Genetic characterization of dengue viruses prevalent in Kerala State, India

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INTRODUCTION

Dengue fever is an important emerging tropical disease infecting about 50–100 million people annually (Gubler, 2006; Halstead, 2007) and distributed among about 120 countries globally. Its incidence has increased about 30-fold in the past 50 years (Zorlu & Fleck, 2011). Dengue is caused by four serologically distinct types of dengue virus (DENV), DENV-1, DENV-2, DENV-3 and DENV-4, belonging to the family Flaviviridae, genus Flavivirus, species Dengue virus. These positive-sense single-stranded RNA viruses are transmitted to human beings by Aedes mosquitoes such as Aedes aegypti and Aedes albopictus. About half of the global population lives under the risk of infection of this arboviral disease (WHO, 2009). An increase in global incidence has been reported, caused by complex epidemiological factors such as globalization, unplanned urbanization, microevolution of the viruses and climatic change. The reported number of dengue cases has doubled since the last decade (Kroeger & Nathan, 2006), and about 25 000 people die of this mosquito-borne disease annually. However, no chemotherapeutic drugs or vaccines are currently available for this emerging viral disease.

The entire population of India lives under the risk of dengue fever. Since the first reported case in Calcutta in 1963, cyclical outbreaks of dengue have been recorded in this country. Since 2001, India has been recording an increase in the number of cases annually (Supplementary Fig. S1a, available in the online journal). In 2010, the maximum number of suspected cases (28 055) since 1991 was recorded. At the time of writing, only provisional figures were available for 2011. The cyclical trend of dengue outbreaks recorded previously (Singh et al., 2005) is being transformed to an endemic disease status across the country.

Kerala state, located in the southernmost tip of India, recorded a massive outbreak of chikungunya fever, another Aedes-borne arboviral disease caused by chikungunya virus (CHIKV), in 2006–2007, and was the state worst affected by this disease in 2007 (Kumar et al., 2008). The epidemic caused by this arboviral infection continued until 2011 in different regions of the country. The dengue fever problem in Kerala has shown an increasing trend since 2006 (Supplementary Fig. S1b available in the online journal). This state, despite having an area of only ~1.5 % of the country, contributed 9.2 % of the cases of dengue fever in 2010. The population density of Kerala is among the highest in the country, with 859.11 persons km-2 (Census of India, 2011; http://censusindia.gov.in). The thick forest...
cover with a rich biodiversity found in this state may facilitate the spread of zoonotic diseases, as the fringe areas of forest overlap human dwellings. Recent studies have indicated invasion of *Ae. albopictus*, a forest species, over the entire state, which would have supported the recent widespread outbreaks of chikungunya fever in this state (Kumar et al., 2011).

Dengue fever was first recorded in Kerala in Kottayam District in 1997 (Kalra & Prasittisuk, 2004). This district continues to contribute the maximum number of cases of dengue fever in the state, next to Trivandrum District in the south every year. Topographically, Kottayam District is a semi-forested region with hills and hillocks located in the foothills of the Western Ghats. This district is the abode of rubber plantations in the country and grows rubber in ~60% of its net area under agriculture. *Ae. albopictus*, the vector of dengue fever, is abundant throughout the plantation belt of Kerala, including Kottayam District (Kumar et al., 2011). No studies on the genetic characterization of DENV

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**Fig. 1.** Analysis of CprM gene sequences of the DENV-positive cases for serotype determination. Bar, 0.05 nucleotide substitutions per site.
have been carried out in this region so far. As the distribution of serotypes and genotypes has been reported to be a major contributor towards the severity of the disease, we studied the genetic structure of viral strains involved in dengue fever outbreaks in 2008–2010 in Kerala. This also provided an insight into the phylogeographical trends of the virus in the region, which is essential for devising an integrated strategy towards the management of this emerging arboviral disease in Kerala and the rest of India.

**METHODS**

**Serum samples.** Blood samples (n=89) collected from acute fever patients admitted to different hospitals and who were clinically suspected to be infected with DENV were sent to us to determine their arboviral infection status by different institutions under the Department of Health Services, Kerala State, India, during 2008–2010. These included Kerala State Institute of Virology and Infectious Diseases, District Health Office of Kottayam District, Kerala, and Government Primary Health Centres, Pallippuram, Alappuzha District, Kerala. Private hospitals located in Kottayam District also contacted us with samples collected from acute cases of suspected dengue fever. These samples were collected during 2008–2010 from seven districts in the state: Kottayam, Alappuzha, Idukki, Thrissur, Kozhikode, Malappuram and Kasaragod (Supplementary Fig. S2, available in the online journal). Blood samples were collected in 2 ml sterile Eppendorf tubes and transported to the Vector Control Research Centre Field Station, Kottayam, on icepacks at 0–4 °C. Informed consent from patients for characterization of the viral isolates for case management was obtained in the respective hospitals, following hospital guidelines. The results of the study were communicated to the hospitals and patients.

