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Emergence of the Asian genotype of DENV-1 in South India

D. Cecilia[a,*], J.A. Patil[a], M.B. Kakade[a], A. Walimbe[a], K. Alagarasu[a], B. Anukumar[b], A. Abraham[c]

[a] Dengue Group, ICMR-National Institute of Virology, 20-A Ambedkar Road, Pune 411001, Maharashtra, India
[b] ICMR-NIV Kerala Unit, Allappuzha, Kerala, India
[c] Christian Medical College, Vellore, Tamil Nadu, India

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ABSTRACT

A large outbreak of dengue occurred in Tamil Nadu, South India in 2012 with 12,000 cases and CFR of 0.5%. Molecular characterization of virus present in the sera of dengue patients was undertaken to determine if there were changes in the virus population. All four serotypes were circulating but DENV-1 was dominant, present in 52% of the serotyped samples. Furthermore, the genotype of only DENV-1 had changed; the Asian genotype had displaced the American/African. Phylogenetic analysis revealed that the Asian genotype was introduced from Singapore and shared 99% similarity with viruses, associated with large outbreaks in Singapore and Sri Lanka. We report for the first time the emergence of the Asian genotype of DENV-1 in southern India causing an extensive and severe outbreak. The study proves how movement of DENV can affect dengue outbreaks and underscores the need for close molecular monitoring of DENV.

1. Introduction

Presently dengue affects more than 100 countries. The World Health Organization (WHO) reports 50–100 million cases/year of which 1% progress to severe disease (WHO Fact Sheets, 2017). The number of dengue cases increased from 2.2 million in 2010 to 3.2 million in 2015, which was attributed partially to improved diagnosis and reporting. During the last five years, the National Vector Borne Disease Control Programme (NVBDCP) reported 80,725 cases/year and average case fatality rate (CFR) of ~ 0.24% (http://nvbdcp.gov.in/dengu1.html). The NVBDCP has a network of 542 sentinel centres distributed over 36 states of India and records serologically confirmed cases of dengue.

Dengue virus (DENV) is represented by four antigenically defined serotypes and each serotype has multiple genotypes with several lineages/clades based on phylogenetic analysis of envelope (E) or whole genome sequences. DENV causes a spectrum of disease, ranging from subclinical infection to severe fatal disease. Both host and viral factors have been incriminated in dengue pathogenesis. As there are no animal models for dengue disease, the role of virus has been indicated largely by epidemiological and evolutionary studies. The associations of certain DENV genotypes with outbreaks that are unusually large or severe have indicated the importance of virus genetics. The introduction of the Asian /American genotype of DENV-2 in the Americas in the 1980s was considered responsible for DHF outbreaks (Rico-Hesse et al., 1997). Genotype III of DENV-3 which originated in India and spread to Sri Lanka, Latin America and East Africa was associated with the emergence of DHF outbreaks (Messer et al., 2003; Patil et al., 2012). Sequence analysis of the E gene of DENV circulating over a period of two years in Singapore indicated that *in situ* evolution could lead to improved fitness of viruses and switching of clades within genotypes (Lee et al., 2012). In India, occurrence of DHF outbreaks was coincidental with changes in the genotypes/lineages of all four serotypes in the late 1980s (Cecilia, 2014). Genotype shifts for DENV-2, American to Cosmopolitan (Kumar et al., 2010) and DENV-4, genotype V to I (Cecilia et al., 2011) and lineage changes in AM/AF genotype of DENV-1 (Patil et al., 2011) and in genotype III of DENV-3 were observed. No further change in genotype has been reported during the last 20 years in India. There have been changes in lineages within the AM/AF genotype of DENV-1 (Kukreti et al., 2008; Anoop et al., 2010), the Cosmopolitan genotype of DENV-2 (Afreen et al., 2016) and genotype III of DENV-3 (Manakkadan et al., 2013), but these changes were not associated with virulence. The Tamil Nadu outbreak in 2012, with 12,000 cases was larger than the average outbreak in India and the case fatality rate (CFR) of 0.5% was higher than the average CFR reported in India. It was possible that the outbreak was caused due to the introduction of a new genotype of DENV. Therefore, molecular characterization of the virus circulating in Tirunelveli, the epicentre of the outbreak was undertaken.

