<u>Research Article</u>

Phylogenetic analysis of Dengue Virus Serotype 1 Isolated from Clinically Suspected Pediatric Patients in Chennai, Tamilnadu.

Shanthi. G, Indu Purushothaman and Rajarajan .S

PG & Research Department of Microbiology & Biotechnology,
Presidency College (Aut.) Chennai, Tamilnadu, India

ABSTRACT

The evolution of Dengue virus has a major impact on their virulence in humans and on the epidemiology of dengue disease around the world. In order to perform disease surveillance and to understand DENV evolution, the molecular studies with Bioinformatics tools are required. Hence this study aimed to focus the genotype identification of the isolated dengue virus which is very important to find out the analogous strain circulating in the area. In this present study, we sequenced C-prM gene junction of one strain isolated from pediatric dengue virus patients. About 100 serum samples were collected among clinically suspected pediatric patients reporting to children's hospital from August 2011 to October 2011 in Chennai, Tamilndau, India. All the collected samples were analyzed for RT-PCR for identification of positive cases along with serotype prediction. Among the RT-PCR results, 10 cases were detected for dengue 1 which is predominant and 2 cases were dengue 3 serotype. Due to cost effectiveness, one predominant serotype was sequenced to identify the genotype of dengue virus to predict the dengue virus evolution. The Sequence of the isolate were also published in NCBI and the Phylogenetic tree were drawn with the help of appropriate Bioinfomatic tools. Our study showed that Dengue 1 was dominant in positive samples of dengue virus infection. The Dengue 1 isolate sequenced in our study belong to the genotype III

Key words: Dengue virus, Sequencing, phylogenetic tree, genotype, Chennai-India, Serotypes

INTRODUCTION

Dengue, one of the life threatening mosquito borne diseases in human, is caused by four dengue serotype namely DENV1, 2, 3 and 4, positive strand RNA virus. Dengue virus is an arbovirus belonging to family Flaviviridae and is responsible for a wide range of clinical manifestations in human, including an acute flu like illness called dengue fever (DF) which is first likely to be asymptomatic stage in few case concerns. Later, when it is severe illness with hemorrhages characterized with plasma leakage known as Dengue Haemorrhagic fever (DHF) and when progresses it may lead to hypovolemic shock with circulatory failure are called Dengue Shock syndrome (DSS).

India is endemic for dengue and has witnessed several dengue outbreaks in the past. All the four known seroytpes have been implicated in these outbreaks, but the major outbreaks have been caused by Dengue 2. However, Dengue 1 was isolated from different parts of India at regular intervals, since its first isolation from southern India (Vellore) in 1956. It in increasingly being implicated as a major

serotype during recent outbreaks in India, including the Delhi outbreak in 2006 (Sanjeev et al 2012)

The genotype of dengue virus serotypes is not clearly studied and also there is no end of the finding of genotypes for all the serotypes. The prevalence of four serotypes of dengue virus has risen dramatically in recent years accompanied by an increase in viral genetic diversity. The evolution of DENV had a major impact on their virulence in human and on the epidemiology of dengue disease around the world. In order to perform dengue surveillance on dengue genotype evolution, an efficient and accurate method for genotype identification is required.

The four DENV serotypes are defined based on limited cross-reactions in various serological tests. Initial genetic characterizations of DENV in all serotypes identified was by RNase fingerprinting. Later, nucleic acid sequencing confirmed the homology of the four serotypes as well as their conserved genetic organization, and allowed for the most precise and broad classification of DENV into genetically distinct groups or genotypes within each serotype (Rico-Hesse, 1990). Rico-Hesse defined DENV "genotypes" as clusters of DENV with sequence divergence not greater than 6% within the chosen genome region (in this case the E/NS1 junction), which was based on the clustering of strains for which associations could be inferred on epidemiological grounds (Scott CW and Nicos V 2009). Based on sequence analysis of the E/NS1 region, and using a cut-off of 6% divergence, each DENV serotype can be divided in different genotypes (Alvaro et al 2010).

Phylogenetic analysis have elucidated the origins and forces underlying the molecular evolution of DENV in different geographic regions of the world. Molecular characterizations of these viruses are necessary to identify the molecular subtype/ genotype and to determine the introduction of any new lineages. Moreover, it also helps to gain insight into the degree of genetic variability, rates and pattern of evolution. Genotype I and II of Dengue 1 have never been found in India. In Delhi, till 2003, the predominant serotype was DV-2 (genotype IV) (Nivedita Gupta 2012) None of the Indian strains could be classified as genotype I or II (Sanjeev T, et al 2012) including ours which was isolated from pediatric patients in Chennai, India.

