Genetic characterization of dengue virus serotypes causing concurrent infection in an outbreak in Ernakulam, Kerala, South India

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Dengue fever, a mosquito-borne viral infection, causes significant morbidity and has become endemic in the Indian subcontinent. Virus strains currently circulating in many parts of the country are not well studied at the molecular level. In the present study, genetic characterization of virus strains from a dengue outbreak that occurred in and around a tertiary care hospital in Ernakulam, Kerala in the year 2008 has been reported. By reverse transcription polymerase chain reaction (RT-PCR), 37 out of 75 (49.3%) clinically suspected cases were positive for dengue viral RNA. Among these, 21 (56.8%) samples showed concurrent infection with multiple serotypes of the virus. Majority of the combined infections were caused by dengue serotype 2 and 3. Co-infections with type 1 and 2 in two patients, and type 1, 2 and 3 in one patient were also observed. The core-pre-Membrane (CprM) junction nucleotide sequencing and phylogenetic analysis revealed that the type 1 strains were related to the viral strains reported from Delhi-2001 and Gwalior-2002 dengue outbreaks, while the type 2 strains were related to the strains from Gwalior-2001 epidemic. Sequences of type 3 strains did not show clear relation to any of the previous Indian isolates, and in the phylogenetic analysis, they formed a distinct lineage within the Indian type 3 strains. This study indicates hyperendemicity of dengue in the region with the presence of multiple serotypes and high rates of co-infection, and local genomic evolution of the viral strains involved in this outbreak.

Keywords: Co-infection, Dengue, Genotyping, Multiplex RT-PCR

Infection by dengue virus, a positive stranded RNA virus belonging to the family Flaviviridae, is a leading cause of morbidity in the tropical and subtropical areas. It affects around 50 million people globally. Most parts of the Indian subcontinent are endemic to dengue and all the four serotypes of the dengue virus have been reported from India. Primary infection caused by any of the four serotypes of dengue virus usually results in dengue fever (DF), a benign disease. Secondary dengue infections can lead to life-threatening dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS). The phenomenon of antibody dependent enhancement (ADE) of viral replication is considered as a major reason for severity of secondary dengue infections. The genotype of the virus causing the disease and the host genetic factors, such as polymorphisms in HLA and other candidate genes have also been implicated in pathogenesis of severe dengue.

Dengue outbreaks wherein combined infection of the same patient with more than one serotype occurs have been identified and in such cases different combinations of the viral serotypes were observed as the cause of concurrent infection. The rates of concurrent dengue infections varied from 5.5% in Puerto Rico, 9% in Southern Taiwan to 19% in Northern India. It is hypothesized that concurrent infection predisposes the affected patients to develop DHF or DSS.

Disease outbreaks caused by dengue virus and presence of the virus in the Aedes vector mosquitoes in Kerala have been previously reported. However, there is no information on serotypes and genotypes of the virus circulating in this part of the country. This information would be useful in understanding the dengue virus evolution, and may help in correlating disease severity to viral serotypes or genotypes during future outbreaks. In the present investigation, molecular typing of the virus involved in a recent outbreak of dengue fever in Ernakulam in

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Kerala state was carried out by reverse transcription-polymerase chain reaction (RT-PCR) and nucleic acid sequencing. Interestingly, the study revealed the involvement of three viral serotypes, Type 1, Type 2 and Type 3 in this outbreak and concurrent infection in a large number of patients.

Materials and Methods

Clinical Samples—Serum samples of 75 patients admitted to General Hospital, Ernakulam, Kerala during a fever-outbreak in November, 2008 with high fever for ≤ 7 days, and two or more of the associated symptoms like headache, back-ache, retro-orbital pain and myalgia were used in the study. They were clinically diagnosed as dengue fever as per WHO guidelines. Samples were collected under informed consent and were processed as per procedures approved by the Institutional Bio-safety Committee.