* Corresponding author at: Dengue Group, ICMR-National Institute of Virology, 20-A Ambedkar Road, Pune 411001, Maharashtra, India.
E-mail address: cecilia.dayaraj@gmail.com (D. Cecilia).

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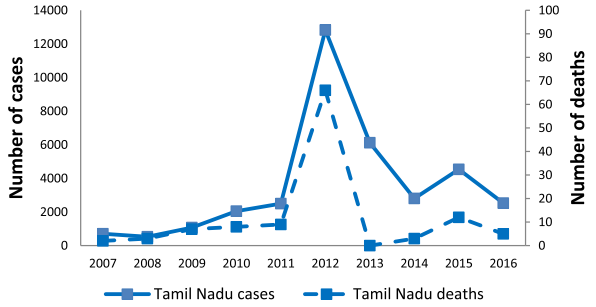

Fig. 1. Dengue in Tamil Nadu during 2007–2016. The number of cases and deaths due to dengue reported by NVBDCP in Tamil Nadu, India.

2. Materials and methods

2.1. Samples

The serum samples analysed in the present study were from one of the largest dengue outbreaks that occurred in Tamil Nadu in May-June 2012 with its epicentre in Tirunelveli. There were 12,826 cases reported with 66 deaths (CFR of 0.51%). The number of dengue cases and deaths as reported by National Vector Borne Disease Control Programme (NVBDCP) (http://nvbdcp.gov.in/den-cd.html) for ten years (2007–2016) for the state of Tamil Nadu is shown in Fig. 1. The average number of cases excluding 2012 is 2539/year and the average number of deaths is 5.44/year (0.21%). Thus, it is evident that the 2012 outbreak was one of the largest in Tamil Nadu.

The National Institute of Virology (NIV) is the Apex Reference Centre for the NVBDCP and provides serotyping/genotyping for the programme. NIV was requested by the Special Secretary to Govt. Health & Family Welfare Department, Tamil Nadu to assist in virological investigations during the 2012 outbreak. Serum samples were collected preferably within the first few days after onset of fever and brought to NIV. Serotyping/genotyping was also carried out for serum samples received from NIV Kerala Unit, Kerala in 2013 and from Christian Medical College (CMC), Tamil Nadu in 2015.

Serum samples from suspected dengue patients were tested for the presence of viral nonstructural protein 1(NS1) and DENV-specific IgM using ELISA based kits manufactured by Pan Bio (Australia) and NIV (India) respectively. NS1 is present in the serum of dengue patients from 1 to 10 days post onset of symptoms and IgM appears 5–7 days after onset of illness (WHO). Viral RNA is present in samples from 0 to 7 days post onset. Therefore, samples which were NS1 positive and IgM negative were selected for the study as they were, more likely to be positive for viral RNA.

3. Serotyping and phylogenetic analysis

The serotype was determined by multiplex RT-PCR (Lanciotti et al., 1992) using viral RNA purified from the serum sample. Representative positive samples were processed for sequencing of envelope (E) gene using primers designed previously (Patil et al., 2011, 2012). The phylogenetic relationship was determined by constructing maximum likelihood (ML) tree using MEGA v. 6.0 (Tamura et al., 2013). For phylogenetic analysis, the envelope (E) gene sequences of 24 Indian and 77 global sequences of DENV-1, 38 Indian and 52 global sequences of DENV-2, 21 Indian and 75 global sequences of DENV-3, and 14 Indian and 47 global sequences of DENV-4 were included. In addition, phylogenetic analysis was also carried out using the C-prM region sequence for DENV-1. The deduced amino acid sequences of the E protein of DENV-1 (Asian) viruses from Tirunelveli, Kerala, Vellore, Sri Lanka and Singapore were compared.

4. Phylogeographic analysis for DENV-1 Asian genotype

There was more than 99% identity at nucleotide level in the E gene sequences of the seven DENV-1 (Asian) viruses from Tirunelveli. Therefore, only two sequences from Tirunelveli, one each from Kerala and Vellore from the present study along with 110 world-wide E gene sequences of DENV-1 (Asian), available in GenBank, were used for analysis. The year of sample collection ranged from 1943 to 2015.

