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Molecular Epidemiology of Dengue Virus Genotype Circulation in Tamil Nadu, India, 2011-2014

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Abstract

Aim: Dengue fever is the most important vector borne viral disease in many tropical and subtropical countries. In this study we have analyzed the molecular epidemiology of dengue in Tamil Nadu to improve understanding of the evolution for the past four years from June 2011 to June 2014, by testing Dengue outbreak samples from twenty districts of Tamil Nadu. The serum collected from suspected Dengue patients were analyzed for Dengue specific IgM antibodies by Mac IgM antibody capture enzyme linked immunosorbent assay (ELISA) using NIV kit and detection of NS1 antigen & IgG antibodies using Pan Bio kits. NS1 positive samples were subjected to Dengue serotyping by RT-PCR. World Health Organization case definition was adopted to categorize the Dengue cases.

Results: The total number of samples screened during the period was 690, out of which 211 (79 Ns1 & 132 IgM) (30.58%) were positive for Dengue and RT-PCR results revealed that all the four Dengue serotypes were in circulation in different combinations during this study period. Re-emergence of Dengue 2 after nine years in Chennai, Tamil Nadu in 2014 was a notable feature.

Conclusion: Our analysis exhibited that in Tamil Nadu outbreaks occurred during monsoon and post monsoon season and most likely cause was by endemic virus strains that had been circulating in South East Asia for several years. In developing countries like India, public heath containment activities play a pivotal role in the control of outbreaks.

Keywords: Dengue virus; Re-emerging; Mac IgM; ELISA; RT-PCR

Introduction

Dengue virus is a leading cause of illness and death in the tropics and subtropics. In recent years, Dengue transmission had increased in urban and semi urban areas [1]. CDC estimated that 400 million people are infected annually, with over 50% of the world's population living in regions at the risk of contracting Dengue fever [2,3]. Dengue is caused by any one of the four related genotypes transmitted by mosquitoes. Dengue virus is an enveloped positive sense RNA virus. The genomic RNA is approximately 10.7 kb in length and is composed of three structural protein genes that encode for nucleocapsid or core protein (C), a membrane-associated protein (M), an envelope protein (E) and seven non-structural protein genes including NS1 protein. NS1 is a highly conserved glycoprotein which appears essential for virus replication [4]. Dengue virus attachment to the host cell surface is mediated by the viral attachment protein (VAP), the glycoprotein E present on the viral membrane [5]. The routine laboratory diagnosis of dengue virus infection is primarily achieved by the isolation of virus in tissue culture, serodiagnosis by detection of NS1/IgM/IgG antibodies followed by molecular detection by the demonstration of viral RNA by RT-PCR in the early phase of infection [6-8].

Dengue fever has been reported from India over a long time, but Dengue Haemorrhagic Fever was first reported in 1963 from Kolkata city [9]. Since then several outbreaks of Dengue fever was reported from India, with a major epidemic of Dengue Haemorrhagic Fever that occurred in Delhi in 1996 when 10,252 cases and 423 deaths were reported. Out of 35 States/Union-territories in the country 29 have reported cases and deaths due to Dengue [10]. It was mainly due to Dengue serotype 2. In New Delhi, Dengue fever outbreaks were also reported in 1967,1970,1982,1996, 2003 and 2006 [11].

In South India, particularly in Tamil Nadu DF and complications have been well documented. The first evidence of Dengue fever in the country was reported from Vellore district, Tamil Nadu in 1956 [12]. Of the 30 districts in Tamil Nadu, Dengue cases have been reported from 29 districts between 1998 and 2005 which include DF/DHF outbreaks in Chennai in 2001, Nagerkoil and Thiruchirapalli in 2003. Though uncomplicated Dengue fever (DF) was prevalent in adults and children in Chennai, since 2005 Chennai has been witnessing Dengue Haemorrhagic fever among young and adults [13]. During July 2007, suspected cases of Dengue were reported from two rural areas/hamlets near Madurai, Tamil Nadu [14]. In another study, by Bhuvaneswari et al. a total number of 200 samples were screened, out of which 79 (39.5%) were positive for IgM and IgG antibodies and 65 (32%) for IgM antibodies only. By clinical evaluation, amid 43 cases of Dengue fever, 18 had DHF manifestations and 4 patients had progressed to DSS. Though Dengue Sock Syndrome & Dengue Heamarageic fever were present in 22 patients, all of them recovered well [15]. The incidence of Dengue cases was the highest in 2012 during the span of four years. Tamil Nadu reported the highest number of cases in the