Dengue virus specific RT-PCR—Dengue viral nucleic acid detection was done by multiplex RT-PCR as previously reported. The single-step RT-PCR mix contained 1× FideliTaq RT-PCR buffer (USB Corp., USA), 4 µl viral RNA isolated using QiaAmp Viral RNA kit (Qiagen, Germany), 15 picomoles each of the forward (D1F) and reverse primers NTS-1, NTS-2, NTS-3 and NDEN-4 (Table 1) in a 25 µl volume. An initial reverse transcription for 1 hr at 42°C was followed with a denaturation step at 94°C for 2 min, and 35 PCR cycles with denaturation at 94°C for 30 sec, primer annealing at 60°C for 1 min and extension at 68°C for 1 min. PCR contamination was avoided by spatially separating the pre- and post-amplification steps and to detect possible contamination, a no-template, negative control was incorporated in all the PCR reactions. Amplified products were checked by 1.5% agarose gel electrophoresis, and the gel purified PCR products were directly sequenced using the specific primers using the Big Dye Terminator kit (Applied Biosystems, USA) as per the manufacturers directions in an ABI 3730 Genetic Analyzer automated DNA sequencer.

RT-PCR amplification of Den-2 CprM region—A 654bp fragment of the CprM region was amplified from Den-2 positive samples using the primers D1F and DencomR2 (Table 1) by RT-PCR, which was sequenced as described above. DencomR2 primer is a degenerate primer designed based on the consensus nucleotide sequences of the CprM region of all the four dengue serotypes available in the GenBank.

Phylogenetic Analysis of Dengue virus CprM sequences by Bioinformatics—The sequences were analyzed using BioEdit program. Phylogenetic analyses were carried out by Neighbor-Joining (NJ) method using the MEGA 3.1 programme with Kimura-2 parameter distance correction and 10000 boot-strap replications.

Results

RT-PCR detection of Dengue viral nucleic acid—Of the 75 samples tested by multiplex RT-PCR, 37 (49.3%) were positive for dengue viral RNA. This multiplex PCR using the type-specific primers were originally designed to amplify a 489bp fragment for Den-1, 123bp for Den-2, 296bp for Den-3 and 395bp for Den-4 from the C-prM region of the Dengue viral genome. So, based on the amplification product size 16 samples (43.2%) showed infection with a single serotype (Serotype 2). The remaining samples (21 nos., 56.75%) showed co-infection with more than one serotype (Fig. 1). Presence of RNA for dengue serotype 2 and 3 were detected in 18 of these samples. Samples from 2 patients showed presence of Type 1 and 2, whereas sample from one patient showed presence of three serotypes viz. Type 1, 2 and 3.

Comparative sequence analysis of the amplified CprM region—A 362-bp nucleotide sequence analyzed from this region was identical in all the three

Table 1—List of primers used in the study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
<th>Location (with respect to ref. sequence)</th>
<th>Reference sequence (GenBank Ac. No)</th>
<th>Reference (Publication)</th>
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<tr>
<td>D1F</td>
<td>TCAATATGCTGAACGCGAGAAACCG</td>
<td>132-159</td>
<td>NC_001477</td>
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<tr>
<td>DecomR2</td>
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<td>783-765</td>
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<tr>
<td>NTS1</td>
<td>CTGGTTCCGGTCTCATGATCCCCGGGG</td>
<td>620-595</td>
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<td>NTS2</td>
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<td>AY858096</td>
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<td>NTS3</td>
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<td>NC_001475</td>
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<td>NDen4</td>
<td>CTCTGTGTTCTAAAACAAGAGGCTC</td>
<td>527-502</td>
<td>NC_002640</td>
<td>Harris et al., 1998</td>
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</table>
Den-1 positive samples. With the prototype Den-1 strains, the sequence had 96.96% identity with 1956 Indian prototype (P23086; GenBank Accession No. EU626489) and 95.58% identity with the Singapore 8114/93 prototype (GenBank Accession No. AY762084). Maximum homology of the Ernakulam (Ekm) Den-1 sequence (98.34% at nucleotide level and 99.16% at predicted amino acid level) was to that of strain F37.D1.05 (GenBank Ac No. EU005247) that was isolated in Finland from a dengue fever patient returning from India. With other closely related Indian strains (Fig. 2), the sequence had an average nucleotide level identity of 97.79%, and predicted amino acid level identity and similarity of 97.9%. The A143V non-synonymous substitution in the prM region was a common change observed in the predicted amino acid sequence in Ekm Den-1, GWL-14, D1/1 and F37.D1.05 strains (Fig. 2). The only unique predicted amino acid change in Ekm Den-1 was the M48K substitution in the capsid protein region sequence.

