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Isolation and molecular characterization of dengue virus clinical isolates from pediatric patients in New Delhi

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ABSTRACT

Objective: To characterize the in vitro replication fitness, viral diversity, and phylogeny of dengue viruses (DENV) isolated from Indian patients.
Methods: DENV was isolated from whole blood collected from patients by passaging in cell culture. Passage 3 viruses were used for growth kinetics in C6/36 mosquito cells. Parallel efforts also focused on the isolation of DENV RNA from plasma samples of the same patients, which were processed for next-generation sequencing.
Results: It was possible to isolate 64 clinical isolates of DENV, mostly DENV-2. Twenty-five of these were further used for growth curve analysis in vitro, which showed a wide range of replication kinetics. The highest viral titers were associated with isolates from patients with dengue with warning signs and severe dengue cases. Full genome sequences of 21 DENV isolates were obtained. Genome analysis mapped the circulating DENV-2 strains to the Cosmopolitan genotype.
Conclusions: The replication kinetics of isolates from patients with mild or severe infection did not differ significantly, but the viral titers varied by two orders of magnitude between the isolates, suggesting differences in replication fitness among the circulating DENV-2.

Introduction

Dengue virus (DENV) is a common arbovirus that causes about 60 million apparent infections every year, leading to over 10 000 deaths. The number of dengue cases doubled every 10 years between 1990 and 2013 (Stanaway et al., 2016). Antibody-dependent enhancement of viral infection is a major driver of severe dengue infections, but pre-existing antibodies do not always lead to severe infection for all serotypes (Endy et al., 2004). The antibody response in primary infections, which does provide cross-protection albeit transiently, has also been shown to differ between serotypes, suggesting that the genotype and serotype of DENV isolates elicit differential immune responses (Chaudhury et al., 2017; Clapham et al., 2016; VanBlargan et al., 2013). Serotype-specific differences in efficacy have been reported for the only licensed live-attenuated dengue vaccine, which is constructed on a yellow fever virus backbone (Capeding et al., 2014). Some of the reasons for these differences could be the lack of T-cell epitopes of DENV non-structural regions and possible divergence in sequence between the strains used in the vaccine and the circulating serotypes in the endemic countries where the vaccine trials were conducted (Guy et al., 2015; Juraska et al., 2018).

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We and others have reported that a significant proportion of primary dengue cases also result in severe dengue disease in endemic countries, suggesting that the circulating viral strains and intrinsic host responses play an important role in disease outcomes (Balmaseda et al., 2006; Ngwe Tun et al., 2013; Nunes et al., 2018; Singla et al., 2016). Cohort studies tracking dengue epidemics over many years have identified viral evolution as a key player that impacts serotype-specific responses. Different clades of DENV-2 were found to lead to different disease outcomes in children who had prior immunity to DENV-1 or DENV-3 (OhAinle et al., 2011). Genetic variation in DENV sequences is of significance for both epidemiology and vaccine development. It has been well documented in many earlier reports that the replication fitness, infection dynamics, and pathogenicity vary among DENV isolates (Balmaseda et al., 2006; Borges et al., 2018; Fontaine et al., 2018; Leitmeyer et al., 1999; Vaughn et al., 2000; Watts et al., 1999).

India contributes about 34% of the global DENV cases and there is limited information on the evolution of DENV strains circulating in India (Bhatt et al., 2013; Dias et al., 2018). Considering the recent progress in dengue vaccine development, it is important to understand the drivers of positive selection and the evolution of DENV strains in India to better prepare for vaccine trials (Wichmann et al., 2017). In this study, DENV was isolated from patient blood and viral growth kinetics were characterized in vitro; furthermore, complete DENV genomes were recovered from plasma and compared, in order to create a molecular profile of the DENV strains circulating in India.

Materials and methods

The study population, isolation of viral RNA from whole blood or plasma, and estimation of viremia have been described previously; a description is provided in the Supplementary Material.

Virus isolation from whole blood

Blood samples were diluted 1:10 in minimal essential medium (MEM) containing 2% fetal bovine serum (FBS) (Gibco) and added to a cultured monolayer of C6/36 cells for 1 h with gentle rocking in a 28 °C CO_2 incubator. After 1 h, the inoculum was removed and cells were cultured for 7 days in MEM with 2% FBS and antibiotics. The culture supernatant was collected on day 7 and used to infect a new batch of C6/36 cells, as above. This was repeated three times to achieve passage 3 (P3) isolates. Viral titers in the supernatant were measured by plaque assays on BHK-21 cells, as described previously (Agrawal et al., 2013).

