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Co-circulation of dengue virus serotypes in Central India: Evidence of prolonged viremia in DENV-2

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ABSTRACT

In view of paucity of information on serotype distribution of Dengue virus (DENV) in Central India, we undertook a cross-sectional study to identify clinical and virological characteristics of DENV serotypes that circulated in this region during the 2016 outbreak. Suspected cases were screened by ELISA for NS1 antigen and anti-DENV IgM antibodies. Serologically confirmed cases were subjected to RT-PCR based detection and serotyping. The RT-PCR results were confirmed by nucleotide sequencing. Genome-wide association was undertaken with DENV sequences from ViPR database and the immune evasion potential of infecting serotypes was ascertained by computing antigenic variability in B cell and Cytotoxic T cell (CTL) epitopes of all DENV proteins. The immunological basis of more prolonged viremia in DENV2-infected patients was also addressed through sequencing of NS2a gene and comparing the CTL activity in NS2a sequences identified among patients with ≤5 days and > 5 days of illness. Among 166 serologically confirmed Dengue patients, 75 were positive for DENV RNA. Serotyping revealed predominance of DENV-1 and DENV-2, followed by DENV-3. Co-infection with multiple serotypes was observed in 15.5% of cases. In ~40% cases, DENV RNA was detectable beyond 5 days, among whom majority were DENV-2 infected (p = .044). Highest prevalence of antigenic variability was observed in B cell and CTL epitopes of DENV-2. The potential association between prolonged viremia and higher ability for immune evasion in DENV-2 patients was further corroborated with the observation of poorer HLA-I binding affinity in CTL epitopes observed in NS2a sequences retrieved from patients with > 5 days of illness, compared to those with ≤5 days. This is the first report from central India revealing circulation of all DENV serotypes and high prevalence of co-infection with multiple serotypes. We also observed prolonged viremia upon DENV-2 infection, which could be potentially associated with its superior immune evasion potential.

1. Introduction

Dengue has been reported to be the most rapidly spreading mosquito-borne viral disease that causes approximately 400 million cases and 25,000 deaths each year (www.cdc.gov/dengue/). In sync with increasing incidence, the disease has also spread to newer regions of the world with endemicity being observed in > 100 countries (WHO Factsheet # 387, March 2014). India too has witnessed a rising burden of Dengue; with the number of cases rising by > 230% in 2015–16, compared to the annual average of previous 5 years. The public health importance of Dengue is also contributed by the development of life-threatening complications like Dengue Hemorrhagic Fever and Dengue Shock Syndrome in approximately 25% of infected individuals. According to National Vector Borne Disease Control Programme, 111,880 Dengue cases and 227 deaths have been recorded in 2016 (http://nvbdcp.gov.in/den-cd.html). In absence of definitive treatment and effective vaccines, local viral surveillance could aid in better understanding of disease pathogenesis and formulation of improved management strategies.

India experienced its first Dengue epidemic in Kolkata during 1963–64, since then many outbreaks are documented in India (Gupta et al., 2006). In 1996, Delhi and adjoining areas witnessed one of the largest outbreak of DENV-2 (Dar et al., 1999). In 2003, Delhi witnessed another outbreak due to the emergence of DENV-3 (Dash et al., 2006).

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In 2008, DENV-1 replaced DENV-2 and DENV-3 in Delhi and became the predominant serotype (Chakravarti et al., 2010). DENV-4 emergence was first reported from Andhra Pradesh, South India in 2007 (Dash et al., 2011). Now, all the four serotypes are co-circulating in most parts of India with the changing predominant serotype (Gupta and Ballani, 2014). Geographical and temporal variation in the distribution of serotypes is regularly observed, with co-circulation of multiple serotypes being reported as an indicator of increased endemicity (Vinodkumar et al., 2013; Saha et al., 2016). Despite the importance of knowledge regarding regional serotype distribution, majority of such studies from India have been confined to northern and southern Indian states and reports from central India have been rather limited (Vinodkumar et al., 2013: Afreen et al., 2014; Prakash et al., 2015; Reddy et al., 2017). Considering such paucity of information on DENV serotypes responsible for Central Indian outbreaks and in view of the importance of local virological surveillance, we undertook serological and molecular characterization of DENV that circulated in and around Bhopal region in the Central Indian state of Madhya Pradesh during the most recent outbreak in 2016 and compared the circulating serotypes with the ones identified previously. We also compared clinical correlates of infection with the different serotypes and observed relatively longer viremia in case of infection with DENV-2, which could be attributable to the higher frequency of antigenic variability in this serotype.

2. Materials and methods

2.1. Study design

This cross-sectional study was performed with the patients presenting with fever to Medicine and Pediatrics Outpatient Department of our tertiary care teaching hospital from September 2016 to April 2017. Samples were received from 7 different districts within the Central Indian state of Madhya Pradesh. Dengue-suspects, among these patients, were identified on the basis of WHO case definitions.

