

## Lineage shift of dengue virus in Eastern India: an increased implication for DHF/DSS

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### SUMMARY

Dengue fever, a mosquito-borne viral disease, has become a major public health problem with marked expansion in recent decades. Dengue has now become hyperendemic in India with co-circulation of all the four serotypes. Herein, we report an unprecedented outbreak which occurred during August to October 2011 in Odisha, eastern India. This is the first report of a large epidemic in Odisha. Detailed serological and molecular investigation was carried out to identify the aetiology. Almost half of the samples were found to be dengue antigen (NS1) positive. Further molecular assays revealed circulation of mixed dengue serotypes (DENV-2 and DENV-3). Cosmopolitan genotype of DENV-2 and -3 were identified as the aetiology by phylogenetic analysis. Interestingly, a new lineage of DENV-3 within cosmopolitan genotype was incriminated in this outbreak. The emergence of the unprecedented magnitude of the dengue outbreak with the involvement of a novel lineage of DENV in a newer state of India is a major cause for concern. There is an urgent need to monitor phylodynamics of dengue viruses in other endemic areas.

**Key words:** Dengue virus, diagnosis, epidemiology, Odisha, phylogeny, serotype.

### INTRODUCTION

Dengue viruses (DENVs) are important arthropod-borne viruses of global public health significance. They belong to the genus *Flavivirus* (family Flaviviridae) and are transmitted by *Aedes* (*Stegomyia*) spp., primarily *A. aegypti* and *A. albopictus*, [1]. There has been an explosive upsurge in dengue infections in most parts of tropics and subtropics over last three decades. Currently, one third of the global

human population are at risk of infection. This expansion is primarily attributed to uncontrolled urbanization; inadequate water supply, sewage and waste management; rapid movement of humans, animals and goods via air and sea routes, as well as unsustainable vector control programmes [2]. Recently, DENV has also been reported from some temperate parts of the world including France, Croatia, China, and Madeira/Portugal, which has raised serious concerns among public health authorities as well as the scientific community [3, 4]. The WHO estimates that about 50–100 million global dengue infections per year were presented in a spectrum of clinical manifestations [3]. The majority of infections are either asymptomatic or commonly manifested as a

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self-limiting flu-like disease [dengue fever (DF)]. About 1–2% of DF cases lead to a severe form of the disease [dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS)], characterized by vascular leakage and/or haemorrhage, requiring hospitalizations under intensive care with a case-fatality rate of about 5% [5].

DENVs are classified into four antigenically and genetically distinct serotypes (DENV 1–4) with a positive polarity, single-stranded RNA as their genomic material. Each serotype is further classified into 4–5 distinct genotypes based on their genetic make-up [6]. Further, multiple lineages are also identified within each genotype based on their distinct phylogenetic classification [7]. Infection with one dengue serotype confers lifelong immunity against homologous serotypes and no or limited immunity against heterologous serotypes. Markedly, some genotypes and lineages are particularly attributed to a severe form of dengue infection. These genetic variants with subtle changes are also responsible for causing major outbreaks due to their higher transmission in both humans and mosquitoes [8, 9].

Many Southeast Asian countries are now hyperendemic with circulation of all four DENV serotypes, and rates of severe dengue being 18 times higher in this region compared to the Americas [10]. The first DHF outbreak in India occurred in the national capital Delhi in 1996 where DENV-2 was identified as the aetiology [11]. At present, all four DENV serotypes are found circulating in most parts of Southeast Asia including India [10, 12]. DENV-2 and DENV-3 were identified as the aetiological agent in the majority of DENV outbreaks in India [13, 14]. Dengue has been more prominent in northern India since 2000 with regular post-monsoon outbreaks reported from Delhi, Uttar Pradesh, Madhya Pradesh, Haryana, Rajasthan and Punjab [15]. Since 2006 onwards, the southern states of Kerala and Andhra Pradesh began reporting massive dengue outbreaks along with chikungunya [12]. The western Indian states, including Maharashtra, also reported dengue outbreaks at regular interval [16, 17]. However, the eastern Indian state of Odisha had never reported a dengue outbreak prior to 2011. During 2011 a large number of districts in Odisha reported dengue outbreaks with more than 10 000 cases [18]. The genetic information of DENV from India remained restricted to northern, western and southern parts, due to the higher number of outbreaks. The DENVs circulating in eastern and northeastern India are poorly

characterized. The present study was conducted to investigate the explosive unprecedented outbreak using serological and molecular analysis.

