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Continued Circulation of DENV-2 (Genotype IV) in Delhi, India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Background: Dengue is one of the rapidly emerging arboviral infection in many parts of the world including India. The metropolitan city, Delhi is one of the worst affected areas by dengue. In the last two decades, it has witnessed frequent outbreaks with the continuous changing trend of circulating serotype(s)/genotype(s). The genetic characterization of circulating serotype(s)/genotype(s) is essential to establish the molecular epidemiology of the virus.
Aims: The present study was undertaken to elucidate the molecular epidemiology of dengue virus (DENV), circulated during the post-monsoon period of 2014.
Study Design: Total 112, dengue suspected samples were included in the study. Samples, positive in the NS1 antigen detection assay were processed for reverse transcription polymerase chain reaction (RT-PCR) and partial nucleotide sequencing for capsid-premembrane (CprM) gene region.
Results: Serotypic and genotypic analysis revealed cases of DENV-2 (genotype IV) 58.33%, DENV-3 (genotype III) 33.33% and DENV-1 (genotype III) 8.33%. Presence of DENV-2 (genotype IV) in majority of the cases (58.33%) indicated its pre-dominance. Multiple sequence alignment and phylogenetic analysis revealed the presence of a new strain of DENV-2 (genotype IV);

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differentiated from pre-existing strain by the substitutions; Ala102Val in capsid and Ile49Val in premembrane protein.
Conclusion: This study reports, continued circulation of DENV-2 (genotype IV) in 2014. The results also indicated circulation of a new strain of DENV-2, similar to Hyderabad isolate "1392" (JX475906), along with the pre-existing strain. It is proposed that, this strain has been introduced recently and circulating at low key in the capital.

Keywords: Dengue; capsid-premembrane; outbreak; molecular epidemiology.

1. INTRODUCTION

Dengue fever (DF), a mosquito borne arboviral infection is emerging as a major public health threat on a global scale. A recent study estimated one third contribution of India in global dengue infection [1,2]. The infection from dengue virus (DENV) can cause mild fever (DF) to severe disease conditions; dengue hemorrhagic fever (DHF) /dengue shock syndrome (DSS) [3]. DENV (genus *Flavivirus* and family *Flaviviriadae*) is enveloped, single stranded positive sense RNA of nearly 11 kb length. The genomic organization consists of three structural (capsid, C; membrane, M; and envelope, E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) genes flanked by non-coding region at 5' and 3' ends [4]. DENV circulate as four antigenically distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Serotypes are further classified into genetically related forms, termed as genotypes which may differ in their virulence and pathogenesis.

Delhi, the capital city is majorly affected area by dengue. The city has witnessed a continuous changing trend of circulating serotypes(s)/ genotype(s) during the last two decades [5]. The present study was undertaken to detect the circulating serotype(s)/genotype(s) in Delhi during the post-monsoon period of 2014. To achieve this partial molecular characterization of DENV was performed on the basis of capsid-premembrane (CprM) gene region.

2. MATERIALS AND METHODS

2.1 Study Samples

Acute phase serum samples of suspected dengue patients with fever of less than 5 days and prominent clinical symptoms such as body aches, headache, myalgia and rash were included in the study. Samples were referred to the National Centre for Disease Control from different locations in Delhi during the post-monsoon period of 2014.

2.2 NS1 Antigen Detection Assay

For detection of DENV, NS1 antigen detection assay was carried out by DENV Detect NS1 ELISA kit (InBios, USA) according to the manufacturer's instruction. Optical density was read at 450 nm. Immune status ratio (ISR) was calculated from the ratio of optical density obtained from test sample and the mean optical density of the cut-off control. ISR ≥ 1 considered positive for NS1 antigen.

2.3 RNA Extraction

Viral RNA was isolated from 140 µl serum, by spin column technique according to the manufacturer's instructions. For this purpose QIAamp Viral RNA Mini kit (Qiagen, Germany) was used. RNA was eluted in 50 µl nuclease free water and stored at -80ºC.

2.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Nearly 511 bp CprM gene region (from 134 to 644) was amplified using Access Quick one-step RT–PCR kit (Promega, USA) [5]. RT-PCR was carried out in a 25 µl reaction volume containing PCR master mix, AMV RT and gene specific, forward (D1: 5'-TCAATATGCTGAAA CGCGCGAGAAACCG-3' and reverse (D2: 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3') primers [6]. Thermal profile consisted a reverse transcription step (42℃ for 45 min) followed by initial denaturation (95℃ for 2 min) and 35 cycles of denaturation (95℃ for 1 min), annealing (55℃ for 1 min) and extension (72℃ for 2 min). Final extension step was carried out at 72℃ for 10 min. Amplified products were visualized on 1.2% agarose gel, stained with ethidium bromide.