**Viral RNA extraction.** Blood serum separated from these samples was processed using a viral RNA extraction kit (Qiagen). Briefly, 150 μl serum samples were used for extraction of viral RNA following the manufacturer’s protocol. After the incubation step for lysis, the samples were passed through Qiagen columns and the viral RNA bound to silica was eluted in 30 μl deionized RNase- and DNase-free water after two washing steps. The RNA extracts were stored at −80 °C until further processing.

**cDNA synthesis and PCR amplification.** A Titan One Step RT-PCR kit (Roche Diagnostics) was used for amplification of arboviral diagnostic fragments. The enzyme mix (avian myeloblastosis virus reverse transcriptase and an Expanded High Fidelity enzyme mix consisting of Taq DNA polymerase and Tgo DNA polymerase) permitted the reaction to be carried out in a single step, using the same buffer for both cDNA synthesis and PCR amplification. As both alphaviruses and flaviviruses were reported in the region of Kerala, PCR was initially performed to characterize these two genera of arboviruses. The multiplex primers used were forward M2W and reverse cM3W primers (Pfeffer et al., 1997), and forward FG1 and reverse FG2 primers (Fulop et al., 1993), which amplify the non-structural protein 1 (NSP1) gene of Alphaviruses and the non-structural protein 5 (NS5) gene of Flaviviruses, respectively. As these primers do not amplify the gene for CHIKV, we also included a CHIKV non-structural protein 1 (NSP1) primer set (Hasebe et al.,

**Fig. 2.** Genetic analysis of CprM gene sequences of DENV-1 serotype isolates from Kerala. Bar, 0.005 nucleotide substitutions per site.

http://jmm.sgmjournals.org
2002) in the reaction. Briefly, the protocol was as follows: reverse transcription at 50 °C for 30 min, followed by an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min 20 s and elongation at 68 °C for 1 min 30 s, with a final elongation step at 68 °C for 6 min. The alphaviruses (except CHIKV) produced a 434 bp fragment and the flaviviruses produced a 958 bp fragment, whilst CHIKV produced a 354 bp fragment (Hasebe et al., 2002). The flavivirus-positive 958 bp fragment was further processed by amplification of the capsid pre-membrane (CprM) gene (~511 bp) using the DNA primers D1 and D2 reported for DENV (Lanciotti et al., 1992) to check for dengue infection status, following the protocol of Lanciotti et al. (1997).

Serotyping and genotyping of DENV infections. The amplified ~511 bp CprM amplicon was processed further to determine the serotype involved in infection using nested PCR (D1 forward primer along with reverse primers TS1, TS2, TS3 and TS4; Lanciotti et al., 1992). The ~511 CprM fragment was also custom sequenced and the sequences were analysed to determine the genotypes of the viruses. A phylogenetic analysis of all CprM sequences was carried out along with representatives of the four serotypes reported from India for confirmation. Phylogenetic analysis of CprM sequences of each serotype was also carried out separately along with representatives of different genotypes to characterize the genotypes of the isolates in the study and to gain an understanding of the evolutionary trends of the species. Sylvatic strains were also included in the analysis. The whole envelope gene (1435 bp) of DENV-4-positive isolates recorded in the study was also amplified as two fragments using two primer pairs, D4742/D4CP1838 and D41236/D4CP2536 (Lanciotti et al., 1997) to determine the genotypic evolution of this rare serotype in India. The protocol followed was modified from that described by Lanciotti et al. (1997). The one envelope gene and all 29 CprM gene sequences generated in the study were submitted to GenBank. Phylogenetic analysis was carried out using MEGA version 4.0 (Tamura et al., 2007) and DNASP version 5.0 (Librado & Rozas, 2009).

