Tropical Medicine and International Health doi:10.1111/tmi.12898

VOLUME 22 NO 7 PP 871–880 JULY 2017

Genetic signatures coupled with lineage shift characterise endemic evolution of Dengue virus serotype 2 during 2015 outbreak in Delhi, India

Manish Chandra Choudhary[1], Ekta Gupta[1], Shvetank Sharma[2], Nadeem Hasnain[1] and Pragya Agarwala[1]

1 *Department of Virology, Institute of Liver and Biliary Sciences, New Delhi, India*
2 *Molecular and Cellular Medicine Department, Institute of Liver and Biliary Sciences, New Delhi, India*

Abstract

OBJECTIVE In 2015, New Delhi witnessed a massive outbreak of Dengue virus (DENV) resulting in high morbidity and mortality. We report the molecular characterisation of the dominant circulating DENV strain to understand its evolution and dispersal.

MATERIALS AND METHODS DENV infections were diagnosed by detection of IgM/NS1 antigen, and serotyping was performed by C-PrM PCR. Envelope gene was amplified, and variation(s) in envelope gene were analysed. Phylogenetic tree construction, time-based phylogeny and origin of DENV were analysed. Site-specific selection pressure of envelope gene variants was analysed.

RESULTS Confirmed DENV infection was observed in 11.34% (32 of 282) cases, while PCR positivity for C-PrM region was observed in 54.16% (13 of 24) of NS1 antigen-positive cases. All samples belonged to serotype 2 and cosmopolitan genotype. Phylogenetic analysis using envelope gene revealed segregation of cosmopolitan genotype strains into specific lineages. The Indian strains clustered separately forming a distinct monophyletic lineage (lineage III) with a signature amino acid substitution viz., I162V and R288K. Selection pressure analysis revealed that 215D, 288R and 304K were positively selected sites. The rate of nucleotide substitution was 6.93×10^{-4} substitutions site-1 year-1 with time to most common ancestor was around 10 years with JX475906 (Hyderabad strain) and JN030345 (Singapore strain) as its most probable ancestor.

CONCLUSION We observed evolution of a distinct lineage of DENV-2 strains on the Indian subcontinent with possible changes in endemic circulating dengue strains that might give rise to more pathogenic strains.

keywords dengue virus, India, outbreak, cosmopolitan genotype, MCMC, lineage shift

Introduction

Dengue, one of the most important mosquito-borne viral diseases, is a global health problem [1]. The virus belongs to the *Flaviviridae*, genus Flavivirus and consists of a single-stranded, positive-sense RNA as its genetic material [2]. Based on sequence diversity, it is classified into four distinct serotypes, each serotype is further grouped into various genotypes [3]. Infections by Dengue virus (DENV) are either asymptomatic or self-limiting, presenting with acute febrile illness often accompanied by rash. However in some cases, serious complications may arise: high fever, irreversible damage to blood vessels, hepatomegaly, bleeding from nose and gums and failure of the circulatory system. Infection with any of the serotype leads to lifelong immunity to that particular serotype [4]. All four serotypes have been found to be responsible for DENV epidemics [4]. Secondary infection caused by a different serotype of DENV is often more dangerous due to antibody-dependent enhancement (ADE) resulting in high viraemia [5, 6].

The first outbreak in Delhi was reported in 1996 [7]. In the last 15 years, DENV fever outbreaks in Delhi have been occurring every 3–4 years [8–10]. Many factors have been attributed to the spread of DENV, which has led to wider circulation of DENV in India in the past few decades [11]. With 15 867 cases and 60 deaths, the 2015 DENV outbreak in Delhi was the worst in 20 years (http://www.nvbdcp.gov.in/den-cd.html).

DENV serotype 2 and serotype 3, the 'virulent' serotypes, have mostly been associated with outbreaks [12]. Studies have shown the association of enhanced severity of dengue during outbreaks with serotype (DENV-2 to DENV-1) and genotype (DENV-2 genotype IV to

genotype I) shifts [13–15]. Even changes at the lineage level have been attributed to disease severity in dengue virus, as observed with DENV-3 serotype in Sri Lanka [16, 17]. Circulating DENV from Delhi has also been reported to shift in serotypes between outbreak and non-outbreak infections [18].

This study focuses on genetic characterisation of DENV serotype circulating in the 2015 outbreak in New Delhi, using the Envelope (E) gene and studying its molecular evolution in the context of global DENV-2 serotype sampled across the period 1944 to 2016. E gene was selected for analysis as it is known to be under immune selection pressure [19].

Materials and methods

Patient selection and sample collection

This was a retrospective study carried out from August 2015 to December 2015 in a tertiary care liver hospital in New Delhi, India. All consecutive patients with signs and symptoms (according to WHO guidelines) suggestive of DENV infections visiting the OPD or admitted in IPD were tested for DENV infection [20]. Plasma was separated from blood samples and stored at −80 °C till further testing.

DENV diagnosis

DENV was diagnosed with Dengue NS1 Ag if the sample was collected within the first 5 days of onset of clinical symptoms, and with Dengue IgM capture ELISA if the sample was collected after 5 days of onset of clinical symptoms. Dengue IgG testing was performed using Dengue IgG capture ELISA to detect prior exposure. Dengue NS1 Ag ELISA, IgM and IgG ELISA tests were purchased from PANBIO (Gyeonggi-do, ROK).

