OVERALL DISCUSSION

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CONCLUSION
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The present study set out to understand the evolution, distribution and dispersion of Dengue virus (DENV) in India in context to the global scenario. Dispersion of DENV right across Asia Africa and North America in the 18th century, is believed to have occurred due to the import of labour from Africa and India (Gubler et al., 1978) and later in the beginning of the 20th century due to the two World Wars (Sabin., 1952). It is evident that India would have played a major role in the dispersion of dengue. Studies on the evolution of dengue so far included a majority of sequences from south-east Asia and South America with a few recent sequences from India (Leitmeyer et al., 1999, Cologna and Ricco-Hess, 2003, Diaz et al., 2006, Messer et al., 2003). The value of this study is the inclusion of Indian isolates from the 1960s and the recent years. For DENV-1 and 2 isolates from the intervening decades were also included.

The results of the phylogenetic and molecular clock analysis of envelope (E) gene sequences obtained for Indian isolates clearly indicated that the isolates from the 1960s for all serotypes had a role in the evolution of the currently predominant genotypes of DENV. Inclusion of the 1960s isolates resulted in identification of a new lineage India III in AM/AF genotype of DENV-1 (Patil et al., 2011), a genotypic shift from American to Cosmopolitan for DENV-2 (Kumar et al., 2010), a new lineage F in Gill of DENV-3 and a new genotype, GV for DENV-4 (Patil et al., 2012). Recombination between an Indian isolate of GV and a Sri Lankan isolate of Gl of DENV-4 was detected. Recombination has been reported infrequently but has been observed for all serotypes (Worobey et al., 1999, AbuBakar et al., 2001, Tolou et al., 2001, Uzcategui et al., 2001, Ming Wei Su et al., 2012).

The genotype shift in DENV-2 was associated with a change in disease profile from mild disease in the 1960s to moderate disease (with hemorrhagic manifestations) in the present (Kumar et al., 2010). For DENV-3, lineage F was at the root of Gill (Patil et al., 2012). Gill is the virulent and most widespread genotype now (Sharma et al., 2011, Kanakaratne et al., 2009). GV seeded Gl of DENV-4 (Patil et al., 2012). Gl is considered as a genotype with greater transmission potential (Dash et al., 2011). The molecular analysis also indicated that there was import of DENV into India from Sri Lanka, Singapore and Africa and export of viruses from India to Sri Lanka, America and Thailand. The tMRCA that was calculated (which varied from 123 years to 200 years) matched the earlier published values for all serotypes (Holmes and Twiddy, 2003). FOR DENV-3 and DENV-
the values of tMRCA had increased precision because of the inclusion of Indian isolates (Patil et al., 2012). The nt substitution rate was on an average $6.5 \times 10^{-4}$ for DENV-1, 2 and 4 as been noted before (Twiddy et al., 2003). DENV-3 had a higher rate of mutation of $1 \times 10^{-3}$. The DENV-3 had higher rate of nucleotide (nt) substitution which could have been due to lack of data for the intervening years (1960-2000). For DENV-2 positive selection pressure was observed at two sites, previously reported to be important for defining cellular tropism (Roehrig., et al., 1994). For DENV-1, 3 and 4 there was no positive selection pressure observed. DENV have been characterized by low selection pressure because of the functional constraints imposed by the dual host life cycle of DENV. (Holmes., 2003).

The results of the phylogenetic and molecular clock analysis carried out with whole genome (WG) sequence were in agreement with the results obtained with E gene sequences. Analysis with individual genes revealed prM and core (C) genes to be unsuitable for phylogenetic analysis. There were changes in topology of the phylogenetic tree with NS2b and NS4B genes indicating that they are prone to genetic variation. Diversity in nt and amino acid (aa) sequence was highest for DENV-2. On analysis of p-distance values for individual genes, it was found that higher nt and aa diversity present in the C and Non Structural (NS2B, NS4A and NS5) genes contributed to genotype shift and diversity in the NS3, NS4A and NS4b were associated with lineage changes.

The aa sequence analysis revealed that a large proportion of the aa substitutions that occurred were conservative and probably did not affect the structure of the protein. The non-conservative substitutions were very often unstable and a small proportion was fixed. The aa sequence analysis revealed that the C and prM had maximum mutations in DENV-2. The E protein had mutations in all four serotypes. Of the NS proteins, NS2A/2B and 4A/4B had just one or two stable mutations. NS1 on the other hand had significantly larger number of mutations in DENV-2 while NS3 and NS5 had mutations in all serotypes. In NS3 the mutations were located in the helicase domain. In NS5 the maximum mutations were in the polymerase domain. The mutations in NS3 and NS5 may increase the enzyme activity of the protein by affecting the structure which may alter its binding efficiency to RNA thereby increasing the replication efficiency of the virus.
The study therefore has successfully delineated the genotypes/lineages of the four serotypes of DENV that were and are circulating in India. The study has also demonstrated the key role of Indian isolates in the evolution of DENV. Events of importations, exportations, recombination and mutation have all contributed to the emergence of viruses with greater virulence and transmissibility.