# Shift of Dominance of Circulating Dengue Virus Serotypes During the Period 2014-17 in Kolkata

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# ABSTRACT

**Introduction:** Dengue virus is consists of four antigenically distinct viruses DENV-1, -2, -3 and -4, belongs to the genus *Flavivirus* of family *Flaviviridae*. Dengue infection cause a self limiting mild dengue fever (DF) which often leads to dengue with warning signs (DWS) and/or more complicated severe dengue (DS). Co-circulation of multiple serotypes is reported in every major outbreak. This study is focused to detect sero-prevalence of dengue fever among the fever cases attending the Virology unit of CSTM, Kolkata and to find out the prevailing serotypes and genotypes circulating during the year 2014-17.

**Material and methods:** Plasma samples were collected from suspected dengue cases and tested for NS1 antigen (fever < 5 days) or dengue specific IgM (fever  $\geq 5$  days). Dengue positive samples were taken for serotyping and genotyping analysis by real-time RT-PCR and sequencing.

**Results:** In 2014-15 the prevalent serotype was DENV-2 (Cosmopolitan genotype) and DENV-3 (genotype III) respectively with co-circulating DENV-1 (American African). In 2016, the dominant serotype was DENV-1 (American African). Again in 2017, the dominant serotype was DENV-2 (Cosmopolitan and American genotype).

**Conclusion:** Kolkata is a hyper-endemic zone for Dengue with circulation of multiple serotypes. This study helps to conclude that there is a continuous change of circulation of multiple serotypes in Kolkata. However, a shift of dominance has been observed in every year. This dominance shift may be responsible for Dengue outbreaks in Kolkata and increasing number of complications.

Keywords: Dengue Fever, Dengue Virus, Epidemic, Serotype.

# **INTRODUCTION**

Dengue fever is one of the most rapidly spreading arboviral diseases in tropical and subtropical regions. Over the last 50 years up to 30-fold increase of dengue fever incidence has been reported. About 2.5 billion people live with high risk of dengue fever in over 100 endemic countries. More than 50 million reported infections occur every year with 22,000 deaths mainly among children.<sup>1</sup>

Dengue virus, is transmitted by the mosquito *Aedes aegypti* and *Aedes albopictus*, belongs to the family *Flaviviridae*. It is a positive strand RNA ~11kb in length with single open reading frame (ORF) that carry genes for three structural (C-prM-E) and seven non-structural (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) proteins. There are four serotypes of dengue virus i.e. DENV-1,-2,-3 and -4. More than one serotype are found to be circulating in dengue endemic

countries like India, Pakistan, China.<sup>2-4</sup> Series of outbreaks have been reported regularly in different states of India such as West Bengal, Delhi, Tamil Nadu, Punjab, Rajasthan.<sup>5-10</sup> Dengue fever with warning signs (DWS), severe dengue (DS), the life threatening form of dengue infection, is strongly associated with sequential infection by different serotypes which are more common in India.<sup>2</sup> This paper is dedicated to present a comprehensive report of dengue infection among febrile cases attending the viral OPD as well as indoor facility of Calcutta School of Tropical Medicine (CSTM) during the period January 2014 to December 2017 and also to provide information about the circulating serotype/s and genotype/s of dengue virus.

Kolkata, capital of West Bengal is reported as hyper endemic region for circulation of multiple dengue serotypes.<sup>11</sup> In 1824, dengue infection was first documented in Kolkata (Calcutta). Since then several major outbreaks took place during the year 1836,1906,1911,1972, affecting about 40% of the city population.<sup>12</sup> Recent large outbreaks were documented in 2005<sup>13</sup>, 2012.<sup>11</sup> A shift in the dominance of predominant serotypes with continuous co-circulation of multiple other serotypes has been observed. Dominant serotype in 2010 was DENV-2.<sup>14</sup> A shift of dominance from DENV-2 to DENV-1,-3 was reported in 2012 outbreak.<sup>11</sup> This study was done to detect sero-prevalence of dengue fever among the fever cases attending the Virology unit of CSTM, Kolkata and to find out the prevailing serotypes and genotypes circulating during the year 2014-17.