RNA extraction, RT-PCR for and sequencing

RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen, GmBH Germany) according to manufacturer's instructions. DENV detection and typing were performed according to Lanciotti *et al.* with slight modifications [21]. cDNA was synthesised using Quantitect reverse transcriptase kit (QIAGEN, GmBH Germany) according to manufacturer's instructions. 511-bp product of C-PrM gene was amplified with D1 and D2 primers using Phusion high-fidelity DNA polymerase (Thermofisher Scientific, MA, Waltham) (Table S1). Envelope gene amplification was performed using *in-house* designed primers (Table S1). PCR amplicons were gel-purified using QIAquick gel extraction kit (QIAGEN, GmBH Germany) as per manufacturer's instructions. Purified amplicons were subjected to Sanger sequencing using ABI 3730 DNA analyzer (Thermofisher Scientific, MA, Waltham).

Sequence analysis and phylogenetic reconstruction

Forward and reverse sequence reads were aligned and assembled using DNA Baser v3.5.1 (Heracle BioSoft SRL, Romania) and subjected to similarity search using Nucleotide BLAST program. Sequences were aligned using Clustal W program in BioEdit software (v7.2.5). A phylogenetic tree was constructed using the Maximum likelihood method in MEGA7 with 1000 bootstrap replicates [22].

Selection pressure analysis using Selecton server

SELECTON server was used to assess sites undergoing positive selection at a single amino acid site of DENV-2 E gene. Two data sets were used for this analysis, one data set comprising Indian cosmopolitan strains and another comprising all cosmopolitan strains. The ω ratios (ka/ks) were calculated using M8 [23], M8a [24], M7 [25], M5 [26] and MEC [25, 26] models.

Molecular clock analysis using Bayesian MCMC

Data set for molecular clock analysis comprised of 13 Indian DENV-2 sequences reported in this study and 136 DENV-2 global sequences retrieved from PubMed. Sequences with query coverage <90% and sequence similarity >99.5% were excluded from the study. Rate of nucleotide substitution and time to most recent common ancestor (TMRCA) of DENV-2 strains were calculated by using Bayesian MCMC approach implemented in BEAST v1.8.4 [27]. Best fit nucleotide substitution model was determined by Akaike information criterion, corrected (AICc) using MODELTEST in MEGA7 [28]. We employed relaxed uncorrelated exponential molecular clock with constant population growth model as described earlier [29]. MCMC chain was run for 50 000 000 steps with parameter values sampling at every 1000 steps. Three independent MCMC analyses were performed, and the resulting log files were combined using Log-Combiner v1.8.1 with 10% burn-in sets for each run. Log files were visualised using Tracer v1.6 to check chain convergence and also to ascertain whether an effective sample size (>200) had been reached for all parameters (http://evolve.zoo.ox.ac.uk). 95% HPD intervals were assessed to ascertain uncertainty in the parameter estimates. Tree Annotator v1.8.1 was used for

generating maximum clade credibility tree. Figtree v1.4.2 was used for visualising resultant tree file. Bayesian skyline plot (BSP) was constructed using BEAST program.

Ethics

The study was approved by the Institute Ethics committee (IEC) of ILBS. As this was a retrospective study of leftover clinical samples sent to virology laboratory for routine diagnosis for DENV, as per standard protocol of IEC of ILBS, informed patient consent was not required.

Results

Dengue virus typing and Envelope gene sequence analyses

C-PrM PCR revealed that all samples (13 of 13) belonged to DENV serotype 2 and a cosmopolitan genotype. Next, we amplified 1.485 kb amplicon corresponding to envelope gene from 13 DENV-2 serotype samples which were further sequenced (Genbank accession numbers: KX061419-KX061430). The percentage nucleotide identity of Indian DENV-2 E gene with that of global DENV-2 E gene was in the range of 94–99% and the deduced amino acid sequence identity ranged from 97.4% to 99.8%. Single-nucleotide substitutions were scattered randomly along the entire length of the amplicon, while insertions and deletions were absent. Transitions were more frequent than transversions with the overall transition/transversion ratio of 14.92 (209/14). Comparison of deduced amino acid sequence of envelope showed substitution along the entire length of the amplicon. Most of these substitutions were genotype-specific, and based on these substitutions, these genotypes were segregated into specific lineages. Some of the signature substitutions in cosmopolitan genotypes using AY449677 sequence as reference were I46T, Q52H, E71A, H149N, I164V, S203D and N390S (data not shown). All 13 samples from this outbreak had a signature substitution, namely I162V reported for the first time (data not shown). Three samples (KX061428, KX061429 and KX061431) had a unique R288K change in the envelope protein.

Phylogenetic reconstruction based on 1.48 kb amplicon

The 1.485 kb Envelope gene was used for reconstructing a phylogenetic tree by maximum likelihood method using the Tamura-Nei substitution model with Gamma-distributed (G) rate with invariant among sites. 149 DENV-2 serotype sequences including three sylvatic strains (as outlier) were used in the analysis with a clear delineation into five genotypes evident from the phylogram (Figure 1). These five genotypes were Asian-I, Asian-II, American, Asian-American and Cosmopolitan, together with sylvatic strains (Figure 1). Of 146 DENV-2 serotype sequences, five sequences clustered in the Asian-I, 10 in the Asian-II, six in the American, 14 in Asian-American and 111 sequences in the cosmopolitan group (Figure 1). All Indian sequences reported in this study clustered within the cosmopolitan genotype with high bootstrap values (>70%). Further, based on branching pattern and amino acid sequence variation, these isolates were subdivided into specific lineages within cosmopolitan genotypes. The cosmopolitan genotype was segregated into four lineages with further sublineages based on branching pattern and specific signature changes in the envelope region (Table 1). All changes reported here are in context of AY449677 as a reference sequence.

