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Circulation of Dengue Virus -2 in Chennai-2014, India.

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Abstract:
Background:*Dengue infection is the most rapidly emerging serious health problem in Tamil Nadu. We report emergence of the dengue 2- strain in Chennai, Tamil Nadu, India.*
Background: *This study aims to describe the molecular characterization of circulating DENV serotypes between Dec 2013-Jan 2014 by identifying dengue specific NS1, IgM and IgG antibodies by NS1 , IgM&IgG capture Enzyme Linked Immuno Sorbent Assay (ELISA) using PanBio kit(NS1 &IgG) andIgM antibody capture ELISA by using NIV kit.*
Results:*A total of 304 fever cases with clinical signs and symptoms suggestive of dengue were tested in Chennai district. Serodiagnosis revealed that 29 (9.5%) were positive for IgM antibodies, 23 (7.6%) were dengue IgG antibodies positive and 3 (1 %) for IgM antibodies only. RT-PCRtest revealed circulation of dengue 2 serotypes.*
Conclusion:*These results show the reemergence of dengue 2 sero type in Chennai, Tamil Nadu. There is no literature evidence for Dengue 2 virus circulation in Chennai since 2001. The study of various serotypes in the dengue endemic region may act as predictive marker to define the range of manifestation in future.*
Keywords: *Flavivirus, Dengue virus, ELISA, RT-PCR, Chennai.*

I. Introduction

Dengue, an endemic arthropod -borne flavivirus infection of humans is caused by four serologically distinct viruses namely dengue virus -1, 2, 3 and 4[(1)].,which belong to the Flaviviridae family, genus Flavivirus[(2, 3)]. The outbreak and prevalence of the dengue infections have increased in recent decades, and has emerged as an important arboviral disease in humans. The infection with DENV causes dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)which are serious public health problems in the tropics [(4)]. Dengue virus produces a wide spectrum of clinical illness in tropical and sub tropical areas where the vectors Aedesaegepti and A.albopictus are abundant [(2, 5)].

In India, dengue virus infections has been frequently encountered in epidemic proportions in several States [(6, 7)]. In south India, the disease has been reported in Tamil Nadu [(8)], Karnataka [(9)]. In Tamil Nadu dengue cases were reported in 29 districts out of 30 districts during 1998-2005**.** The DENV-2 strain was isolated in South India over a time span of more than 50 years (1956-2005) [(10)]. In 2001 DF outbreaks were reported in Krishnagiri and Dharmapuri districts and the dengue strain isolated was dengue virus-2 [(11)].The subsequent years marked a changed trend in DV strain circulation in Tamil Nadu. The dengue cases were reported from 33 units in 2006 and in 2007 and DENV -3 genotype was isolated in rural areas of Madurai [(12)].During July 2003, 2011 suspected cases of dengue fever were reported from certain rural areas of Kanyakumari,Trichy and Thanjavur district, Tamil Nadu [(11, 13)].

In Tamil Nadu, there has been an increase in the number of Dengue reporting units during the last nine years. In 1998, Dengue cases were reported only from 4 units, which had been increased to 33 units in 2006 [(5, 11)].

II. Materials and Methods

This tests were performed at King Institute of Preventive Medicine and Research, Chennai, Tamil Nadu, India. The sera samples were collected from patients at different phases of illness and age from the three government tertiary care hospitals in Chennai . The Dengue suspected individuals were classified based on WHO guidelines (1) and were observed for the symptoms of arthralgia, rashes, myalgia, hemorrhagic manifestations and leucopenia. The.samples were collected 2-7 days from the onset of illnessdengue from cases characterized by clinical case definition of WHO. Approximately 5 ml of blood sample was collected and sera were separated by centrifugation. The sera samples were subjected to testing by dengue NS1 capture ELISA test

(PanBio kit), IgMcapture ELISA (NIV kit), IgG capture ELISA (PanBio kit). Those serum samples collected < 5 days of onset from the suspected dengue cases were subjected to Reverse Transcriptase PCR, for the identification of serotype.