The best evolutionary distance model, as implemented in MEGA 6, was found to be TN93+G (Tamura-Nei with gamma-distributed rates of variation among sites). The Bayesian Markov Chain Monte Carlo method, available in BEAST 1.8.2 (Drummond and Rambaut, 2007) was applied to estimate the rate of nucleotide substitution and evolutionary time scales on the basis of the year of sample collection associated with each sequence. Among different clock models, the relaxed log normal clock model with Bayesian Skyline tree was chosen as the best fit model on the basis of log marginal likelihood estimated by using path sampling and stepping stone sampling methods (Baele et al., 2012; Baele et al., 2013).

For phylogeographic analysis, the name of the country of sample collection for each sequence was used as "Trait". The standard continuous-time Markov chain (CTMC) model with the Bayesian stochastic search variable selection (BSSVS) was fitted to the data (Lemey et al., 2009) in order to estimate the most probable ancestral country for different nodes of the Maximum Clade Credibility (MCC) tree.

The SPREAD 1.0.3 software (Bielejec et al., 2016) was used to identify significant transmission links between different countries. Three independent runs of the Markov chain were performed with 500 million generations and 50,000 as sampling frequency.

5. Results

5.1. Serotypes detected

A total number of 143 samples, which were positive for viral antigen NS1, were brought from Tirunelveli in 2012. Most of the samples were collected within 4 days of fever (n = 102). The age of patients ranged from 11 months to 70 years with median age of 11.5 years. There was a larger representation of cases from the < 10 yr age group (n = 66) followed by the 11–20 yr age group (n = 43) and > 20 yr age group (n = 34). The female:male ratio was 1:1.4. Serotyping by multiplex RT-PCR revealed the presence of all four serotypes in Tirunelveli. Of the 143 serum samples tested, 96 sera could be serotyped, of which six represented co-infections with two serotypes. Amongst the single serotype infections, there were 44 (48.9%) DENV-1, 17 (18.9%) DENV-2, 25 (27.8%) DENV-3 and four (4.4%) DENV-4 cases. Amongst the dual infections, there were three cases of DENV-1+DENV-2, two cases of DENV-1+DENV-3 and one case of DENV-1+DENV-4. DENV-1 was present in 62.7% of < 10 yr old cases, and in 43% of the > 10 yr old cases.

As part of the regular monitoring of viruses carried out by NIV, 25 sera from dengue suspected cases were serotyped at the Kerala Unit of NIV during the 2013 outbreak. There was equal representation of three serotypes; eight samples were positive for DENV-1, nine for DENV-2 and eight for DENV-3. Of the 20 DENV NS1-positive sera received from CMC, Tamil Nadu in 2015, nine could be serotyped; two were found to be DENV-1, four were DENV-2, two were DENV-3 and one was DENV-4.

6. Phylogenetic analysis

The E gene was sequenced for 27 samples; 16 from Tirunelveli (7 DENV-1, four DENV-2, three DENV-3 and two DENV-4), two from Kerala (one each of DENV-1 and DENV-2) and all nine from Vellore (four DENV-2, two each of DENV-1 and 3 and one of DENV-4. The ML