Lineage I had samples mainly from Burkina Faso & Indonesia with the sampling year ranging from 1975 to 2003. Lineage I was subdivided into Ia (reference sequences) and Ib (I322V). Lineage II had sequences from East-Asian countries namely, Philippines, China, Taiwan and Singapore with the sampling year ranging from 1993 to 2013. These included sequences from two outbreaks: Taiwan, 2001, and Singapore, 2008. Further, lineage II was subdivided into lineage IIa (I46T, Q52H, I164V, S203D, N390S) and lineage IIb (Q52H, I164V, N390S). Lineage III had sequences from Singapore, India and Ethiopia with the sampling year ranging from 2009 to 2016. All sequences reported in this outbreak clustered into lineage III with high bootstrap values (100%). The changes in amino acid sequences in lineage III vis-à-vis reference sequences were V129I, I162V, I164V, R288K and N390S. The signature lineage III changes were I162V (all lineage III samples) and R288K (KX061428, KX061429, KX61431). Lineage IV was represented by maximum number of sequences with samples mainly from China, Pakistan, Singapore, Saudi Arabia, Sri Lanka, Bhutan and India with samples ranging from 1999 to 2014. Lineage IV had sequences from two major outbreaks: Pakistan, 2011, and China, 2014. Further, lineage IV was subdivided into four sublineages: IVa had sequences from China, India, Bhutan, Singapore (V129I, I141V, I164V, I322V & N390S). Lineage IVb had sequences mainly from the 2011 dengue outbreak in Lahore, Pakistan [29]. AB194883 (Sri Lanka, 2004) & GQ252676 (Sri Lanka, 2003) were two closely related sequences to Pakistan outbreak sequences and branched with high bootstrap values (88%). The specific amino acid changes compared to reference sequences were V129I, I141V, I164V, T226I, I322V and N390S. Lineage IVc had sequences from India, Singapore, China,

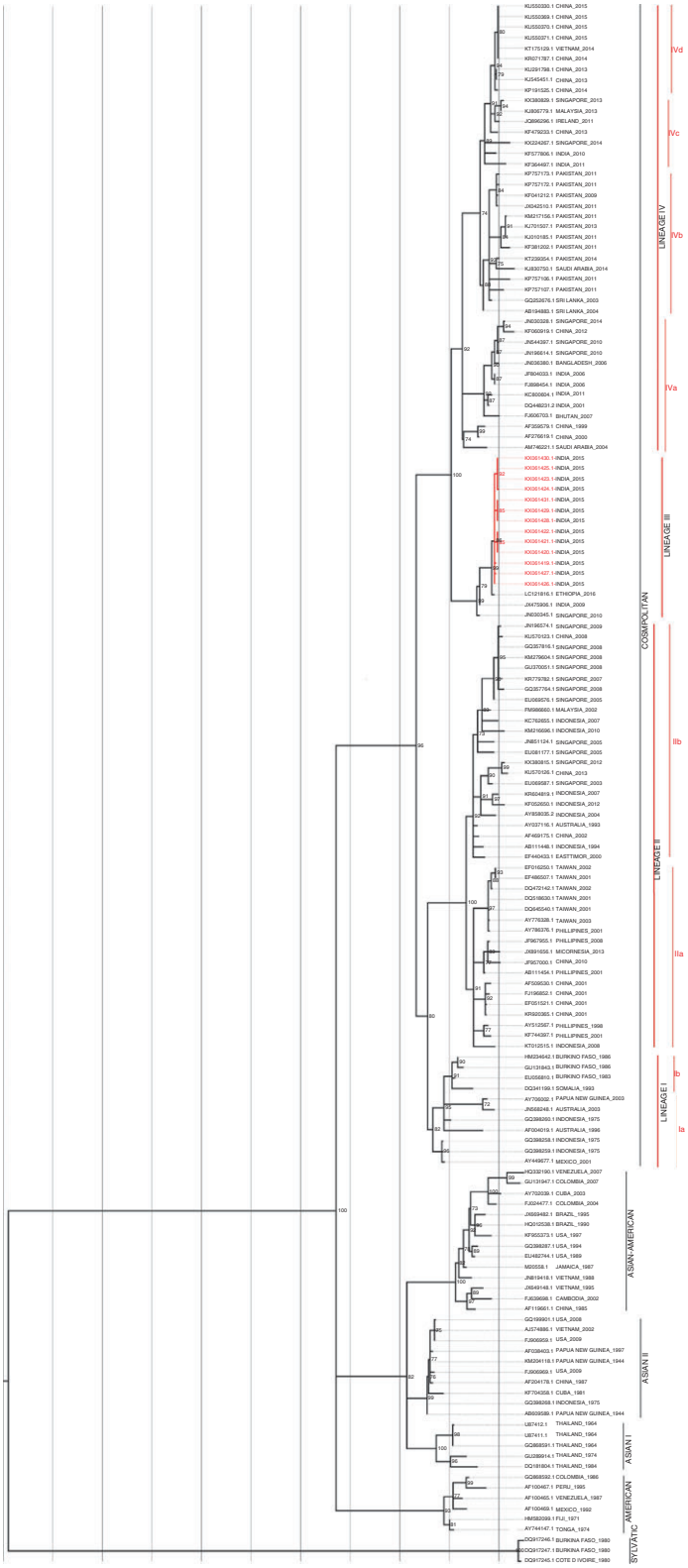

Figure 1 Molecular Phylogenetic analysis of DENV-2 strains by maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model. Numbers at the nodes indicates bootstrap values generated by 1000 replicates. Only bootstrap values >70 were considered for constructing tree. Strains sequenced in the study have been highlighted with red. Evolutionary analyses were conducted in MEGA7. The analysis involved 149 nucleotide sequences.