## METHODS

### Clinical samples

Blood samples collected from 97 patients clinically suspected of dengue during August to October 2011 from Ipat General Hospital (IGH), Rourkela and 21 samples from the National Vector Borne Disease Control Programme (NVBDCP), Odisha, were transported to the Virology laboratory under cold conditions. This laboratory is one of the National Apex referral laboratories designated by the Ministry of Health & Family Welfare, Government of India for laboratory confirmation of acute dengue infections. Informed consent was obtained from all patients prior to sample collection. Serum was separated from the blood samples and stored at  $-80^{\circ}\text{C}$  until required.

### SD BIOLINE dengue duo test

The SD Bioline Dengue Duo kit (Standard Diagnostics Inc., South Korea) is a rapid, *in vitro* immunochromatographic one-step assay designed to detect both DENV NS1 antigen and antibodies to DENV (IgG/IgM) in human serum, plasma or whole blood. All the samples were tested using this kit according to the manufacturer's protocol. Briefly, 100  $\mu\text{l}$  and 10  $\mu\text{l}$  of suspected sample was applied for detection of NS1 and IgM/IgG antibodies, respectively. The result was recorded within 15–20 min following sample application.

### One-step dengue multiplex RT-PCR (mRT-PCR)

Viral RNA was isolated from 140  $\mu\text{l}$  of serum samples suspected of dengue, employing the QIAamp viral RNA mini kit (Qiagen, Germany) and used as template for mRT-PCR. One-step single-tube serotype-specific multiplex PCR was performed with RNA extracted from patients' serum samples suspected of DENV following a protocol published previously [19]. Briefly, the amplification was performed in a 50  $\mu\text{l}$  total reaction volume with Access quick RT-PCR kit (Promega, USA) according to the manufacturer's protocol, along with five primers, i.e. dengue group-specific forward primer (D1) and four serotype-specific reverse primers (TS1, TS2, TS3, TS4). The

Table 1. Serological profile of dengue virus RT-PCR positive samples

Sample no.	Sample ID	RT-PCR/serotype	NS1 antigen	Antibody status
1	6	Positive/dengue-2	+	IgG +ve
2	8	Positive/dengue-2	+	-ve
3	20	Positive/dengue-2	+	IgM & IgG +ve
4	46	Positive/dengue-3	+	IgM +ve
5	47	Positive/dengue-2	+	IgG +ve
6	64	Positive/dengue-2	+	-ve
7	67	Positive/dengue-2	+	-ve
8	80	Positive/dengue-2	+	-ve
9	82	Positive/dengue-2	+	-ve
10	2 095	Positive/dengue-2	+	-ve
11	2 112	Positive/dengue-2	+	-ve
12	2 138	Positive/dengue-2	+	-ve
13	2 141	Positive/dengue-2	+	-ve
14	2 137	Positive/dengue-3	+	-ve
15	2 103	Positive/dengue-3	+	-ve
16	2 124	Positive/dengue-2	+	-ve
17	58	Positive/dengue-2	-	IgM +ve

amplicons were verified by 2% standard agarose gel electrophoresis.

#### Nucleotide sequence analysis

Double pass sequencing of six DENVs was performed with a BigDye Terminator Cycle Sequencing ready reaction kit (Applied Biosystems, USA) according to the manufacturer's instructions [13]. Briefly, each sequencing reaction was performed in a final volume of 20  $\mu$ l by mixing the terminator mix containing the thermostable *AmpliTaq* DNA polymerase, dNTPs and four distinct dye-labelled ddNTPs and ~25 ng purified PCR amplicons, and 3.2 pmol respective sense or anti-sense primers. Following cycle sequencing, the reaction mixture was column purified and dried in vacuum. The DNA pellet was resuspended in 15  $\mu$ l Hi-Di formamide, heated at 95 °C for 5 min and loaded on the ABI 3130 DNA sequencer (Applied Biosystems).