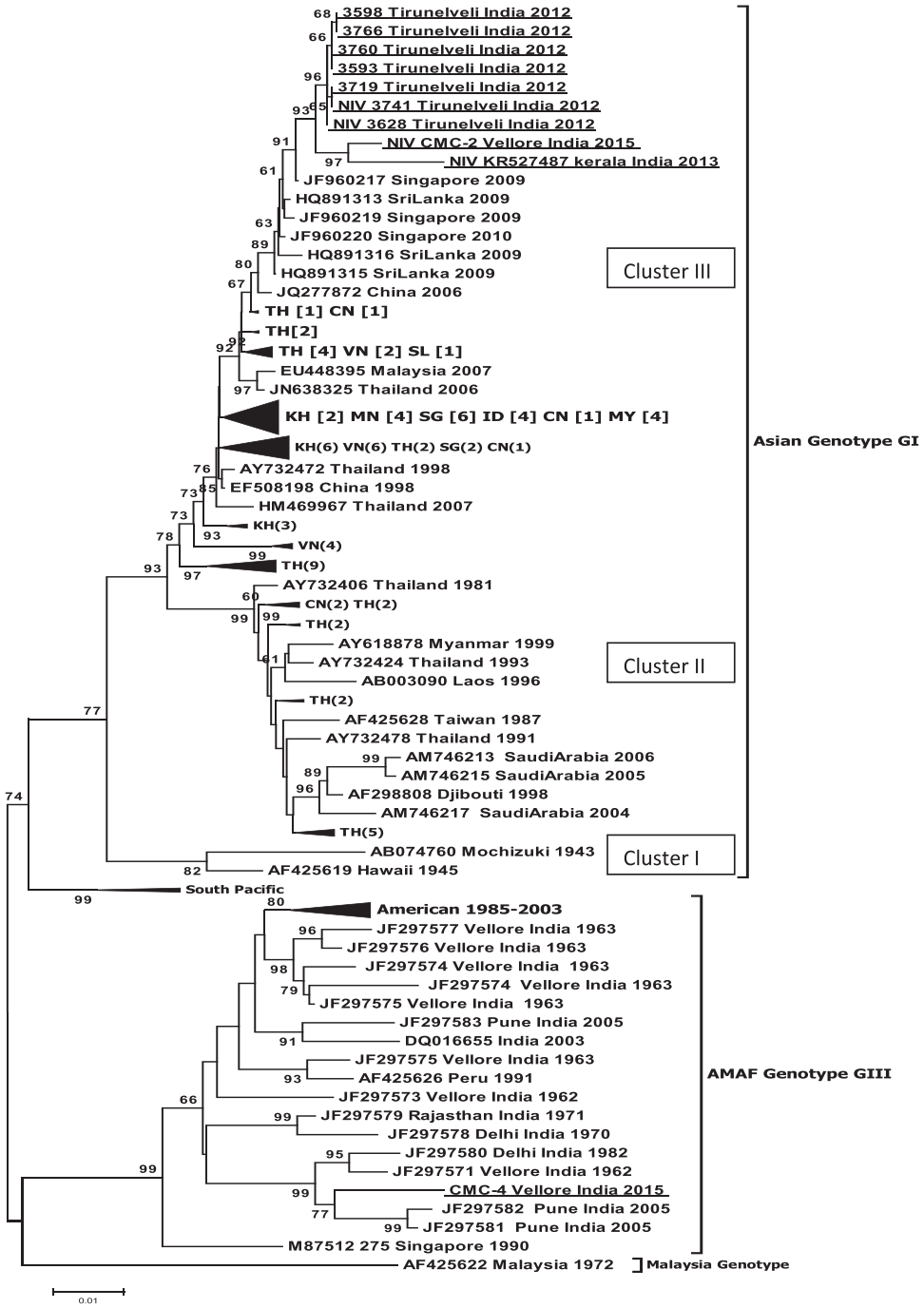

Fig. 2. Phylogenetic tree of DENV-1 Asian genotype. The complete E gene sequences were analysed by Maximum Likelihood (ML) method using MEGA v. 6.0.

tree for DENV-1 (Fig. 2) revealed that all the Indian isolates of DENV-1 from 1962 to 2005 and one from Vellore (2015) belonged to the American/African (AM/AF) genotype, whilst all the viruses from Tirunelveli, Kerala and one from Vellore belonged to the Asian or GI genotype. There were three major clusters in the Asian genotype. The Hawaii (1945) and Mochizuki (1953) viruses in cluster I were the outliers at the root. Cluster II contained isolates from Saudi Arabia, Africa, China and Thailand. Cluster III represented viruses from South/Southeast Asia. The viruses from South India sequenced in the present study formed a tight monophyletic group in Cluster III with 93% bootstrap support (Fig. 2). To ensure inclusion of larger number of sequences from India, additional analysis was carried out using C-prM sequences. The sequence set included 17 from Tirunelveli, 54 from India and 68 global sequences. Excluding the current isolates from South India, all the DENV-1 sequences from India clustered with the AM/AF genotype (Fig. S1). However, there were four sequences from Genbank (Accession nos. AY584591 to AY584594) submitted from Delhi that grouped with the outlier cluster I of Asian genotype. These sequences, submitted without publication, were very similar to Hawaii 1944 virus, which is used as the prototype virus in many laboratories and could have been a source of cross contamination. As this data has not been included in most of the publications from India, it was excluded from the present analysis.