Whole genome sequencing

Specific cDNA synthesis was performed using a primer specific for the 3′ untranslated region (UTR) of flaviviruses (5′ GGGTCTCCWCTAACCTCTAGTCCT 3′) using the Thermofisher Scientific Maxima H Minus Reverse Transcriptase. Second strand synthesis was performed using the NEBNext Second Strand Synthesis module. Resulting DNA was purified using magnetic beads. Sequencing library preparation was performed using the Nextera XT Illumina sequencing kit with the 96 barcodes. About 10 pM DNA from pooled libraries was taken for sequencing of the MiSeq platform. Fastq reads were extracted from the run, demultiplexed, and used for de novo assembly using SPAdes (ver. 3.12.0) (Nurk et al., 2013).

Reference recruitment to the closest Blastn hit and genome editing was performed using Geneious software (ver. 11.15), with coverage of a least 9× and majority rule for calling the consensus. Resulting consensus sequences were annotated by transferring annotations from the reference sequence (RefSeq) and submitted to GenBank. The GenBank accession numbers are provided in the Supplementary Material.

Molecular phylogenetic analysis

Multiple sequence alignment was performed for representative sequences from known genotypes (obtained from the Virus Pathogen Database and Analysis Resource, ViPR), for which complete genomes are available (the accession numbers of the sequences are provided in Table T4 of the Supplementary Material), using MUSCLE. The phylogenetic trees for whole genome sequences were constructed using the maximum likelihood method in MEGA X software (Kumar et al., 2018). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. Bootstrapping with 1000 replicates was used to improve the statistical significance of each node. Around 91% of the nodes in all trees had bootstrap values >90%. The phylogenetic tree for the envelope protein of DENV-2 was constructed using the neighbor-joining method with 100 bootstraps. FigTree (v1.4.3) was used for graphical presentation of the phylogenetic trees.

Statistical methods

Appropriate statistical tests were performed, as indicated in the figure legends and tables. Prism 7 was used for graphical representation of the data.

Results

Dengue cohort

The characteristics and clinical parameters of the 119 patients enrolled in the study are presented in Table T1 of the Supplementary Material. As expected, most patients with severe dengue disease came to the hospital at later days of fever as compared to patients with mild dengue (DI) and dengue with warning signs (DW). Patients with severe dengue had significantly lower platelet counts at the time of enrolment as compared to DI and DW cases. Based on the IgM and IgG ELISA, 56 (47%) patients were classified as either seronegative or having primary infections and the other 63 patients (53%) as having secondary infections.

Isolation of DENV RNA is more efficient from whole blood

Patients were enrolled into the study based on a positive dengue NS1 test using a point-of-care kit. To further confirm DENV positivity, determine the infecting DENV serotype, and to estimate viremia, RNA was isolated from whole blood sampled from patients at the time of enrolment. Furthermore, to determine whether RNA isolation from the plasma preparation of the same samples is equally efficient, RNA isolation was performed using plasma samples from most of these patients. Total RNA isolated from blood or plasma was used for the detection and quantitation of viral RNA as described previously (Agrawal et al., 2013).

Viral RNA was detected in a significantly higher number of whole blood samples as compared to plasma samples. Overall, 92% of the RNA samples isolated from whole blood had detectable levels of viral RNA, whereas the same was 39% for plasma samples (Supplementary Material, Figure S1A). This suggests that the efficiency of RNA isolation is higher for whole blood samples, which may be due to the presence of cellular RNA. Although carrier

RNA was added as per the manufacturer's instructions during RNA isolation from plasma, the process was not as optimal for the detection of viral RNA under our conditions.

The DENV genome copy numbers in whole blood did not differ significantly between the different disease severities (Supplementary Material, Table T1), as reported previously (Singla et al., 2016). However, viral genome copy numbers were significantly higher in plasma samples as compared to whole blood samples, as the total RNA obtained from plasma is likely to be predominantly of viral origin as compared to whole blood RNA which will be dominated by cellular RNA (Supplementary Material, Figure S1B).

Eighty percent of the patients were infected with DENV-2 as determined by serotyping PCR (Supplementary Material, Table T2). About 11% of the samples were either negative or undetermined by serotyping PCR, but they were confirmed to be dengue-positive by antibody ELISA or viral RNA detection or virus isolation experiments.