2.2. Ethics statement

The study protocol was duly approved by the Institutional Human Ethics Committee (IHEC), AIIMS Bhopal. Demographic and clinical details of the patients, including their age; gender; presenting manifestations and salient laboratory findings, were collected by the clinicians and entered in a pre-designed case record form after obtaining written informed consent. For child participants, parents/guardians provided consent on their behalf.

2.3. Sample collection

Blood samples were collected aseptically in the heparinized vacutainers by the trained phlebotomists within our tertiary care hospital. The samples were transported to our Regional Virology Laboratory for serological diagnosis. Upon receiving, blood samples were centrifuged at 3000 rpm for 10 min at 4 °C to separate serum. The serum sample was stored at 4 °C until further use.

2.4. Serological diagnosis

Serum samples were tested by Dengue IgM capture ELISA (supplied by National Institute of Virology, Pune, India) and Dengue NS1 antigen ELISA (PanBio Diagnostics, India) following manufacturer's protocols.

2.5. RT-PCR based dengue virus detection

Serologically positive samples were subjected to RT-PCR. For this, viral RNA was extracted from serum using QIAamp viral RNA mini kit (Qiagen, Germany) following kit instructions. The RNA was eluted in a final volume of 60 µl of elution buffer and stored in −80 °C until further use. RT-PCR was carried out using SSIII platinum one-step RT-PCR kit (Invitrogen, USA) and DENV group-specific consensus primers (D1 and D2) (Lanciotti et al., 1992). The PCR cycling condition were as follows: 1 cycle of Reverse transcription; 45 °C: 30 min, 1 cycle of Denaturation; 94 °C: 2 min, 40 cycles of PCR amplification: 94 °C for 15 s, 55 °C for 30 s, 68 °C for 1 min, 1 cycle of Final extension; 68 °C for 5 min. After amplification, the PCR products were electrophoresed on 1% agarose gel and visualized on a Gel Documentation system (Syngene, USA). The sample positive for DENV was identified on the basis of 511 bp band.

2.6. Serotyping

Samples positive by RT-PCR were further subjected to multiplex RT-PCR for serotype identification. For this, semi-nested RT-PCR was carried out using 1:100 dilution of the previous RT-PCR product as template and five primers viz., forward D1 and four serotype specific reverse primers (Ts1, Ts2, Ts3 and Ts4) (Lanciotti et al., 1992). PCR products were electrophoresed on 1% agarose gel and serotypes were identified by differential band sizes viz. 482 bp, 119 bp, 290 bp and 392 bp for DENV-1, 2, 3 and 4 respectively.

Samples found to be infected with multiple serotypes were further confirmed in singleplex reactions using the primers of corresponding serotypes viz., D1-Ts1 (DENV-1), D1-Ts2 (DENV-2), D1-Ts3 (DENV-3) and D1-Ts4 (DENV-4).

2.7. Nucleotide sequencing for confirmation of serotypes

DENV serotypes were also confirmed by nucleotide sequencing using Sanger's method. For this, BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was used on 3730xl DNA Analyzer (Applied Biosystems, USA) following the manufacturer's protocol. For this, five representative samples of DENV-1, four representative samples of DENV-2 and two representative samples of DENV-3 were selected for serotype confirmation and the amplicons already obtained by the consensus primers were submitted for sequencing. Following sequencing, the nucleotide sequences were subjected to BLAST analysis in the NCBI database to identify the serotype. The nucleotide sequences of the serotypes and other necessary information were submitted in GenBank.

2.8. Phylogenetic analysis

The nucleotide sequences of DENV-1, DENV-2 and DENV-3 obtained in the present study were aligned with different Indian sequences of these serotypes using MEGA 7.0 software (Kumar et al., 2016). Following alignment, a phylogenetic tree was constructed using Maximum Likelihood method based on Tamura Nei model (Tamura and Nei, 1993). The tree topologies were confirmed using 1000 bootstrap replicates.

2.9. In-silico analysis of genome wide variability

To undertake genome-wide identification of variable B cell and T cell epitopes, full length polyprotein and individual protein sequences for all DENV serotypes (DENV-1 = 1787, DENV-2 = 1351, DENV-3 = 900, DENV-4 = 207) were extracted from Virus Pathogen Resource (https://www.viprbrc.org/).

In order to calculate the antigenic variability at every position of the polypeptide/proteins, the sequences for each protein were first aligned using ClustalW (Thompson et al., 1994) and Wu-Kabat's Variability Coefficient (Garcia-Boronat et al., 2008) was calculated using following formula:

$$\text{Variability} = N^{*}k/n,$$

where N denoted the number of sequences in the alignment, k

denoted number of different amino acids at a given position and n represented the number of times that the most common amino acid at that position was present.