PCR:

Extraction of RNA:

The collected serum samples from dengue suspected patients were subjected to RNA extraction using QIA amp viral RNA mini kit (Qiagen). Positive control consisted of equal volume of RNA extracted from the cultured cells infected with DENV of known serotypes. (Den 1, 2, 3, 4) was used and for negative control uninfected cultured cells were used. The extracted RNA was then stored at -20^0 C.

III. Amplification

Veriti^{TM}Dx 9700 Fast Thermal Cycler; IQ (Applied Biosystems®) was used for the study of the conventional PCR in the assay. The DENV RNA was isolated by RT-PCR using standard method and electrophoresis was employed. The RT-PCR was used as it is highly specific and sensitive. RT-PCR was performed using primers against E1 region for Pan Dengue [(6)]. The positives were subjected to dengue genotyping. The amplification of RT-PCR was assayed using 25 µl reaction mixture containing 5 µl of RNA, 25 pmol of the D1, D2, D3 and D4 primers and components of a one-step RT-PCR kit (QIAGEN). The amplification involved the following steps: Reverse transcription at 50°C for 30 minutes; one cycle of initial denaturation of the reverse transcriptase and activation of the HotStartTaq polymerase at 95°C for 15 minutes, 55°C for 15 seconds, and 72°C for 30 seconds; 34 cycles at 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 30 seconds; and a 10-minutes, 72°C extension. 5 µl of the amplified product was analyzed by using agarose gel electrophoresis with 1.5% agarose gel containing ethidium bromide. The serotypes are determined by all positive amplicon size targeting E1 region. For every RT-PCR run, a positive control (a confirmed positive Dengue isolate) and a negative control was included. An aliquot of 10 µl of the amplified PCR product was analyzed using 1.5% agarose gel electrophoresis containing ethidium bromide and visualized under UV light illuminator. The amplified PCR product of the known serotypes DEN-1, DEN-2, DEN-3, and DEN-4 was 208, 119, 288, and 260 respectively and for Pan Dengue E1 the size was 511 bp. The PCR gel was stained with ethidium bromide was run at 100 volts and the resulting bands were captured using Polaroid camera.

Serological analysis

The data presented were analyzed using Chi-square test for proportion and the Chi- square test for linear trend using Graph pad prism 6.03 program. Results were statistically significant when $p<0.05$.

Nucleotide sequence accession numbers. Nucleotide sequences were submitted to GenBank under accession no. seq1(KJ511225) and seq2 (KJ511226).

Phylogenetic analyses.

Phylogenetic tree derived from E protein gene nucleotide sequences and representative sequences from the GenBank library. Viruses are listed by Epidemiological type followed by abbreviation for country and year (Table 2) and were aligned using the EMBOSS Transeq program of the Genetics Computer Group with default gap penalties. GenBank sequences of endemic DEN-2 strains were included in our analysis to provide information on the relative divergence levels in comparison to other DEN serotypes. Nucleotide sequences were aligned manually based on codon homology. Phylogenetic analyses of the aligned nucleotide and amino acid sequences were performed using the EMBOSS Transeq (version 6.6.0) D. and neighbor-joining program implemented in this package.

IV. Results

A total of 304 cases were screened from December 2013 to January 2014, 162 (53.29%) were males and 142 (46.71 %) were females. out of which 29 (9.5%) were positive for IgM antibodies, 52 (17.1%) were dengue IgG antibodies and 8 (2.6 %) were positive for both IgM and IgG antibodies (Table 1). Among the IgM positives, 16 (55.2%) were pediatric cases and 13 (44.8%) were adults (Table 1). Positivity is insignificant among males when compared to females. An age wise breakup revealed that maximum number of cases36.51 % was in the age group of 6-12 years followed by 32.67% from the age group of >18 years while 26.02% were in the age group 13–18 years. Those less than 1 year of age constituted 12% as seen in Table 1. All cases (100%) presented with fever and was the most common symptom followed by headache (92%), myalgia (84%), vomiting(42%), abdominal pain (51%), breathlessness 21%, skin rash (15%) and altered sensorium (13%) (Fig 2). Figure 3 shows the distribution of dengue cases during the study period, in the month of December 2013, 8.3% were IgM positives with gradual increase to 10.4% in January 2014. Among the 7 samples subjected to RT PCR using group specific primers, 3 were positive for pan Dengue; of this all the 3 samples were positive for