For the other serotypes, based on E gene sequence, no change was detected in the genotypes; DENV-2 clustered with GIV (Cosmopolitan), DENV-3 with GIII (Indian subcontinent) and DENV-4 with GI (supplementary data Fig. S2a–c). Similar findings were obtained with CprM sequences (n = 14) (data not shown).

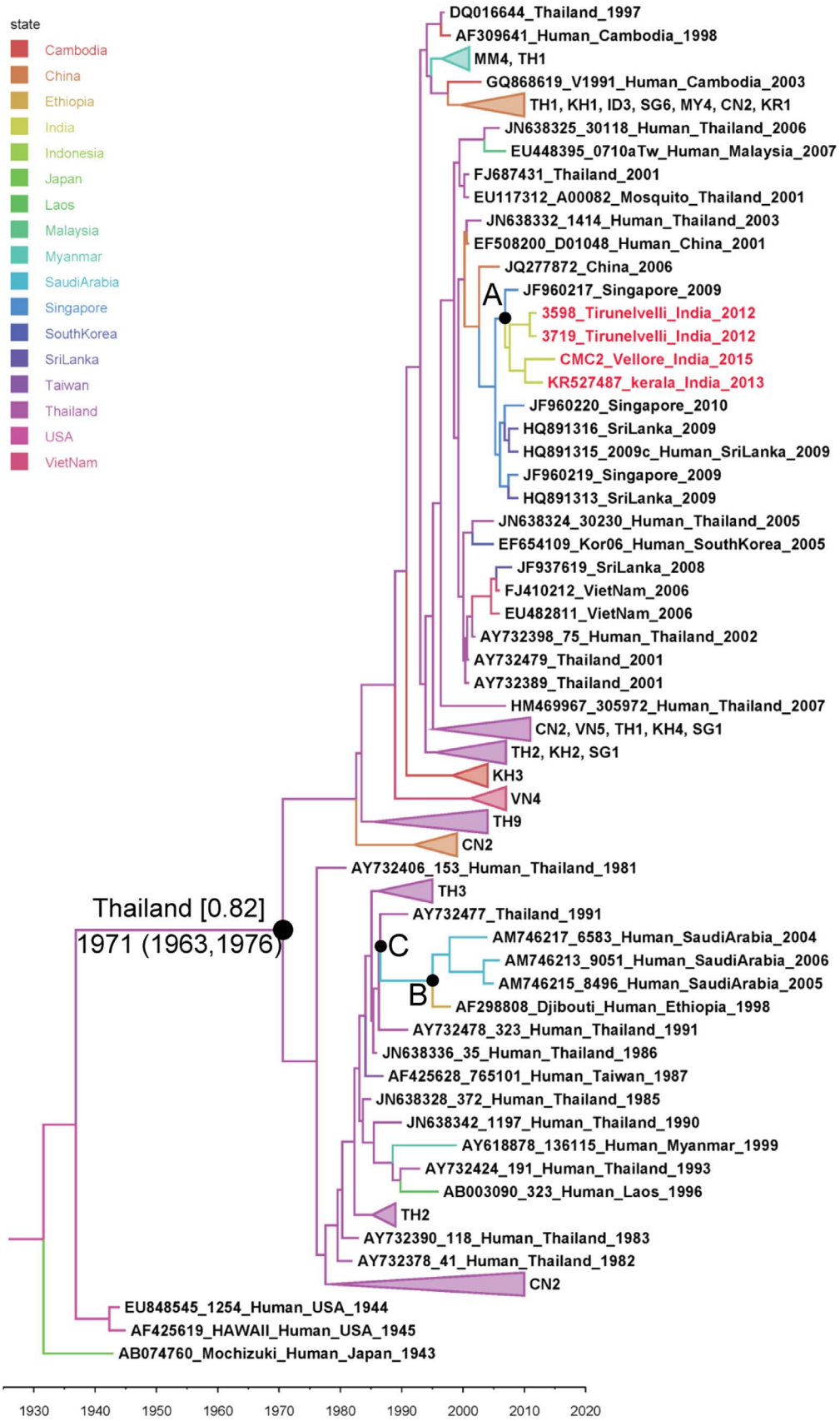

Fig. 3. Maximum Clade Credibility (MCC) tree under relaxed lognormal clock. Most probable ancestral location with posterior probability in square brackets and time to most recent common ancestor (tMRCA) with 95% HPD limits is mentioned at particular nodes. Indian sequences from this study are shown in red colour. The branches are coloured according to the most probable ancestral location. The name of countries and number of sequences from each country are mentioned wherever a cluster is collapsed. Node A: Singapore [0.9]; 2007 (2005,2009); Node B: Saudi Arabia [0.54]; 1995 (1992,1998); Node C: Thailand [0.99]; 1987 (1985,1989). *ISO 3166-1-alpha-2 codes used for country names: - CN: China, ID: Indonesia, KH: Cambodia, KR: South Korea, LK: Sri* Lanka, MM: Myanmar, MY: Malaysia, SG: Singapore, TH: Thailand, VN: Viet Nam.

Comparison of nucleotide sequences of the viruses within cluster III containing the South India viruses revealed very low sequence diversity (0.1–0.5%) between Tirunelveli, Singapore and Sri Lanka viruses. There was indeed greater diversity between Tirunelveli and Vellore 2015 1.2–1.3%) and Kerala 2013 (1.9%) sequences. Comparison of the deduced amino acid sequences revealed that one Tirunelveli isolate was identical to the Singapore and Sri Lanka viruses, four isolates had a single conservative (C) change V491A in the transmembrane region and two had a T276A change in Domain II. There were no non-conservative (NC) changes. In contrast, the Kerala virus had 13 changes of which eight were unique and five were shared with the Vellore virus. Of the eight mutations in Kerala viruses, there were two C (T145I, G146A) and two NC (V24E, D147A) changes in domain I and four NC (G106E, Q243L, D244F, S273F) changes in domain II. The five mutations common to Kerala and Vellore were present in all domains; domain I had one C (T163I) and one NC (K285I) change; domain II had one C (M272T) and one NC (K284N) change and domain III had one C (V320I) change. In addition Vellore virus had a unique C change, V55I in domain II.