2.10. Immune epitope prediction

For the identification of immune epitopes/ antigenic sites in the DENV proteins, we predicted epitopes for two different sets of proteins, viz. (a) Structural proteins (capsid, membrane and envelope) for humoral response (b) All structural & non structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b & NS5) for cell-mediated response. BepiPred-2.0 server (http://www.cbs.dtu.dk/services/BepiPred-2.0/) was used for B cell epitope prediction (Jespersen et al., 2017) and IEDB Analysis Resource (http://tools.iedb.org/mhci/) for prediction of HLA I binders (Kim et al., 2012).

2.10.1. B cell epitope prediction

BepiPred is a Random Forest-based prediction algorithm for linear B cell epitopes and identifies residues involved in B cell epitope formation. We obtained B cell epitope scores for each position in all the sequences. The average B cell epitope score was calculated for each position in the proteins in alignment. Taking the variability score of every position in the alignment along with B cell epitope score (default threshold of 0.5 as given in web server), the number of variable B cell epitope residues were computed for each of the proteins.

2.10.2. HLA I binding prediction

Similarly, the HLA I binding prediction for all the proteins from each serotypes was performed on a set of 27 reference alleles (as provided by IEDB Analysis Resource). The average IC_{50} (nM) was obtained from the predicted IC_{50} of binding for every running window of 9-mers in an alignment. The HLA I binders for each alignment were identified at IC_{50} threshold ≤ 50 nM, as provided in the web server. The highest variability score of any of the 9 residues was considered as the variability score for each 9-mer since a single highly variable residue may change the immunological property of epitope drastically (Dédier et al., 2000). After computing the average IC_{50} of HLA I binding and single variability score of each 9-mer peptide, the number of variable HLA I binders were obtained at different variability thresholds.

In view of the abundance of 2 and 3 substitutions at every position in the whole polypeptide, we increased the stringency and performed our studies on higher variability thresholds such as 4, 5 and 6. We selected the variability range from 4 to 6 as most of the positions in proteins were having variability below 4 and very few positions displayed variability > 6.

2.11. Nucleotide sequencing for confirmation of antigenic variability in our DENV-2 strains

In order to confirm if the higher antigenic variability in NS2a protein of DENV-2 is leading to immune escape due to reduced cytotoxic T cell response, we performed nucleotide sequencing of NS2a gene (as NS2a protein showed highest antigenic variability among all other proteins) of DENV-2 strains obtained in our study and compared the cytotoxic T cell activity (number of HLA-I binders) among NS2a sequences observed in patients with ≤ 5 days of illness (n = 5) and > 5 days of illness (n = 4). The cytotoxic T cell activity/ HLA binders were predicted with the same in-silico tools (IEDB Analysis Resource) as described above. Moreover, in addition to strong HLA-I binders (≤ 50 nM), intermediate HLA-I binders (> 50 nM & ≤ 500 nM) were also identified and compared. The nucleotide sequences of the NS2a gene of DENV-2 isolates has been submitted in GenBank.

2.12. Statistical analysis

Categorical variables like temporal and gender distribution, presence of different clinical manifestations, relative abundance of serotypes, detection of viremia beyond 5 days of onset of fever were compared using chi-square test. Continuous variables like age distribution and duration of illness were compared by independent two-tailed t-test. The mean variability scores for each protein were calculated and the relative variability of each of the ten proteins was compared between the serotypes using ANOVA and posthoc tests. The relative frequency of B cell and T cell eiptopes and the frequency of antigenic variability within these epitopes were compared between the different serotypes by chi-square test. Similarly, the relative prevalence of intermediate- and strong- affinity CTL epitopes in NS2a sequences retrieved from patients ≤ 5 days and > 5 days was compared using chi-square test with Yates' correction wherever applicable. $p < .05$ was considered to be statistically significant.

3. Results

3.1. Demographic characteristics, clinical manifestations and temporal variation

A total of 846 dengue-suspects, who fulfilled the inclusion criteria, were recruited in this study. Of them, 166 patients were serologically positive for dengue and 75 were positive by RT-PCR. The study layout has been represented in Fig. 1.

Majority of the RT-PCR positive cases belonged to the age group of 16–30 years, with the mean (± SD) age being 24 (± 12) years. Comparison of the patient particulars between the RT-PCR positive and negative cases revealed that majority of the clinical manifestations, including non-specific febrile symptoms and warning signs of Dengue, were similarly distributed between the two groups (Table 1). Headache,