Dengue 2 only.RT-PCR revealed circulation of dengue 2 serotypes (Figure 3). During the last 12 years (2001-2013) there is no evidence of dengue 2 serotype circulation in Tamil Nadu. These results show that the emergence of dengue 2 sero type in Tamil Nadu after 12 years. [(5)]

From the serological tests it was deduced that 29 cases had primary Dengue (IgM positive) and 8 had secondary Dengue (IgM and IgG positive). Among the secondary Dengue infections, four were reported in pediatric cases. By clinical evaluation, Dengue fever was in 29 cases, eighteen cases had hemorrhagic manifestations. No patients had evidence of DSS. The mortality rate was nil during the study period due to timely diagnosis and management ..

A total of 2 E gene sequences were obtained from the Chennai DEN-2 virus isolates in 2014, from Tamil Nadu state. These were compared with all the available published E gene sequences from global isolates of DEN-2 viruses deposited in GenBank. A maximum likelihood tree of all 22 sequences is presented in Fig. 4 and shows that the Chennai isolates fall into one distinct group. The results show four major distinct groups from USA (2011), UK (1992), Pakistan (2011) and France (2006). Of the Chennai isolates, 3 were closely placed together along with one other strains isolated from, Pakistan (2011), Vellore (2001) and Finland (2005). Three other group isolates, USA (2011), UK (1992) and France (2006) were more distant. Hence, the viruses we isolated and subsequently documented in Chennai seem to be ultimately derived from isolates circulating in Pakistan (2011).

V. Discussion

Dengue is an important emerging arboviral disease in tropical and sub tropical areas. In India dengue has been first reported in 1964 and the circulating serotype was DENV 1 and 4. [(6, 7)] There were intermittent reports of dengue infections from Ludhiana [(14)], Delhi [(15)], Kolkata [(16)], Chennai [(17)], Mangalore [(18)], Assam [(19)], Nagaland and Vellore [(20)] during the past few decades. The last major dengue outbreak occurred in India in 2006 involving about 12000 cases and 154 deaths. There was a steady decrease in the number of people affected by the dengue in year 2009, 2010 and 2011based upon our statistics. This may be due to the increasing public awareness and control measures. Dengue virus serotypes may influence the clinical expression of the disease. This antigen dependent enhancement is considered as the major factor in the emergence of the dengue. The transmission of the dengue was observed in the monsoon and in post monsoon period. The dengue strain 2 outbreaks in Krishnagiri and Dharmapuri districts in 2001 have been reported and there was no evidence of the DENV-2 outbreaks in Tamil Nadu in subsequent years [(5, 11).] Our study on the molecular characterization of the circulating serotypes and their genotypes will help in addressing the control of DSS/DHF incidence in future.

The serological tests in the study revealed that 29 cases had primary dengue (IgM positives) and 8 (IgM&IgG positives) suffered from secondary dengue. The four cases of the secondary dengue infection were found to be pediatric cases. In the clinical evaluation of the 29 cases with the primary dengue and 18 cases were found to have hemorrhagic manifestations and none of the patients evidenced with DSS. In the study during 2001 the dengue outbreak in Dharmapuri district, the total cases were found to be 256 had fever. Among these 13 out of 31 cases and 14 out of 52 cases had the dengue strain 2 infections [(12)]. In our study the cases reported were low may be due to timely diagnosis and unfavorable environmental conditions for the transmission. Dengue IgM and IgG testing help in diagnosis of secondary infections, genotype specific diagnosis is possible with RT-PCR.

The various serotypes are more frequent on global base circulation of dengue strains in the past years. The co- circulation of the various dengue serotypes has been reported in various parts of the world. The recombinant potential may lead to more virulent strains. There is no evidence of such Dv-2 outbreak in Chennai district, for past years. The report predicts the emergence of dengue strain-2 in Chennai.

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Fig-1

Month-wise distribution of suspected cases of Dengue fever and Dengue IgM positive cases during the period of Dec-13- Jan-14.