#### **METHOD AND MATERIALS**

This study was done to detect sero-prevalence of dengue among the fever cases attending the virology unit of

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CSTM, Kolkata during the period 2014-2017 and to find out the prevailing serotype(s) among the dengue fever cases using molecular based techniques. This study protocol was approved by institutional ethical committee. All adult volunteers provided written consent. Parents or legal representatives authorized the participation of their children in the study on behalf of them.

# Sample collection

Suspected dengue fever cases of all ages and either sex were referred from outpatients department (OPD) and also from inpatient department of CHTD, STM, Kolkata to the Virology Unit and enrolment was done as per WHO criteria, namely, 1) high grade fever with two or more signs at the time of enrolment: arthralgia, myalgia, headache, retroorbital pain, nausea/vomiting, abdominal pain, rash and haemorrhagic tendency. A standardized questionnaire was used to collect the socio-demographic and clinical data at the time of blood sample collection. Patients were classified as self limiting dengue fever (DF), Dengue fever with warning signs (DWS) and severe dengue (DS) according to the 2009 WHO classification scheme.

## Serology

3 ml venous blood was collected in EDTA coated vial. Plasma samples were separated by centrifugation at 4°C and aliquotted in two vials. One was used for ELISA test and other was kept at -80°C for further analysis. Plasma samples from suspected dengue fever cases  $\leq$ 5 days were tested for NS1 antigen using Panbio Dengue Early ELISA kit (Standard Diagnostics, Republic of Korea) and fever cases of  $\geq$ 5 days were tested for dengue specific IgM using MAC-ELISA kits prepared by National Institute of Virology, Pune strictly following the manufacturer's protocol.

## **RNA Extraction and Serotyping**

Viral RNA was extracted from 200µl acute phase plasma using Viral RNA extraction kit (Qiagen Hilden, Germany) according to manufacturer's protocol. Serotyping was done by real-time RT-PCR using superscript III platinum one step qRT-PCR kit (Invitrogen, Waltham, MA, USA). For serotype specific primer-probe set CDC DENV 1-4 Real time RT-PCR assay set was used which was kindly supplied by CDC, Atlanta. Multiplex real-time PCR was done as per CDC protocol.

## Sequencing of CprM region

Sequencing of Capsid-premembrane (C/prM) region was done on 61 Kolkata isolates. Amplicons were generated using Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol using D1 and D2 as forward and reverse primers respectively<sup>15</sup> specific for C/prM gene junction for all four serotypes. The 511bp PCR products were observed in 2% agarose gel stained with ethidium bromide. PCR products were purified using clean sweep PCR purification kit (ThermoFisher Scientific, Waltham, MA, USA). Sequencing was done in both directions by using BigDye Terminator Cycle Sequencing Ready Reaction kit version 1.1 containing 3µl PCR product as template (Applied Biosystem, Foster city, CA, USA). The sequencing reaction was performed in 3500Dx Genetic Analyser (Applied Biosystem, Foster city, CA, USA).

# **Phylogenetic Analysis**

Nucleotide sequences of CprM genes were submitted in GeneBank and were aligned with serotype specific reference sequences, selected and retrieved from GeneBank by using BioEdit (version 7.2.5). Dengue genotypes were identified phylogenetically using MEGA softwere (version 6). Phylogenetic tree was constructed using neighbour joining method and the robustness of the tree was assessed by means of bootstrap analysis with 1,000 replicates. Pair wise evolutionary distances were computed using the Kimura two-parameter model.

