Genetic and Phylogenetic characterization of Dengue Virus among Clinically Suspected Patients in Eastern India

Abstract

Introduction: Dengue is an acute systemic viral disease well known globally in both endemic and epidemic transmission cycles. Particular serotypes/genotypes and lineages of dengue virus (DENV) are associated with more severe outbreaks. Many reports from India have shown an association between change in DENV genotype/lineage and magnitude of the outbreak and disease severity.
Moreover, molecular monitoring of viruses circulating in the locality provides baseline data on the circulating serotypes/ genotypes of dengue virus (DENV). So this monitoring and early detection of changes in the circulation pattern may help in the prediction of DENV outbreaks and can augment disease control efforts.

Material & Methods: This cross- sectional study (first report from Bihar) was aimed to investigate the circulating DENV serotypes and genotypes in Bihar during year 2022 outbreak from July to December. Combination of serological assays (NS1 antigen and IgM antibody ELISA) was used for early diagnosis. For genetic characterization of prevalent serotypes, real-time reverse transcription polymerase chain reaction assay was used. Representative samples were sequenced for the envelope (E) gene.

Results: All four prevalent serotypes, DENV-1, DENV-2, DENV-3 and DENV-4 were found to be circulating in Bihar with dominance of DENV-2. Mixed infection cases of DENV-2 with DENV-3 and DENV-1 with DENV-2 were also seen. Phylogenetic analysis based on C-prM gene revealed DENV-1 sequences to be 92% similar with Vietnam genotype I (2003) strain, DENV-2 isolates clustered with genotype IV of North India strains, Haryana (1996), Delhi (1996) and Jammu (1993) with 95%, 94% and 94% sequence similarity respectively. DENV-3 isolate was more closely related to genotype III of Indian origin (2003), Gwalior and Delhi with 98% and 99% sequence similarity respectively.

Conclusion: With this background data, more extensive study is recommended to help clinicians, government, and policy makers to take effort towards disease prevention and execution of epidemiological control measures.

Introduction

Dengue is an acute systemic viral disease well known globally in both endemic and epidemic transmission cycles. Acoording to WHO report, 2016, 3.9 billion people, in 128 countries has been estimated at risk of infection with dengue viruses of which 96 million (67–136 million) manifest clinically [1]. The Indian population as a whole is at a risk of succumbing to this disease with reported annual average of 20,474 dengue cases between 2006-2012 [2]. Dengue spreads by *Aedes* mosquitoes which also transmits chikungunya, yellow fever and zika infection [3].It is an enveloped virus with positive-sense single-stranded RNA belonging to family *Flaviviridae*, genus *Flavivirus* with four distinct serotypes, DENV-1, DENV-2, DENV-3 and DENV-4. They produce a spectrum of clinical illnesses ranging from a classical Dengue Fever (DF) to potentially fatal complications known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)[4]. Dengue is widespread throughout the tropics, with local variations in risk influenced by climatic change, unplanned rapid urbanization and microevolution of viruses. Since the last decade the reported number of dengue cases has been doubled and 25,000 people die of this mosquito born disease annually[5]. Though there is high morbidity and mortality rate, there is no specific drug or vaccines are available for its treatment. India has witnessed several dengue outbreaks in the past since 1780 and thus it is endemic for dengue [6]. All the four known serotypes have been implicated in this outbreak. From 2001 onwards there has been shift in the cause of these outbreaks from DENV-2 to DENV-3, which was found to be predominant serotype circulating in northern and some parts of southern India[7] [8]. Although in India majority outbreak are of DENV-2 and DENV-3 however DENV-1 is increasingly being implicated as major serotype since 2005 [9].

The geographic, climatic and densely populated environment of Bihar favors the vector borne disease like dengue to flourish but till now there is no published report regarding serotype and seroprevelence of dengue in Bihar. Thus, this is the first study from Patna, Bihar regarding seroprevalence, serotype and genetic characterizing of DENV which will help the physicians to consider possibility of dengue cases for proper management to avoid fatal complications and implementation of epidemiologic analysis.