country (9,249), followed by West Bengal which reported 6,067 cases. The mortality in Tamil Nadu was also high wherein 60 succumbed to the disease, followed by fifty nine in Maharashtra [16]. This study was undertaken to analyses the Dengue outbreaks in the state over a period of time, to detect the circulating strains and to study the molecular epidemiology of the Dengue virus.

Materials and Methods

Specimen collection

This was an outbreak based retrospective study conducted at King Institute of Preventive Medicine & Research, Guindy, Chennai from June 2011 to June 2014. A total of 690 serum samples were collected from clinically suspected dengue cases from Government General Hospitals and Primary Health Centres in twenty districts of Tamil Nadu. All Serum samples were shipped to State ArboViral referral Centre (KIPM&R) Guindy, Chennai. The samples were tested immediately for Dengue NS1, Dengue IgM and IgG. IgM Capture ELISA was performed using National Institute of Virology kit and NS1 antigen detection and IgG ELISA (<5 days of illness for NS1 ELISA, ≥ 5-6 days of illness IgM&IgG ELISA) was performed using Panbio Dengue Duo, Australia. The tests were performed according to the kit protocol.

Dengue RT-PCR

All NS1 positive serum samples were subjected to Dengue viral RNA extraction using the QIA amp viral RNA mini extraction kit (Qiagen,Germany) as per manufacture's instruction. Veriti DX® Thermal Cycler (Applied Biosystems®) was used throughout this study for the RT-PCR assay. Initially, extracted viral RNA was amplified using primers against Envelope Protein1 region for pan Dengue and those which were positive were subjected to Dengue genotyping. The RT-PCR amplification was performed with a 20 µl reaction mixture and 5 µl of RNA. The reaction mix have 0.5 µl of 25 picomole concentration of each forward and reverse primer of the D1, D2, D3 and D4 primers and components of a one-step RT-PCR kit (ABI). The amplification included following four steps: RT (Reverse transcription) at 50˚C for 30 min; one cycle of initial denaturation and activation of the Hot Start Taq polymerase at 95˚C for 15 min, 55˚C for 15 s and 72°C for 30 s followed by 40 cycles at 95˚C for 15 s, 55˚C for 15 s and 72˚C for 30 s; and 2˚C extension for 10 min [15].

After completion of RT-PCR, 10 µl RT-PCR product and 2 µl of loading dye were loaded into 1.5% ethidium bromide stained agarose gel and run at about 100 volts for 45 min. The gel was viewed under Alpha Imager (Alpha Innotech San Diego, California, USA) and the resulting bands were captured with a Polaroid camera. The genotype was determined by the size of amplicon in comparison with that of marker. The size of the RT-PCR products from the amplification of Dengue (Dengue 1,2,3 and 4) were 208 bp, 119 bp, 288 bp, and 260 bp respectively [17]. For every RT-PCR run positive control and negative controls were included [18].

Phylogenic tree construction

The PCR products were sequenced in both forward and reverse directions by commercial sequencing services (Samved Biotech, Ahmedabad, India). The similarity of the sequences were checked and confirmed by NCBI BLAST. The forward and reverse sequences were aligned and manually edited in BioEdit (v2.7.000) software to obtain the consensus sequence [19]. The sequences obtained in the present study and other similar sequences were retrieved from the GenBank and were aligned in ClustalW2. Phylogenetic tree were constructed using Maximum Likelihood method in MEGA 6.06 software [20]. The evolutionary distances were computed using the Neighbor-joining method [21]. The robustness of the tree was assessed with 1000 bootstrap replicates.

Statistical analysis

The data presented were analyzed using Chi-square test for proportion and the Chi-square test for linear trend using the Epi Info version 6.03 programs. Results were considered statistically significant at P value <0.05.

