Acta Scientific Microbiology (ISSN: 2581-3226)

Volume 7 Issue 3 March 2024

Research Article

Molecular Characterization of Dengue Viruses Causing Hemorrhagic Fever from the Atypical Dengue Cases in Chennai, India

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DOI: 10.31080/ASMI.2024.07.1356

Received: January 22, 2024
Published: February 11, 2024

Abstract

Objectives: The classic presentation of dengue fever has expanded its horizon by involving severe forms as atypical manifestations and hemorrhagic fever with reason unknown. This study aimed to characterize dengue viruses (DENV) causing hemorrhagic fever.

Methods: Clinically suspected cases of dengue fever were confirmed by NS1 antigen, IgM and IgG antibody. Following clinical confirmation dengue virus isolates were subjected to reverse transcriptase polymerase chain reaction (RT-PCR), sequenced, aligned using Clustal W and serotype based phylogenetic analysis was performed.

Results: A total of 308 cases were screened, during 2015-18 of which 106 (34%) samples were positive for dengue infection. 42 (40%) cases showed atypical dengue manifestations, 14 (13%) presented with hemorrhagic fever. Among the atypical manifestations, hepatomegaly and splenomegaly were the most common, followed by ascites 12 (11%), pleural effusion 9 (8%), bradycardia 6 (6%), meningitis 6 (6%), acalculous cholecystitis 04 (4%), haemoptysis 3 (3%) and acute pancreatitis 2 (2%). Dengue hemorrhagic fever (n = 14) was caused by, DENV-1 (n = 7), DENV-2 (n = 5) and DENV- 4 (n = 2). Serotype DENV-3 was not isolated in any of the samples. A new clade of genotype I (GI) was predominant in DENV-1; Genotype IVC (GIVC)was predominant in DENV-2 showing mixed clustering suggesting lineage C; clade B of lineage C, genotype I (GI)was seen in DENV-4. The amino acid changes were observed in M51I, L72F, S93N in DENV-1 and M104V, V112A in DENV-2 of C-prM regions of hemorrhagic isolates when compared to standard strains.

Conclusions: Multiple amino acid changes may have influenced the emergence of new clade, lineages and resulted in higher magnitude of severity dengue and presented as hemorrhagic fever (DHF).

Keywords: Atypical Manifestation; Characterization; Dengue Viruses; Hemorrhagic Fever; Phylogenetic Analysis

Introduction

Dengue fever (DF) is the most important mosquito borne viral disease in humans and poses a serious public health problem in tropical and sub tropical regions of the world [1]. The World Health Organization (WHO) estimates that around 2.5 - 3 billion people are presently living in dengue transmitted zones [2] and India contributes the majority of clinically apparent infection, placing the country as the global epicenter for dengue [3]. Dengue, is normally a self resolving febrile illness but can progress to severe dengue, defined by plasma leakage that may lead to shock (dengue shock), severe bleeding (hemorrhagic fever) and severe organ impairment [4,5].

Dengue virus is classified into four serotypes (DENV-1 to DENV-4) and all four dengue virus serotypes are further determined into different genotypes. There are different lineages and clades within the genotype [6]. The pathogenesis of severe dengue manifestations is very complex and not completely understood. Studies suggest that the genomic variation of dengue virus and related shift in circulating genotypes or even lineages in a geographical region may increase the incidence of severe dengue cases [7,8]. Hence, this study was sought to address this knowledge gap by molecular characterization of hemorrhagic fever causing DENV isolates from patients during 2015 - 2018 in a tertiary care hospital in Chennai, India, by sequencing the *C-prM* region.

Material and Methods

Patient recruitment and sample collection

Between 2015 - 2018, patients suspected with dengue infection who was admitted in the General Medicine department at Sri Muthukumaran Medical College Hospital and Research Institute in Chennai, southern India, was included in this study. Ethical clearance (IEC/SMMCHRI/46/2016) and informed consent was obtained from all the dengue positive patients. Severe dengue cases were transferred to Intensive care unit (ICU) and pertaining data were collected until the patients got discharged. Enrolled patients were classified as per WHO 2012 dengue classification guidelines as Group A (dengue without warning signs), Group B (dengue with warning signs) and Group C (severe dengue) [1] and grades of DHF classified in to DHF-1 to DHF-4 [9]. Venous blood samples (3 - 5 mL) were drawn, serum separated and tested for NS_1 antigen, IgM, and IgG antibody by rapid card method (J. Mitra and Co. Pvt. Ltd., India). The positive results were confirmed by ELISA. The samples were collected on days 0 to 7 after onset of fever (3 days on average) and serum stored at -86°C until use.

Virus isolation

Dengue was isolated from the serum sample, after sterilization through a 0.2µm syringe filter, onto Vero cells maintained in Minimum Essentials Medium with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100µg/ml streptomycin. The cells were cultured at 37°C at 5% CO_2, observed for 10 days and harvested when the cytopathic effects (CPE) became prominent. The culture harvests were aliquoted and stored at −86°C until processed further. Two blind passages were performed on all CPE-negative tissue cultures.

PCR Amplification and automated DNA sequencing

Viral genomic RNA was extracted from 140µl of serum sample by using a QIA amp viral RNA mini kit (Qiagen, Germany). The extracted total RNA was quantified and converted to complementary DNA (cDNA) by using there verse transcriptase kit (Thermo fisher, USA). Full length of the 511 bp *C-prM* region was amplified from cDNA by using specific primers as per Fatima Z., *et al*. 2011 [10]. The amplified PCR products were sequenced as per standard protocol [11].