Materials and Methods

Patient Sample

This serosurveillence study was conducted in Molecular Biology Laboratory, Indira Gandhi Institute of Medical Sciences (IGIMS) Patna, Bihar. The variables in the present study included age, gender, month and patients were selected according to the following inclusion and exclusion criteria. The study included patient aged above 2 months with symptoms of fever (38.5–41.4°C) and with more than or equal to two of the following 1) joint pain, 2) rash, 3) myalgia, 4) headache, 5) retro-ocular pain 6) abdominal pain and 6) hemorrhagic manifestation. All clinical samples collected from Indira Gandhi Institute of Medical Sciences (IGIMS) Patna, Bihar. The study excluded children less than 2 months old, patient with fevers of known cause, and those patients who were unable or unwilling to give a written consent. In case of minors and children below 12 years of age, the consent has been taken by their parents**.** Clinical and demographic data were collected by a structured assessment form. Venous blood samples were aseptically drawn from the study participants. Disposable transfer pipette was used to transfer serum into two sterile screw-capped cryo tubes and stored at -80°C until testing.

Serology

Serum sample were separated and subjected to panel of tests. Dengue NS1 antigen capture ELISA make InBios (Seattle, Washington 98104 USA) and IgM antibody capture (MAC) ELISA), using a kit procured by InBios (Seattle, Washington 98104 USA).

Viral RNA extraction

Viral RNA was extracted from 140µl of specimen using the Viral RNA kit (QIAamp® DSP Viral RNA Mini Kit (Qiagen). Extraction of RNA was performed according to the manufacturer's instructions.

Multiplexing of Prime Probe by Real Time PCR

The extracted samples were then subjected to all four serotype studies by multiplex PCR in single run by the RealStar® Dengue Type RT-PCR Kit 1.0 and 2.0 make Altona Germany

Gel purification

Gel purification was done using the QIAquick Gel Extraction Kit (Qiagen, Germany) and the purified product was eluted in 25µl of autoclaved milli-Q water which was used as a template for sequencing reaction.

Sequencing reaction

DNA sequencing was performed on gel purified PCR products using dye-terminator method of ABI (Applied Biosystems, Foster City, CA). The nucleotide sequence was resolved by 377 automated DNA sequence (Applied Biosystems).

Nucleotide and Phylogenetic analysis

The raw sequences of DENV were annotated using *Finch Tv*, [11], BLAST was carried out to confirm the identity and submitted to DDBJ (http://www.ddbj.nig.ac.jp) [12-14].

Statistical analysis

The data analysis was done using *SPSS v.21*. Chi-square analysis was used to determine the relationship between gender, age group with dengue IgM and NS1 antigen. Logistic regression analysis was used to examine various factors related to a positive dengue IgM and NS1 antigen, p-value of <0.05 was considered statistically significant

Result

Suspected dengue fever cases were 602 during 2022-23, all were found positive for dengue NS1. Mean age of febrile cohort was 39.2 years ranging from 5 to 82 years.

Confirmation of DF by NS1Ag ELISA and selected variables

Out of the 602 study subjects all were found to be NS1 positive. The most prevalent incidence of NS1 antigen was observed in males (18.64%, 95% CI (12.28-26.72) compared to females (13.98%, 95% CI 10.48-19.22) significant association was seen for NS1 positive subjects and gender (p = 0.02). The incidence of NS1 was high in the age group of 21-30 years 19.28 %, 95% CI 13.79-25.21) compared to the children age group ≤ 10 years and older people aged ≥51 years. No statistical relationship was observed between NS1-positive subjects and age groups (p = 0.53)**.**

Clinical Signs & Symptoms of Dengue-Infected Patient

The clinical signs and symptoms of dengue patient, were statistically significant with fever (p =0.001), headache (p =0.00), joint pain (p =0.006), and fatigue (p =0.007) respectively

Monthly distribution of dengue cases and deaths in Patna

A high incidence of dengue was observed in October to December (n=136), 20 cases were detected in July to September, and no single case was detected in January 2022 to June2023 (**Table 1).**

Table 1 : Monthly distribution of dengue cases and deaths in Patna

Month	2022	2023
January-March	00	00
April- June	06	18
July –September	46	122

October –December	160	250
Total	212	390

Seasonal incidence of dengue fever in Patna

The seasonal determination surveys demonstrated that the most affected period (n = 370, 61%) was post-monsoon followed by the monsoon period (n = 18, 28%) and rest positive case was noted in the pre-monsoon period **.**