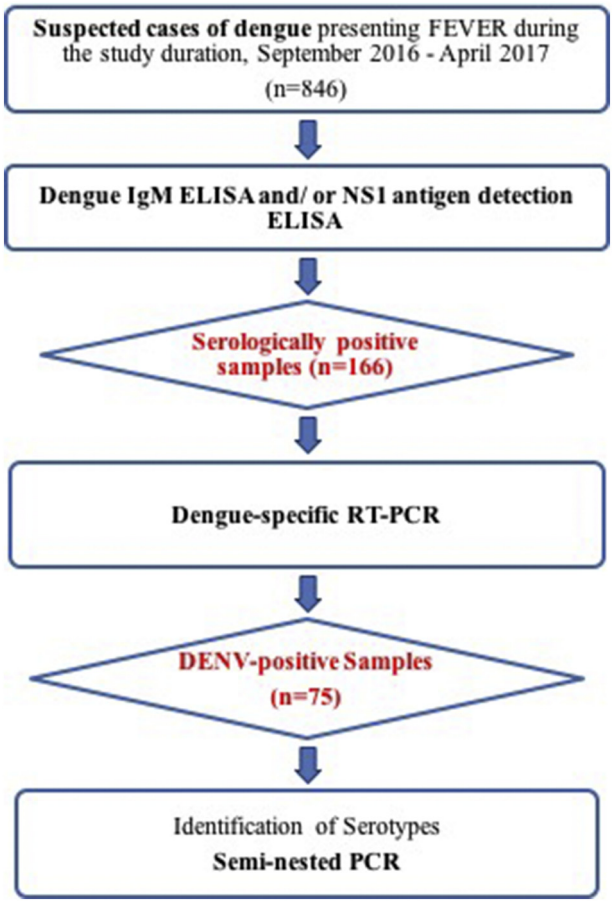

Fig. 1. Study outline representing the algorithm for diagnostic work-up.

Table 1
Demographic characteristics and clinical manifestations in DENV infected patients.

Variables	Cases positive by RT-PCR (n = 75)	Cases negative by RT-PCR (n = 91)	p-Value
Age	24 ± 12	25 ± 14	.163
Gender			
Male	58 (49.6%)	59 (50.4%)	.079
Female	17 (34.7%)	32 (65.3%)	
Serotype distribution	DENV 1 = 29 DENV 2 = 22 DENV 3 = 9 Mutiple = 11	Not Applicable	–
Clinical features			
Chills	61 (49.2%)	63 (50.8%)	.074
Headache	61 (51.7%)	57 (48.3%)	.008
Bone/Joint Pain	45 (50.0%)	45 (50.0%)	.175
Myalgia	35 (45.5%)	42 (54.5%)	.947
Malaise	31 (41.3%)	44 (58.7%)	.366
Rigors	25 (55.6%)	20 (44.4%)	.102
Rash	15 (41.7%)	21 (58.3%)	.632
Retro-Orbital Pain	9 (50.0%)	9 (50.0%)	.664
Haemorrhagic Manifestations	2 (100.0%)	0 (0.0%)	.117
Thrombocytopenia	5 (62.5%)	3 (37.5%)	.315
Joint Swelling	1 (33.3%)	2 (66.7%)	.677

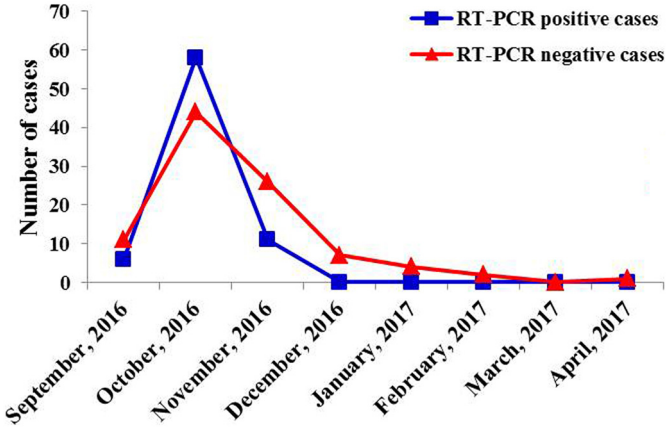

Fig. 2. Temporal distribution of cases during the outbreak.

however, was reported more commonly in the RT-PCR positive group. Haemorrhagic manifestations were observed in only 2 patients and no patient presented with manifestations of Dengue shock syndrome. The temporal distribution of the cases showed a post-monsoon distribution with the peak incidence being observed in the month of October, both among RT-PCR positive and negative cases. However, RT-PCR positive cases were not detected beyond the month of November; while serologically reactive cases were observed till April. This change in the temporal distribution of RT-PCR positive cases was found to be statistically significant (p = .0014) (Fig. 2).

3.2. Serological analysis

Among the 846 samples tested, 124 cases (14.6%) were detected positive by Anti-Dengue IgM ELISA and 62 cases (7.3%) were detected positive for NS1 antigen. Mean (± SD) duration of illness for patients with detectable NS1 antigen was found to be 5 (± 3) days and for patients positive for IgM antibodies, the same was 8 (± 12) days (p = .025) (Table 2).

3.3. Serotype distribution and duration of illness

Of the 75 samples (45.2%) positive for viral RNA, 60 cases were

Table 2
Diagnostic yield of different diagnostic modalities stratified according to the duration of illness.