Phylogenetic analysis and protein sequences alignment

Phylogenetic analysis was carried out on nucleotide sequences of all the DENV serotypes (except serotype 3) of *C-prM* gene region by comparing with sequences selected from GenBankby BLAST-EXPLORER program that uses the neighbor-joining method with1000 bootstrap replicates [12]. Alignment of *C-prM* residues sequences between dengue serotypes and the references strains, were analyzed by MEGA-10 [13].

Definition and Genbank accession numbers

Genotypes are defined based on the nucleotide divergence of 6 - 8% within each serotype. With a genotype, the group of DENV strains which form phylogenetically distinct monophyletic clusters with bootstrap support of at least 75% and a nucleotide divergence of 2.4 - 4.9% are classified as lineages and the term clade is used to characterize strains clustered under lineages [3]. All the nucleotide sequences were submitted to GenBank and their accession numbers are provided in table 1.

Table 1: Details of Hemorrhagic fever presenting dengue cases.

Age/Sex	GenBank Accession	Serotype	Classical symptoms	Significant Hematological parameter	Atypical Manifestation	Hemorrhagic Presentation and Type
25/F	MK328806	1	Fever, Headache, Myalgia	Platelet↓, Hematocrit↑ WBC↓, Monocyte↑	Ascitis	Rashes in skin (DHF – I)
33/M	MK328807	1	Fever, Headache, Myalgia, Retro-orbital pain	Platelet↓, Hematocrit↑ WBC↑, Monocyte↑	Pleural effusion	Gingival bleeding (DHF – II)
28/M	MK328808	1	Fever, Headache, Myalgia, Abdominal Pain	Platelet↑, Hematocrit↓, WBC↑, Monocyte↑	Acute pancreatitis	Bleeding in GIT (DHF – II)
61/M	MK328811	1	Fever, Headache, Myalgia, Nausea, Vomiting	Platelet↓, Hematocrit↓, WBC↑, Monocyte↑	A. Cholecystitis	Rashes in skin (DHF – I)
45/F	MK328812	1	Fever, Headache, Myalgia, Diahorrea	Platelet↓, Hematocrit↑, WBC↓, Monocyte↑	Pleural effusion	Petechiae (DHF – II)
36/M	MK328880	1	Fever, Headache, joint pain hypotension	Platelet↑, Hematocrit↑, WBC↓, Monocyte↓	Haemoptysis	Intracranial bleeding (DHF – III)
19/F	MK328879	1	Fever, Headache, Myalgia, Burning micturition	Platelet↓, Hematocrit↓, WBC↑, Monocyte↑	A. Cholecystitis	Hematuria (DHF – II)
54/F	MF489720	2	Fever, Headache, Myalgia, Dry cough, vomiting	Platelet↓, Hematocrit↑, WBC↑, Monocyte↑	Ascitis	Blood in stool (DHF – II)
13/M	MK328813	2	Fever, Headache, Myalgia, hypotension, restlessness	Platelet↓, Hematocrit↑, WBC↓, Monocyte↓	Meningitis	Intracranial bleeding (DHF – III)
39/M	MK328810	2	Fever, Headache, Myalgia, Retro-orbital pain	Platelet↓, Hematocrit↓, WBC↑, Monocyte↑	A. Cholecystitis	Gingival bleeding (DHF – II)
28/F	MK328814	2	Fever, Headache, joint pain	Platelet↓, Hematocrit↑, WBC↓, Monocyte↑	Bradycardia	Petechiae (DHF – II)
49/M	MK328815	2	Fever, Headache, Myalgia	Platelet↓, Hematocrit↑, WBC↓, Monocyte↑	Ascitis	Bleeding in Venipunture (DHF – II)
64/M	MF489721	4	Fever, Headache, Myalgia, Nausea, Vomiting, hypotension, restlessness	Platelet↓, Hematocrit↑, WBC↑, Monocyte↑	Pleural effusion	Intracranial bleeding (DHF – III)
29/F	MK328809	4	Fever, Headache, Myalgia, Abdominal Pain	Platelet↓, Hematocrit↑, WBC↓, Monocyte↑	Ascitis	Bleeding in GIT (DHF – II)

Result

Amongst 308 cases screened for suspected dengue fever 106 (34%) were laboratory confirmed dengue cases and their varied clinical features are listed (table - 2A). Among the 106 that were dengue confirmed by RT-PCR, DENV-1, 48 (45%) was predominant, while DENV-2,33 (31%) and DENV-4, 18 (17%) were less common followed by DENV-3,7 (7%). Out of 106, 42 (40%) cases showed varied atypical manifestations and their serotype distribution is listed (table - 2B). Of atypical manifestations, hepatomegaly and splenomegaly were the most common, followed by ascites 12 (11%), pleural effusion 9 (8%), bradycardia 6 (6%), meningitis 6 (6%), acalculous cholecystitis 04 (4%), haemoptysis 3 (3%) and acute pancreatitis 2 (2%). Among the 42 cases of atypical manifestations, 14 (13%) had hemorrhagic manifestation, their atypical manifestations and serotypes are detailed (table - 2C). The classical symptoms, significant hematological parameter, atypical manifestations and nature of hemorrhagic presentations of 14 dengue cases were listed (Table 1). The symptoms of fever, headache, myalgia were most predominant followed by nausea, vomiting and retro orbital pain. There was a significant rise in hematocrit and rapid reduction in platelet counts. Among the atypical manifestation, ascitis and pleural effusion were the most common in all the serotypes followed by acalculous cholecystitis, acute pancreatitis, bradycardia and haemoptysis which were seen in DENV 1 and 2 infections. All the 14 cases manifested various bleeding sites and most common was the gingival, Intracranial, GIT followed by rashes on the skin and type II and III DHF were common. There was no mortality as none of the patients had dengue shock syndrome in our study.