Serotyping of analyzed samples

Out of 602 NS1 positive samples of 2022-2023, all tested positive for dengue viral RNA by RT-PCR. Multiplex PCR was utilized for serotype analysis for all 602 positive cases, 459cases have DENV2 serotype infection and 6 had concurrent infection with two DENV serotypes (**Fig 2**). The overall prevalence of concurrent infections of DENV-1, DENV-3and DENV-4 virus serotype was 18, 4and 01 . Thus, DENV-2 and DENV-1 are the most common serotype combination observed during the outbreak. The highest dengue virus positive cases were observed in the age group of 20-30 years (33.75%) followed by the age group 10-20 years (28.75%). Clinical recovery was observed in all these patient affected with DENV-2.

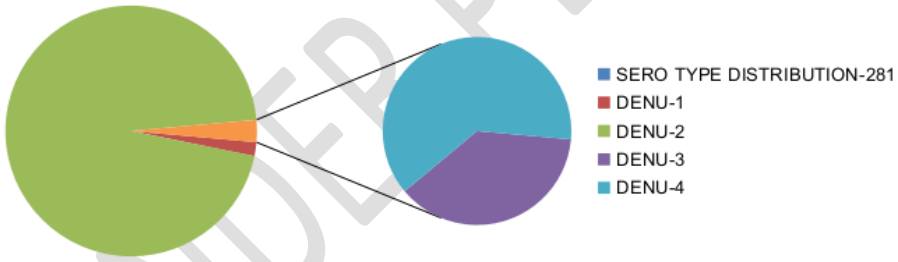

Fig.1showinig all four prevalent serotypes, DENV-1, DENV-2, DENV-3 and DENV-4 with DENV2 most common serotype

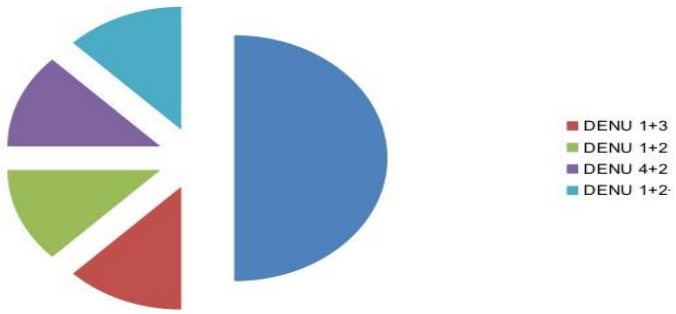

Figure.2 showing mix mixed infection cases of DENV-1 with DENV-3, DENV-1 with DENV-2 and DENV-2 with DENV-4

Phylogenetic analysis of DENV-1, DENV-2 and DENV-3 Sequences

The accession numbers of the submitted sequences are LC114463, LC114464 and LC114465 for DENV-1, DENV-2 and DENV-3 respectively. Phylogenetic tree was constructed with 23 sequences of DENV-1, 27 sequences of DENV-2 and 20 sequences of DENV-3 using CprM gene region from India and different parts of the world. Analysis revealed that DENV-1 clustered into three genotypes in which DENV-1/IGIMS_Patna_India belongs to genotype I, which is closer to vietnam (GQ199793.1 and GQ199831.1) having an average of 92% identity. DENV-2/IGIMS_Patna_India clustered with, genotype IV as shown in **Fig3b**, that is closely related with Haryana strain having 95% homology.