MATERIALS AND METHODS
Clinical Sample Collection
About 100 serum samples were collected from clinically suspected pediatric patients from Children hospitalized at Institute of Child Health, Egmore, Chennai, Tamilnadu during an outbreak, which started from July 2011 with proper ethical clearance obtained from an Ethical Review committee of the Madras Medical College, Chennai. The collected serum samples were stored at -70 C until its use.

Determination of positive dengue cases
All the collected samples were processed by RT-PCR for confirmation of dengue cases and also for serotype identification.

RNA Extraction:
Viral RNA from 140 µl was isolated directly extracted using QIAamp viral RNA Extraction kit (Qiagen, India) as per the manufacturer's protocol. The RNA was eluted in 60 µl of elution buffer and stored at -80 C.

RT-PCR
Dengue group-speciifc degenerative primers were designed targeting C-prM gene junction described by Lanciotti et al 1992. Serotype specific primers used by Zaren et al 2011 were used. With these primers Reverse transcriptase Polymearse chain reaction (RT-PCR) was performed for serotyping analysis of the collected samples. The template was amplified by 20 µl of mixture which contains 5 µl of extracted RNA, 15 µl of PCR mixture which contains forward and reverse primers, dNTPs, $MgCl_2$ and Taq DNA Polymerase. The thermal profile was followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 2 minutes. The same thermal profile was followed for dengue serotype specific

confirmation except the annealing was carried out at 54°C for 45 seconds in 35 cycles.

The resulting serotype specific DNA products were visualized on 1.5% agarose gel as serotype specific DNA band of Dengue 1 (411bp) and dengue 3 (453bp) by ethidium bromide staining with available markers for comparison of molecular weight. Amplified products were purified by agarose gel electrophoresis, and was eluted in 30 μl of double distilled water which was used as template for sequencing reaction .

Sequencing

Among the positive cases the predominant dengue 1 was sequenced for further study. DNA sequencing was performed on gel purified PCR products. Sequencing Reaction (10μl) was performed using Big Dye Terminator version 3.1"cycle sequencing kit containing 4μl of big Dye Terminator ready reaction mix and to that 1μl of template (100ng/μl), 2μl of primer (10pmol), 3μl of Milli Q water was added. Initial denaturation was carried out at 96°C for 1min followed by denaturation at 96°C for 10 Sec, hybridization at 50 °C for 5 Sec and elongation at 60 °C for 4 min for 25 cycles using POP 7 polymer 50 cm Capillary Array in Applied Biosystem Micro Amp Optical 96-Well Reaction plate. The obtained chromatogram was analyzed using Seq Scape v 5.2

Phylogenetic Analysis by Bioinformatic Tools

The C-prM gene region sequences of DENV in this study were submitted to Genebank and accession numbers were acquired prior to which BLAST search was carried out to confirm the virus type. Relationships between the aligned amino acid sequences of the Dengue virus were determined using Mega version 5.0. The Kimura two-parameter algorithm was applied to calculate the evolutionary distances and the neighbour-joining method was used to construct the phylogram, which was viewed using the tree view program. Bootstrap analysis was performed on 1000 replicas using the programs seqboot and consense to ascertain support for

the major branches of the tree. The reference sequences of C/prM from India and from different geographical location across the globe were retrieved from NCBI GenBank excluding the sequences collected from mosquitoes and were analyzed for the construction of phylogenetic tree to deduce the clade of the subjected query sequence.

RESULTS

Enumerating the prevalence in 100 samples by molecular technique confirms serotype of dengue virus which is promising. We could identify the virus in 12 (12%) serum samples and could not find the dengue virus in remaining 88 (88%) samples that are PCR Negative. Of these, 12 positive PCR samples were further processed for serotype confirmation and confirmed as 10 positive samples for dengue1 serotype which is predominant and the remaining two positive samples for dengue 3 serotype.

Sequencing of Dengue 1 clinical isolate

Owing to high expenditure for sequencing, only one predominant serotype (Dengue 1) was sequenced and published in NCBI (Accession number – KC954624). The C-prM gene of dengue 1 isolate has been chosen for sequencing. The length of the amplified product was 313 bp for sequenced Dengue serotype 1. The BLAST search was done and the sequence of serotype 1 were found close to a Delhi strain (GenBank: EU846231.1) with an average of 99% homology.