Table 2A: Clinical features of Dengue positive cases.

Clinical Features	Total No. of Patients (n = 106)	Percentage (%)
Fever	106	100
Headache	96	90
Myalgia	76	72
Body pain	48	45
Vomiting	47	44
Joint pain	46	43
Dry cough	42	40
Nausea	32	30
Abdominal Pain	29	19
Diahorrea	19	18
Retro-orbital pain	15	14
Burning micturition	12	11
Rashes	03	03

Table 2B: Atypical clinical manifestations in Dengue patients.

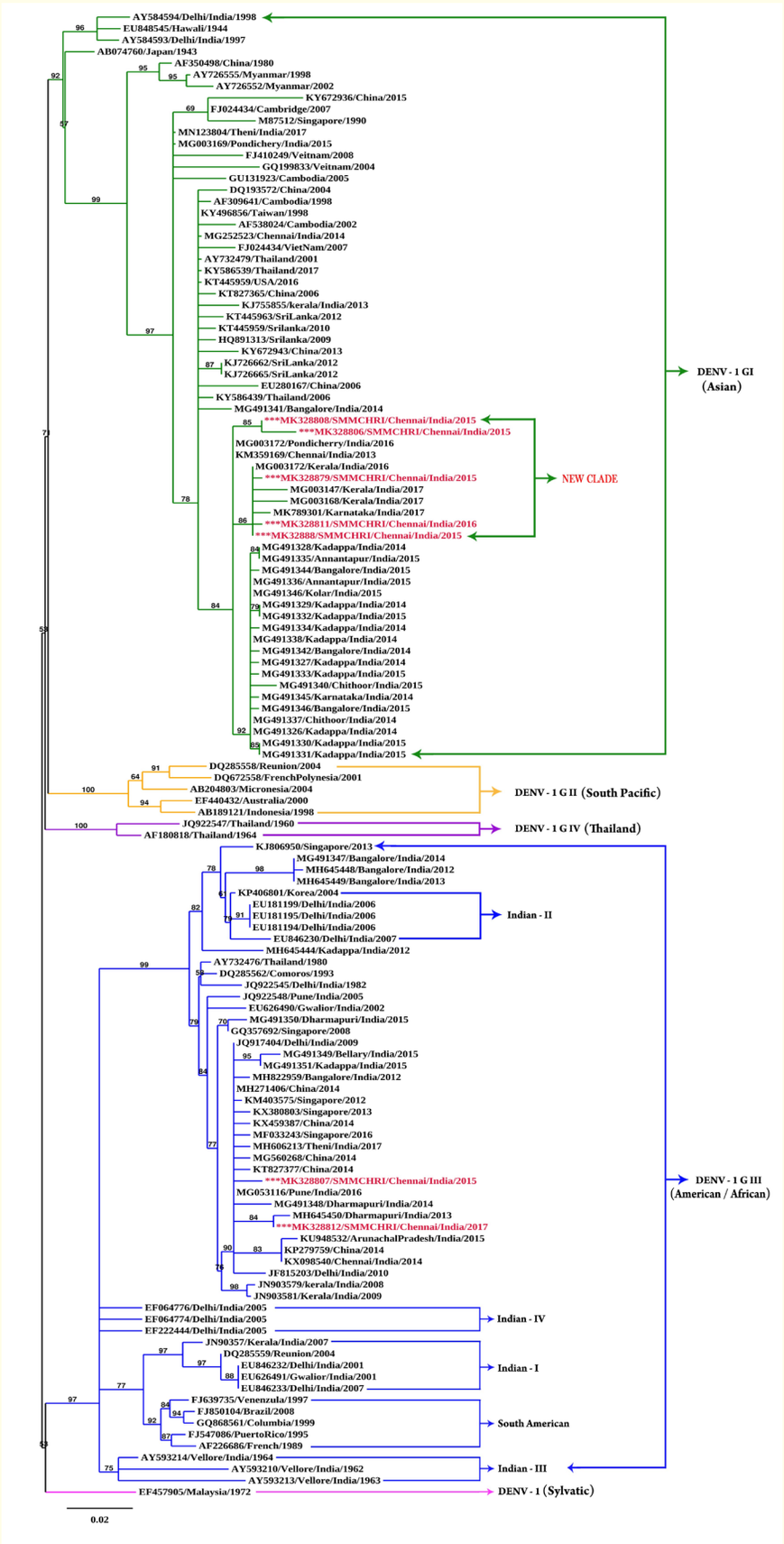
Clinical Feature	Atypical manifestations (42/106)	Total %
Ascitis	12 (S_1-6, S_2-4, S_3-0, S_4-2)	8.2
Pleural effusion	9 (S_1-4, S_2-2, S_3-1, S_4-2)	6.1
A. Cholecystitis	4 (S_1-3, S_2-0, S_3-0, S_4-1)	2.7
Meningitis	6 (S_1-4, S_2-1, S_3-0, S_4-1)	4.1
Acute pancreatitis	2 (S_1-2, S_2-0, S_3-0, S_4-0)	1.3
Bradycardia	6 (S_1-4, S_2-1, S_3-0, S_4-1)	4.1
Haemoptysis	3 (S_1-3, S_2-0, S_3-0, S_4-0)	2