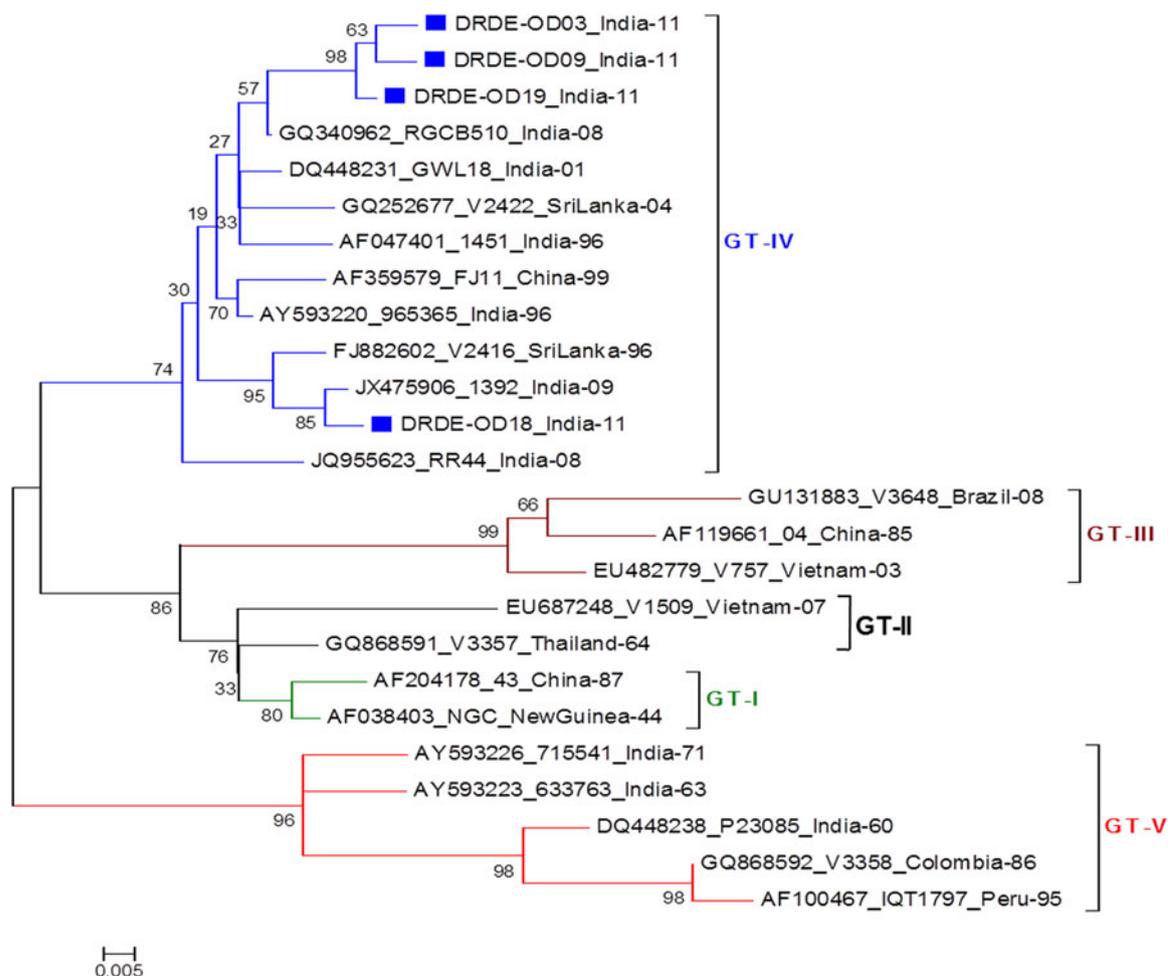
#### Phylogenetic analysis

The nucleotide sequences were edited and analysed with the EditSeq and MegAlign modules of the Lasergene 5 software package (DNASTar Inc., USA). Phylogenetic analysis was conducted using MEGA v. 5.03 [20]. The Tamura-Nei model of nucleotide substitution with gamma-distribution rates available in MEGA was used to construct the Neighbour-Joining tree. The tree topologies were evaluated using 10 000 replicates of the dataset.

## RESULTS

A major dengue outbreak was reported in Odisha during the post-monsoon season (August to October 2011), affecting more than 10 000 persons [18]. A total of 118 clinical samples were collected from the affected areas in this study. The clinical history revealed that all patients had suffered from acute onset of fever with headache and joint pain. Common symptoms were fever with chill, headache, joint pain, nausea, anorexia and abdominal pain. Thrombocytopenia was observed in 73% of patients. The sex ratio of the infected individuals was 1.7:1 (male:female). The majority of infected individuals were found to be in the 15–55 years age group.