Days of illness	Samples tested by Dengue IgM/NS1 ELISA	Samples positive for NS1 Antigen	Mean duration of illness (NS1 Ag positive)	Samples positive for Anti-Dengue IgM	Mean duration of illness (IgM positive)	Samples tested by RT-PCR	Samples positive for DENV RNA	Mean duration of illness (RT-PCR positive)
0–3 days	264	10	5 ± 3 days	19	8.0 ± 12 days	28	15	6 ± 3 days
4–5 days	214	26		28		48	25	
> 5 days	312	22		67		77	28	
Duration not known	56	4		10		13	7	
	846	62 (7.3)		124 (14.7)		166	75 (45.2)	

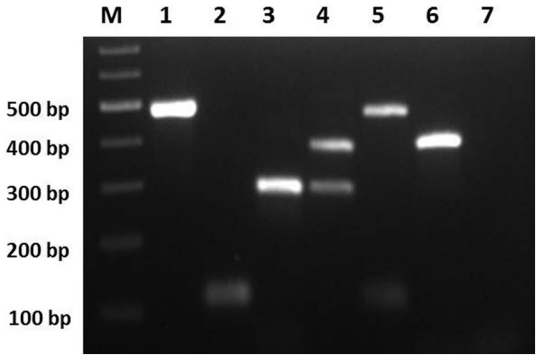

Fig. 3. Detection of DENV serotypes in five representative samples by nested PCR. Lane M; 100 bp DNA marker, lane 1; DENV-1 infected sample (482 bp), lane 2; DENV-2 infected sample (119 bp), lane 3; DENV-3 infected sample (290 bp), lane 4; DENV-3 & DENV-4 co-infected sample (290 bp and 392 bp), lane 5; DENV-1 & DENV-2 co-infected sample (482 bp and 119 bp); lane 6; positive control (DENV-4 RNA extracted from the harvested supernatant of DENV-4 infected C6/36 cell lines), lane 7; negative control.

found to be infected with single DENV serotype, 11 cases with multiple serotypes and the results were inconclusive in 4 cases. Mean (± SD) duration of illness for RT-PCR based viral detection was 6 (± 3) days; while the corresponding figure for RT-PCR negative cases was 9 (± 14) days (p = .045).

DENV-1 was found to be the commonest serotype (n = 29), followed by DENV-2 (n = 22) and DENV-3 (n = 9) (p = .037). Among patients positive for multiple serotypes, co-circulation of DENV-2 and DENV-3 was the most common (n = 6) and 1 patient each was found to be infected with DENV-1 and DENV-2, DENV-1 and DENV-3, DENV-3 and DENV-4. In 2 patients, all the 3 prevalent serotypes, i.e. DENV-1, DENV-2 and DENV-3, were detected. A representative gel image is shown in Fig. 3.

The serotype identification performed through RT-PCR was validated by sequencing of a randomly selected subset of samples. BLAST analysis of the sequencing data demonstrated complete agreement with RT-PCR results. Accession numbers of the sequences submitted in GenBank are MH051267, MH051268, MH051269, MH051270, MH051271, MH051272, MH051273, MH051274, MH051275, MH051276 and MH051277.

Phylogenetic analysis of the present strains with the different Indian strains arranged all the three DENV serotypes into three separate clusters. Within these, DENV-2 and 3 are emerging from a single branch showing more genetic closeness among them than DENV-1. All the Bhopal strains are showing closeness to recent strains from Delhi and Pune (Fig. 4).

No increase in disease severity or duration of illness was observed between patients infected with single serotype and those infected with multiple serotypes (p = .095). Both the patients with haemorrhagic manifestations were RT-PCR positive, with 1 patient being infected with DENV-2 and the other patient being co-infected with DENV-2 and 3.

We next analyzed the serotype-wise duration of illness among the RT-PCR positive cases, in which 27 cases were found to be viremic beyond 5 days of fever onset. There was a significantly higher representation of DENV 2- infected patients among them, with 12 of these 27 cases being positive for this serotype (p = .044) (Table 3).

3.4. Genome wide identification of variable epitopes responsible for immune evasion

In view of the significantly higher prevalence of DENV-2 among patients with viremia exceeding 5 days, we sought to determine if this relatively prolonged persistence in blood could be due to its greater propensity for immune evasion. In this regard, we first computed the