Results

Out of 690 Dengue suspected cases during the four years, 211(30.58%) were positive for Dengue. 52.03% (359) of male were positive and 47.97% females (331) were positive (Table 1). In this study, 30.81% of dengue positivity was among pediatric cases. 17.06% adolescents, 42.65% adults and 9.47% from geriatric age group were affected. In this outbreak study, adults were the most infected group rather than the pediatric group. Breeding of mosquitoes which routinely started during the monsoon (July-August), rapidly increased during the period of post monsoon or autumn season (September-November) which gradually subsided from the month of January. The overall percentage positivity of dengue infection was high in the month of November 7.54% followed by 7.0% in October and 6.23% in September in Tamil Nadu. Though sporadic incidence of dengue was noticed during the period from January to May every year no outbreaks were documented (Table 2).

Month/ Year		June	July	August	September	October	November	December	Total
2011	Tested (Positive)	-	20 (5)	21 (6)	32 (15)	31 (12)	32 (11)	16 (4)	152 (53)
2012	Tested (Positive)	12 (0)	6 (3)	7 (7)	20 (15)	102 (20)	113 (27)	54 (15)	314 (88)
2013	Tested (Positive)	-	10 (2)	16 (4)	43 (13)	41 (16)	49 (14)	45 (12)	204 (61)
2014	Tested (Positive)	5 (4)	15 (5)	-	-	-	-	-	20 (9)
Total (Positive)		17 (4)	51 (15)	44 (18)	95 (43)	174 (48)	194 (52)	115 (31)	690 (211)

Table 1: Dengue distribution in month wise during the period of 2011-2014.

Year	2011		2012		2013		2014		Total
Gender	Male positive	Female positive	Male positive	Female positive	Male positive	Female positive	Male positive	Female positive	
0-5	6	5	4	3	7	5	0	1	31
6-12	5	5	7	6	5	4	1	1	34
13-18	4	4	8	9	5	4	1	1	36
19-30	5	4	11	8	9	5	1	1	44
31-45	4	3	11	7	6	4	1	0	36
46-55	2	3	6	5	3	2	1	0	22
56+	2	1	1	2	1	1	0	0	8
Total	28	25	48	40	36	25	5	4	211
The 19-30 age group comprising of young adults were predominantly positive for Dengue.									

Table 2: Dengue distribution in Age wise during the period of 2011-2014.

A total of 690 outbreak samples were collected from twenty districts of Tamil Nadu (Chennai, Coimbatore, Cuddalore, Erode, Kanchipuram, Kanyakumari, Madurai, Nagapattinam, Perambalur, Pudukottai, Salem, Sivagangai, Thanjavur, Trichy, Thirunelveli, Thiruvannamalai, Vellore, Villupuram, Ariyalur and Thiruvarur). Out of 690 cases, 211 samples were positive for dengue from different districts of Tamil Nadu. In Central Tamil Nadu 33.66% of Dengue positivity was observed followed by Northern districts (33.2 %), 29.38% in the East 27.38% in the South and 24.36% in the West (Tables 3 and 4).

Year/ sex	Male		Female	Total
2011	Tested (Positive)	80 (28)	72 (25)	152 (53)
2012	Tested (Positive)	161 (48)	153 (40)	314 (88)
2013	Tested (Positive)	107 (36)	97 (25)	204 (61)
2014	Tested (Positive)	11 (5)	9 (4)	20 (9)
Tested (Positive)		359 (117)	331 (94)	690 (211)
The number of males who were positive was more when compared to females during all the four years.				

Table 3: Dengue distribution in Gender wise during the period of 2011-2014.

S.No	Region	Districts Covered	No.of Samples Collected	Total Samples Tested	Total No.of Dengue Positives	% of Positivity
1	North	Chennai	134	250	50	39.34%
		Salem	8		2	
		Kancheepuram	63		21	
		Vellore	20		6	
		Tiruvannamalai	25		4	
2	South	Madurai	37	84	5	10.09%
		Kanyakumari	15		6	
		Tirunelveli	32		12	
3	East	Cuddalore	31	177	8	24.65%
		Nagapattinam	35		18	
		Sivaganga	13		4	
		Thanjavur	98		22	
4	West	Pudukottai	20	78	3	9.01%
		Thiruvarur	29		6	
		Erode	11		4	
		Ariyalur	8		3	
		Coimbatore	10		3	
5	Central	Trichy	55	101	22	16.11%
		Perambalur	10		2	
		Villupuram	36		10	
	Total		690	690	211	

Table 4: Dengue distribution in Region wise during the period of 2011-2014.