As the small fragment of the Den-2 CprM (123 bp) amplified in the multiplex PCR does not give much information in sequence analysis, a 654bp CprM

Fig. 1—Multiplex RT-PCR-based dengue typing profiles showing single and combined infections. Representative results are shown. M: 100bp ladder (NEB). Sample numbers are indicated.

Fig. 2—Amino acid sequence alignment of Ekm Den-1 strain, selected Indian strains and reference strain-Singapore 8114/93. GenBank accession number of the strains, followed by the strain name is given. Identical amino acids are represented by “.” Amino acids are numbered with respect to the polyprotein. Identified genotypes and lineages in Den-1 strains are also indicated.
region was RT-PCR amplified and sequenced from the sixteen samples that showed infection with Den-2 alone. Sequence of a 350bp region (nucleotide positions from 215 to 564, with respect to the Den-2 reference sequence New Guinea C; GenBank accession No.AF038403) of five representative samples with unique sequences (Table 2) was used in the analysis. At nucleotide level, four of the Ekm strains (RGCB432, 457, 488 and 507) had 92.76% identity, whereas the strain RGCB510 had 94.28% identity, with the New Guinea C strain. The average nucleotide level identity of Ekm Den-2 strains with that of Indian strains were 98.7%. The number of nucleotide substitutions in these strains varied from 20-26 with respect to the reference strain, and most of them were synonymous changes. Ekm Den-2 strains had the M104V and V112A change as in other Indian Den-2 strains (Fig. 3). A unique amino acid substitution was observed at position 119 (T to R) in the prM region in the strain RGCB507.

Den-3 positive samples in the study had co-infection with other serotypes. So a 242bp sequence (from nucleotides 186 to 427, with respect to the Den-3 reference sequence H-87, GenBank Accession No.M93130) closer to the CprM junction, obtained directly from the 296bp amplified region in the multiplex PCR, was used in the analysis. Ekm Den-3 sequences had a nucleotide level identity of 92.9% with the H-87. With selected Indian Den-3 strains (Fig. 4), Ekm strains had a mean identity of 97.8% at the nucleotide level. In the predicted amino acid sequence, the K86R and M108I changes that were observed in most of the Indian Den-3 strains were not seen in the Ekm strains (Fig.4).  

**Phylogenetic analysis of Dengue sequences**—In the phylogenetic analysis (Fig. 5), Den-1 sequence from our study fell into the Den-1 (genotype III) cluster, and grouped together with the 2001 isolates from Delhi (D1/1 and D1/2) and one of the 2002 isolates from Gwalior (GWL-14), within the India-4 lineage. Analysis of the Ekm Den-2 strains and other Indian Den-2 strains revealed that the Ekm strains formed a distinct cluster within the Indian Genotype IV strains, showing a closer relation to the 2001 isolates from Gwalior outbreak. Within the Ekm Den-2 strains, the RGCB510 showed more divergence and formed a separate clade. In the phylogenetic tree, all the Ekm Den-3 sequences analysed, except RGCB486, formed a separate lineage clearly discreet from other reported Indian Den-3 sequences (Fig. 5). This separation was supported with a high bootstrap value (94%), indicating that these strains are genetically distinct. This clade formation resulted from the presence of two conserved amino acids (lysine at position 86 and Methionine at position 108 of the capsid protein) in the Ekm Den-3 strains and the reference Den-3 strain (H-87) of 1990, whereas in the North Indian strains they were replaced with Arginine and Isoleucine, respectively (Fig. 4). The RGCB486 sequence that showed more divergence was placed in a separate branch together with GWL-60 strain. Ekm strains indicated closer relation with a 2003 isolate from Puerto Rico (GenBank Accession No. FJ390372). However, the boot-strap support for this relation was very low (Data not shown). All the three Ekm strains (Den-1, Den-2 and Den-3) showed the same genetic relationship even in the analysis that included sequences of isolates from different parts of the world (Data not shown).