2.5 Sequencing and Phylogenetic Analysis

Amplified PCR products were purified, using the QIAquick PCR purification kit (Qiagen, Germany). For sequencing BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) was applied. Purification of sequencing PCR product was done, using Centri-Sep™ Spin Column (Princeton Separations, USA). Purified products were lyophilized, subsequently reconstituted in HiDi Formamide (Applied Biosystems, USA) and loaded in 3130xl Genetic Analyzer, (Applied Biosystems, USA) for capillary electrophoresis. The sequences thus obtained were submitted to GenBank and accession numbers were acquired (Table 1). A BLAST search was carried out to confirm serotype(s)/genotype(s) of DENV. For sequence comparisons, multiple sequence alignment was performed with other isolates (DENV-1, 2 and 3) of diverse geographical origin, with the help of Clustal W tool available in Bio edit version 7.0.5.3. The phylogenetic tree was constructed in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0.

Table 1. Dengue virus isolates used for sequence alignment and phylogenetic tree (DENV isolates sequenced in the study are shown in bold accession numbers)

S. no.	Virus isolate	Country	Year	Accession numbers	Serotype
1.	ThD1_0442_80	Thailand	1980	AY732476	DENV-1
2.	ThD1_0673_80	Thailand	1980	AY732474	DENV-1
3.	Comoros 04.329/93	Comoros	1993	DQ285562	DENV-1
4.	D1/2CprM/Del01	India	2001	EU846233	DENV-1
5.	DENV-1/CO/BID-V3379/2001	Colombia	2001	GU131948	DENV-1
6.	GWL-14	India	2004	EU626491	DENV-1
7.	DENV-1/VE/BID-V3558	Venezuela	2005	GU131837	DENV-1
8.	04/1/del2006	India	2006	EF126999	DENV-1
9.	05/1/del2006	India	2006	EF127000	DENV-1
10.	Delhi-24	India	2010	JF815193	DENV-1
11.	14/Del/2011	India	2011	KJ420619	DENV-1
12.	58/Del/2011	India	2011	KJ420622	DENV-1
13.	3/D1/Del/2012	India	2012	KJ438859	DENV-1
14.	4/D1/Del/2012	India	2012	KJ438860	DENV-1
15.	RR57	India	2009	JQ917404	DENV-1
16.	RGCB585	India	2009	JN903580	DENV-1
17.	RGCB592	India	2009	JN903581	DENV-1
18.	Delhi-27	India	2010	JF815195	DENV-1
19.	1/D1/Del/2013	India	2013	KT180231	DENV-1
20.	3/D1/Del/2013	India	2013	KT180233	DENV-1
21.	**1/D1/Del/2014**	**India**	**2014**	**KT180255**	**DENV-1**
22.	NGC 44	New Guinea	1944	AF038403	DENV-2
23.	980	India	1996	AF047396	DENV-2
24.	841	India	1996	AF047394	DENV-2
25.	FJ11/99	China	1999	AF359579	DENV-2
26.	DENV-2/LK/BID-V2416/1996	Sri lanka	1996	FJ882602	DENV-2
27.	FJ-10	China	1999	AF276619	DENV-2
28.	GWL228 INDI-01	India	2001	DQ448237	DENV-2
29.	06DEL03	India	2003	AY706094	DENV-2
30.	D2/SG/05K3295DK1/2005	Singapore	2005	EU081177	DENV-2
31.	D2/SG/05K3330DK1/2005	Singapore	2005	EU081178	DENV-2
32.	RR44	India	2009	JQ955623	DENV-2
33.	1392	India	2009	JX475906	DENV-2
34.	D2/Pakistan/78/2009	Pakistan	2009	KF041237	DENV-2
35.	VCRC/DEN2/12/10	India	2010	JN935393	DENV-2
36.	VCRC/DEN2/11/10	India	2010	JN935392	DENV-2
37.	Den 13/Odisha	India	2011	KC800607	DENV-2
38.	Den 14/Odisha	India	2011	KC800608	DENV-2