All 118 serum samples were tested by SD Bioline Dengue Duo test kits (Standard Diagnostics) for the detection of NS1 antigen, anti-dengue IgM and IgG antibodies in dengue-infected serum samples. All the samples were also tested by RT-PCR. The results of the dengue duo test with respect to RT-PCR are given in Table 1. Out of 118 serum samples 58 (49%) were positive for DENV NS1 antigen. The serological analysis revealed an overall seropositivity of 41.5%. The antibody profile revealed 12.7% IgM, 6.7% IgG and 22% IgM and IgG antibodies. Further analysis of these samples by mRT-PCR indicated 14.4% (17/118) positivity. The serotyping by multiplex PCR revealed the presence of mixed serotypes. A total of 14 and three



**Fig. 1.** Phylogenetic tree among dengue-2 viruses generated by the Neighbour-Joining method based on the C-prM gene junction. Each strain is abbreviated with the GenBank accession number followed by strain ID, country of origin with last two digits of year of isolation. The dengue-2 viruses sequenced in this study are indicated by a solid square (■). Bootstrap values are indicated at the major branch points.

samples were found positive for DENV-2 and DENV-3, respectively.

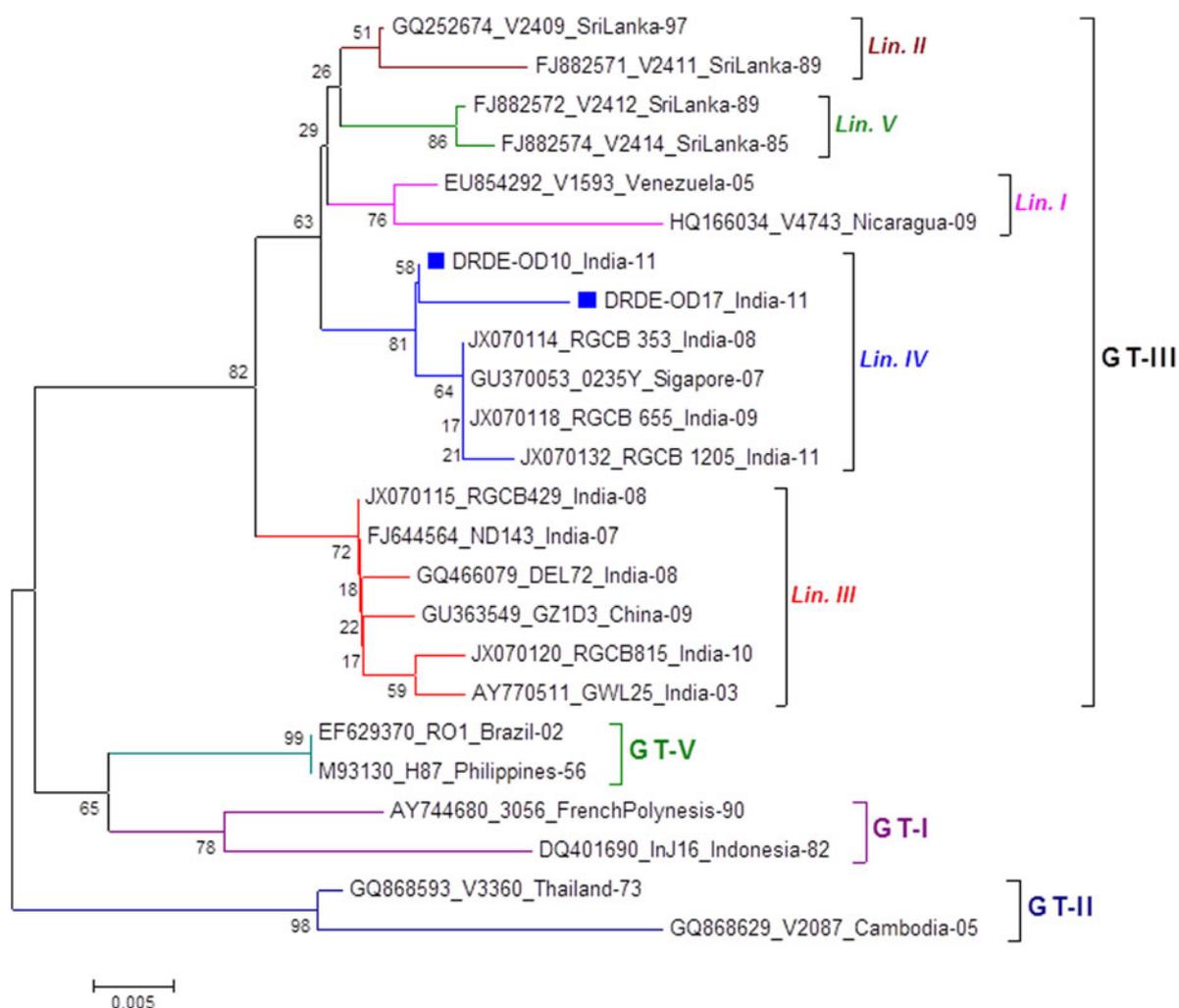
The phylogenetic analysis classified DENV-3 into four genotypes (GT). Both the DENV-3 sequenced in this study were grouped into GT-III (Fig. 1). Further GT-III was grouped into five distinct lineages. DENV-3 from Odisha was found to belong to lineage IV along with isolates from Kerala isolated during 2008–2011. However, the isolates from other parts of India belonged to lineage III.