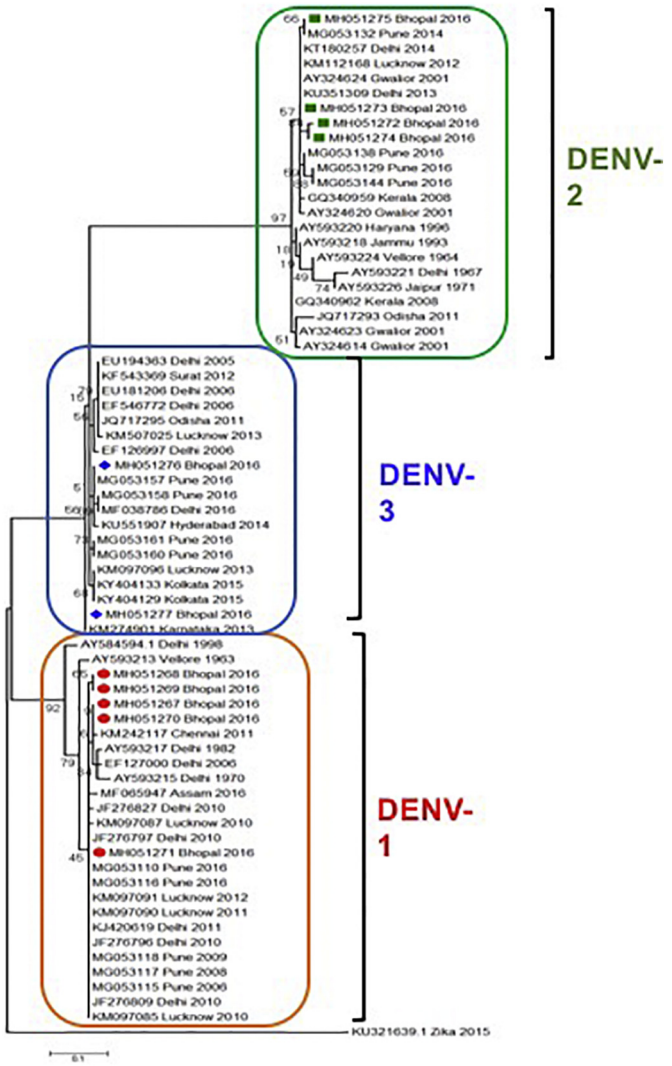

Fig. 4. Phylogenetic analysis of CprM gene sequences of DENV-1, 2 and 3. The tree is constructed on the basis of CprM gene sequences using Maximum Likelihood method. Each strain is represented by GenBank accession number followed by city and year of isolation. Sequences of the present study are represented by solid symbols.

variability coefficient for each position in DENV polyprotein of all four serotypes. We observed that the average variability in DENV-2 polyprotein was higher as compared to other serotypes (p < .05) (Table S1). We also observed that NS2a protein of DENV-2 displayed highest average variability among all the proteins (Table S1). Next, we aimed to analyze the frequency of variability in the sequences of B cell epitopes and HLA I binders which might be responsible for the evasion of humoral immunity and cytotoxic T cell responses respectively. Between the serotypes, the number of B cell epitopes for all the structural proteins were comparable (p = .32). However, the number of variable B cell epitopes were significantly different between serotypes and highest in DENV-2 for variability scores ranging from 4 through 6 (Tables 4 and S2).

Similarly, on analyzing the serotypic distribution of T cell epitopes in all DENV proteins, number of HLA I binder (9-mers) were comparable between serotypes (p = .13). However, the total number of variable HLA I binders were significantly different (p < .05) between the four serotypes and maximum in case of DENV-2 at variability thresholds 4, 5 and 6 (Table 5). The relative paucity of T cell epitopes in DENV-2 could be responsible for more successful evasion of CD8 T cell activity, while the maximum variability in T cell epitopes could make

Table 3
Serotype distribution in DENV RT-PCR positive samples stratified according to duration of illness.

	DENV-1	DENV-2	DENV-3	DENV-4	Multiple serotypes	Serotyping not conclusive
0–3 days	4	3	2	0	4 (DENV-1, DENV-3 - 1) (DENV-2, DENV-3 - 3)	2
4–5 days	14	5	3	0	3 (DENV-2, DENV-3 - 1) (DENV-3, DENV-4 - 1) (DENV-1, DENV-2, DENV-3 - 1)	0
> 5 days	8	12	4	0	3 (DENV-2, DENV-3 - 2) (DENV-1, DENV-2, DENV-3 - 1)	1
Duration not known	3	2	0	0	1 (DENV-1, DENV-2 - 1)	1
Total	29	22	9	0	11	4

Table 4
Distribution of variable B cell epitopes in structural proteins of different DENV serotypes.

	DENV-1 (1787)	DENV-2 (1351)	DENV-3 (900)	DENV-4 (207)	p-Value
B cell epitopes	240	235	231	210	.323038
Var = 4	23	27	9	0	< .001
Var = 5	2	13	2	0	< .001
Var = 6	3	10	1	0	< .001

Figures in parentheses indicate the number of protein sequences belonging to the particular serotype in Virus Pathogen Resource (ViPR) database.

Table 5
Distribution of variable T cell epitopes (HLA I binders) in all proteins of different DENV serotypes.

	DENV-1 (1787)	DENV-2 (1351)	DENV-3 (900)	DENV-4 (207)	p-Value
HLA I binders	510	467	492	533	.13214
Var = 4	216	234	129	38	< .001
Var = 5	64	143	34	8	< .001
Var = 6	38	79	12	2	< .001

Figures in parentheses indicate the number of protein sequences belonging to the particular serotype in Virus Pathogen Resource (ViPR) database.

the pre-existing activated CD8 T cells functionally redundant.