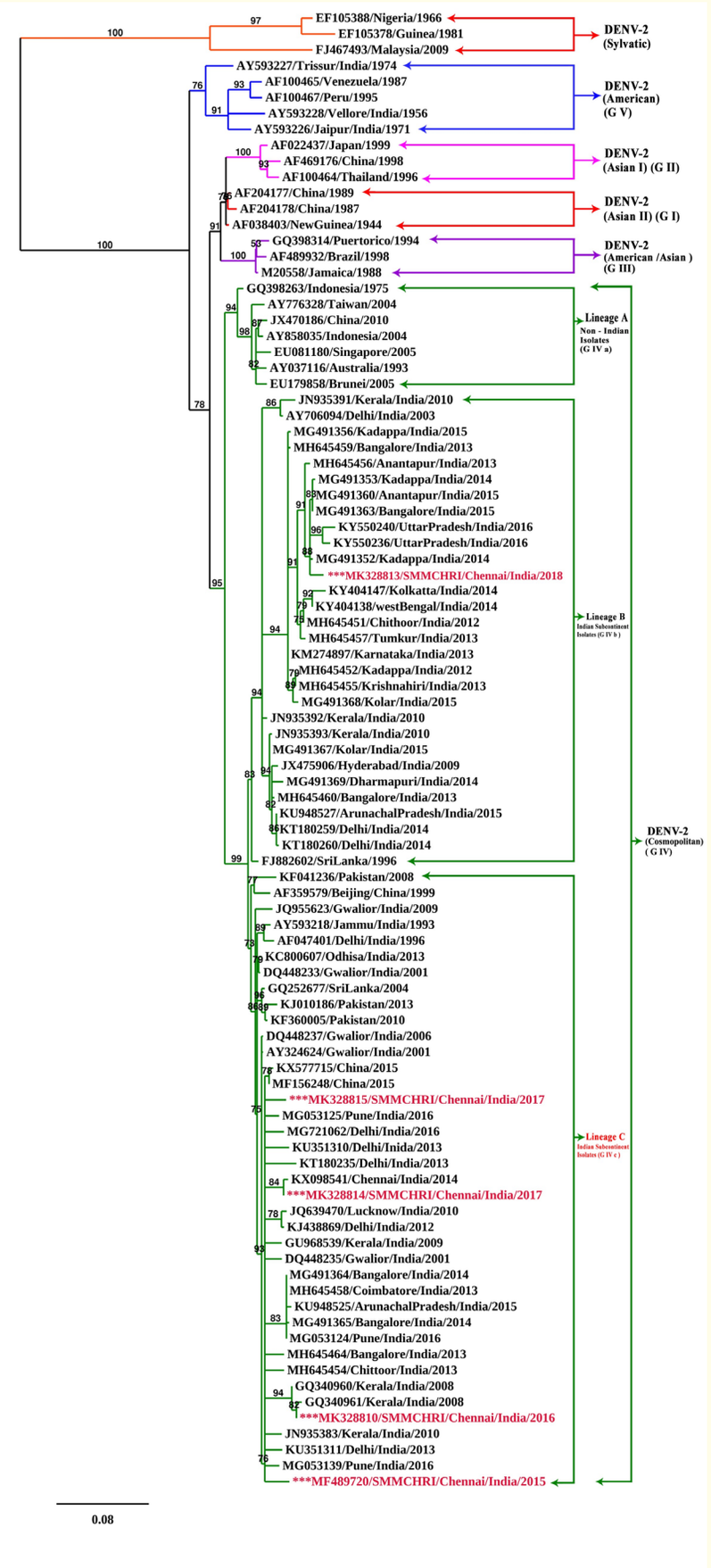
S_1 – Serotype 1, S_2 – Serotype – 2, S_3 – Serotype -3, S_4 – Serotype – 4.

Table 2C: Molecular characterization on serotypes of Dengue viruses.

Clinical Feature	Serotypes			
	DENV-1	DENV-2	DENV-3	DENV-4
	7 (50 %)	5 (36 %)	Nil	2 (14 %)
Ascitis	1	2	-	1
Pleural effusion	2	1	-	1
A. Cholecystitis	2	-	-	-
Meningitis	-	1	-	-
Acute pancreatitis	1	-	-	-
Bradycardia	-	1	-	-
Haemoptysis	1	-	-	-

Serotype specific phylogenetic analysis was performed for all the 14 hemorrhagic fever causing isolates. 7 (DENV-1), 5 (DENV-2) and 2 (DENV-4)of study isolates along with the 75 GenBank references sequences clearly distinguished five different genotypes of DENV-1, 66 GenBank reference sequences, was able to differentiate six different genotypes of DENV-2 and 52 GenBank reference sequences showed pattern of five genotypes of DENV-4, respectively. Out of 7 DENV-1 isolates sequenced, 5 isolates were genotype I (GI) which formed a new clade with tight cluster of Chennai-2013, Kerala-2016, Pondicherry-2016, Karnataka-2017 strains and the remaining 2 isolates did not fall into any of the previously described Indian lineages but clustered with other strains from India (Kadappa-2015, Delhi-2009, Kerala-2009), China-2014 and Singapore-2016, showing 95-98% sequence identity (Figure - 1A). The 5 DENV-2 isolates sequenced in this study belonged to genotype IV (cosmopolitan genotype). Genotype IV classified into non Indian isolates (lineage A - GIVa) and Indian subcontinent isolates (linage B - GIVb). One sequence clustered with previously reported lineage B strains from India with 94% nucleotide identity. Re-