DENV isolation from patient samples

Like all other RNA viruses, dengue viruses evolve rapidly, and changes in the genome regions encoding the T cell and B cell epitopes have an impact on vaccine development and the immune response. Therefore, focus was next placed on the isolation of DENV from patient samples collected in this study. A flow diagram of the process that was followed is shown in Figure 1.

Whole blood was used for the isolation of viruses by three blind passages in C6/36 cells. Sixty-four isolates with measurable viral titers in P3 supernatants were obtained. Segregation of these isolates based on the day of fever (DOF) of the original sample showed that 72.5% of the samples collected between DOF 1 and DOF 3 yielded isolates and 44% of the samples collected between DOF 4 and DOF 6 yielded isolates, clearly demonstrating that samples collected between DOF 1 and DOF 6 would be ideal for virus isolation (Supplementary Material, Figure S2A). The number of virus isolates obtained and the viral titers at P3 did not differ significantly between samples obtained from DI, DW, or severe dengue patients (Supplementary Material, Figure S2B, S2C).

Forty-six of the isolates were confirmed to be of DENV-2 serotype by RT-PCR and 25 of these isolates had plaque assay titers high enough for growth curve assays in C6/36 cells ($\geq 10^5$–10^8 pfu/ml). The 3-day growth kinetics of P3 isolates were assessed by infecting C6/36 cells with 0.1 MOI (multiplicity of infection) of P3 supernatants. At 24 h, 48 h, and 72 h post-infection, supernatants were collected and viral titers were measured by plaque assay. Of the 25 isolates, it was possible to study 22 isolates in C6/36 cells. The growth rates of the isolates differed drastically and the viral titers on day 3 post-infection from the clinical isolates ranged from 10^3 pfu/ml to 10^6 pfu/ml (Figure 2A). The highest titers ($>10^6$ pfu/ml) were associated with the samples from a patient with severe dengue who had died of the disease and from a DW case. Nevertheless, viral titers on day 3 post-infection did not differ significantly between viruses isolated from patients with different disease severities (Figure 2B and C). Of the three isolates that failed to amplify, one was from a DI patient and two were from patients with severe dengue, and all three samples were obtained within 3 days of fever. These in vitro data suggest that the isolation efficiency and growth kinetics of clinical isolates from DENV patients with disease of different severity are not significantly different in cell culture conditions.

Whole genome sequencing of clinical DENV isolates

Next, viral RNA was isolated from 84 plasma samples and the amount of DENV RNA was determined by quantitative RT-PCR. Thirty-five samples with copy numbers exceeding 10^5 DENV genome equivalents per milliliter were processed for whole genome sequencing. A modified RNA sequencing protocol was used to generate the whole genome sequences (see Methods). Using de novo assembly followed by reference recruitment (mapping), complete genomes were recovered with greater than 9× coverage across the genome from 21 RNA samples. These

