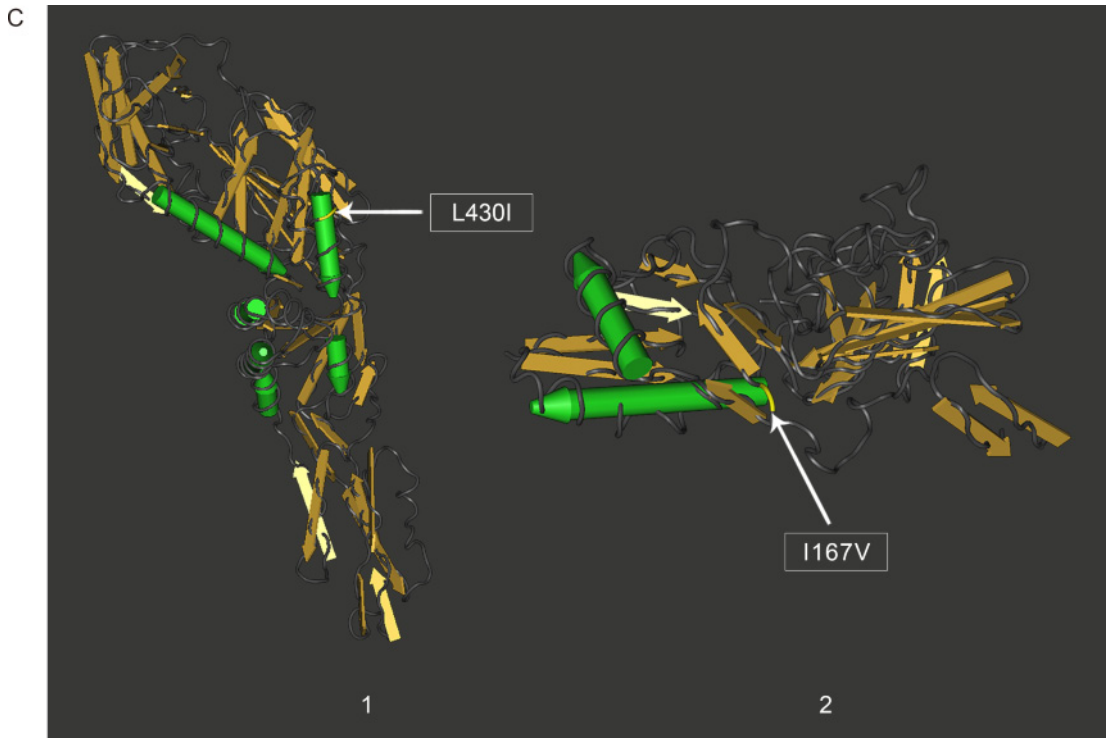

Figure 1. (A) Phylogenetic tree of DENV-3 based on 981 bp (nucleotide 1418 to 2398) *E* gene region generated by the neighbor-joining (NJ) method (1000 Bootstrap replications). Each sequence is denoted by serotype, country of origin, followed by the last two digits of the year of isolation and GenBank accession number. Isolates sequenced in the study are shown in bold (symbol with ▲). (B) Phylogenetic tree of DENV-3 based on 823 bp (nucleotide 2498 to 3320) *NS1* gene region generated by the NJ method (1000 bootstrap replications). Each sequence is denoted by serotype and country of origin, followed by the last two digits of year of isolation and the GenBank accession number. Isolates sequenced in the study are shown in bold (symbol with ●). (C) 3D representation using Cn3D. C-1: The position of the L430I substitution inferred from the three-dimensional (3D) structure of the E protein (PDB sequence; 3J6S_A). C-2: The position of I167V substitution inferred from the 3D structure of the NS1 protein (PDB sequence; 406B_A).