AY584594/Delhi/India/1998
EU848545/Hawali/1944
AY584593/Delhi/India/1997
AB074760/Japan/1943
AF350498/China/1980
AY726555/Myanmar/1998
AY726552/Myanmar/2002
KY672936/China/2015
FJ024434/Cambridge/2007
M87512/Singapore/1990
MN123804/Theni/India/2017
MG003169/Pondichery/India/2015
FJ410249/Veitnam/2008
GQ199833/Veitnam/2004
GU131923/Cambodia/2005
DQ193572/China/2004
AF309641/Cambodia/1998
KY496856/Taiwan/1998
AF538024/Cambodia/2002
MG252523/Chennai/India/2014
FJ024434/VietNam/2007
AY732479/Thailand/2001
KY586539/Thailand/2017
KT445959/USA/2016
KT827365/China/2006
KJ755855/kerala/India/2013
KT445963/SriLanka/2012
KT445959/Srilanka/2010
HQ891313/Srilanka/2009
KY672943/China/2013
KJ726662/SriLanka/2012
KJ726665/SriLanka/2012
EU280167/China/2006
KY586439/Thailand/2006
MG491341/Bangalore/India/2014
***MK328808/SMMCHRI/Chennai/India/2015
***MK328806/SMMCHRI/Chennai/India/2015
MG003172/Pondicherry/India/2016
KM359169/Chennai/India/2013
MG003172/Kerala/India/2016
***MK328879/SMMCHRI/Chennai/India/2015
MG003147/Kerala/India/2017
MG003168/Kerala/India/2017
MK789301/Karnataka/India/2017
***MK328811/SMMCHRI/Chennai/India/2016
***MK32888/SMMCHRI/Chennai/India/2015
MG491328/Kadappa/India/2014
MG491335/Annantapur/India/2015
MG491344/Bangalore/India/2015
MG491336/Annantapur/India/2015
MG491346/Kolar/India/2015
MG491329/Kadappa/India/2014
MG491332/Kadappa/India/2015
MG491334/Kadappa/India/2014
MG491338/Kadappa/India/2014
MG491342/Bangalore/India/2014
MG491327/Kadappa/India/2014
MG491333/Kadappa/India/2015
MG491340/Chithoor/India/2015
MG491345/Karnataka/India/2014
MG491346/Bangalore/India/2015
MG491337/Chithoor/India/2014
MG491326/Kadappa/India/2014
MG491330/Kadappa/India/2015
MG491331/Kadappa/India/2015
DENV - 1 GI (Asian)
NEW CLADE
DQ285558/Reunion/2004
DQ672558/FrenchPolynesia/2001
AB204803/Micronesia/2004
EF440432/Australia/2000
AB189121/Indonesia/1998
DENV - 1 G II (South Pacific)
JQ922547/Thailand/1960
AF180818/Thailand/1964
DENV - 1 G IV (Thailand)
KJ806950/Singapore/2013
MG491347/Bangalore/India/2014
MH645448/Bangalore/India/2012
MH645449/Bangalore/India/2013
KP406801/Korea/2004
EU181199/Delhi/India/2006
EU181195/Delhi/India/2006
EU181194/Delhi/India/2006
EU846230/Delhi/India/2007
Indian - II
MH645444/Kadappa/India/2012
AY732476/Thailand/1980
DQ285562/Comoros/1993
JQ922545/Delhi/India/1982
JQ922548/Pune/India/2005
EU626490/Gwalior/India/2002
MG491350/Dharmapuri/India/2015
GQ357692/Singapore/2008
JQ917404/Delhi/India/2009
MG491349/Bellary/India/2015
MG491351/Kadappa/India/2015
MH822959/Bangalore/India/2012
MH271406/China/2014
KM403575/Singapore/2012
KX380803/Singapore/2013
KX459387/China/2014
MF033243/Singapore/2016
MH606213/Theni/India/2017
MG560268/China/2014
KT827377/China/2014
***MK328807/SMMCHRI/Chennai/India/2015
MG053116/Pune/India/2016
MG491348/Dharmapuri/India/2014
MH645450/Dharmapuri/India/2013
***MK328812/SMMCHRI/Chennai/India/2017
KU948532/ArunachalPradesh/India/2015
KP279759/China/2014
KX098540/Chennai/India/2014
JF815203/Delhi/India/2010
JN903579/kerala/India/2008
JN903581/Kerala/India/2009
DENV - 1 G III (American / African)
EF064776/Delhi/India/2005
EF064774/Delhi/India/2005
EF222444/Delhi/India/2005
Indian - IV
JN90357/Kerala/India/2007
DQ285559/Reunion/2004
EU846232/Delhi/India/2001
EU626491/Gwalior/India/2001
EU846233/Delhi/India/2007
Indian - I
FJ639735/Venezuela/1997
FJ850104/Brazil/2008
GQ868561/Columbia/1999
FJ547086/PuertoRico/1995
AF226686/French/1989
South American
AY593214/Vellore/India/1964
AY593210/Vellore/India/1962
AY593213/Vellore/India/1963
Indian - III
EF457905/Malaysia/1972
DENV - 1 (Sylvatic)
0.02