RESULTS

A total of 89 samples from hospital-admitted cases with suspected acute dengue fever (collected within 5–6 days of the onset of fever) were processed in this study, 3 from 2008, 38 from 2009 and 48 from 2010. These included 50

Fig. 3. Genetic analysis of CprM gene sequences of DENV-2 serotype isolates from Kerala. Bar, 0.02 nucleotide substitutions per site.
from Kottayam District, 17 from Kasaragod District, 12 from Alappuzha District, 5 from Kozhikode District, 4 from Thrissur District and 1 from Malappuram District of Kerala state. Among these, we recorded 29 cases (Supplementary Table S1, available in the online journal) that were DENV infected (positive by DENV diagnostic RT-PCR). These positive specimens were obtained from four districts, Kottayam (19), Kozhikode (5), Kasaragod (4) and Alappuzha (1) (Supplementary Fig. S2 available in the online journal). The numbers of positive specimens recorded for different years (2008, 2009 and 2010) were 1, 8 and 20, respectively. The year-wise distribution of serotypes of the virus recorded was: DENV-3 in 2008, DENV-2 and DENV-3 in 2009, and all four serotypes in 2010. Among the 29 DENV-positive samples, the major serotype recorded was DENV-2 (n=15; 51.72%), followed by DENV-3 (n=12; 41.38%). One case each of DENV-1 and DENV-4 were also isolated. Kottayam District recorded all four serotypes in 2009–2010, whilst in 2009 DENV-3 was recorded and in 2010 DENV-2, DENV-1 and DENV-4 were recorded. Kozhikode and Kasaragod Districts recorded co-circulation of both DENV-2 and DENV-3. The case from Alappuzha District (imported from Trivandrum District) was found to be DENV-3. The phylogenetic analysis of the CprM gene of all 29 DENV-positive samples along with representatives from GenBank of the different serotypes is shown in Fig. 1. The phylogenetic analysis based on nucleotide sequences of the CprM gene for DENV-1 (Fig. 2), DENV-2 (Fig. 3) and DENV-3 isolates (Fig. 4) determined these serotypes to be genotypes III, IV and III, respectively.

The lone DENV-4 isolate was found to be of genotype I, based on analysis of the envelope full-gene sequence (Fig. 5). No multiple genotypes were recorded for any of the four serotypes circulating in Kerala State.

**DISCUSSION**

The incidence of dengue fever, a major emerging arboviral disease transmitted by *Aedes* mosquitoes, has been increasing in India, and in Kerala State in particular, since 2001. Dengue cases had a restricted geographical distribution in Kerala during 1997–2002. A major outbreak of this arboviral disease occurred during 2003 in this state. Since then, all 14 administrative districts of the state continue to have disease outbreaks during the pre-monsoon season (May–July). Since 2007, the number of dengue cases reported in the state has been increasing, with the maximum number of cases recorded in 2010 (2597 cases), which made it the third most affected state in the country. Kerala has an abundant *Ae. albopictus* population (Kumar et al., 2011), an established vector for dengue fever in Asia (De Figueiredo et al., 2010; Vasilakis et al., 2011), and restricted prevalence of *Ae. aegypti* in urban situations. Kottayam District from which dengue cases have been reported since 1997 continues to record cases of dengue every year with an increasing trend in the number of cases. In the present study, we recorded all four serotypes circulating in Kottayam District, Kerala State. We found the DENV-3 serotype circulating in Kerala to be of

![Fig. 4. Genetic analysis of CprM gene sequences of DENV-3 serotype isolates from Kerala. Bar, 0.005 nucleotide substitutions per site.](http://jmm.sgmjournals.org)
Genotype III (Fig. 4). Genotype III of DENV-3 is of Indian origin (Messer et al., 2003), and this strain has been suspected to cause more fatal infections (Dash et al., 2005). Two lineages of this genotype were recorded in the present study in Kerala. That recorded in 2008 from Alappuzha, which was an imported case from Trivandrum District, had a different lineage compared with the other 11 isolates recorded from Kottayam, Kozhikode and Kasaragod Districts (Fig. 4). The former was found to be more closely related to Singapore and Sri Lankan strains, whilst the latter isolates had a lineage related to Indian strains reported from Delhi and Gwalior. All the isolates from Kottayam District branched out as a separate cluster with threonine at position 112 of the structural polyprotein instead of alanine found in all other Indian strains of genotype III isolates. The evolutionary significance of this amino acid mutation in the 3' end of the capsid gene requires further analysis.

The DENV-2 isolates from Kerala did not show any specific fingerprints on their CprM genome sites (Fig. 3) and belonged to genotype IV with a lineage genetically related to the widely circulated genotype in North India such as the Gwalior and Delhi strains (Dash et al., 2004). This serotype and genotype has been recorded to be the most predominant one in the country. One of the cases (a 26-year-old female) infected with this serotype included in the study had severe manifestations resulting in mortality.