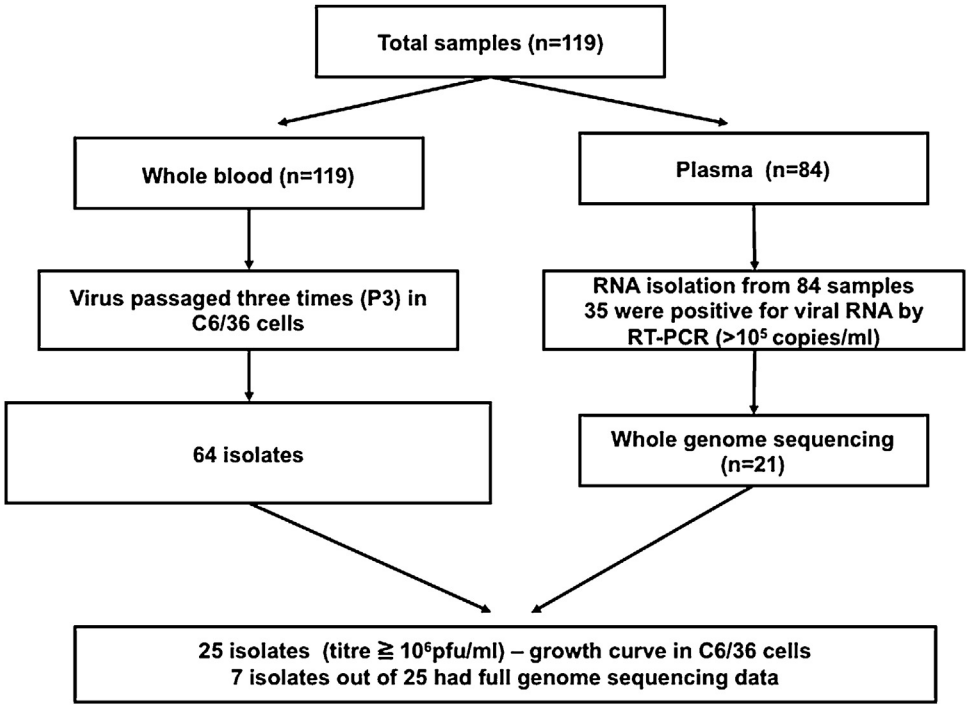

Figure 1. Work flow of the study. Flowchart of activities, depicting virus isolation and growth curve analysis from whole blood/plasma in C6/36 cells, viral RNA isolation from plasma, and whole genome sequencing.

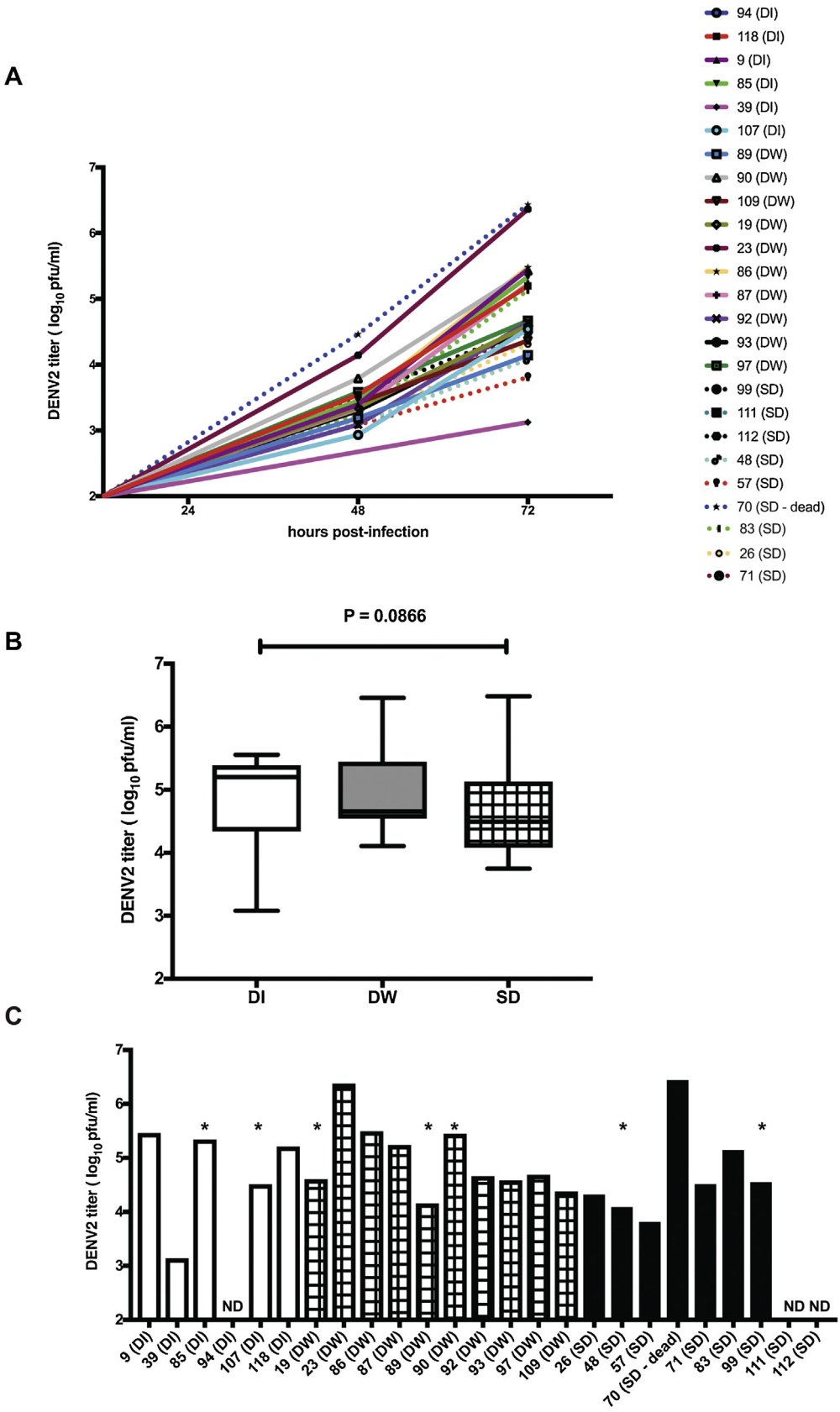

Figure 2. Growth kinetics of patient isolates (passage 3). (A) C6/36 cells were infected at a multiplicity of infection (MOI) of 0.1 and culture supernatants were collected on days 1–3. Viral titers were estimated by plaque assays. (B) Comparison of day 3 viral titers from the above isolates according to the disease classification of the samples at origin. (C) Samples indicating individual viral titers on day 3. ND, not detected. The asterisk (*) indicates those isolates for which whole genome sequences were obtained.