# RESULT

# Serology

Out of total 10682 samples collected during the year 2014 - 2017 (2014-1625 samples, 2015-1900 samples, 2016-4048 samples, 2017- 3109 samples) 17.85%, 23.05%, 29.45% and 21.71% cases were positive for dengue NS1 and IgM in 2014,2015, 2016 and 2017 respectively. In total 37.38% cases were NS1 ELISA positive, 44.35% cases were IgM ELISA Positive. 18.15% of cases were positive for both NS1 and IgM ELISA. The highest number of infection was recorded in age group 21-30 years (32.25%) followed by 11-20 years (28.76%) (Figure1A). The proportions of male patients were 63.93% as compared to 36.07% female cases. The highest number of positive cases was recorded during the months of October –November (Figure 1B), which establishes the fact that dengue cases mostly occur during post-monsoon period, and declines after the onset of winter.

# **PCR Results**

From 2014 to 2017, total 354 acute fever samples (nonhaemolysed, NS1/IgM positive, <7days fever) were taken for serotyping, out of that 72.03% samples (i.e. 255/354) were PCR positive. In 2014 – 71.23% out of 73 samples, in 2015- 85.18% out of 54 samples, in 2016- 90.74% out of 54 samples and in 2017- 62.43% samples out of 173 samples were PCR positive. Dominant dengue serotype in 2014 was DENV - 2 (28/52 i.e.53.85%), in 2015 was DENV - 3 (18/46 i.e. 39.13%), in 2016 was DENV-1 (20/49 i.e. 40.81%) and DENV-2 in 2017 (61.11% i.e. 66/108). In 2014, 5 samples were infected with DENV-3 while only 4 samples were positive for DENV-1. In 2015, DENV-2 was found in 11 samples, DENV-1 was found in only 2 samples. In 2014, 19.23% cases were found to have dual infection with DENV-2,-3 but only 4 samples were positive for DENV-1,-2 dual infection. The percentage of DENV- 1,-2 and DENV-2, -3 dual infected cases were 21.74%, and 8.69% respectively in 2015. The other identified serotypes in 2016 were DENV-2 in 10 samples and DENV-3 in 2 samples. 17 samples had mixed infection with multiple serotypes. Twelve cases were found to have dual infection with DENV-1& -2, four had dual infection with DENV-2 &-3, while only one case with DENV-1&-3. DENV- 4 serotype was not present during the period 2014-2016. Surprisingly in 2017, DENV-2 reemerges as dominant serotype (61.11% i.e. 66/108) with simultaneous co-circulation of DENV-1 (16.67%), DENV-3 (15.74%) and DENV-4 (3.70%) [Table I].

#### **Nucleotide Sequence Analysis**

Total 61 isolates from Kolkata (DENV1 – 10; DENV2- 28; DENV3- 19, DENV4- 04) have been sequenced (approx.



**Figure-1A:** Age wise distribution of total dengue positive cases during the study period 2014-2017 (X-axis- Age in years).

455bp.) and analyzed during this time period (Genebank No.-KY404121-KY404150, MG049788-Accession MG049799, MG973726-MG973744). These sequences were compared with 25 geographically diverse reference sequences. DENV-1 sequences showed 99.99% homology with china isolate (KT187559). DENV-2 and DENV-3 isolates were close to Indian strains from Delhi (JX475906, JX891649) with average 99.98% and 99.99% homology respectively. DENV-4 isolates were closely related to Pune 2009 isolate JQ922560 (99.99% homology). These sequences were taken as prototype respectively for further analysis. The CprM regions of all the four serotypes were found to be rich in A and G. Majority of mutations were silent in nature and transition in type. The deduced amino acids revealed that the amino acid changes were conservative in nature. In both DENV-1 and DENV-3 serotypes this fragment was rich in leucine, lysine, for DENV-2 it was leucine and arginine and for DENV-4 it was leucine followed by Threonine.