3.5. Validation of NS2a antigenic variability as immune escape mechanism in our strains

In-silico prediction of HLA-I binders in NS2a gene of our strains demonstrated a higher number of strong HLA-I binders (n = 13) exclusively present in NS2a sequences retrieved from patients with ≤5 days of illness as compared to strong HLA-I binders (n = 4) observed in the same from patients with > 5 days of illness (p = .01). Similar pattern was observed when we compared the HLA-I binders including those with intermediate affinity, where we found 28 HLA-I binders to be uniquely present in the former group compared to only 10 HLA-I binders in the latter group (p = .001) (Tables 6 and S3). The accession numbers of the NS2a sequences submitted in GenBank are MH077528, MH077529, MH077530, MH077531, MH077532, MH077533, MH077534, MH077535 and MH077536.

4. Discussion

In this paper we have undertaken molecular characterization of DENV serotypes circulating in Central India during the 2016 outbreak. We observed a high prevalence of co-infection with multiple serotypes of DENV in 15.5% of the infected patients. We also report, for the first time, significantly longer period of viremia, particularly in case of infection with DENV-2. Compared to other serotypes, DENV-2 demonstrated highest frequency of antigenic variability in B cell and T cell epitopes, which was compatible with increased efficiency in evading host immunity and could lead to more persistent viremia. The possible association of prolonged viremia and immune evasion potential was corroborated by the observation of relatively poorer HLA-I binding activity in CTL epitopes observed in NS2a sequences retrieved from DENV2-infected patients with > 5 days of illness, compared to those with ≤5 days of illness.

During primary infection, neutralizing antibodies are produced against the infecting serotype and cross reactive/non-neutralizing antibodies produced during secondary infection lead to severe manifestations (Diamond and Pierson, 2015). Secondary infections are very common among populations residing in Dengue-endemic regions (Halstead, 2007). Thus, knowledge of DENV serotype associated with a particular attack of Dengue could be helpful in identifying patients with higher likelihood of severe outcomes. Moreover, co-circulation of multiple DENV serotypes at a particular location could potentially lead to variations in DENV genomes through genetic recombination (Perez-Ramirez et al., 2009) and characterization of circulating DENV serotypes could enable better understanding of the evolutionary basis of their emergence. Also, in the absence of antivirals or vaccines against Dengue, viral surveillance could offer insights in epidemiological dynamics of viral transmission and lead to improved strategies for outbreak containment.

Molecular studies revealed the presence of all four DENV serotypes in this outbreak with predominance of DENV-1, 2 followed by DENV-3. DENV-1, 2 and 3 are prevalent in most of North (Afreen et al., 2014; Prakash et al., 2015; Saswat et al., 2015) and South India (Ahamed et al., 2017; Reddy et al., 2017). These reports reinforce our previous knowledge regarding the existence of spatial and temporal variation in the prevalence of DENV serotypes. Limited reports available from focal outbreaks in Central India have demonstrated the presence of DENV-4 in 2010 (Barde et al., 2012), DENV-1 during 2012 (Barde et al., 2015a) and DENV-2 during 2013 (Barde et al., 2015b). The serotype distribution observed in our study suggests a continuation of the previous trend in this region, along with the replacement of DENV-4 with DENV-3. We understand that the central location of this study site within the country could explain the finding of all serotypes that have been independently reported from northern and southern regions of India. High incidence of Dengue cases has been reported from Asia Pacific region also (Wartel et al., 2017). Recent studies have demonstrated the circulation of all four serotypes in Pakistan (Suleman et al., 2017). Importation of DENV-1, 2 and 3 in China led to their predominance (Shi et al., 2016).

Table 6
Propensity of HLA-I binders in NS2a protein of DENV-2 strains of our study.

HLA-I binders exclusively found in ≤5 days or > 5 days viral strains	Viral strains with ≤5 days of illness (n = 5)	Viral strains with > 5 days of illness (n = 4)	p-Value
Total no. of unique peptides as strong HLA-I binders	13 (13/5 = 2.6)	4 (4/4 = 1)	.01
Total no. of unique peptides including strong & intermediate HLA-I binders	28 (28/5 = 5.6)	10 (10/4 = 2.5)	.001

Epidemiological shifts and spatial distribution is also evident within other countries like Indonesia where all serotypes were circulating, with the predominance of DENV-1 in Surabaya in 2012 (Wardhani et al., 2017) and DENV-3 in Bali in 2015 (Megawati et al., 2017).