Phylogenetic analysis of Dengue 1 isolate

A Phylogenetic tree was constructed using a pairwise comparison of the DENV-1 c-prM gene sequenced in this study with the 61 sequences around the globe out of which 24 sequences taken for the study was from India. After the analysis the study revealed that the isolate of the present study fall under the genotype III, which was found to closely related to the two Indian strains isolated during the 2006 epidemic and the other strains clustered in the genotypes (I, II, & III). It's also evident that the all the Indian strains from the period of 1963 to 2011 included in the study

were also from the genotype III. So the genotype circulating in India is genotype III despite various outbreaks. It was interesting to see that all the strains isolated from India belong to the same genotype along with the few strains from Singapore, Thailand and Comoros. All the Indian strains belong to the genotype in different subclades.

DISCUSSION

As we all know dengue virus pathogenesis is not yet clearly understood. In India disease is caused by all the four prevalent serotype which are known to be circulating either singly or in combination among serotypes which remains in major risk factors of DHF. The incursion of new genotype into an area is also being attributed to the severe form of the disease (Ricco Hesse et al 1990). All these facts drive the scientific community to pay more attention towards the genetic nature of dengue viruses and their spread in the population.

Like other RNA virus, dengue virus reveals strong genetic diversity. Different regions of the dengue genome like Envelope, E-NS1, C-prM and complete genome has been utilized for the genotyping (Zhang et al 2005). We have utilized the sequence information of C-prM gene junction in this study. The genotyping based on the C-prM gene junction has been adopted by several researchers, including us in the recent past (Kumar M et al 2004). This results in faster and economical genotyping due to utilization of a single set of primer pair for both amplification and sequencing (Lanciotii et al 1992).

Based on pairwise comparison, the phylogenetic analysis of sequenced isolate fall in genotype III. Totally 61 sequences were used for comparing the sequences. Of these 24 sequences belong to Indian study, which was be very helpful for comparison. Among these retrieved sequences Genotype I, II and III were clustered with their respective similarity basis. In earlier Sanjeev Tripathi et al 2012 studied the phylogenetic study of Dengue virus 1 circulating in Lucknow, India. They also studied their sequences with 54 previously published sequences to study their genotype which included 23 Indian sequences. Genotype I, II and III were only the genotypes derived from the Phylogenetic tree.

The phylogenetic analysis clearly revealed the continuous circulation of genotype III viruses over the last 50 years in India. Interestingly, the majority of the Indian genotype III viruses is found to be phylogenetically quite distinct. It is surprising to find that the recent viruses recovered in quick succession in 2001–02, 2004 and 2006–07 from northern India also belong to separate lineages. In contrast, this type of distinct lineage pattern was not observed among Indian DENV-2 and 3 (Dash et al 2006).

In this analysis the isolated strain Genotype III was found to be closely related to two Indian strains (Delhi sequence) which was isolated in 2006. Which are in the same clusters. The remaining subclades are also fall with Indian strains except Singapore, Thailand and Comoros strain. In earlier studies Sanjeev Tripathi et al 2012 reported that all Indian Dengue virus strains in the study and retrieved sequences for comparison from different outbreaks, clustered in genotype III along with viruses Mexico, Colombia, Brazil and Venezuela but they all are classified into distinct subclade

Also it is important to note hilariously that the strain analysed in this study has been isolated from Chennai, South India because most of the sequences under genotype III reported till date were mainly from North India, including strains from South India (Vellore) recovered during 1962-1964 which do not cluster with any other Indian and global isolates (Gupta E et al 2006). Where as strains in the present study shows a very good homology similarity to the strains isolated from North India, which is the very best evidence of circulating the dengue virus from a different region to confirm the Dengue virus spread for the cause of the epidemic.

Genotype I and II have never been found in India. According to the sequence retrieved in this study, Genotype I were clustered mainly in

Thailand, Dijibouti, Vietnam, china Cambodia, Seychelles and Reunion Island. Where as Genotype II sequences were clustered by Seychelles, Reunion, China, Japan and Australia.