included 18 DENV-2, two DENV-1, and one DENV-3 genomes (Supplementary Material, Table T3). GenBank accession numbers are provided in Table T4 of the Supplementary Material.

Phylogenetic analysis

MEGA X (Kumar et al., 2018) was used to reconstruct the evolutionary tree(s) for the 21 near-complete DENV genome sequences. The DENV-3 samples mapped to genotype III and the DENV-1 sample mapped to genotype V (Supplementary Material, Figure S3A, S3B). Since most of the whole genome sequences recovered belonged to the DENV-2 serotype, focus was placed on further analysis of these samples.

The DENV-2 serotype has six genotypes that are largely spatially related, namely American, American-Asian, Cosmopolitan, Asian-I, Asian-II, and Sylvatic (Waman et al., 2016). Based on the neighbor-joining (NJ) tree of the envelope protein (E), it was observed that the Indian DENV strains from pre-2000 were of the American genotype, while the recent strains (post-2000) mostly seemed to be dominated by the Cosmopolitan genotype (Figure 3A). Even within the Cosmopolitan genotype, recent studies have reported the prevalence of three lineage clusters in India (Afreen et al., 2016). Clustering of the DENV-2 genomes with two distinct lineages within the Cosmopolitan genotype was also observed. Lineage III sequences were largely prevalent in the second half of the epidemic (2014–15) and lineage I sequences dominated the first half of the epidemic (2012–13) while co-circulating at the same time in the Delhi region. The maximum likelihood phylogenetic tree was then constructed using the whole genome sequences of the DENV-2 from GenBank (Figure 3B). DENV-2 genomes from the present study largely followed the phylogenetic analysis of the E protein sequences, suggesting insignificant recombination in the lineages.

Sequence diversity from global DENV-2 strains

Next, the level of diversity between the present DENV-2 sequences and the DENV-2 Cosmopolitan genotype was evaluated. A consensus DENV-2 Cosmopolitan genome was first generated using the 115 whole genomes in the GenBank database (Supplementary Material, Table T5). Synonymous single nucleotide polymorphisms (SNPs) in the polyprotein coding region of the virus genome were high (1051 mutations) and uniformly distributed across the whole genome, as expected for RNA viruses. The non-synonymous mutations were rare but significant (138 mutations) and spread non-uniformly across the polyprotein (**Supplementary Material**, Figure S4; **Supplementary Material**, Table T6). Most of the changes in the E protein were in the B cell epitopes, and two of the changes in the NS3 region were part of the CD8 T cell epitope (**Supplementary Material**, Table T6) (Vaughan et al., 2010). We next located the amino acid changes in the E protein from the consensus and the 16881 DENV-2 sequence that has been adapted to generate the DNA infectious clone (Kinney et al., 1997). Almost all of the E protein mutations mapped to the exposed face of the E protein in its dimeric conformation, possibly suggesting immunological selection being the driving force (Figure 4A and 4B). For example, a large proportion of the mutations spanned the EDI/II region, which is the binding epitope for human monoclonal antibody (hMAb) 3F9, whereas the mutation H346Y mapped to the EDIII binding epitope recognized by hMAbs 2D22 and 1L12 (Gallichotte et al., 2018). We also compared the UTRs of the genomes since they are reported to be critical for virus replication (Gebhard et al., 2011; Ng et al., 2017). The largely conserved 5′ UTR among the Cosmopolitan genotype carried a C–U mutation that lies in the RNA polymerase binding SLA loop (**Supplementary Material**, Figure S5). The 3′ UTR on the other hand displayed several mutations that fell into two categories: one set that was common among all the Cosmopolitan genotype and another set of mutations that set the two lineages apart within the genotype (Supplementary Material, Figure S6).