The rate of nucleotide substitution for Asian genotype was 1.09×10^{-3} (with 95% Highest Probability Density limits (HPD) 0.094, 1.24). Based on the phylogeography analysis (Fig. 3), the Asian genotype of DENV-1 originated in US/Japan during 1932 with 95% HPD limits (1922, 1939). However, the US/Japan strains of the 1940s formed an outgroup and may not have contributed to dissemination of the Asian genotype. The most probable ancestral location of all other Asian genotype viruses was Thailand with probability of 0.82 and time to the Most Recent Common Ancestor (tMRCA) 1971 (HPD limits: 1963, 1976). The Indian strains circulating during 2012–2015 in Tirunelveli, Kerala and Vellore (South India) probably descended from a common ancestor that existed in Singapore (probability = 0.9) during the period 2005–2009 (Node A). The strains belonging to Saudi Arabia and Ethiopia formed a monophyletic group with the most probable ancestral origin as Saudi Arabia at Node B (probability = 0.54) during 1990s. Interestingly it seems that the seed virus was dispersed from Thailand at Node C (probability = 0.99).

The rate map shows a significant migration link between Singapore and India (Bayes Factor (BF) = 54.9) and between Singapore and Sri Lanka (BF = 115.8). Overall from the rate map it can be concluded that Thailand is an important source or hub for the dissemination of Asian DENV-1 viruses.

7. Discussion

The present study has shown the emergence of the Asian genotype (GI) of DENV-1 in South India during one of the largest outbreaks in Tamil Nadu in 2012 with greater number of cases and higher CFR. Although all four serotypes were circulating, DENV-1 Asian genotype was predominant as it was detected in ~ 52% of the cases that were serotyped. Co-infections were detected in six cases, the dominant DENV-1 with either DENV-2, 3 or 4. Co-infections of DENV-1 with other serotypes have been reported in a few instances (Loroño-Pino et al., 1999; Vong et al., 2010; do Carmo Alves Martins et al., 2014), especially from regions where dengue is endemic. Circulation of two different genotypes, Asian and AM/AF was observed in Vellore. Most outbreaks in India are characterized by the presence of all four serotypes (Dar et al., 2006; Mishra et al., 2015; Cecilia, 2014) whereas the presence of two different genotypes in the same outbreak is rarer (Tao Jiang et al., 2012).