Table 1 Amino acid changes observed in cosmopolitan genotype samples vis-à-vis Lineage Ia cosmopolitan samples as a reference sequence. Wild-type amino acid is denoted next to its respective position, whereas the substitution is indicated in the box at its respective position. Outbreak samples are denoted by √, while signature amino acid change is mentioned, wherever it was identified. Extent of amino acid selection is indicated by colour scale with 1 indicating positive selection, while 7 indicates purifying selection

		Amino acid position (Lineage Ia reference sequence)							
Lineages	Outbreak samples	I46	Q52	V129	I141	I164	I322	N390	Signature amino acid change
Lineage Ib							V		
Lineage IIa	√	T	H			V		S	N203D
Lineage IIb			H			V		S	
Lineage III	√			I		V		S	I162V
Lineage IVa				I	V	V	V	S	
Lineage IVb	√			I	V	V	V	S	T226I
Lineage IVc				I	V	V	V	S	
Lineage IVd	√			I	V	V	V	S	T182A

Malaysia, Ireland with sampling year from 2010 to 2014. The distinct amino acid changes were V129I, I141V, I164V, I322V and N390S. Lineage IVd was represented by sequences mainly of 2014 dengue outbreak in China and was closely related to lineage IVc sequences. The distinct amino acid changes were V129I, I141V, I164V, I322V and N390S.

All outbreak sequences segregated distinctly, forming a monophyletic lineage in cosmopolitan genotype, and each of the outbreak sequences was characterised by signature amino acid change (Table 1).

Selection pressure analysis

Site-specific selection pressure was analysed by likelihood procedures and calculating significance between null model and alternate model. Two data sets were employed as follows: the first data set consisted of lineage III sequences within cosmopolitan genotype (n = 16); the second data set consisted of all cosmopolitan genotype sequences (n = 111). Four sites were found to be undergoing positive selection in lineage III cosmopolitan genotype, namely 215D, 288R, 314K, 361K (Figure 2a). Among 16 lineage III cosmopolitan genotypes, 13 sequences had 288R and three sequences (KX061428, KX061429, KX061431) had 288K. At position 314, all sequences except KX061427 (314K) had 314D, whereas at position 361, all sequences had 361K, except LC121816 (361R) as shown in Figure 2a. Selection pressure analysis using the second data set revealed many sites undergoing positive selection. Sites that were strongly associated with positive selection were 46I, 52Q, 61I, 93K, 131P, 150A, 162I, 181T, 254G, 288R, 308V, 321I, 359T, 360G, 361K, 443I and 491V (Figure 2b). It is interesting to note that most of the sites undergoing positive selection are also the sites that incorporated changes while undergoing lineage shifts in cosmopolitan genotypes (Figure 1).

Bayesian-based molecular clock analysis

Time-based phylogenetic tree reconstruction, nucleotide substitution rates and the time to most recent common ancestor (tMRCA) were inferred from a total of 149 DENV-2 E gene-dated sequences including 13 Indian DENV-2 reported in this outbreak. Posterior probability of the tree was in the range of 0.78–1, which was indicative of fully resolved branched tree with a higher degree of confidence. The approximate age of the entire tree was estimated to be about 141.29 years (95% HPD; 101.66–196.05) including sylvatic strains, whereas it was 98.54 years (95% HPD; 83.45–119.24) as shown in Figure 3. Among DENV-2 sequences from humans, American strains rooted out first around 49.61 years ago (95% HPD; 45.03–56.38), followed by Asian-American strains 44.62 years ago (95% HPD; 37.83–53.76) followed by Asian-I and Asian-II. The most common ancestor for DENV-2 cosmopolitan genotype was AY449677 from Mexico. Phylogenetic analysis comprising of DENV-2 cosmopolitan genotype by Bayesian MCMC algorithm revealed a bifurcation in divergence with two distinct branches: clade 1 represented by lineage I and lineage II samples, and clade 2 represented by lineage III and lineage IV samples. Clade 1 appears to have diverged earlier, around 59 years ago (95% HPD; 50.16–70.79), while clade 2 diverged around 37 years (95% HPD; 28.37–49.26). Among clade 2, lineage III from India rooted out around 9.49 years back (95% HPD;

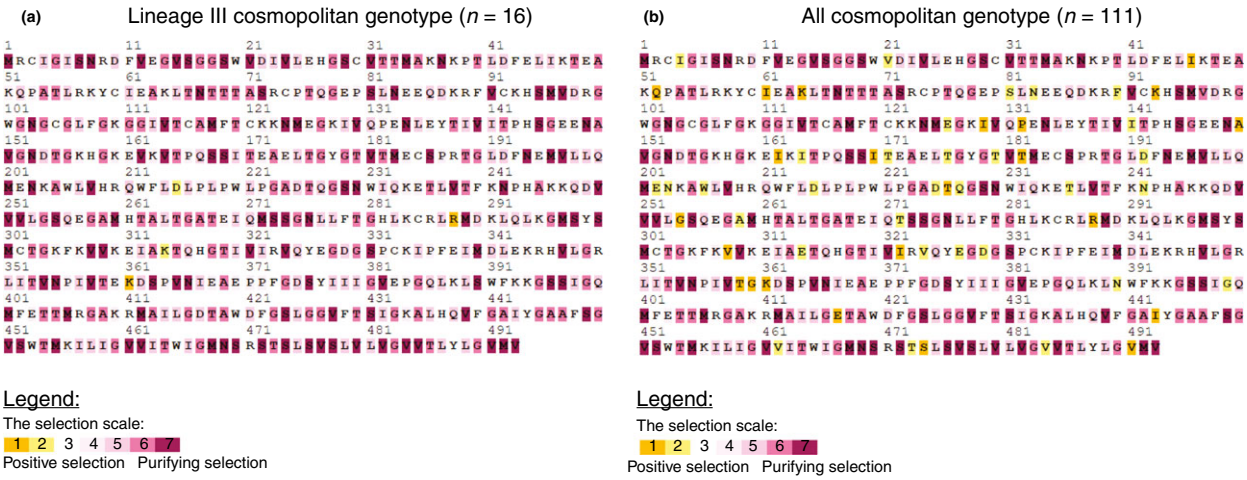

Figure 2 Selection pressure analysis using SELECTON server. Colour-coded selection pressure analysis was performed on two data sets. Data set 1 comprised of lineage III cosmopolitan genotypes (n = 16), while data set 2 consists of all cosmopolitan genotype strains (n = 111). Extent of positive selection is indicated by colour-coded scheme on a scale of 1–7; 1 with dark-yellow indicating sites undergoing positive selection, while 7 with magenta colour indicated sites under purifying selection.