S. no.	Virus isolate	Country	Year	Accession numbers	Serotype
39.	D2/Pakistan/2011-3/2011	Pakistan	2011	KF041233	DENV-2
40.	5/D2/Del/2012	India	2012	KJ438868	DENV-2
41.	6/D2/Del/2012	India	2012	KJ438869	DENV-2
42.	1/D2/Del/2013	India	2013	KT180234	DENV-2
43.	2/D2/Del/2013	India	2013	KT180235	DENV-2
44.	3/D2/Del/2013	India	2013	KT180236	DENV-2
45.	**1/D2/Del/2014**	**India**	**2014**	**KT180256**	**DENV-2**
46.	**2/D2/Del/2014**	**India**	**2014**	**KT180257**	**DENV-2**
47.	**3/D2/Del/2014**	**India**	**2014**	**KT180258**	**DENV-2**
48.	**4/D2/Del/2014**	**India**	**2014**	**KT180259**	**DENV-2**
49.	**5/D2/Del/2014**	**India**	**2014**	**KT180260**	**DENV-2**
50.	**6/D2/Del/2014**	**India**	**2014**	**KT180261**	**DENV-2**
51.	**7/D2/Del/2014**	**India**	**2014**	**KT180262**	**DENV-2**
52.	D3/SG/05K3927DK1/2005	Singapore	2005	EU081212	DENV-3
53.	D3/SG/05K3305DK1/2005	Singapore	2005	EU081199	DENV-3
54.	25/3/del2006	India	2006	EU181211	DENV-3
55.	D3/2CprM/Del07	India	2007	EU846235	DENV-3
56.	ND143	India	2007	FJ644564	DENV-3
57.	D3/3CprM/Del07	India	2007	EU846236	DENV-3
58.	GZ2D3	China	2009	JN662391	DENV-3
59.	ZJYW2009	China	2009	JF504679	DENV-3
60.	4/D3/Del/2012	India	2012	KJ451724	DENV-3
61.	8/D3/Del/2012	India	2012	KJ451728	DENV-3
62.	11/D3/Del/2012	India	2012	KJ451731	DENV-3
63.	13/D3/Del/2012	India	2012	KJ451733	DENV-3
64.	13GDZDVS30E	China	2013	KF954949	DENV-3
65.	1/D3/Del/2013	India	2013	KT180250	DENV-3
66.	4/D3/Del/2013	India	2013	KT180253	DENV-3
67.	5/D3/Del/2013	India	2013	KT180254	DENV-3
68.	**1/D3/Del/2014**	**India**	**2014**	**KT180263**	**DENV-3**
69.	**2/D3/Del/2014**	**India**	**2014**	**KT180264**	**DENV-3**
70.	**3/D3/Del/2014**	**India**	**2014**	**KT180265**	**DENV-3**
71.	**4/D3/Del/2014**	**India**	**2014**	**KT180266**	**DENV-3**

3. RESULTS AND DISCUSSION

Out of 112 samples, 12 were positive for NS1 antigen. The mean age of the samples (6 male and 6 female) was 22.05 years (SD ±13.51 years). The platelet count equal or less than 150,000 cells per mm^3 was observed in positive samples. All the 12 samples were amplified for the CprM gene region by RT-PCR. Amplified PCR products were sequenced and serotype was confirmed through BLAST analysis. Of the 12 samples 7 were DENV-2 (58.33%), four were DENV-3 (33.33%) and 1 was DENV-1 (8.33%). In the phylogenetic tree (Fig. 1), sequences grouped into three separate clades, DENV-1 (genotype III), DENV-2 (genotype IV) and DENV-3 (genotype III). Single DENV-1 sequence of 2014 (KT180255) was clustered with recent DENV-1 isolates of Delhi (JF815193, JF815195, KJ420619, KJ420622, KJ438859, KJ438860, KT180231 and KT180233) in close proximity to Comoros isolate (DQ285562). Analysis of DENV-2 revealed clustering of 4 sequences (KT180256, KT180257, KT180261 and KT180262) with pre-existing strains of 2012 and 2013 (KJ438868, KJ4388869, KT180234 KT180235 and KT180236). However, three sequences (KT180258, KT180259 and KT180260) formed a separate clade and clustered with Indian isolates from Hyderabad (Andhra Pradesh) and Kerala (JX475906 and JN935393). The sequences (KT180258, KT180259 and KT180260) were closely related to Sri Lankan isolate (FJ882602) with average sequence identity of 97.07%. The multiple sequence alignment of DENV-2 isolates (from nucleotide 172 to 615) with the prototype strain NGC 44 (AF038403) revealed mostly synonymous substitutions with no base insertion or deletion. Deduced amino acid alignment revealed two amino acid substitutions i.e. Alanine (Ala) to Valine (Val) at position 134 (Fig. 2) and

Isoleucine (Ile) to Valine (Val) at position 195 (Fig. 2) in three sequences (KT180258, KT180259 and KT180260). The substitutions were corresponding to position 102 of the capsid (Ala102Val) and 49 of the premembrane (Ile49Val) proteins respectively.