Co-circulation of multiple DENV serotypes in a particular area is an indicator of endemicity (Messina et al., 2014), which is influenced by growing urbanization, deforestation and changing weather conditions (Vasilakis et al., 2011; Ebi and Nealon, 2016). Our study unveiled high prevalence of co-infection with multiple serotype (15.5%) in Central India. Among the multiple infections, DENV-2 and DENV-3 were the most commonly observed serotypes during the outbreak. Similar finding was also reported from Karnataka in Southern India, where multiple serotypes were circulating simultaneously but DENV-2 and DENV-3 were the most commonly encountered co-circulating serotypes (Vinodkumar et al., 2013). There are no previous reports from Central India which demonstrate circulation of multiple serotypes. In early 2000's only two reports from Delhi region of India have shown the co-circulation of multiple serotypes (Dar et al., 2006; Bharaj et al., 2008). However, more frequent reports on co-circulation of multiple Dengue virus serotypes have been observed in India since 2011. For instance, 14% samples were found to be co-infected with DENV-1, 2 and 3 in a study conducted in New Delhi in 2013 (Afreen et al., 2014), while during 2014, DENV-1 was the predominant serotype followed by DENV-2, with concurrent infection in 43% of the positive samples (Tazeen et al., 2017). All serotypes were detected with co-infection rates of 6.6% from the southern states of Karnataka, Andhra Pradesh and Tamil Nadu in 2014–15 (Ahamed et al., 2017). Similarly, in Odisha (Eastern India) and Hyderabad (Southern India) during the 2014 Dengue season, all the four serotypes have been found to be circulating (Mishra et al., 2017; Vaddadi et al., 2017). In a recent study, all four DENV serotypes have been found to be co-circulating during a single season of 2016 in Pune (Western India) with the predominance of DENV-2 (Shrivastava et al., 2018). However, only DENV-3 has been found to be circulating in New Delhi during 2016 outbreak indicating a sudden change in epidemiological trend (Parveen et al., 2019). Exceptionally high co-infection rates (100%) were observed in 2013–15 in the southern state of Kerala, where all the samples were co-infected with multiple DENV serotypes (Reddy et al., 2017). This could be due to the coastal location of Kerala providing optimal climatic conditions for mosquito breeding, as evidenced by the presence of both *Ae. aegypti* and *Ae. albopictus* vectors across the state (Anoop et al., 2010). Acquisition of multiple blood meals by a mosquito during its lifetime can lead to infection by different serotypes. In absence of credible vector surveillance data from Central India, it is difficult to identify the incriminated vectors in this region. Our finding of high co-infection rate is, therefore, in sync with other reports from India and hint at the increasing endemicity of this disease in India.

DENV genome can be detected in serum for 4–5 days after the onset of illness (http://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf). But in this study, we detected viremia in ~40% cases beyond the usual duration after fever onset. Interestingly, such delayed clearance of viremia was significantly more among DENV-2 infected cases. To the best of our knowledge, this is the first report citing prolonged viremia in DENV-2 infected patients. Since mutation in the antigenic sites is a known mechanism for immune evasion in viruses (Burke and Cox, 2010), we performed in-silico study with worldwide DENV sequences to ascertain if the prolonged viremia observed in DENV-2 followed the same mechanism. While the number of B and T cell epitopes were similar among all serotypes, DENV-2 showed maximum variability in both types of epitopes across all coefficients of variability. This observation is suggestive of the improved efficiency of DENV-2 in escaping pre-existing neutralizing antibodies (Chiappelli et al., 2014) and virus-specific cytotoxic $CD8^+$ T cells (Pagni and Fernandez-Sesma, 2012) and is consistent with our finding regarding its relatively prolonged persistence in bloodstream. The progression of disease severity and prolonged viremia has already been associated previously with escape mutants (Lim and Ng, 1999; Guzmán et al., 2000).

Our hypothesis that antigenic variability might be the reason for immune escape of DENV-2 which further leads to prolonged viremia was experimentally validated by nucleotide sequencing performed on our viral isolates. The sequencing of NS2a gene of DENV-2 was performed as it displayed maximum antigenic variability among all other viral proteins. The higher propensity of predicted HLA-I binders or CTL epitopes in NS2a sequences retrieved from patients with ≤ 5 days of illness, as compared to those with > 5 days of illness, supports the possible association between successful immune evasion and prolonged viremia.

The study suffered from the limitations of a small sample size and the possibility of selection bias. Being a hospital-based study, our findings might not be completely representative of the serotype distribution in the community. In addition, genotypic analysis of all the circulating strains could not be performed in this study due to resource constraints. Being focused on the characterization of the strains associated with the acute outbreak, we did not look for the presence of anti-Dengue IgG antibodies in our patients and thus failed to discriminate between primary and secondary Dengue cases. However, in a separate unpublished study we observed the presence of secondary Dengue in 25% of Dengue patients presenting to our hospital (our unpublished data). Confirmation of RT-PCR data on serotype distribution was done through sequencing of a randomly selected subset of patients, since facilities for the WHO-recommended PRNT assay were not available in our laboratory.

To conclude, this study reflects the rising endemicity and the increased prevalence of multiple co-circulating DENV serotypes in Central India. In addition, this paper reports the occurrence of maximum variability in B cell and T cell epitopes of DENV-2, which is consistent with the more prolonged viremia observed in infection with this serotype.

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Disclosure statement

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.02.024.

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