Discussion

Previous reports have compared DENV detection using different RNA isolation methods from serum, plasma, or whole blood (Anwar et al., 2009; Dettogni and Louro, 2011). This study confirms the results of previous studies that have reported viral detection to be more efficient with whole blood as compared to plasma (Klungthong et al., 2007). Dengue serotyping using rapid diagnostic tests has also demonstrated the convenience and utility of using whole blood for the isolation of viral RNA (Vongsouvath et al., 2016). Another study that used cellular components of whole blood, serum, clot specimens, and plasma from blood bank donors who were DENV RNA-positive also demonstrated that the detection of viral RNA by RT-PCR was more consistent with cellular components of blood (Añez et al., 2016). In the present study, it was found that viral RNA isolation was more efficient using whole blood and in samples collected within 6 days of fever.

Blood viremia did not differ significantly between samples obtained from patients with different disease severities; however, the study has a caveat in that most of the patients with severe dengue sought clinical care at later stages of infection when viremia is on the decline. It is possible that the high viral load in the early stages of infection leads to higher inflammation, resulting in severe disease. Therefore, estimation of viremia as a function of time starting from the earliest possible time upon infection until hospitalization/recovery would provide a clearer picture of the role of viral load in disease progression.

About half of the patients enrolled in this study had a primary/seronegative status based on IgG and IgM ELISA. Thirty-two percent of the severe dengue cases were also primary infections (no dengue IgG at the time enrolment), suggesting that in a subset of patients, severe disease was a consequence independent of infection-enhancing antibodies.

Very few studies have characterized clinical isolates from India and it is necessary to determine whether the antibodies generated from previous natural infections or vaccine trials are capable of neutralizing circulating viruses in the country. In addition, sequencing information and corresponding virus neutralization data will also provide clues to the emergence of escape variants. Clinical isolates minimally passaged in vitro have been used to assess replication fitness in cell culture models of infection and in immunocompetent and immunocompromised mouse models or rhesus macaques (Borges et al., 2018; Ferreira et al., 2010; Sarathy et al., 2018; Tuiskunen et al., 2011a,b). It has been demonstrated that isolates from patients with severe or fatal dengue disease demonstrate enhanced replication and induce inflammation at a much higher level (Murgue et al., 1997; Silveira et al., 2011). The present study revealed that the low passage clinical DENV-2 isolates showed different growth kinetics in cell culture but that the differences were not associated with the disease severity in the patients. Although these isolates were passaged only three times in cell culture to limit adaptive mutations, the possibility that the sequence of the virus obtained after the third passage was not identical to that infecting the patient cannot be ruled out. Further sequencing of P3 isolates will clarify this issue.

Full genome sequencing data from recent reports suggest that in most parts of South America and Latin America, the circulating DENV-2 strains belong to the American/Asian genotype and that phylogenetically close viruses are circulating between these countries (Lizarazo et al., 2018; Stewart-Ibarra et al., 2018; Williams et al., 2014). Similarly, the full genome sequence of a