The sign and symptoms in dengue cases included fever 100% (Continuous fever 95.51% and intermittent fever (4.49%), headache (93.48%), myalgia (84.20%) rash (69.57%), followed by abdominal pain (44.20%), vomiting (26.95%), hepatomegaly (15.51%). Hepatomegaly and splenomegaly was mainly observed in patients with DHF/DSS. Bleeding manifestations & rash being the most commonly observed symptoms and rarer atypical manifestations included diarrhea (7.54%)

and altered sensorium (1.11%). Signs and symptom wise distribution in all age groups was found to be statistically significant with P value <0.0001. Other clinical manifestations were similar in all age groups irrespective of gender. A radical decrease was noticed in the platelet count among the patients especially in the pediatric age group. Among the 211 Dengue positive patients, 15 (7.11%) had platelet counts between 20,000 and 50,000/mm^3, 165 (78.2%) had counts between 50,000 and 100,000/mm^3, and 31 (14.69%) had a platelet count of <100,000/mm^3 (Table 5). In our study, 73.98% of samples were Dengue fever (DF) cases and 14.40% Dengue suffered from DHF. A marginal rise in DSS was also observed (11.61%). In this outbreak, majority of samples were collected during the early onset of illness (3 to 5 days) which indicated public awareness of Dengue disease in Tamil Nadu. In our study, no mortality was noticed during this period which attributed to timeline diagnosis and public awareness of dengue (Figure 1).

Clinical characteristic	Dengue patients (n=684)
Symptom	
Duration of fever (days)	5 ± 1
Characteristic of fever	
Continuous	659 (96.34)
Intermittent	25 (3.66)
Rash	480 (70.18)
Headache	645 (94.29)
Myalgia	581 (84.94)
Abdominal pain	305 (44.59)
Nausea	405 (59.21)
Vomiting	586 (79.82)
Diarrhea	152 (22.22)
Physical finding	
Temperature (˚F)	98.7
Hepatomegaly	107 (7.07)
Gum bleeding	121 (17.69)
Platelet count (× 10^3/μmL)	85 ± 74
Outcome of hospitalization	
Recovered or left against medical advice	100

Table 5: Sign and symptom wise distribution of Dengue.