EF105388/Nigeria/1966
EF105378/Guinea/1981
FJ467493/Malaysia/2009
DENV-2 (Sylvatic)
AY593227/Trissur/India/1974
AF100465/Venezuela/1987
AF100467/Peru/1995
AY593228/Vellore/India/1956
AY593226/Jaipur/India/1971
DENV-2 (American) (G V)
AF022437/Japan/1999
AF469176/China/1998
AF100464/Thailand/1996
DENV-2 (Asian I) (G II)
AF204177/China/1989
AF204178/China/1987
AF038403/NewGuinea/1944
DENV-2 (Asian II) (G I)
GQ398314/Puertorico/1994
AF489932/Brazil/1998
M20558/Jamaica/1988
DENV-2 (American /Asian) (G III)
GQ398263/Indonesia/1975
AY776328/Taiwan/2004
JX470186/China/2010
AY858035/Indonesia/2004
EU081180/Singapore/2005
AY037116/Australia/1993
EU179858/Brunei/2005
Lineage A Non - Indian Isolates (G IV a)
JN935391/Kerala/India/2010
AY706094/Delhi/India/2003
MG491356/Kadappa/India/2015
MH645459/Bangalore/India/2013
MH645456/Anantapur/India/2013
MG491353/Kadappa/India/2014
MG491360/Anantapur/India/2015
MG491363/Bangalore/India/2015
KY550240/UttarPradesh/India/2016
KY550236/UttarPradesh/India/2016
MG491352/Kadappa/India/2014
***MK328813/SMMCHRI/Chennai/India/2018
KY404147/Kolkatta/India/2014
KY404138/westBengal/India/2014
MH645451/Chithoor/India/2012
MH645457/Tumkur/India/2013
KM274897/Karnataka/India/2013
MH645452/Kadappa/India/2012
MH645455/Krishnahiri/India/2013
MG491368/Kolar/India/2015
Lineage B Indian Subcontinent Isolates (G IV b)
JN935392/Kerala/India/2010
JN935393/Kerala/India/2010
MG491367/Kolar/India/2015
JX475906/Hyderabad/India/2009
MG491369/Dharmapuri/India/2014
MH645460/Bangalore/India/2013
KU948527/ArunachalPradesh/India/2015
KT180259/Delhi/India/2014
KT180260/Delhi/India/2014
FJ882602/SriLanka/1996
DENV-2 (Cosmopolitan) (G IV)
KF041236/Pakistan/2008
AF359579/Beijing/China/1999
JQ955623/Gwalior/India/2009
AY593218/Jammu/India/1993
AF047401/Delhi/India/1996
KC800607/Odhisa/India/2013
DQ448233/Gwalior/India/2001
GQ252677/SriLanka/2004
KJ010186/Pakistan/2013
KF360005/Pakistan/2010
DQ448237/Gwalior/India/2006
AY324624/Gwalior/India/2001
KX577715/China/2015
MF156248/China/2015
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MG053125/Pune/India/2016
MG721062/Delhi/India/2016
KU351310/Delhi/Inida/2013
KT180235/Delhi/India/2013
KX098541/Chennai/India/2014
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JQ639470/Lucknow/India/2010
KJ438869/Delhi/India/2012
GU968539/Kerala/India/2009
DQ448235/Gwalior/India/2001
MG491364/Bangalore/India/2014
MH645458/Coimbatore/India/2013
KU948525/ArunachalPradesh/India/2015
MG491365/Bangalore/India/2014
MG053124/Pune/India/2016
MH645464/Bangalore/India/2013
MH645454/Chittoor/India/2013
GQ340960/Kerala/India/2008
GQ340961/Kerala/India/2008
***MK328810/SMMCHRI/Chennai/India/2016
JN935383/Kerala/India/2010
KU351311/Delhi/India/2013
MG053139/Pune/India/2016
***MF489720/SMMCHRI/Chennai/India/2015
Lineage C Indian Subcontinent Isolates (G IV c)
0.08