7.34–12.81). Phylogenetic analysis using relaxed molecular clock approach revealed that the most closely related DENV-2 ancestors were JN030345 (Singapore) and JX475906 (India). The mean evolutionary rate of the DENV-2 envelope genes was 6.93×10^{-4} substitutions/site/year (95% HPD 5.26×10^{-4}–8.71×10^{-4}).

Evolutionary demography of DENV-2 Indian outbreak data set

The Bayesian skyline plot was constructed using both global DENV-2 sequences and Indian 2015 DENV-2 outbreak sequences. Analysis using the global DENV-2 data set revealed that the number of infections remained relatively constant until the 1980s, as when there has been increase in effective number of infections until 2000s (Figure 4a). This was followed by a decrease in population size of global DENV-2 sequences from 2000 till date; however, levels of Neτ were higher in the period 2000 to till date than at the start of the plot. A similar trend was seen when the analysis was performed using Indian DENV-2 outbreak sequences. The number of dengue infections remained constant until 1996, thereafter the effective population size increased marginally until 2001–2002. This was followed by a biphasic dip with the first dip starting around 2005–2008 and lasting until around 2010–2014 after which it has stabilised till date (Figure 4b). Levels of Neτ were higher in the period post-2000 to till date than at the start of the plot, indicating that the diversity of the virus has increased.

Discussion

Delhi is a metropolitan city in the northern part of India which has become hyperendemic for DENV infection [30]. In the present study, we analysed dengue sequences from 2015 outbreak in New Delhi. Phylogenetic analysis using E gene revealed that it belonged to DENV-2 serotype and further clustered within a cosmopolitan genotype. During the first dengue epidemic in Delhi, 1996, DENV-1 was the predominant serotype [31]. Phylogenetic analysis revealed that it belonged to cosmopolitan genotype IV. DENV-2 was the predominant serotype until 2003, when for the first time all four serotypes were reported, changing it to a hyperendemic state [32], followed by DENV-3 predominance in 2005 [33]. Within the DENV-2 serotype, cosmopolitan genotype has been reported in India after the 1974 DENV-2 infections [29, 34].

In practice, any genes of the dengue genome can be used for evolutionary analysis as the substitution rate is uniform for almost every gene; be it structural or non-structural [35, 36]. In the current analysis, we selected the envelope region as it is known to accumulate adaptive mutations under selection pressure [35]. Sequence analysis of the 13 sequences from 2015 Delhi outbreak samples vis-à-vis global DENV-2 sequences revealed two unique changes: I162V and R288K (data not shown). 162 aa is a critical residue in the formation DI-DII hinge region in the envelope protein of dengue virus which mediates post-pH-triggered reorganisation of these two