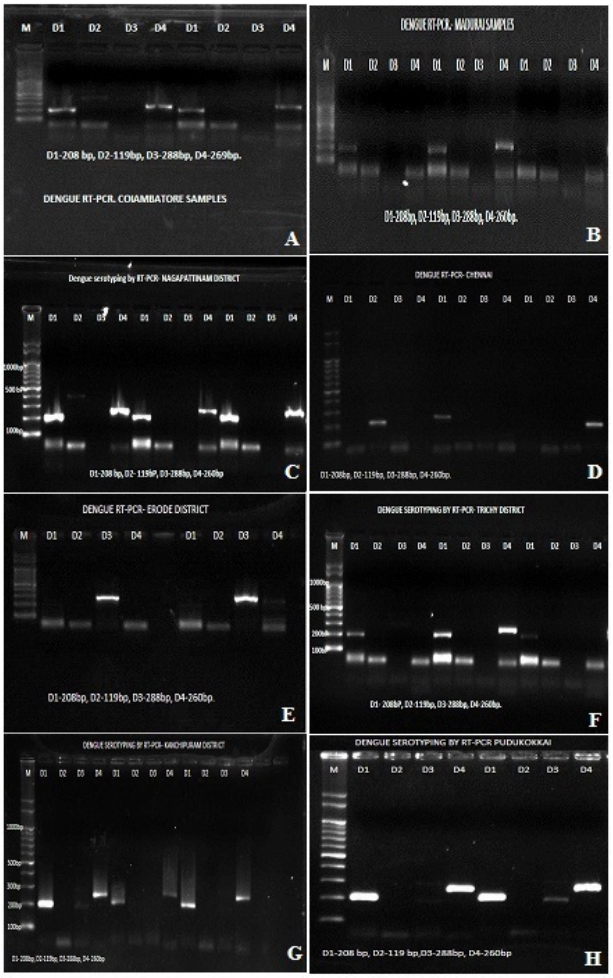

Figure 1: Sensitivity of Dengue one-step RT-PCR in the absence (-) and presence (+) of E1 specific nucleic acid band. D1, D2, D3 & D4-Dengue 1,2,3 & 4 serotype specific primer amplification. M: Marker; [A]: RT-PCR for Coimbatore samples; [B]: RT-PCR for Madurai samples; [C]: RT-PCR for Nagapattinam samples; [D]: RT-PCR for Chennai samples; [E]: RT-PCR for Erode samples; [F]: RT-PCR for Trichy samples; [G]: RT-PCR for Villupuram samples; [H]: RT-PCR for Pudukottai samples.

Dengue RT-PCR serotyping revealed that Dengue 1, Dengue 2, Dengue 3 and Dengue 4 were in circulation in different combinations. Dengue 1 was prevalent in Chennai, Kanchipuram, Thirunelveli and Thiruvarur districts of Tamil Nadu in 2011. However, during the same time period (2011), Dengue 1 & 4 co-circulated in Thanjavur, Trichy.

A distinct pattern of co circulation of Dengue 1 and Dengue 4 was observed in Coimbatore,Kanyakumari, Madurai, Sivagangai, Thirunelveli, Thiruvannamalai, Villupuram, Ariyalur and Thiruvarur while the combination of Dengue1&4 were observed in Chennai, Kanchipuram, Nagapattinam, Pudukottai, Thanjavur, Trichy &Vellore in the year of 2012. Dengue 3 separately and also co-circulated with Dengue 1&4 in Cuddalore, Erode, Kanchipuram, Nagapattinam & Pudukottai districts of Tamil Nadu.

Dengue 4 circulation was entirely replaced by Dengue 1 and Dengue 3 during the year of 2013. In 2005, Dengue 2 was in circulation in the entire country as per the reports of ICMR, India [22] and after a span of 9 years, re-emergence of Dengue 2 was observed in Chennai during 2014 instead of dengue 1&3 in 2013.

Molecular characterization of Dengue strains detected during the 2011-2014 outbreaks was also carried out by sequencing of the envelope protein gene, E-1 gene of 67 DENV-1,2 DENV-2, 2 DENV-3 and 1 DENV-4 strains were sequenced in the study. The sequences were accepted in the GenBank database that were assigned following accession numbers; GenBank: KJ947881-KJ947930,KJ937886-KJ9378894&KJ918755-KJ918762 (DENV 1strains), GenBank: KJ511225 & KJ511226 (DENV-2 strains), GenBank: KJ997936 & KJ918758 (DENV-3 strains), GenBank: KM505037 (DENV-4 strains). Phylogenetic analysis of the DENV strains was carried out based upon the sequenced region. The analysis involved 64 amino acid sequences of DENV-1 strain including 17 DENV-1 strains sequenced in the present study were aligned. These strains had homology with the South East Asian (Figure 1).

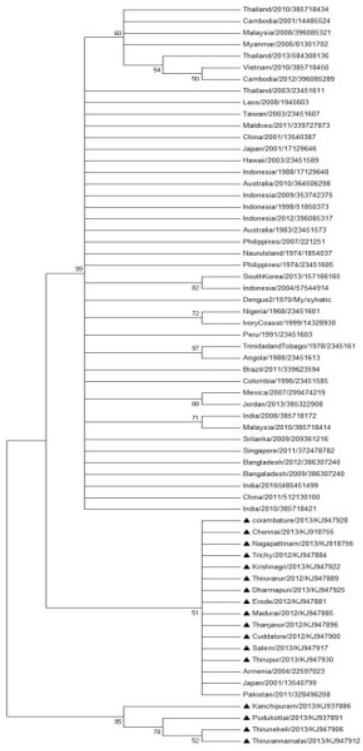

Figure 2: Dengue-1 strains Maximum Likelihood phylogenetic tree. The Phylogenetic tree is based upon partial Envelope protein 1 gene sequences. The sequences obtained in the study are represented by their country followed by year of isolation and GenBank accession number. The numbers on nodes represent bootstrap values generated by 1000 replications. Bootstrap values of >50 are shown. The branch lengths are proportional to the number of nucleotide changes as indicated by the scale bar (0.1 substitutions per site).