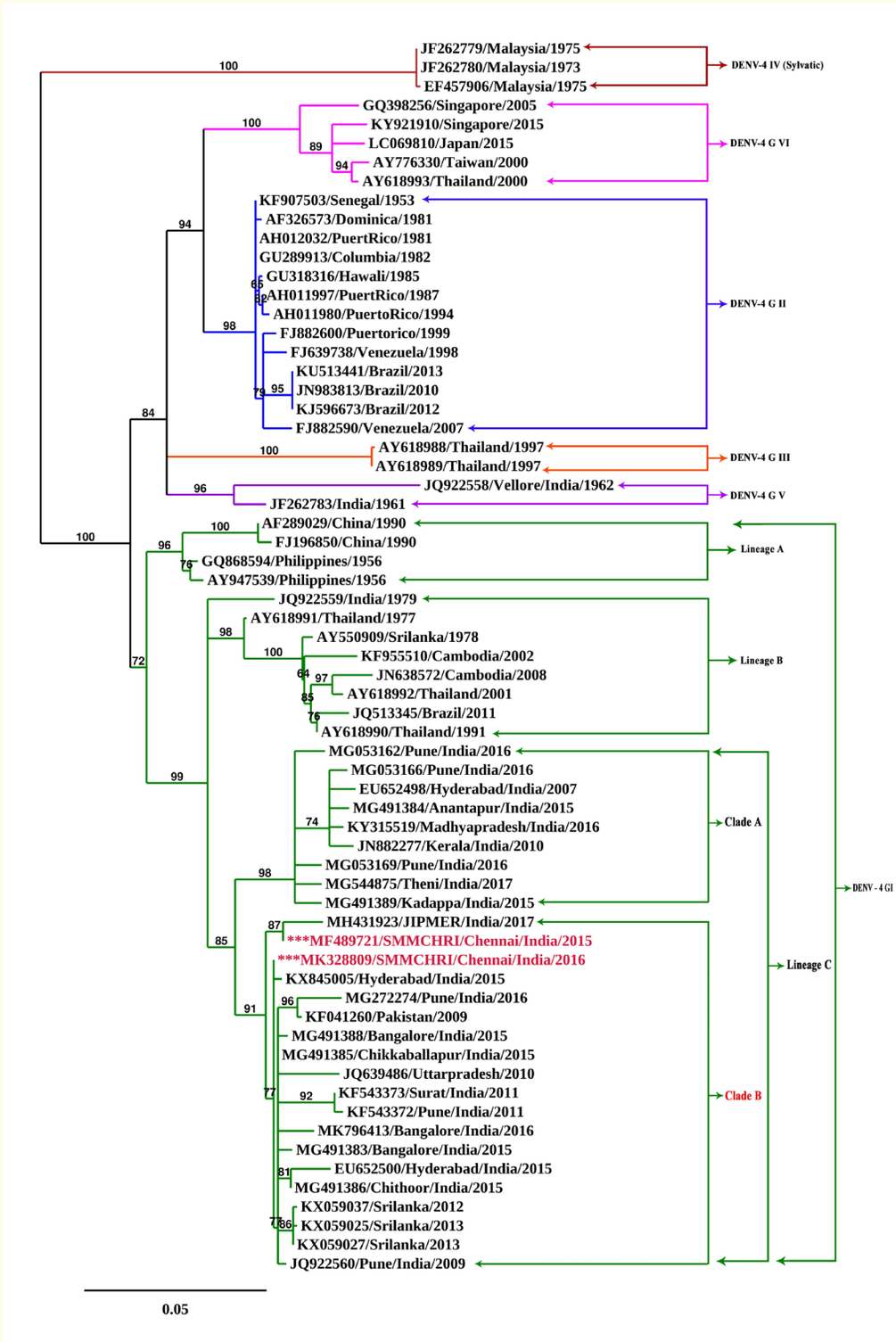

Figure 1: (A - C) Phylogenetic tree of DENV-1 (Figure 1A), DENV-2 (Figure 1B) and DENV-4 (Figure 1C) based on 363bp, 350bp and 400bp of C-prM gene junction (DENV-1, DENV-2, DENV-4, respectively) generated by the maximum likelihood method using Kimura 2-parameter model using 1000 bootstrap replicates. The DENV strains sequenced in this study from 2015-18 are indicated with *** (red color). Bootstrap values (>50) are indicated at the major branch points. Each strain is abbreviated with the GenBank accession number, place of origin and year of isolation.

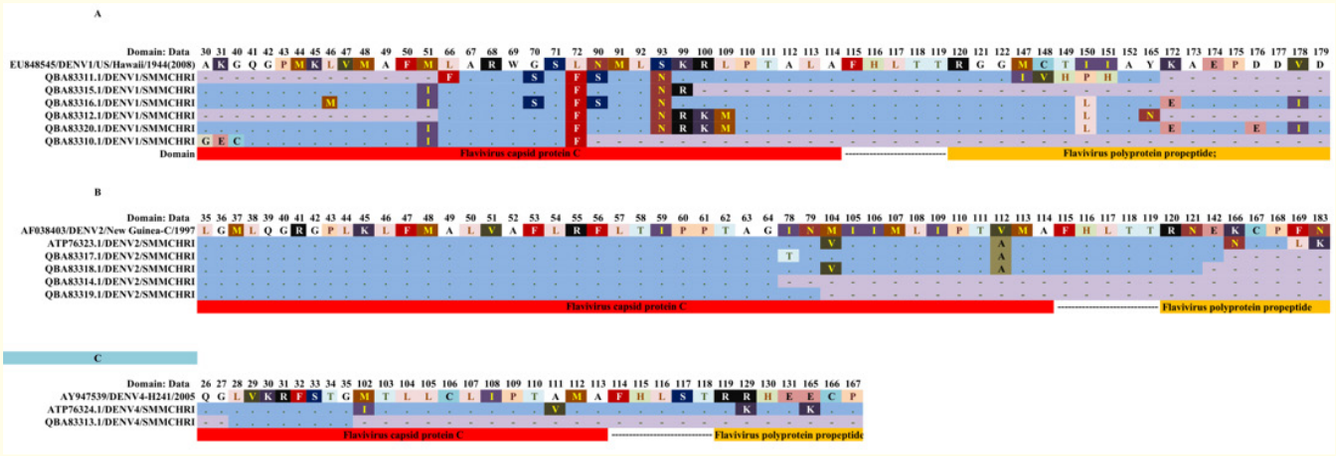

Figure 2: Features of the C-preM protein in DENV-1 (A), DENV-2 (B) and DENV-4 (C).
The amino acid changes were observed in M51I, L72F, S93N in DENV-1 and M104V, V112A in DENV-2 of C-prM regions.

maining four sequences diverged (3.5% sequence) and clustered separately from the previously reported Indian strains forms new lineage C (GIVc) (Figure -1B). The 2 DENV-4 isolates sequenced in this study belonged to clade B of lineage C, genotype I (GI) and exhibited >98% nucleotide identity with Indian strains from Hyderabad-2015, Pune-2016 and Bangalore-2015 (Figure -1C).