The nucleotide diversity per site (Pi) for all sequences isolated was found to be 0.00171. Genetic differentiation between the five lineages for the NS1 gene region was analyzed by Ks = 8.47947, Kst = 0.61084, Ks* = 1.97655, and Kst = 0.32173 and Snn = 1.0. In permutation testing with 1000 replication values, $P < 0.001$ showed statistical significance of nucleotide-based statistics. Nst and Fst for the lineages were recorded as 0.68635 and 0.68296, respectively ($\chi^2 = 260$, $P = 0.0004$). Gene flow (Nm) was estimated as 0.12. Tajima's D test, applied to all the DENV-3 sequences for the *NS1* gene region, showed a neutral evolutionary trend (Tajima's D value = –1.18983, $P > 0.10$).

All the 12 isolates sequenced clustered within lineage III of genotype III. This lineage was formed by recent strains prevalent in India, China, and Pakistan. The presence of different lineages has been explained in an earlier study on the basis of the complete nucleotide sequence of the DENV-3 genome. (Sharma et al., 2011). The nucleotide-based statistics, F statistics, N statistics, and gene flow analysis for both the *E* and *NS1* gene regions advocated genetic differentiation between the phylogenetic lineages. Sequence comparison with prototype strain H87 revealed the presence of a few amino acid substitutions that were also present in other Indian isolates.

A unique amino acid substitution (L430I) in the E protein was observed in 83.33% (10/12) isolates (Supplementary Figure 1). This substitution falls within the α helix of the stem region (Figure 1C-1). The substitution at this site has also been reported in an earlier isolate from Samoa in 1986 (L11435) (Lanciotti et al., 1994). This site is located within the stem–anchor region adjacent to the putative receptor-binding domain (domain III) in E protein. The stem and anchor regions are both predicted to have two α-helices each. Secondary structure prediction of the stem region has suggested that the two consecutive, mostly amphipathic, α-helices are half-buried in the outer lipid leaflet of the viral membrane (Zhang et al., 2003). Their interactions with the lipid

phosphate groups are probably affected by the pH changes, contributing to the forces that cause conformational rearrangements during the time of fusion (Zhang et al., 2003). The substitution L430I is present in the α-helix in the stem region. The presence of a neutral, non-polar, amino acid residue at this site may be essential for such conformational rearrangements. The E protein is a major antigenic determinant. The substitution L430I lies in the MHC complex (epitope I.D 59137) (http://www.iedb.org/refId/1000409) and may have an important role in the host immune response.

Another unique substitution, I167V, in the NS1 protein was observed in a single isolate (KT250588). The substitution lies in the connector subdomain of NS1 (Figure 1C-2), which links the "wing" domain to the central β-sheet through a disulfide linkage (Akey et al., 2014). The *NS1* protein is a protective antigen, and is known to play an important role in viral RNA replication. It has also been suggested to play a role in structural support for providing stability for replication complex (Muller and Young, 2013). Both isoleucine and valine are hydrophobic in nature and prefer to be buried in the hydrophobic cores of protein (Barnes, 2007). It is possible that hydrophobicity at this site is necessary for the structural support and functionality of NS1. However, to confirm this, additional studies are required.

Further analysis also revealed the close identity of the sequenced Delhi isolates to recent isolates (KF954945–KF954948) from China, which is an alarming signal for public health, as this strain has been associated with a dengue outbreak in China (Wang et al., 2015). To prevent the occurrence of such outbreaks and to observe the unique variations accumulating in virus, it is essential to continually monitor the spread of DENV-3 and characterize it at the genomic level.

FOOTNOTES

The authors acknowledge partial financial support from Indian Council of Medical Research (ICMR), Delhi during the course of this study. The authors declare that they have no conflict of interest. The approval of the institutional ethical committee was obtained to carry out the present study. "Waiver of informed consent" was obtained from the committee on the basis of use of "leftover samples after clinical diagnosis" under the 2006 guidelines of the ICMR. This work was carried out in collaboration among all the authors. All authors read and approved the final manuscript.

Supplementary figures/tables are available on the website of *Virologica Sinica*: www.virosin.org; link.springer.com/journal/12250.

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