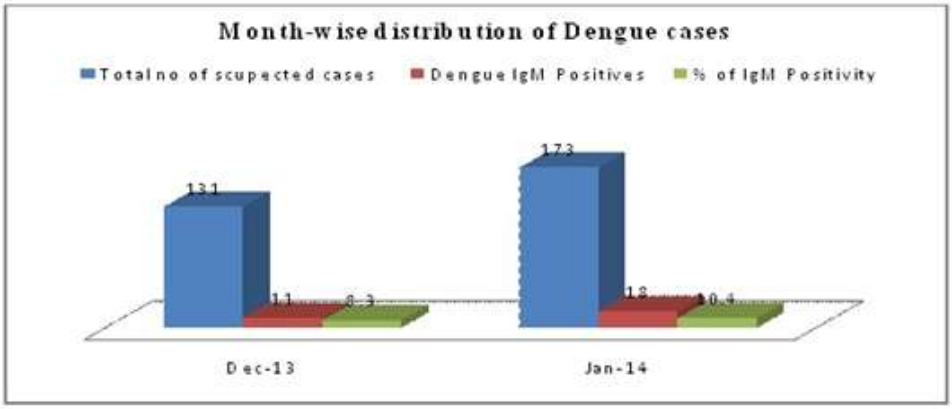

Fig-2

Signs and Symptoms wise distribution of Dengue suspected cases.

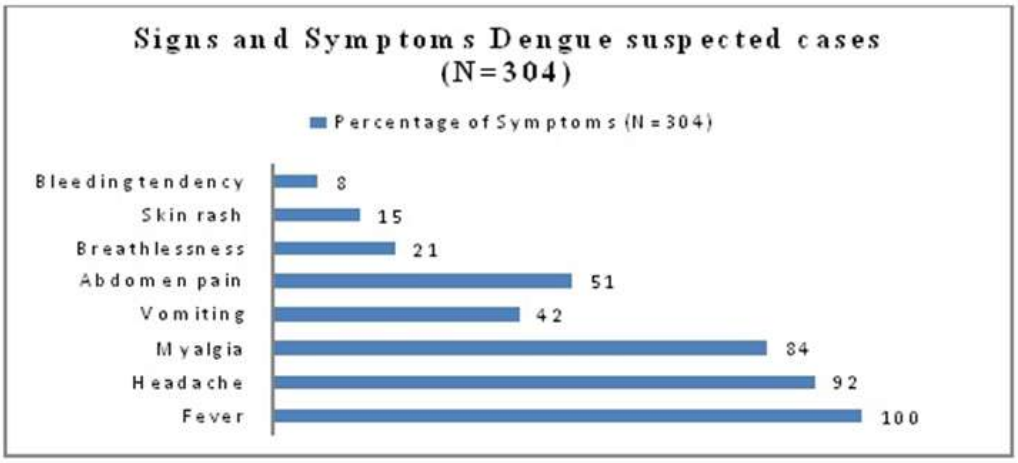

Fig:3 . Phylogenetic analyses.