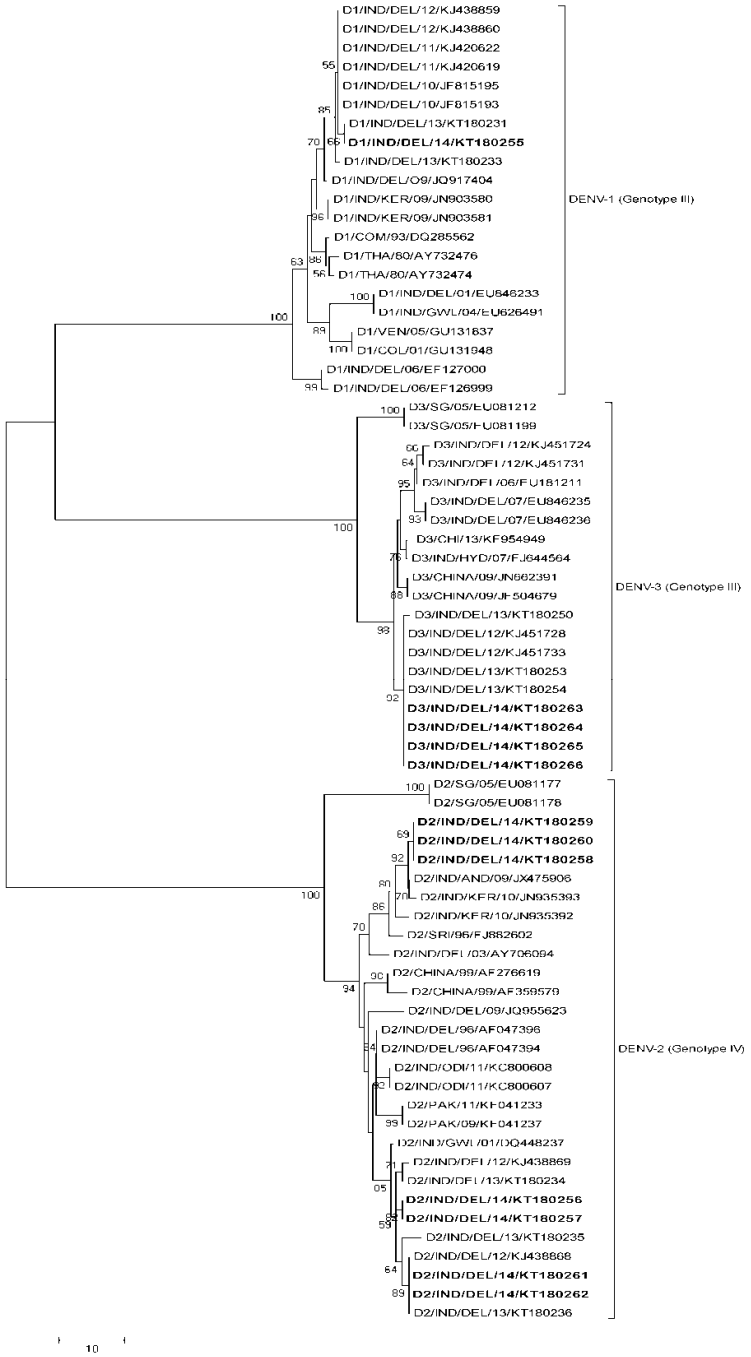

Fig. 1. Phylogenetic tree of DENV based on 360 bp (nucleotide 202 to 561) CprM gene region generated by the Neighbor-Joining (NJ) method (1000 Bootstrap replications). Each sequence is denoted by serotype, country of origin, state name (only for Indian samples) followed by the last two digits of year of isolation and GenBank accession number. Isolate sequenced in the study are shown in bold