CONCLUSION

In this present study the Dengue virus serotype 1 was sequenced and phylogenetic analysis revealed that the genotype that was circulating in the period of 2011 in Chennai, Tamilnadu, South India. Hence, from the available published global sequences phylogenetic tree was constructed using *In silico* methodology to analyze the genotype. The results revealed that the strain isolated in the present study belongs to the serotype 1 and the genotype III, Which showed close similarity with the sequence in Delhi, North India and interestingly the other Indian strains used in the study also belongs the same genotype, but in different clads.Therefore, this brings evidence that the genotype III is frequently circulating in India. The epidemiological population of cocirculation of dengue virus transports the disease severity. Since there is no vaccine and antiviral drugs for dengue, the circulation of dengue virus through vectors must be controlled and that is the only way to eradicate Dengue disease

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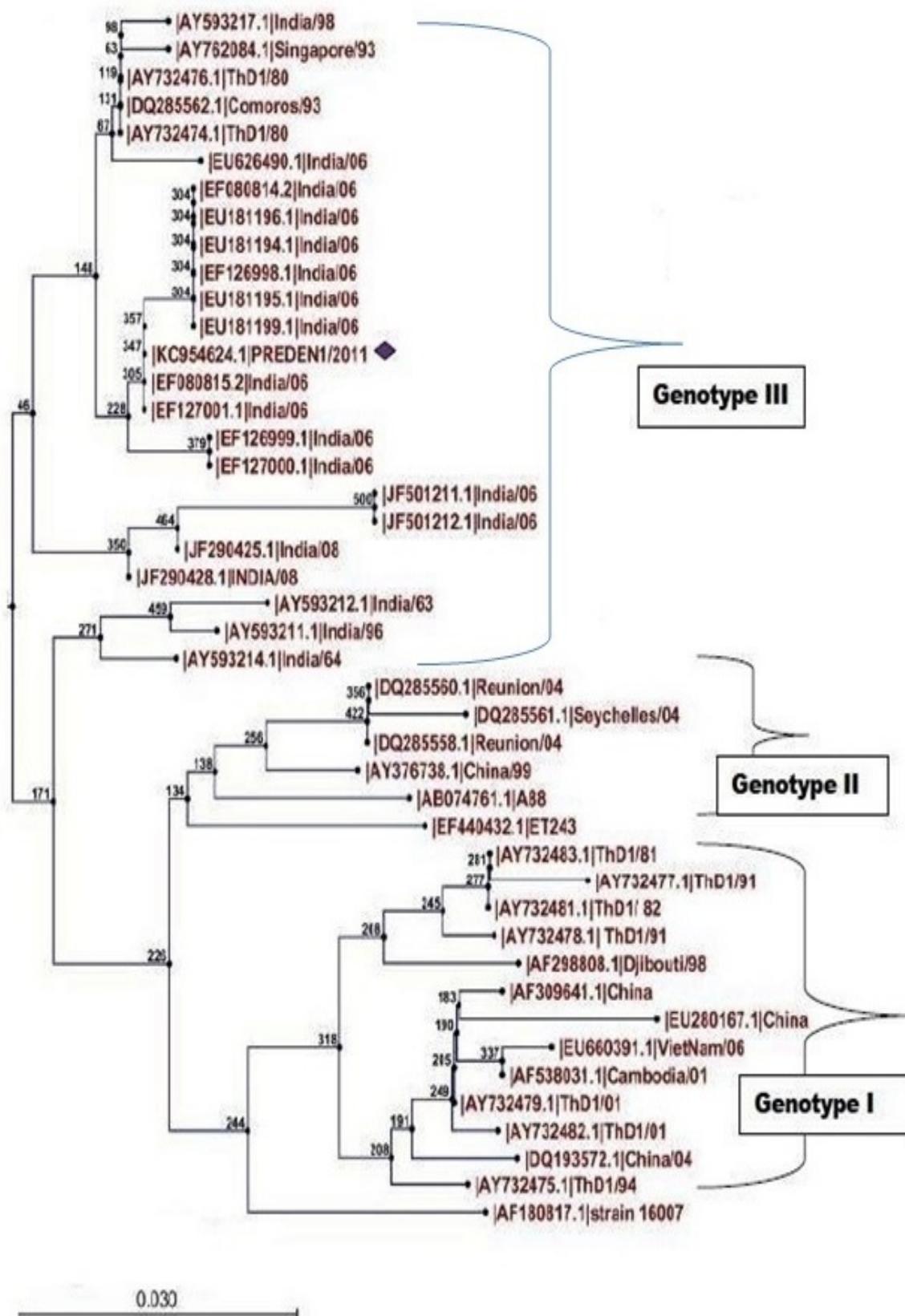
Fig: Phylogenetic tree of DENV-1. Strains in the tree are shown by their GeneBank Accession number, Place/country and Year. Current study strains are marked as