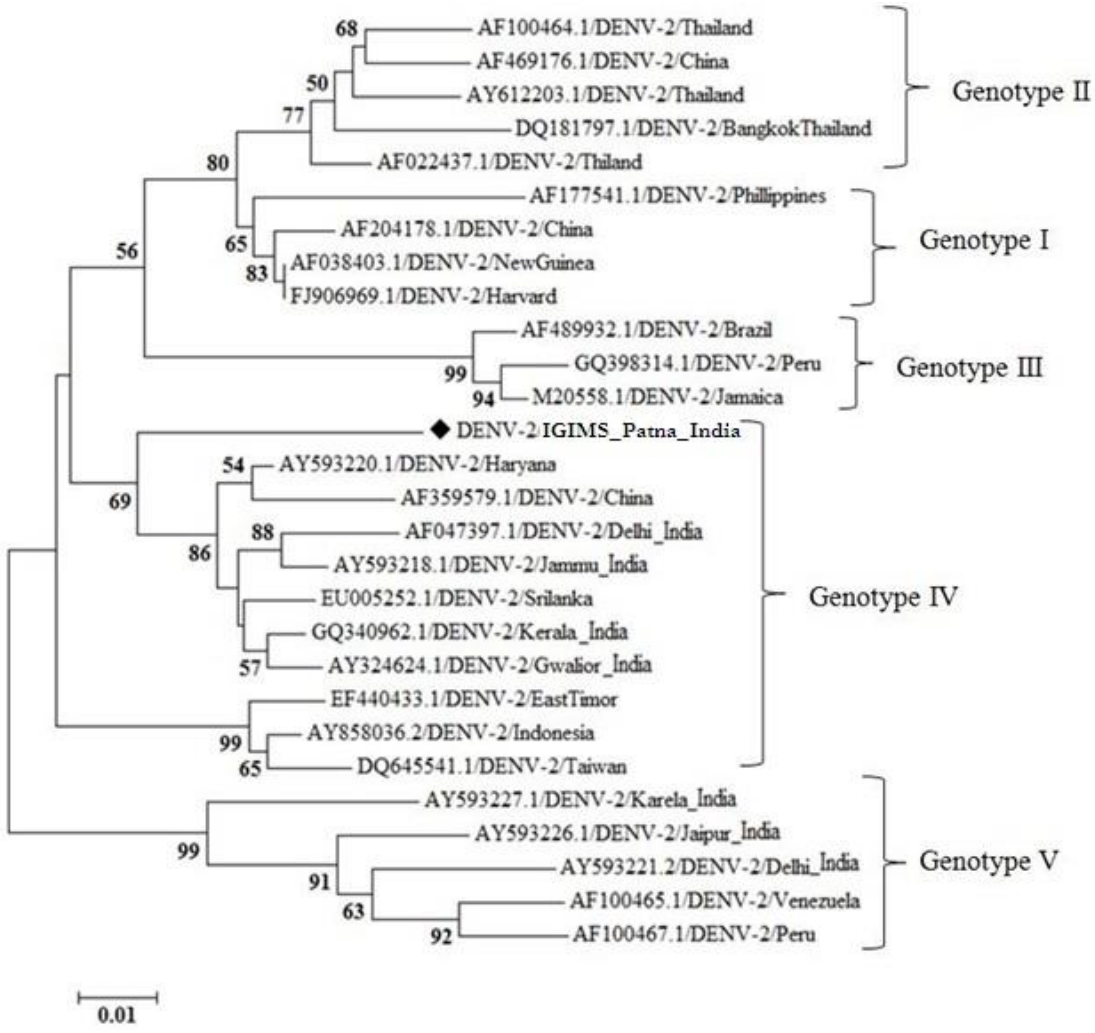

Fig.3a Hierarchical cluster analysis 1

Out of four type genotypes of DENV-3, Patna India sequence belong to genotype III (**Fig3c),** which shared 98% identity with Gwalior India (AY770511.2) that was responsible for major epidemic struck of DHF & DSS in many parts of northern India along with National Capital Delhi and Gwalior, Madhya Pradesh in 2003 [7, 15].