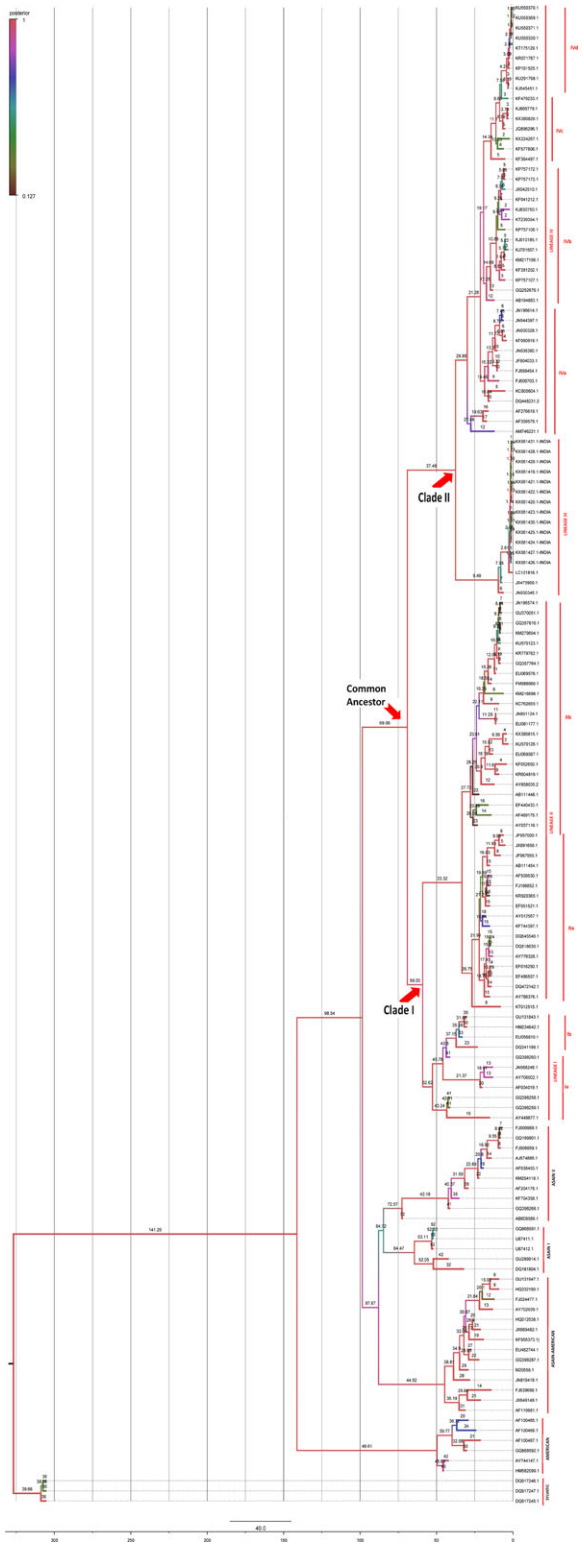

Figure 3 Time-scaled Bayesian-based phylogenetic tree. A time-scaled phylogenetic tree reconstruction was performed for 149 DENV-2 sequences using Envelope region. Each node indicates an isolate and is denoted in the following format: Genbank accession number followed by country and year of isolation. Digits at the nodes indicate age of the node relative to today. Common ancestor and clade 1 and clade 2 are represented by red arrow. The scale at the bottom of the tree represents the time in years with 0 indicating present year. Legend on the upper left side of the image represents posterior values ranging from 0.127 to 1, with 0.127 being the lowest value and 1 being the highest values observed.

domains [37]. Two other sequences had this substitution at position 162, namely, JX475906 from India and JN030345 from Singapore. Further, three sequences displayed an R288K substitution absent from all other DENV-2 sequences. Sequence comparison using translated envelope gene revealed that each genotype of DENV-2 had a specific substitution pattern (Figures 1 and 3). Based on the substitution pattern, we were able to subgroup these genotypes into specific lineages (Figure 1). DENV-2 is the predominant serotype causing infection worldwide and has often been associated with increased disease severity [38]. Whether these substitutions had any role in increased disease severity during this outbreak or not is a question that needs to be answered and warrants further studies.

Lineage-based classification of cosmopolitan genotype of DENV-2 was reported by Afreen *et al.*, but without a substitution pattern [39]. This may be attributed to discrepancy due to sample size included in the study, region of genome used for analysis (partial E gene of 574 bp) and sampling year. Khan *et al.* reported divergence of genotype IV into two geographically distinct sublineages: sublineage IVa, observed in South-East Asia, China and Oceania, and sublineage IVb reported only on the Indian subcontinent [40]. Ali *et al.* suggested subgrouping of geographically distinct cosmopolitan (C) DENV-2 isolates based on full genomes into CIa, CIb, CII and CIII [41]. We have reported lineage replacement of DENV-2 in this study. Among the amino acid substitutions identified, the I162V mutation was a consistent change that fell within domain I of the envelope protein and was observed in all 13 of the isolates studied (data not shown). This change was later identified as a signature substitution of the lineage III sequences as determined by phylogenetic analysis (Figure 1). Each of the outbreak samples reported in this study was associated with a signature substitution/mutation (Table 1).

Lineage replacement has been reported for different serotypes of DENV and has been associated with increased severity in dengue infections [39]. In the present

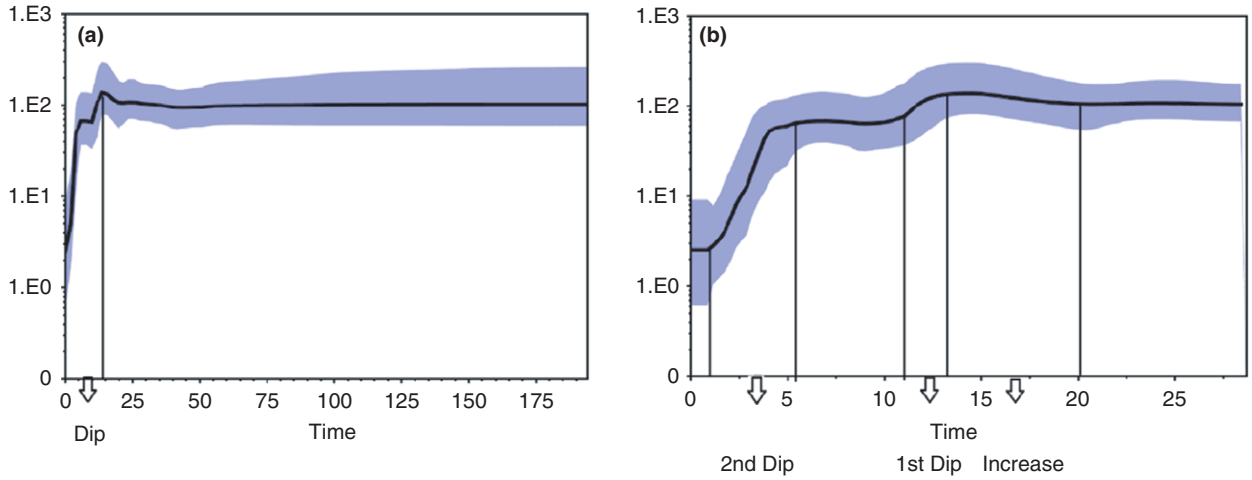

Figure 4 Bayesian skyline plot representing the estimates of the effective number of DENV-2 infections in the studied population. The *y*-axis measures the effective number of infections in log10 scale, while the *x*-axis represents time in years with 0 indicating present year. The solid line represents the median estimate, and the credibility interval based on 95% highest posterior density (HPD) interval is represented by filled area. (a). Bayesian skyline plot analysis using global DENV-2 data set. Dip in effective population size is indicated by arrow at its respective position. (b). Bayesian skyline plot analysis using Indian outbreak DENV-2 samples. Increase and decrease in effective population size is indicated by arrow at its corresponding position.