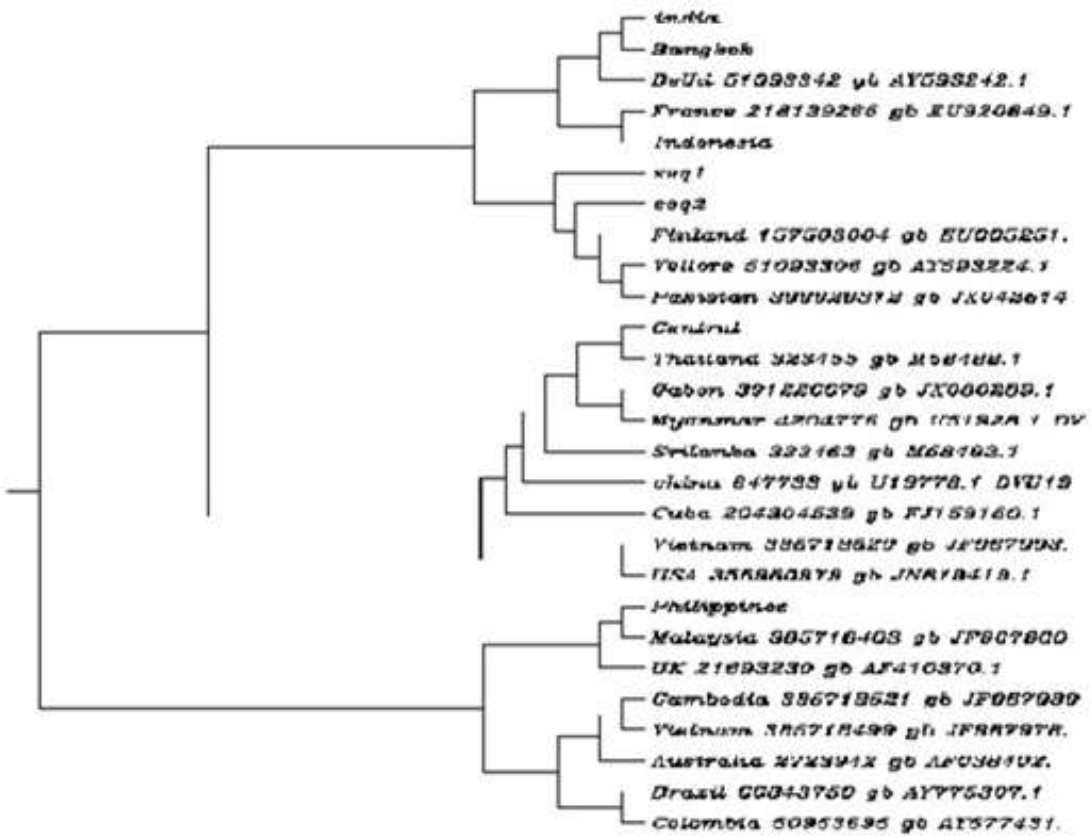

Table-1

Distribution of Dengue positives with respect to age group and serological profile

Duration	Gender	Antibody	<1 (25)	1-5. (42)	6-12. (63)	13-18. (73)	18> (101)	Total(304)
Dec-13 to Jan-14	Male (162) 53.29%	IgM	2	1	5	3	4	15(9.25%)
		IgG	0	2	4	5	12	23(16.19%)
		IgM &IgG	0	0	1	0	2	3(1.8%)
	Female (142) 46.71%	IgM	1	3	4	3	3	14(9.85%)
		IgG	0	5	8	7	11	29(20.42%)
		IgM &IgG	0	2	1	1	1	5(3.5%)
Total			3 (12)	13 (30.95)	23 (36.51)	19 (26.02)	33 (32.67)	IgM 29 (9.54%) IgG 52 (17.11%) IgM & IgG 8 (2.63%)

Table: 2 DEN isolates studied

Epidemiological type	Host	Yr isolated	Country where Isolated	Serotype	GenBank accession no
Endemic	Human	2010	Bangkok	2	AB622062.1
Endemic	Human	2004	Indonesia	2	AB189124.1
Endemic	Human	1967	India	2	AY593244.1
Endemic	Human	1996	Delhi	2	AY593242.1
Endemic	Human	2011	USA	2	JN819419.1
Endemic	Human	1993	Srilanka	2	M58492.1
Endemic	Human	1993	Thailand	2	M58488.1
Endemic	Human	1998	Australia	2	AF038402.1
Endemic	Human	1992	UK	2	AF410370.1
Endemic	Human	2011	Pakistan	2	JX042514.1
Endemic	Human	2006	France	2	EU920849.1
Endemic	Human	2005	Finland	2	EU005251.1
Endemic	Human	2010	Central Africa	2	JX080291.1
Endemic	Human	2007	Gabon	2	JX080289.1
Endemic	Human	2001	Vellore	2	AY593224.1
Endemic	Human	2009	Vietnam	2	JF967993.1
Endemic	Human	2009	Philippines	2	JF967991.1
Endemic	Human	2009	Cambodia	2	JF967989.1
Endemic	Human	2008	Malaysia	2	JF967960.1
Endemic	Human	1997	Cuba	2	FJ159150.1
Endemic	Human	2006	Brazil	2	AY775307.1
Endemic	Human	1999	Myanmar	2	DVU51928