### **Phylogenetic Analysis and Genetic Diversity**

A total of 10 isolates were sequenced and analysed for DENV-1 serotype. These sequences were compared with reference sequences from India (15 isolates from different regions of India) and other geographical regions by constructing phylogenetic tree. The sequences were segregated into two



**Figure-1B:** Monthly distribution of all enrolled febrile cases and dengue positive cases in Kolkata, West Bengal, India during 2014-2017. Peak season for dengue positive cases were the month of October-November i.e. post-monsoon period.

| Year  | 2014        | 2015        | 2016        | 2017        |  |
|---|-------------|-------------|-------------|-------------|--|
| Number of patients  | 73          | 54          | 54          | 173         |  |
| Dengue Positive; n (%)  | 52 (71.73%) | 46 (85.18%) | 49 (90.74%) | 108(62.43%) |  |
| DENV-1; n (%)   | 4 (7.69%)   | 2 (4.35%)   | 20 (40.82%) | 18 (16.67%) |  |
| DENV-2; n (%)   | 28 (53.85%) | 11(23.91%)  | 10 (20.41%) | 66 (61.11%) |  |
| DENV-3; n (%)   | 5 (9.61%)   | 18 (39.13%) | 2 (4.08%)   | 17(15.74%)  |  |
| DENV4; n (%)  | 0           | 0           | 0           | 4 (3.70%)   |  |
| Co-infection; n (%)   | 15 (28.85%) | 15 (32.60%) | 17 (34.69%) | 3 (2.78%)   |  |
| DENV-1 & 3; n (%)   | 1 (6.66%)   | 1 (6.66%)   | 1 (5.88%)   | 0           |  |
| DENV-2 & 3; n (%)   | 10 (66.67%) | 4 (26.67%)  | 4 (23.53%)  | 2 (66.67%)  |  |
| DENV-1 & 2; n (%)   | 4 (26.67%)  | 10 (66.67%) | 12 (70.59%) | 0           |  |
| DENV-3 & 4; n (%)   | 0           | 0           | 0           | 1 (33.33%)  |  |
| Table-1: Changing pattern of serotype predominance during the period 2014-2017. |             |             |             |             |  |





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0.01 Phylogenetic tree

**Figure-2:** Phylogenetic tree of DENV-1, -2, -3, -4 based on 440 bp nucleotide sequences of the CprM gene region. The tree generated by the Neighbour-Joining (NJ) method and Bootstrap support values of 1000.Bootstrap re-sampling values (higher than 60%) are indicated at major nodes. Each strain is denoted by Genbank accession number followed by country of origin and the year of isolation. Only 39 sequences were used to construct the tree to avoid overcrowding. (2A) DENV-1. (2B) DENV-2. (2C) DENV-3 and (2D) DENV-4 study sequences. All study sequences are marked.

distinct clades within American African genotype, India II subgenotype (Clade I and II) (Figure 2A). Clade I was further subdivided into three branches. 2014 isolates from Clade II clustered with other Indian isolates. 2015-17 isolates were

clustered in Clade I which was related, although distantly to isolates sampled in China in 2014 and Delhi, India 2010. Two isolates sampled from two AES patients were clustered in Clade I.

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DENV2 isolates fell into three genotypes; Cosmopolitan, Asian II and American genotype. The isolates clustered into Cosmopolitan genotype grouped into three clades (Figure 2B). Clade I was closely related to JX475906 (Gwalior, India-2009). Two AES causing strains were isolated and sequenced in 2017 and one grouped with Clade I (STM AES688/2017). From Clade II, 2016-17 isolates were closely related to a strain recovered in China in 2013 (KF479233) and two Indian isolates from Delhi, 2012 (KJ438872, KJ438866). Clade III, sampled in the year 2014 was distantly related to China isolates AF276619. STM 20758/2016 grouped in Asian II genotype. An isolate recovered from an AES patient (STM\_AES743/2017) grouped with older Indian isolate (JQ922552 JQ922553), clustered within American genotype. All DENV3 isolates were clustered together and closely related to other isolates circulated in India (Figure 2C). All the isolates belonged to genotype III.

DENV4 serotype was found in 2017 only and the isolated viruses were belonged to genotype I. One isolate was closely related to a strain isolated in Pune, 2016 (MG272273), whereas other three were deviated differently and distantly related to other Indian strains, KU509287 and KX845005 (2009 and 2015 respectively) (Figure 2D).