For DENV-2 sequence, a 249 bp (83 aa) region of E-1 gene of 20 strains including 2 strains sequenced in the present study was aligned. The DENV-2 strains of the present study clustered within the Asian genotypes (Figure 2).

In Dengue 3 sequence, a 201 bp (67 aa) region of E-1 gene of 21 strains including 1 strain sequenced in the present study was aligned. The DENV 3 strains of the present study clustered with the Asian genotype (Figures 2 and 3). In Dengue 4 sequence, a 207 bp (69 aa) region of E-1 gene of 21 strains including 1 strain sequenced in the present study was aligned. The DENV 3 strains of the present study clustered with the Asian genotype (Figure 4).

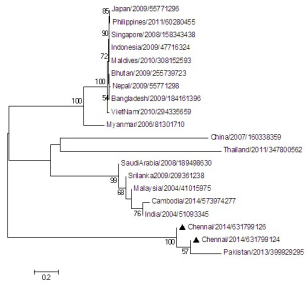

Figure 3: Dengue-2 strains Maximum Likelihood phylogenetic tree. The Phylogenetic tree is based upon partial Envelope protein 1 gene sequences. The sequences obtained in the study are represented by their country followed by year of isolation and GenBank accession number. The numbers on nodes represent bootstrap values generated by 1000 replications. Bootstrap values of >50 are shown. The branch lengths are proportional to the number of nucleotide changes as indicated by the scale bar (0.2 substitutions per site).

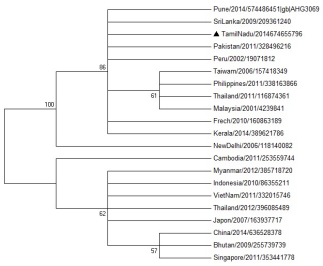

Figure 4: Dengue-3 strains Maximum Likelihood phylogenetic tree. The Phylogenetic tree is based upon partial Envelope protein 1 gene sequences. The sequences obtained in the study are represented by their country followed by year of isolation and GenBank accession number. The numbers on nodes represent bootstrap values generated by 1000 replications. Bootstrap values of >56 are shown. The branch lengths are proportional to the number of nucleotide changes as indicated by the scale bar (0.2 substitutions per site).

Discussion

Dengue fever cases are regular occurrences in Tamil Nadu every year following monsoon. We have investigated an outbreak by conducting dengue Envelope protein specific RT-PCR on 690 suspected dengue samples. Dengue was detected in 30.58% of the samples. The outbreak was mainly caused by Dengue1&3 serotype viruses. Dengue outbreaks were generally caused by a particular serotype as reported from different parts of the world. In 2001, Dengue-3 caused outbreaks in Chennai and Dengue-2 was liable for outbreaks in Dharmapuri [22]. DENV-3 outbreaks have been reported in 2007 in rural areas of Madurai, Tamil Nadu [22].