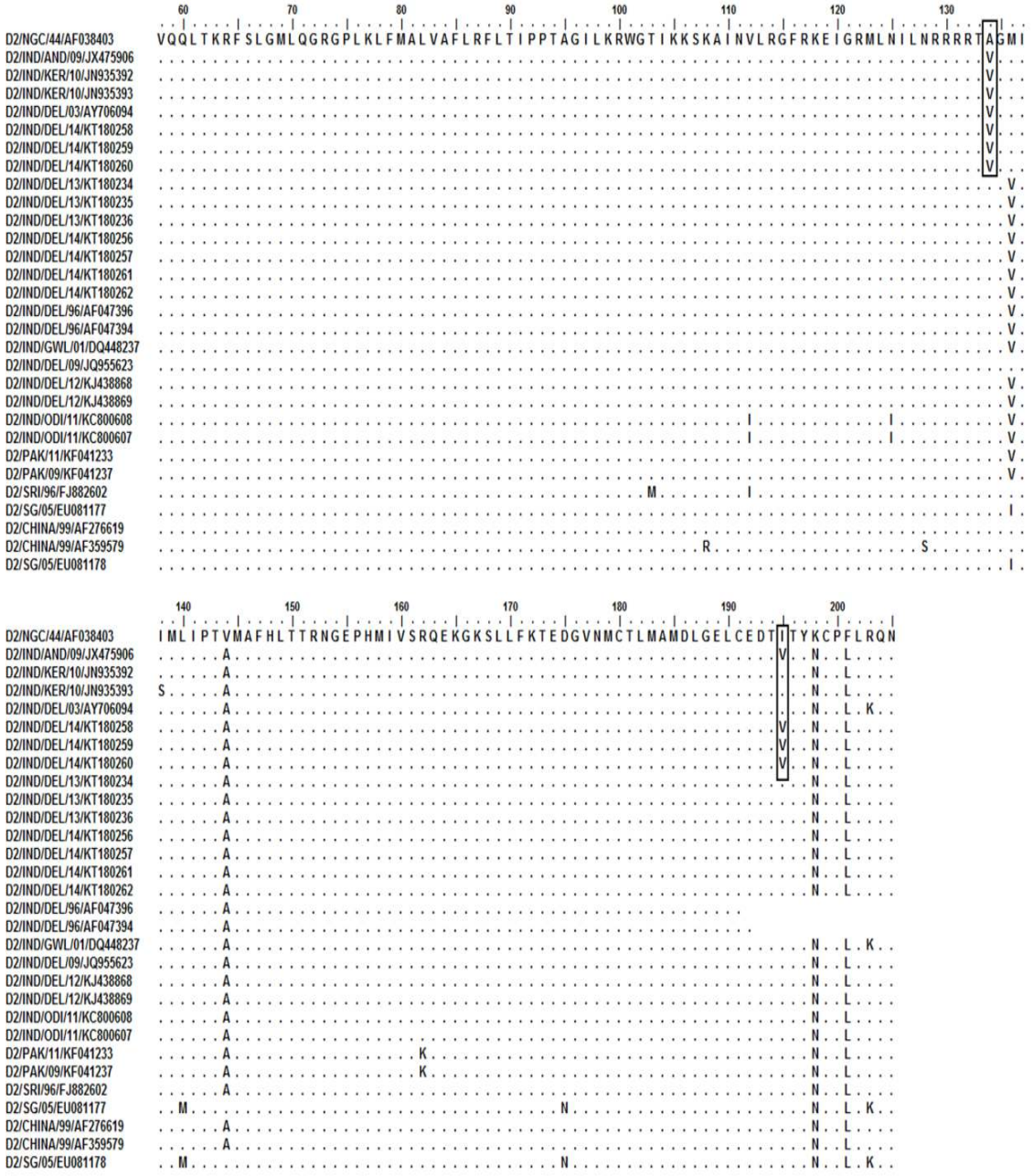

Fig. 2. Deduced amino acid alignment of CprM gene region of all DENV-2 isolates. The numbering of amino acid position corresponds to the ORF of prototype strain NGC 44 (AF038403). Gap ( ) indicates amino acid sequence not available

The substitution, Ala102Val in capsid protein was observed in a Delhi isolate (AY706094), reported in 2003 and Kerala isolates (JN935392 and JN935393) reported later in 2010. However, both substitutions; Ala102Val and Ile49Val were seen in only Hyderabad isolate, "1392" (JX475906) reported in 2009. The average sequence identity of three sequences (KT180258, KT180259 and KT180260) to the Delhi and Kerala isolates; AY706094, JN935392 and JN935393 were recorded 97.71%, 98.65% and 99.18% respectively. The closest identity (99.62%) was observed with Hyderabad isolate "1392" (JX475906).