### DISCUSSION

The danger of dengue fever is continuously increasing now a days with increasing urbanization and climatic changes. Growing incidence of self limiting dengue fever (DF) and DWS/DS indicates that continuous monitoring of dengue fever is very important. In 1964, Haemorrhagic dengue infection was reported in Kolkata.<sup>16</sup> In the same year two serotypes, DENV-1 and DENV-4, were isolated from wildcaught mosquitoes in south India.17 In 1983, DENV3 was reported for the outbreak in Kolkata.<sup>18</sup> Kolkata is a largely endemic zone for dengue fever. The last major outbreak in Kolkata was reported in 2012. In 2012 the dominant circulating serotype was DENV-1.11 Among 19 RT-PCR positive samples 16 cases were reported with monotypic infection. Among which nine cases were DENV-1 while DENV-3 was found in five cases and DENV-4 in two cases. Dual infection with DENV-1,-3 and DENV-1,-2 were found in two and one cases respectively. In 2013 dengue febrile cases dropped drastically. Only 7.63% cases were found to be dengue IgM positive during this year (author's unpublished data). Again in 2014, 2015 dengue febrile cases were found to increase to 17.85% (290/1625) and 23.05% (438/1900) respectively. In 2016, 2017, the proportion of dengue positive cases further increased to 29.45% (1192/4048) and 21.71% (675/3109). Patients with multiple infections had a history of delayed recovery and persistent weakness.

The molecular tests results (conducted on 354 NS1 and/or IgM ELISA positive samples) showed 72.03% positivity. In 2014, 2015, 2016 and 2017 the predominant serotypes were DENV-2, DENV-3, DENV-1 and DENV-2 respectively. Mixed infections or simultaneous infection with more than one serotype was found in all these years (Table 1).

In the present study, sequencing and phylogenetic analysis of

four serotype circulating in Kolkata, India, were conducted on CprM gene junction. Phylogenetic analysis of DENV-1 clustered the newly sequenced isolates in a different branch, although two strains were branched with previously sequenced strains circulating in other parts of India. However the lineage of circulating DENV-1 in Kolkata is India II of American African genotype. Apart from India II sub-genotype, other genotypes were not detected during this study period from this region. DENV1 isolates, in our study, have 99.98% homology with Delhi isolates which caused the 2010 outbreak.<sup>19</sup> DENV-2 isolates grouped with both Indian strains and China strains. However, the most of the isolates belonged to Cosmopolitan genotype. One isolate from 2016 clustered with Asian II genotype, belongs to American genotype and another isolate from 2017 grouped with older Indian isolate (JQ922552).<sup>20</sup> This result shows that multiple genotype of DENV-2 was circulated during this study period. This dengue strain (STM\_AES743/2017) was isolated from a patient suffering from Acute Encephalitis Syndrome which shows 99.92% homology with an old isolate recovered from Kolkata in 1980.

All DENV-3 and DENV-4 isolates grouped with previously reported strains circulating in other parts of India. DENV3 isolates having 99% homology with Delhi isolates which caused the 2004 outbreak.<sup>21</sup>

This data indicates that, sudden change in circulating serotype as well as genotype increase the risk of dengue outbreaks. Although phylogenetic analysis reveals that the circulating genotypes of all four serotypes of dengue virus are closely related to the other circulating genotypes in India and its neighbouring countries but still the isolates from Kolkata have clustered together in a different branch. More than one sub-genotype of DENV2 were circulating during this study period in Kolkata, West Bengal.

# CONCLUSION

This study established that circulation of multiple serotypes are causing the large number of infection. However, a shift of dominance has been observed in every year. This dominance shift also indicates that each serotype has a strong ability to be the causative agent for sudden dengue outbreaks in Kolkata in future. Phylogenetic analysis was also done in this study in a small region of the virus. Characterization of circulating dengue serotypes is underway to ascertain the emergence of new strains. This study will help to design control strategies for epidemics and sudden outbreaks.

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