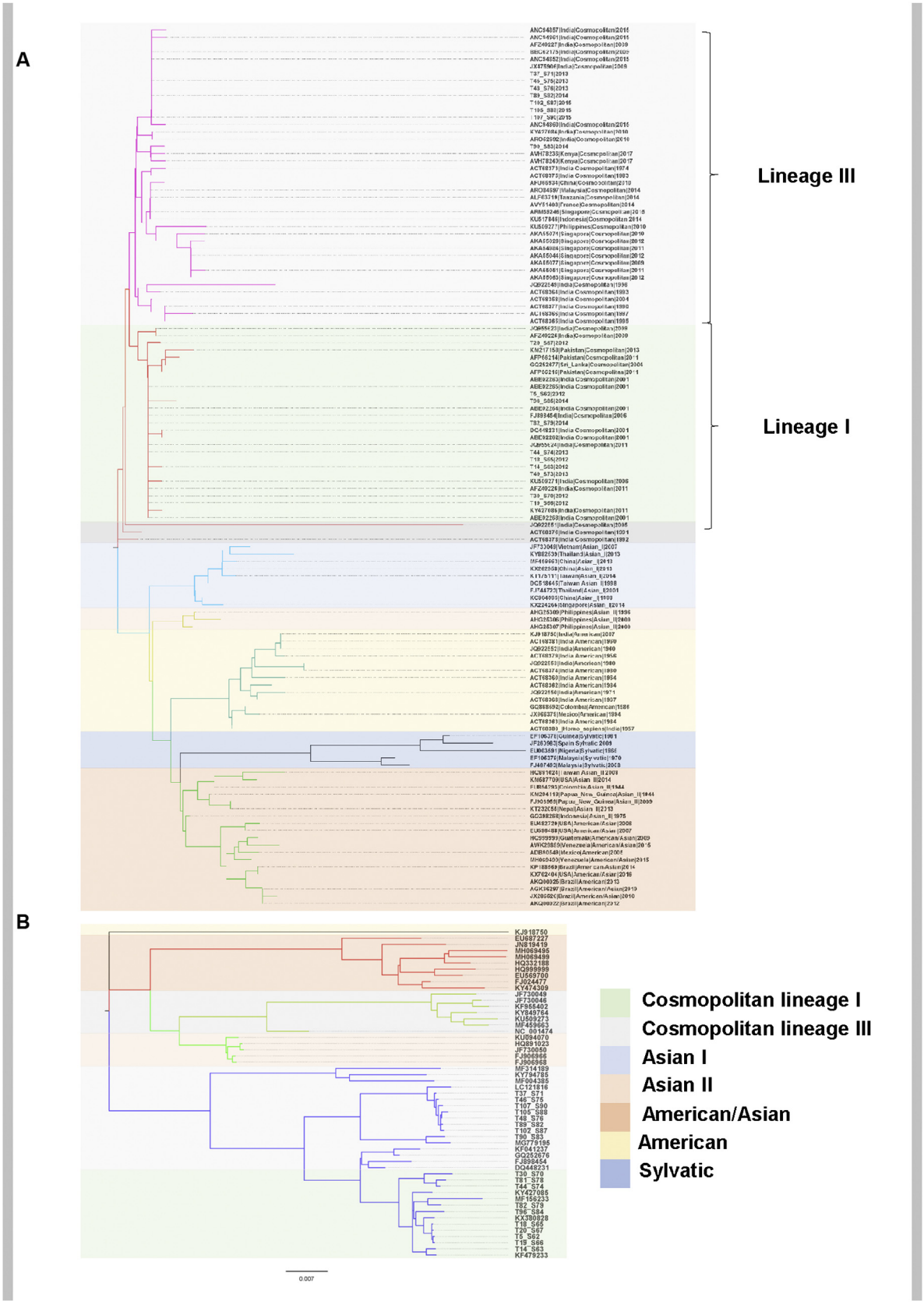

Figure 3. Phylogenetic analysis of DENV-2 clinical isolates. (A) The optimal tree with the sum of branch length = 0.43786479 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of amino acid substitutions per site. The analysis involved 121 amino acid sequences of the E protein. There were a total of 473 positions in the final dataset. (B) Phylogenetic tree for whole genomes of DENV-2 using the maximum likelihood method based on the general time reversible model. The tree with the highest log likelihood (−47856.75) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 53 nucleotide sequences. There were a total of 10 743 positions in the final dataset. Samples from this study are indicated as in Table T3 of the Supplementary Material.