The phylogenetic analysis classified DENV-2 into five genotypes. The four DENV-2 sequenced in this study were grouped into GT-IV along with a large number of recent Indian DENV-2 (Fig. 2). However, the older Indian DENV-2 isolated during 1960s and 1970s belonged to GT-V.

## DISCUSSION

Recent decades have witnessed explosive dengue outbreaks in different parts of India [11–17]. The eastern Indian state of Odisha recorded the first outbreak during August to October 2011, coinciding with the post-monsoon season. The post-monsoon resurgence of dengue and other arboviral infections was reported earlier from other parts of India [11, 14, 18, 21–23]. The favourable mosquito environment during the post-monsoon period is primarily responsible for the rapid spread of dengue. A detailed serological and molecular investigation was initiated in order to identify the aetiology of the unprecedented outbreak.

Serological tests included both antigen (NS1) and antibody (IgM and IgG) assays to pinpoint the causative agent [24–26]. NS1 is now increasingly preferred



**Fig. 2.** Phylogenetic tree among dengue-3 viruses generated by the Neighbour-Joining method based on the C-prM gene junction. Each strain is abbreviated with the GenBank accession number followed by strain ID, country of origin with last two digits of year of isolation. The dengue-3 viruses sequenced in this study are indicated by a solid square (■). Bootstrap values are indicated at the major branch points.

for the early diagnosis of dengue infection. The clinical management of patients is directly related to early diagnosis and NS1 proved to be the best marker for the early febrile stage. As part of the diagnostic algorithm, NS1 in conjunction with antibody assays is advocated for enhanced sensitivity of dengue detection. In the present study, detection of NS1 in 49% of samples confirmed dengue as the aetiology. NS1 protein was found circulating at high levels during the first week of illness, and correlates with the development of DHF in some studies [27]. Further identification of antibody in 41% of the suspected patients clearly indicated dengue infection. The IgM antibody appears on days 4–5 after onset of illness. Although IgG antibody only surfaces after 10 days in primary infection, it can, however, be detected as early as the

third day onwards in cases of secondary infection, which is an important feature in endemic areas [26]. The non-dengue cases were not further investigated for their aetiology and prognosis.

Molecular assays are now widely used in reference laboratories for confirmation of dengue infection. The multiplex PCR adopted in this study led to the detection as well as serotyping of DENV in one operation. The one-step single-tube method has several advantages including cost, time, and immunity from crossover contamination [19]. The identification of both DENV-2 and DENV-3 indicated co-circulation of two dengue serotypes in Odisha. DENV-2 and -3 were already implicated from several major outbreaks in India and considered as the dominant dengue serotypes [11–15]. Further, genotyping of DENV revealed

that DENV-2 and -3 from this outbreak belonged to genotypes IV and III, respectively. These two genotypes are also known as 'cosmopolitan genotypes' due to their circulation in different continents including Asia, Africa, South America and Australia [7, 21, 28]. One of the most striking features was the identification of a novel lineage IV of DENV-3 in the Odisha outbreak. This lineage was recently reported to be circulating in the southern Indian state of Kerala during 2010 [22]. The circulation of multiple lineages of DENV-3 has also been reported from Colombia [29]. So far, all other Indian DENV-3 were found to belong to lineage III [6], thus highlighting the incursion of this novel lineage into the eastern Indian state of Odisha. Incursion of a new genotype/lineage was previously attributed to the emergence of a severe form of dengue infection in other parts of the world as seen in Americas [9, 30]. The extinction and subsequent replacement of lineages were linked to the transmission bottleneck of a virus. The lineage replacement was also recently linked to enhanced virus transmission by a mosquito vector in Thailand [31].

The detailed serological and molecular investigation leading to identification of both dengue viral antigen, antibody and RNA clearly confirms the aetiology of the unprecedented outbreak in Odisha to be DENV types 2 and 3. The emergence of explosive DENV outbreaks in the eastern Indian state of Odisha with a novel lineage of DENV-3 is a major cause of concern, which requires continuous monitoring of viral circulation throughout endemic and non-endemic areas.

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## DECLARATION OF INTEREST

None.

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