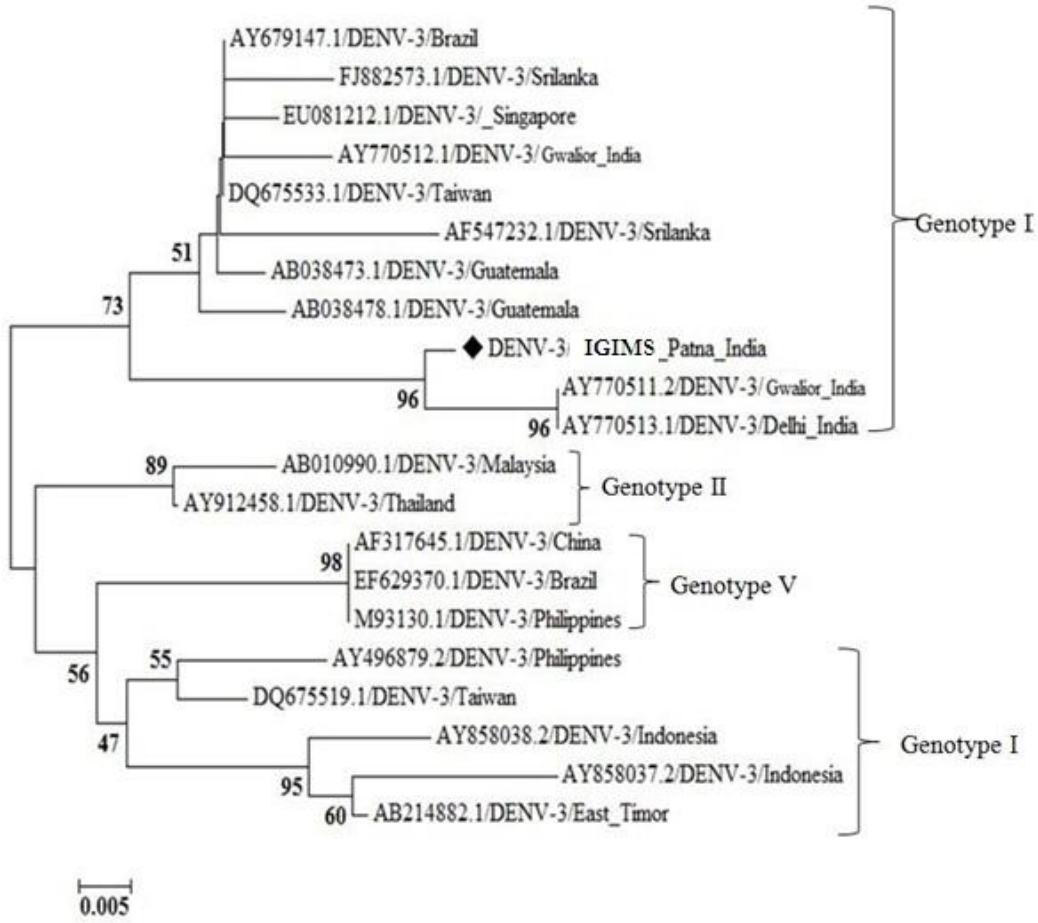

Fig. 3b. Hierarchical cluster analysis 2

Discussion

For over two centuries, dengue infection has been known to be endemic in India as a self-limited disease. But with increasing frequency of outbreaks in recent years, the disease has changed its course manifestation to the severe form. Inspite of increasing incidence of dengue in Bihar, till now only one report of outbreak is published in September 2010. According to government estimation dengue has spread in more than two-third area of this State. District Rohtas was worst affected in 2013 with 1127 reported cases, Bhabua comes second with 305 cases and the state capital, Patna with 105 cases [16]. Seeing the devastating situation of dengue in this state the early diagnostic confirmation in patient is required for timely clinical intervention, etiological investigation and to prevent potential outbreak. Hence, this is the first large scale study for two year (2015-2016) on seroprevalence, serotyping and genotyping of dengue virus circulating in Patna, Bihar. Zhang, H. et al. has reported that