study, we observed a shift from lineage IV to lineage III (Figure 4). There have been earlier reports of lineage shifts caused by clade replacements resulting in the emergence of strains capable of causing more severe forms of the disease [16, 42]. Further, lineage replacement has been linked to improvement in viral fitness ultimately leading to dispersion and its transmission [42, 43]. The close similarity between the lineage III DENV-2 sequences from our study and a Singapore (JN030345) and Hyderabad strain (JX475906) is noteworthy. The signature pattern in each of the outbreak sequences reported in this study (Table 1) together with its resemblance to the Hyderabad and Singapore strains indicate that these sequences have not undergone much evolution and were lying dormant, possibly due to a population bottleneck. Further, emergence of this sequence in this outbreak suggests that the sequence was in circulation locally and required strong positive selection to overcome the population bottleneck. The I162V residue in the envelope protein, identified to be under positive selection in our study, is located within domain I of the protein (Figure 2). This important region undergoes pH-induced conformational change within the cell membrane [37]. R288K was positively selected when two data sets, that is lineage III cosmopolitan genotype and all cosmopolitan genotypes, were analysed.

The nucleotide substitution rate of DENV-2 viruses calculated in this study corroborates rates reported elsewhere using envelope genes [39, 44, 45] and the complete genome [46]. Our nucleotide substitution rate is within the 95% HPD interval reported by these authors. TMRCA estimation of DENV-2 viruses as whole (excluding sylvatic strains), cosmopolitan DENV-2 and Indian outbreak DENV-2 were 98, 69 and 9 years, respectively. These differ from TMRCA reported by Kumar *et al.* [29] and Afreen *et al.* [39], however, within 95% HPD intervals.

The Bayesian Skyline Plot of global DENV-2 sequences and Indian outbreak cosmopolitan DENV-2 sequences revealed that the effective population size decreased in recent years (Figure 4a,b). This is in concordance to the plot reported by Afreen *et al.* for Indian cosmopolitan DENV-2 strains which showed a decrease in effective population size [39].

Conclusion

The evolution of a distinct lineage of DENV-2 strains on the Indian subcontinent with possible changes in endemic circulating dengue strains observed in this study might give rise to more pathogenic strains.

Acknowledgements

We acknowledge Jagat Solanki for compiling Dengue data and Gaurav Singh for collection and archiving of

patient samples and for fetching data related to this manuscript.

References

1. Murray NE, Quam MB, Wilder-Smith A. Epidemiology of dengue: past, present and future prospects. *Clin Epidemiol* 2013: **5**: 299–309.
2. Guzman MG, Gubler DJ, Izquierdo A, Martinez E, Halstead SB. Dengue infection. *Nat Rev Dis Primers* 2016: **2**: 16055.
3. Chen R, Vasilakis N. Dengue–quo tu et quo vadis? *Viruses* 2011: **3**: 1562–1608.
4. Gubler DJ. The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res* 2002: **33**: 330–342.
5. Guzman MG, Vazquez S. The complexity of antibody-dependent enhancement of dengue virus infection. *Viruses* 2010: **2**: 2649–2662.
6. Grange L, Simon-Loriere E, Sakuntabhai A, Gresh L, Paul R, Harris E. Epidemiological risk factors associated with high global frequency of inapparent dengue virus infections. *Front Immunol* 2014: **5**: 280.
7. Gupta N, Srivastava S, Jain A, Chaturvedi UC. Dengue in India. *Indian J Med Res* 2012: **136**: 373–390.
8. Wilder-Smith A, Ooi EE, Vasudevan SG, Gubler DJ. Update on dengue: epidemiology, virus evolution, antiviral drugs, and vaccine development. *Curr Infect Dis Rep* 2010: **12**: 157–164.
9. Singh NP, Jhamb R, Agarwal SK *et al.* The 2003 outbreak of Dengue fever in Delhi, India. *Southeast Asian J Trop Med Public Health* 2005: **36**: 1174–1178.
10. Pandey A, Diddi K, Dar L *et al.* The evolution of dengue over a decade in Delhi, India. *J Clin Virol* 2007: **40**: 87–88.
11. Chaturvedi UC, Shrivastava R. Dengue haemorrhagic fever: a global challenge. *Indian J Med Microbiol* 2004: **22**: 5–6.
12. Rico-Hesse R. Microevolution and virulence of dengue viruses. *Adv Virus Res* 2003: **59**: 315–341.
13. Bennett SN, Holmes EC, Chirivella M *et al.* Selection-driven evolution of emergent dengue virus. *Mol Biol Evol* 2003: **20**: 1650–1658.
14. Yamanaka A, Mulyatno KC, Susilowati H *et al.* Displacement of the predominant dengue virus from type 2 to type 1 with a subsequent genotype shift from IV to I in Surabaya, Indonesia 2008–2010. *PLoS ONE* 2011: **6**: e27322.
15. Barcelos Figueiredo L, Batista Cecilio A, Portela Ferreira G *et al.* Dengue virus 3 genotype 1 associated with dengue fever and dengue hemorrhagic fever, Brazil. *Emerg Infect Dis* 2008: **14**: 314–316.
16. Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM. Emergence and global spread of a dengue serotype 3, subtype III virus. *Emerg Infect Dis* 2003: **9**: 800–809.
17. Ospina MC, Diaz FJ, Osorio JE. Prolonged co-circulation of two distinct Dengue virus Type 3 lineages in the hyperendemic area of Medellin, Colombia. *Am J Trop Med Hyg* 2010: **83**: 672–678.
18. Gupta E, Mohan S, Bajpai M, Choudhary A, Singh G. Circulation of Dengue virus-1 (DENV-1) serotype in Delhi, during 2010-11 after Dengue virus-3 (DENV-3) predominance: a single centre hospital-based study. *J Vector Borne Dis* 2012: **49**: 82–85.
19. Gritsun TS, Holmes EC, Gould EA. Analysis of flavivirus envelope proteins reveals variable domains that reflect their antigenicity and may determine their pathogenesis. *Virus Res* 1995: **35**: 307–321.
20. Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control: New Edition. WHO Guidelines Approved by the Guidelines Review Committee. Geneva 2009.
21. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 1992: **30**: 545–551.
22. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol* 2016: **33**: 1870–1874.
23. Stern A, Doron-Faigenboim A, Erez E, Martz E, Bacharach E, Selecton PT. Advanced models for detecting positive and purifying selection using a Bayesian inference approach. *Nucleic Acids Res* 2007: **2007**: 35.
24. Yang Z, Nielsen R, Goldman N, Pedersen AM. Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics* 2000: **155**: 431–449.
25. Swanson WJ, Nielsen R, Yang Q. Pervasive adaptive evolution in mammalian fertilization proteins. *Mol Biol Evol* 2003: **20**: 18–20.
26. Doron-Faigenboim A, Pupko T. A combined empirical and mechanistic codon model. *Mol Biol Evol* 2007: **24**: 388–397.
27. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007: **7**: 214.
28. Santorum JM, Darriba D, Taboada GL, Posada D. jmodeltest.org: selection of nucleotide substitution models on the cloud. *Bioinformatics* 2014: **30**: 1310–1311.
29. Kumar SR, Patil JA, Cecilia D *et al.* Evolution, dispersal and replacement of American genotype dengue type 2 viruses in India (1956–2005): selection pressure and molecular clock analyses. *J Gen Virol* 2010: **91**: 707–720.
30. Ahmed NH, Broor S. Dengue Fever outbreak in delhi, north India: a clinico-epidemiological study. *Indian J Community Med* 2015: **40**: 135–138.
31. Kurukumbi M, Wali JP, Broor S *et al.* Seroepidemiology and active surveillance of dengue fever/dengue haemorrhagic fever in Delhi. *Indian J Med Sci* 2001: **55**: 149–156.
32. Dar L, Gupta E, Narang P, Broor S. Cocirculation of dengue serotypes, Delhi, India, 2003. *Emerg Infect Dis* 2006: **12**: 352–353.
33. Gupta E, Dar L, Kapoor G, Broor S. The changing epidemiology of dengue in Delhi, India. *Virol J* 2006: **3**: 92.
34. Chakravarti A, Chauhan MS, Kumar S, Ashraf A. Genotypic characterization of dengue virus strains circulating during 2007-2009 in New Delhi. *Adv Virol* 2013: **158**: 571–581.