All the 14 hemorrhagic fever isolates sequences were compared with serotype specific standard strains (EU848545/DENV1/US/Hawali/1944 (2008); AF038403/DENV2/NewGuinea-C/1997 and AY947539/DENV4/H241/2005). The genomic analysis revealed different amino acid substitutions observed in partial *C-preM* gene, especially varying at position M51I, L72F, S93N, K99R, I150Lin DENV-1 isolates; M104Vand V112A in DENV-2 isolates; M102I and R129K in DENV-4 isolates (Figure 2).

Discussion

Dengue symptoms include fever, nausea, vomiting, aches, rash and body pain, while in severe case bleeding and shock can occur and which may be fatal if not diagnosed and treated promptly. The incidence of annual dengue infections range from 284 to 528 million with 96 million of these being apparent cases. Dengue virus is a single stranded positive sense RNA virus with 11 kb genome size that belongs to the genus Flavivirus of the family *Flaviviridae* [14]. The genome encodes for three structural proteins, namely C protein, M protein, and E protein, and seven non structural proteins, namely NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 [15]. There are four commonly circulating dengue viruses in the world, which share a similarity of 65 to 70% of amino acid sequences [16]. Phylogenetic studies have indicated many genotypes of DENV may be associated with severe dengue hemorrhagic fever and dengue shock syndrome [17]. Also, previous studies revealed that genetic alteration may lead to a change in the properties and characteristics of the virus [18,19]. We aimed to investigate phylogenetic analysis and amino acid substitutions in dengue hemorrhagic fever isolates.

Multiple studies revealed that fever; headache and myalgia were the most common clinical manifestation of DENV infection which was also seen in this study [20,21]. Expanded dengue syndrome was coined by WHO to describe cases which do not fall into either dengue shock syndrome or dengue hemorrhagic fever and this has incorporated several atypical findings of dengue [9]. In this study it was observed that 42 (40%) cases showed varied atypical manifestations and among these 14 (13%) had hemorrhagic manifestation. According to a study by Ahlawat RS., *et al*. atypical manifestations was present in 39% and acalculous cholecystitis was the most common manifestation followed by encephalitis and hepatitis [22]. Whereas in this study, the same 40% atypical manifestations were seen but ascitis was the most common followed by pleural effusion, acalculous cholecystitis and meningitis.

Patients can be infected with multiple serotypes of dengue virus and secondary infection with heterologous serotypes is more severe than primary infection, which may be explained by the Antibody Dependent Enhancement (ADE) theory, where entry of the virus into cells was enhanced [23]. In this study all the 14 hemorrhagic dengue cases were with primary infection and there was no history of secondary infection. A study reported that primary infection with DENV-1 caused severe infections compared to other serotypes [24]. Whereas in this study DENV-1 was the predominant

(7/14 cases) followed by DENV-2 (5/14 cases) and DENV- 4 (2/14 cases); no DENV-3 was reported during this time period. Despite DENV-1 being the most common in this study, all the serotypes play a role in causing severe dengue infections.

Dengue hemorrhagic fever can result in major epidemics; these often occur in the Asia and Western Pacific countries such as India, Malaysia, Singapore, Thailand, and Vietnam with a disease cycle of 3 to 5 years [16]. A study documented that DENV-1 GI emerged in 2012 causing a major outbreak in Tirunelveli and Vellore (south India), which then remarkably increased in 2014-15 [25]. Also studies, suggest that these viruses could have been introduced from Thailand to Singapore between 2001-06, then into Sri Lanka and Tamil Nadu through 2009-12 [3,26]. The same kind of DENV-1 GI was isolated in this study during 2015-16. Our results confirm that both DENV-1 GI and DENV-1 GIII circulated during the period of 2015-17 and amino acid substitution could have influenced the new clade of DENV-1 GI and DENV-1 GIII causing hemorrhagic manifestations.

High evolutionary rates of certain DENV genotypes and their increased transmission rate between several human hosts in hyperendemic settings may contribute to the emergence of new lineages [27]. In this study, all DENV-2 sequences belonged to GIV that remains the most widely prevalent genotype in India as reported in previous studies [28]. DENV-2 GIV has been further classified in two lineages (GIVa and GIVb) based on its geographical divergence. A study reports new lineage GIVc, exhibiting minimum nucleotide divergence of 3 to 4% with the closest GIVb strains [3]. In this study, 1 sequence was GIVb, however, 4 sequences diverged and clustered together to form a new lineage 'GIVc'. DENV-4, the least frequently identified serotype has been genetically characterized as GI as well as in other Indian studies and classified into three lineages (A, B and C) [29]. Studies have documented the predominance of clade B over clade A [3] and presence of one or both clades [30]. We observed only clade B in this study.

Conclusion

This study highlights the magnitude of severe dengue infection in Chennai, Tamilnadu by documenting dengue outbreaks during 2015-2018 with manifestation of hemorrhagic fever. This is the first report that demonstrates the molecular characterization of hemorrhagic fever presenting dengue viruses due to, emergence of new clade of genotype I in DENV-1, lineage C in DENV-2 and clade B in DENV-4. The interpretation of our study results is limited by the use of a short fragment of C-prM for phylogenetic analysis and also these results will have to be interpreted in the larger picture.

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