DENV-1 has five genotypes; Asia, South Pacific, Thailand, Malaysia and AM/AF (Cosmopolitan) (Patil et al., 2011). Recently a new sylvatic genotype VI was assigned to a virus isolated from a traveller to Brunei in 2014 (Pyke et al., 2016). Genotyping revealed a displacement of the AM/AF genotype of DENV-1 by the Asian genotype. The AM/AF genotype has been labeled variably as GIII (Anoop et al., 2012; Dash et al., 2015; Kumar et al., 2013), GIV (Tissera et al., 2011) or GV (Pyke et al., 2016) and therefore the geographical nomenclature has been adopted. The AM/AF has been the only genotype reported so far in India; from Delhi (Kukreti et al., 2008; Dash et al., 2015, Afreen et al., 2015), Rajasthan, Pune, Tamil Nadu, Gwalior (Patil et al., 2011), Kerala (Anoop et al., 2012; Kumar et al., 2013), Chattisgarh and Madhya Pradesh (Barde et al., 2015). Therefore, the presence of the Asian genotype in South India is a new introduction into India.

For the other serotypes, there was no change in the genotype, they remained the same as reported earlier; Cosmopolitan genotype (GIV) for DENV-2 (Kumar et al., 2010; Afreen et al., 2016), GIII for DENV-3 (Patil et al., 2012; Manakkadan et al., 2013) and GI for DENV-4 it (Cecilia et al., 2011; Waman et al., 2016).

The DENV-1 Asian genotype virus of Tirunelveli shared 99.5% identity with the Singapore and Sri Lanka viruses which were responsible for huge epidemics in Singapore in 2005 with 14,000 cases and 27 deaths (Koh et al., 2008; Lee et al., 2012) and Sri Lanka in 2009 with 35,008 cases and 346 deaths (Tissera et al., 2011) respectively. The DENV-1 Asian genotype was also the dominant virus in the 2013 outbreak in Myanmar with 20,255 cases and 84 deaths (Ngwe Tun et al., 2016). Based on nucleotide diversity, the Vellore 2014 was closer to the Tirunelveli 2012 virus than the Kerala 2013 virus. The Tirunelveli 2012 virus E protein differed from the Sri Lanka 2009 virus by only one amino acid, the Vellore 2015 by five residues and the Kerala 2013 virus by 13 residues. There were more NC changes in the Kerala virus. According to the functional model presented by Christian et al. (2013), the fusion loop which is critical to virus infectivity is tethered by inter/intra-domain interactions. Three NC mutations in the Kerala virus D147A, Q243L, D244F were part of these interactions and the V24 residue substituted by E was one of the new contact residues for the fusogenic trimer. The fact that DENV-1 (Asian), detected in Kerala and Vellore, was not associated with severe outbreaks indicates that the virus has perhaps mutated to a low virulence phenotype; however this needs to be investigated further.

Phylogeographic analysis revealed Thailand as the seed source of the DENV-1 Asian genotype and a spread through China to South/Southeast Asia. Furthermore it was observed that the Asian genotype was introduced from Singapore into India and in parallel into Sri Lanka during 2005–2009. Ockwieja et al. (2014) suggest that the virus dispersed to Sri Lanka and then to Singapore. The discrepancy in the findings may have been largely due to differences in data sets used for the analysis. The data set in the present study has a better geographical representation and also includes the outlier group, which helps to improve the estimate of topology. DENV-1 Asian genotype was also reported from Jeddah, Saudi Arabia (Azhar et al., 2015), as a probable import from Africa based on only phylogenetic analysis. In contrast, the phylogeographic analysis in the present study indicates that the most probable ancestral origin for the strains from Ethiopia was Saudi Arabia at Node B. The presence of Thailand as the most probable ancestral location at several nodes with high probability indicates it to be an important hub for the dissemination of the Asian genotype of DENV-1.

Further clinical, epidemiological and molecular studies are required to identify the major factors that determined the emergence of this virus and to facilitate the development of control strategies for dengue virus emergence in India and elsewhere.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2017.07.004.

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