The analysis of DENV-3 revealed circulation of the same strain of DENV-3 since 2012. All 4 DENV-3 sequences (KT180263, KT180264, KT180265 and KT180266) were clustered with recent Delhi isolates (KJ451728, KJ451733, KT180250, KT180253 and KT180254). The isolates from China (JF504679 and JN662391) were found closely related to circulating DENV-3 (KT180263, KT180264, KT180265 and KT180266) with average sequence identity of 99.38% and 99.18% respectively.

All the 4 serotypes of DENV circulate in Delhi. During outbreaks, many cases of mixed infection, with more than one serotype have also been documented [7-9]. But so far, only three serotypes have been reported as a main etiological agent in different outbreaks; DENV-1, DENV-2 and DENV-3. The last three outbreaks in 2013, 2010 and 2006 were predominated by DENV-2, DENV-1 and DENV-3 respectively [7-12]. Since the three serotypes (DENV-1, DENV-2 and DENV-3) had also been associated with DF outbreaks throughout the country, it is of prime importance to understand molecular epidemiology of these serotype(s). This study was focused on identifying the circulating serotype(s)/genotype(s) during the post-monsoon period in 2014. A low positivity rate, 10.71% (12/112) was observed in virus detection as compared to the previous years [5,9]. However several factors, one of them could be the presence of DENV NS1 antibodies (secondary infection) in the samples as well as to the fact that the RT-PCR was performed only in the samples NS1 antigen positive. Sequencing and BLAST analysis confirmed the majority of DENV-2 (58.33%) cases as compared to DENV-3 (33.33%) and DENV-1 (8.33%). The single DENV-1 sequence clustered within the same clade with previous DENV-1 isolates, being circulated from 2010 to 2013 (Fig. 1). High sequence identity to these isolates (99.51-99.71%) with no amino acid change confirmed the circulation of the same strain of DENV-1 since 2010. Similar analysis revealed clustering of all the four DENV-3 sequences with previous Delhi isolates (from 2012 and 2013) with no changes in amino acid sequence and hence; circulation of the same strain of DENV-3 for the past three years was observed. Analysis of DENV-2 revealed the circulation of different strains of DENV-2. Four sequences (KT180256, KT180257, KT180261 and KT180262) clustered with pre-existing strain from 2012 and 2013 (Fig. 1) and showed similar genetic make-up at the amino acid level (Fig. 2). The newly introduced strain of DENV-2 (KT180258, KT180259 and KT180260) was characterized by the presence of Ala102Val and Ile49Val substitutions in capsid and premembrane protein. The strain was identical (100%) at the amino acid level to "1392" isolate (JX475906) from Hyderabad (Andhra Pradesh).

4. CONCLUSION

Among all four serotypes DENV-2 is genetically most diverse [13]. Studies have shown existence of 6 different genotypes of DENV-2. Genotype IV of DENV-2 is most widely distributed genotype and has been isolated in different countries of Asia, Africa, American continents and Australia. Due to its global occurrence, it is also known as cosmopolitan genotype [2,14]. The pre-dominance of DENV-2 (genotype IV) during an outbreak has been documented from different areas in the country including Delhi [9,15-17]. This genotype has already been associated with DHF outbreaks in 1996 in Delhi [15]. After being in circulation silently for past few years, its re-emergence in 2012, followed by the pre-dominance in 2013 was recorded [5,9].

This study reports continued circulation of DENV-2 (genotype IV) in 2014. The sequence based studies are helpful to monitor the genomic changes occurring in virus as well as its spread. The persistence of DENV-2 (genotype IV) in the capital is a major concern for public health. Its continuous circulation may give rise to another DF/DHF outbreak. The results also indicated circulation of a new strain of DENV-2, similar to Hyderabad isolate "1392" (JX475906), along with the pre-existing strain. It is proposed that, this strain has been introduced recently and circulating at low key in the capital. The assessment of its impact on the population still needs to be determined. Further study of different gene regions of this strain may be required to detect important mutations and their role in pathogenesis, virulence and outbreak potential, but till then close monitoring of circulating strains along with clinical co-relation will be essential.

ETHICAL APPROVAL

Ethical approval was obtained from the institute to carry out the present study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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