In India, all serotypes of Dengue were reported in Vellore (1968), Ludhiana (1996) and Delhi (2002-06). In South India, particularly in Tamil Nadu DF and DHF have been well documented [11]. Dengue fever have been reported from 29 districts between 1998 and 2005 which include Dengue fever/Dengue Haemorrhagic fever outbreaks in Chennai 2001, Nagerkoil and Thiruchirapalli (2003) and Dengue fever outbreaks in Krishnagari and Dharmapuri Districts in 2001, total of 128 cases and 5 deaths were reported in 1998 which increased to 1600 cases and 12 deaths in 2003 and 1150 cases 8 death in 2005 [13]. Though uncomplicated Dengue fever (DF) was prevalent among children and adults in Chennai, DHF has been largely restricted to infants and children but since 2005 they have been witnessing DHF among young as well as elderly adults in Chennai [11]. During July 2007, Dengue fever suspected cases were reported from two rural areas/hamlets namely O Alankulam, P Alankulam near Madurai Corporations, Madurai, Tamil Nadu [14]. The total number of samples screened during the period was 200, out of which 79 (39.5%) were positive for IgM and IgG antibodies and 65 (32%) for IgM antibodies only. By clinical evaluation, Dengue fever was diagnosed in 43 patients, 18 had hemorrhagic manifestations and four patients had progressed to DSS. Though (DSS+DHF) was present in 22 patients, all of them recovered well [15].

Dengue cases in 2012 highest in four years At 9,249, Tamil Nadu reported the highest number of cases in the country, followed by West Bengal which reported 6,067 cases. The highest number of deaths was also reported in Tamil Nadu where 60 succumbed to the disease, followed by Maharashtra where 59 people died of dengue. In Maharashtra, a total of 1,464 cases were reported, suggesting that a higher percentage of people died in the state. This amounts to four per cent of the patients dying in Maharashtra compared to just 0.6 per cent in Tamil Nadu [16].

All four dengue virus serotypes were detected in the current study. In 2011, Dengue was major circulating serotypes in different part of Tamil Nadu (Table 6). Dengue 1 serotype was mainly responsible for occurrence of last endemic at Delhi in 2010, in which 63% were positive for dengue 1. Dengue 2 outbreak was earlier reported at Delhi in 1996 (25). In our study, PCR report revealed that dengue 2 is a re-emerging viral disease after nine years in Tamil Nadu in 2014 [22].

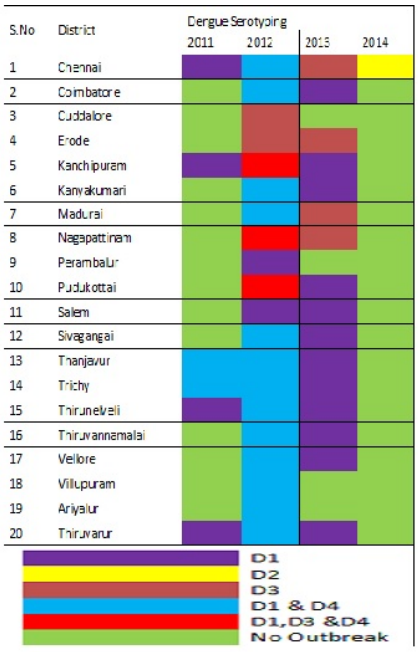

Table 6: Dengue distribution in District wise during the period of 2011-2014.

In 2012, Dengue 3 was predominant serotype next to the dengue 1 in Tamil Nadu. Dengue 3 outbreaks were separately reported in Chennai (2001), Kanyakumari (2003) & Madurai (2007). [22]. Dengue 4 was not separately reported during this study period 2011-2014. We are reporting that Dengue 4 co-circulated with other dengue serotype D1 & D3. Similar studies were reported in Delhi (2006) & Andhra Pradesh (2007). Co-existence of more than one dengue serotype was detected (45%) in the present investigation. In 2005, Kerala, a high percentage of co- infection were reported (56.8%) [23,24].

Conclusion

Serological & Molecular detection of dengue was tested on acute phase & post viremic samples. Dengue 1&4 were reported from 2011-2013. Dengue 2 was predominant in 2014 whereas dengue 3 was most prevalent in 2012 &2013. Dengue virus serotype 2 was detected as the dominant serotype in the outbreak. Co-infection by different serotypes was also detected in the current outbreak. Twenty nine outbreak strains (67 DENV-1, 2 DENV-2, 2 DENV-3 and 1 DENV-4) were sequenced for partial envelope gene. Phylogenetic analysis grouped Dengue 1 strains of Asian genotypes, Dengue 2&3 strains of the Asian (South Central), Dengue 4 Strains of Asian genotypes were detected in the period of 2011-2014 outbreak. This report will helpful in the epidemiological analysis of Dengue in Tamil Nadu.

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