35. Klungthong C, Putnak R, Mammen MP, Li T, Zhang C. Molecular genotyping of dengue viruses by phylogenetic analysis of the sequences of individual genes. *J Virol Methods* 2008: **154**: 175–181.
36. Domingo C, Palacios G, Jabado O *et al.* Use of a short fragment of the C-terminal E gene for detection and characterization of two new lineages of dengue virus 1 in India. *J Clin Microbiol* 2006: **44**: 1519–1529.
37. Christian EA, Kahle KM, Mattia K *et al.* Atomic-level functional model of dengue virus Envelope protein infectivity. *Proc Natl Acad Sci USA* 2013: **110**: 18662–18667.
38. Kyle JL, Harris E. Global spread and persistence of dengue. *Annu Rev Microbiol* 2008: **62**: 71–92.
39. Afreen N, Naqvi IH, Broor S *et al.* Evolutionary analysis of dengue serotype 2 viruses using phylogenetic and Bayesian methods from New Delhi, India. *PLoS Negl Trop Dis* 2016: **10**: e0004511.
40. Khan MA, Ellis EM, Tissera HA *et al.* Emergence and diversification of dengue 2 cosmopolitan genotype in Pakistan, 2011. *PLoS ONE* 2013: **8**: e56391.
41. Ali A, Ali I. The complete genome phylogeny of geographically distinct dengue virus serotype 2 isolates (1944–2013) supports further groupings within the cosmopolitan genotype. *PLoS ONE* 2015: **10**: e0138900.
42. Lee KS, Lo S, Tan SS *et al.* Dengue virus surveillance in Singapore reveals high viral diversity through multiple introductions and in situ evolution. *Infect Genet Evol* 2012: **12**: 77–85.
43. Lambrechts L, Fansiri T, Pongsiri A *et al.* Dengue-1 virus clade replacement in Thailand associated with enhanced mosquito transmission. *J Virol* 2012: **86**: 1853–1861.
44. Anez G, Morales-Betoulle ME, Rios M. Circulation of different lineages of dengue virus type 2 in Central America, their evolutionary time-scale and selection pressure analysis. *PLoS ONE* 2011: **6**: e27459.
45. Zhang C, Mammen MP Jr, Chinnawirotpisan P *et al.* Structure and age of genetic diversity of dengue virus type 2 in Thailand. *J Gen Virol* 2006: **87**: 873–883.
46. McElroy KL, Santiago GA, Lennon NJ, Birren BW, Henn MR, Munoz-Jordan JL. Endurance, refuge, and reemergence of dengue virus type 2, Puerto Rico, 1986–2007. *Emerg Infect Dis* 2011: **17**: 64–71.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Oligonucleotide primers used to amplify Dengue CPrM and Envelope genes.

Corresponding Author **Ekta Gupta**, Department of Virology, Institute of Liver and Biliary Sciences, New Delhi-110070, India. E-mail: ektagaurisha@gmail.com