several approaches were used for laboratory diagnosis of dengue virus infection such as serology, viral isolation, and nucleotide detection; however these tests have their own limitations [17]. So, in this study combination of serological assays have been performed i.e. NS1 antigen ELISA and IgM antibody. NS1 antigen-capture and IgM assays appear to be highly sensitive and complementary, allowing a sufficiently good presumptive (IgM) or definitive (NS1) diagnosis during the acute and the convalescent phase of the disease. This advantage of the combination was also positively demonstrated in the multi-country study by Guzman et. al. [18]. Moreover, both assays are easy, fast, require limited equipments, expertise and are affordable to perform. When the NS1 antigen assay was coupled with MAC-ELISA,the overall sensitivity increases [19]. Age wise analysis on prevalence of dengue infection revealed higher rate in the young age group of when compared to the older age group , which is in congruent with previous studies conducted in other epidemic localities but in contrast with a study conducted in Singapore in which a higher prevalence of IgM and IgG antibodies were observed in older aged people[20-23]. Gender wise observations showed male predominance which is consistent with other studies conducted in tropical/subtropical countries in which males were found to be more susceptible than females [22, 24-26].This difference may be associated with difference in gender-specific contact, according to limited intellectual settings and daily activities in the outdoors. Seasonal observation of two year dengue outbreak in Patna occurred mostly in post-monsoon period (136, 26%), compared to the monsoon period (n = 20, 13.92%) which is in agreement with previous outbreaks in Patna and neighboring eastern India region as shown in **Fig.1.** [27, 28] .The majority of our entire febrile cohort was symptomatic and symptoms such as fever, headache, joint pain, fatigue, and skin rashes were statistically associated to dengue infection which is similar with the study reported by Ali A. et al. and Low et al. respectively [22, 29]. The epidemiology of dengue in Indian subcontinent is very complex and substantially changed over almost past six decades. Disease is caused by all the four prevalent serotype from a subclinical infection to a mild self limiting disease, DF and a severe disease that may be fatal, the DHF/ DSS [6]. Serotypes of the virus kept changing from year to year, and each time either the serotype or the genotype showed a change [15]. The incursion of new genotype into an area is also being associated to the severe form of the disease [30]. All these facts compel us to pay more attention to focus towards the genetic nature of dengue viruses which will provide important information regarding molecular epidemiological analysis of DENV virus in Bihar. We detected three dengue virus serotypes circulating in Bihar, DENV-2 dominated the outbreak while DENV-2, DENV-3 and DENV-4 detected in small number. For genotyping different regions of dengue genome like Envelope, E-NS1, C-prM and complete genome has been utilized [31-34]. We have utilized the sequence information of CprM gene junction in this study, as it is faster and economical due to utilization of a single set of primer pair for both amplification and sequencing [35].

Based on comparison of C-prM gene junction of viral isolates, DENV1 from this study clustered within genotype 1 which is closely related to 2003 Veitnam strain. However this proximity was difficult to explain as during the last 5 decades there has been a endurance of genotype III of DENV-1 in India with genotype I being present only during 2 years i.e. 1997–98 in Delhi. [35] Similar type of genotype I viruses were found circulating predominantly in other Asian countries including Thailand, China,Taiwan, Sri Lanka [35]. Thus, this analysis describes the reemergence of genotype1 to be prevalent in India after 1998 and is foremost report from Bihar, India. Serotype 2 clades into five genotypes (I-V) in which the Indian isolates clustered in genotype IV and V and our DENV-2 isolates belong to genotype IV with the lineage genetically related to widely circulating genotype in North India such as Haryana, Delhi and Jammu as shown in **Fig.3b.** This serotype and genotype has been recorded to be most prominent one in the country, this finding is congruent with Kumar et al.[36], Sharma et al. [37] and Das, B. et al. [38] which also infers that this genotypes is highly virulent and have been associated with repeated dengue outbreaks in India. In this analysis DENV-3 isolate, circulating in Patna is found to be of genotype III of Indian origin, more closely related to Gwalior and Delhi recored in 2003 as shown in **Fig.3c,** also this strain is suspected to cause more fatal infection,widely distributed in South American and Asian countries [39].

Conclusion

According to the present febrile cohort study of two year duration, three serotypes, i.e. DENV-1, DENV-2, and DENV-3and DENV-4 found to be circulating in Patna, Bihar with dominance of DENV-2. Our study revealed dengue infection to be most frequent in the post-monsoon season and relatively higher seroprevalence in young male. DENV-1 isolate clustered as a distinct clad, genotype - I, which signifies reemergence of genotype1 to be prevalent in India and is foremost report from Bihar, India. Thus, this analysis will help for continuous epidemiological surveillance to monitor the incursion and spread of dengue viruses, which will assist to undertake effective control and management strategies at the earliest.

Declaration

The study was reviewed and approved by the ethical committee of the Indira Gandhi Institute of Medical Science Patna India. There is no conflict of interest

Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must. The study was reviewed and approved by the ethical committee of the Indira Gandhi Institute of Medical Sciences (IGIMS) Patna India.

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