DENV-1 belonged to genotype III (Fig. 2), closely related to the Delhi strains reported in 2006 (Kukreti et al., 2009). This serotype has been reported to be circulating in different regions of the country to a lesser extent since its first report from Vellore in south India in 1956.

DENV-4 belonged to genotype I, as described above (Fig. 5). This rare serotype in India was reported recently from Maharashtra (Cecilia et al., 2011) and Andhra Pradesh (Dash et al., 2011). The disease caused by this serotype was described to be severe (grade III) in the first study: one case, which was a primary infection survived, whilst another with a secondary infection died. The second study

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**Fig. 5.** Genetic analysis of envelope gene sequences of DENV-4 in this study. Bar, 0.01 nucleotide substitutions per site.
reported an increasing trend of DENV-4 infections in the state. The case reported by us was collected in 2010. The records of DENV-4 from Kerala State indicate the spread of this serotype in the country after a reported long absence. Analysis of the envelope sequence of the virus isolate showed two specific non-synonymous mutations, 132V and 366S. Both of these mutations were also reported in the Hyderabad strain from 2007 (GenBank accession no. HM237348; Dash et al., 2011), showing a probable lineage relationship of the Kanjirapally isolate to this Hyderabad isolate recorded in 2007. The latter mutation among these was found in the proposed immunodominant gene region of aa 333–368. Among the five sites that have been reported to be selected positively in DENV-4 (Twiddy et al., 2002), our isolate showed only two of these sites (429L and 494Q), similar to the other two isolates reported from India.

No genetic data are available for DENV serotypes in Kerala prior to 2008. A single recent report highlighted the prevalence of DENV-2, DENV-3 and DENV-1 serotypes in a hospital-based study from Ernakulam City (Anoop et al., 2010). Previously, it was reported that DENV-2 was the major serotype circulating in India (Dar et al., 1999; Kumar et al., 2001). Recently, DENV-3 has emerged as the major serotype in different parts of the country (Dash et al., 2005). Due to a lack of studies in Kerala before the 2008 outbreak on the genetic characterization of the virus, we are unable to arrive at a conclusion on the evolutionary trends of different serotypes in Kerala. However, the present study showed that all four serotypes are prevalent in the state and that serotypes 2 and 3 may be the major serotypes involved in the outbreaks. The large-scale movement of people to and from Kerala for multifarious activities including pilgrimage, tourism, employment and education could have contributed to the prevalence and spread of all four DENV serotypes in the state.

Kerala State has a unique topography, with the Western Ghats (the second largest mountain range in India) on its eastern side and the Arabian Sea on its western side. Both south-west and north-east monsoons are active in the state with an annual mean rainfall of >3000 mm. Thick forests with an altitude of >800 m constitute about 27.81 % of the state. Only 12.0 % of the area constitutes coastal land (mean sea level 0–150 m). Ae. aegypti is restricted mainly to the urban settlements in this coastal region. The rest of the area is composed of the semi-forested plantation belt of Kerala where crops such as rubber, pineapple, areca and spices are cultivated. Ae. albopictus has been found to be abundant in the semi-forested plantation sector in Kerala (Kumar et al., 2011), and this species has been already proposed as a bridge vector of sylvatic and human cycles in Malaysia and other south Asian countries (Wang et al., 2000; Cardosa et al., 2009). Thus, spill over of sylvatic cycles to vast semi-forested regions (where Ae. albopictus is the most predominant species) and to coastal regions in Kerala (where Ae. albopictus is prevalent) cannot be ruled out in Kerala during the summer season (with intermittent rainfalls) when the population density of this vector species is at a peak level (Kumar et al., 2011), contributing to dengue outbreaks in the state.

Co-circulation of all the serotypes of DENV in a region as shown in Kerala State can be considered as an important contributing factor to the severity of dengue fever (WHO, 2009). Moreover, the widespread prevalence and abundance of Ae. albopictus in this state suggests a dismal scenario in terms of the health situation of the state. Although the vector capacity of Ae. albopictus had been proposed to be comparatively less than that of Ae. aegypti for DENV (Lambrechts et al., 2010), a scenario in which DENV may undergo crucial mutations that could be naturally selected to overcome this disadvantage, as was recorded for CHIKV during the recent widespread outbreak in Kerala (Kumar et al., 2008), remains a possibility for the dengue situation in this part of India.

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REFERENCES