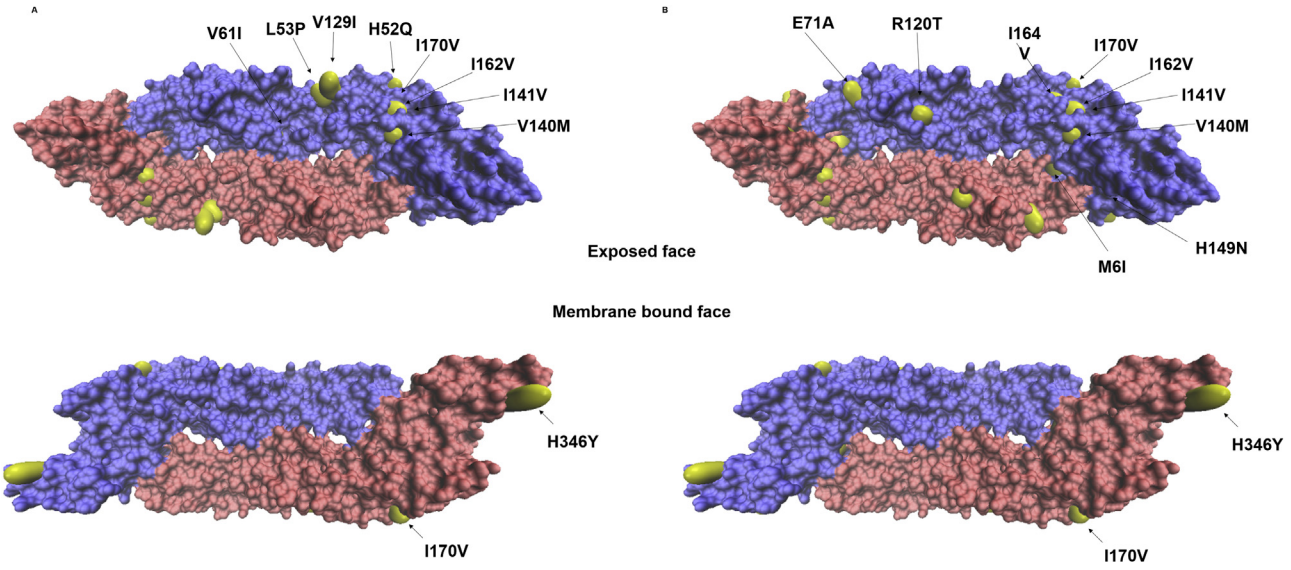

Figure 4. Non-synonymous mutations (yellow) mapped on the envelope protein (Protein Data Bank (PDB) accession code 1OAN) mutations, compared to (A) the consensus generated from 115 Cosmopolitan DENV-2 sequences from all over the world and (B) strain 16881 (NC_001474), are shown.

DENV-2 isolate from Myanmar identified the Asian genotype closely related to the isolates circulating in Vietnam, Thailand, and Cambodia (Zeng et al., 2018). The Asian genotype of DENV-2 dominated in China prior to 2010 and has been replaced by the Cosmopolitan genotype that is similar to the isolates circulating in Singapore (Jiang et al., 2018; Zhao et al., 2012). All four serotypes are circulating in India, although DENV-2 has dominated in recent years in the study region and also in other parts of the country (Mishra et al., 2015; Mukherjee et al., 2017; Shrivastava et al., 2018; Singla et al., 2016). Phylogenetic analysis of DENV-2 sequences from Pakistan suggests that the Cosmopolitan genotype was introduced from India and Sri Lanka about 30 years ago, and a sub-lineage within the Cosmopolitan genotype has emerged in Pakistan and in the Indian sub-continent, causing major epidemics (Akram et al., 2015; Khan et al., 2013). This is further confirmed by DENV-2 sequences (complete genome or the structural region) from India for the period 1960 to 2012, which indicate a shift from American genotype to a unique South Asian clade within the Cosmopolitan genotype (Dash et al., 2013; Mishra et al., 2015). The envelope sequence of DENV-2 isolates from Nepal has shown co-circulation of two DENV-2 genotypes — Asian II and Cosmopolitan IVa and IVb. Among the DENV serotypes, DENV-2 has been the most genetically diverse population. It is the most frequent cause of dengue epidemics worldwide and has also been associated with severe dengue cases (Wei and Li, 2017).

In this study, complete genome sequences of 21 DENV-2 isolates from India isolated between 2012 and 2015 were obtained, which further confirmed a positive selection of Cosmopolitan genotype in India. Fifteen of the 21 sequences were obtained from seronegative patients or those with primary infections, and these were isolated in the early days of fever (median DOF 2.5). This is considered to be a unique feature of the present study and will serve as useful information. The analysis of DENV-2 sequences from primary and secondary infections in Thailand showed that the sequences were homogeneous in secondary infections while the viral diversity was much more heterogeneous in primary infections, suggesting that most primary infections could drive the generation of quasispecies leading to the evolution and selection of dominant isolates during subsequent exposure (Kurosu et al., 2014). It is speculated that non-synonymous mutations in the capsid, NS2A, and NS5 could be acting as a strong driver of positive selection. In addition, some of the mutations in the E protein mapped to B cell epitopes. Further studies should be performed to verify whether any of these isolates are neutralization escape mutants. Future studies should be designed with a focus on capturing the kinetics of viral replication by isolation of viral RNA and viruses from serial samples from both primary and secondary infections, in order to determine the inter-host diversity and evolution of the viral genome with disease progression or recovery.

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Author contributions

MK, AN, AK, SJ, JS, MS, SM, and AP performed experiments and analyzed data. AC, SKK, SK, and RL contributed reagents and analyzed data. RR, CP, and GM conceived the study, designed and performed the experiments, analyzed the data, and wrote the manuscript. All authors have reviewed the final version of the manuscript.

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Ethics statement

The study was approved by the institutional ethics committees of all three participating institutes (Ethics/THSTI/2011/2.1 dated 16 November 2011; AIIMS: IEC/NP-338/2011 dated 17 November 2011; ICGEB/IEC/2011/01 dated 12 November 2011). Written informed consent for the study was obtained from the parents/guardians to collect blood samples at the time of admission.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijid.2018.12.003.

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