<table>
<thead>
<tr>
<th>S. No</th>
<th>Strain name</th>
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<th>Corresponding Serotype</th>
<th>Type of infection</th>
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<tbody>
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<td>GQ340957</td>
<td>Type 1</td>
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<td>Co-infection; 2 &amp; 3</td>
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</table>
**Discussion**

In recent years, Kerala has witnessed widespread outbreaks of chikungunya\(^{25,26}\) and also an increase in the number of dengue\(^{27}\), two major mosquito-borne viral infections. The present study conducted molecular characterization of the virus involved in a Dengue outbreak within and around a tertiary care hospital in Kerala by RT-PCR and virus genotyping. Though conventionally the envelope (E) protein coding region is used for genotyping of the dengue viral serotypes\(^{28}\), a recent study found that sequences of most of the viral genes are suitable for the purpose\(^{29}\). In this study, *CprM* junction region was used for genotype analysis as reported in previous studies of Indian strains\(^{30-32}\).

The study revealed involvement of Serotypes 1, 2 and 3 in the outbreak. Nucleotide sequencing and phylogenetic analysis indicated that the Den-1 and Den-2 strains identified in the present outbreak were predominantly introduced from Northern India. Irrespective of their minor genetic heterogeneity, a close clustering with the Delhi and Gwalior isolates was observed in the analysis. Interestingly, the Den-3 strains involved in the outbreak revealed a greater divergence from other Indian Den-3 strains (Fig. 5), as evidenced by formation of a new clade. Emergence of new clades or lineages of dengue viral strains is considered as a frequent process in dengue endemic areas\(^{33}\). But understanding whether this new lineage of Ekm Den-3 strains circulating in Kerala has emerged locally from the previous Indian strains, or was introduced from other parts of the world would require further analysis of larger genomic regions of the virus. In any case, as reported previously, the persistence or disappearance of these new clades will depend upon the co-circulation of other serotypes in a locality and immune pressure from the host population\(^{33}\).

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**Fig. 3**— Amino acid sequence alignment of Ekm Den-2 strains with that of selected Indian strains and reference strain- New Guinea C. GenBank accession number of the strains, followed by strain name is given. Identical amino acids are represented by “.” Amino acids are numbered with respect to the polyprotein. Previously identified Den-2 genotypes are indicated.
Another striking observation in the study was that majority of the patients (56.75%) had co-infection with multiple serotypes of the virus, with serotypes 2 and 3 as the major strains involved in co-infection. Though it is the first observation of concurrent infection from Kerala, previous studies have documented concurrent multiple-serotype Dengue viral infection in Northern India. The major combination of viral serotypes observed in the North Indian outbreak was serotype 1 and 3 (44%), followed by 1 and 4 (22%). Also, single cases of Den-2 and Den-3 combination were previously reported in Indonesia; in southern Taiwan; in Ceara, Brazil and also in Delhi, India. One patient in the present study had concurrent infection with three serotypes (Den-1, Den-2 and Den-3). Similar incidence has been previously reported in Thai children.

It would be interesting to look into the circumstances that led to a high incidence of concurrent infection in this outbreak in Kerala. Co-circulation of the various virus serotypes and very high vector prevalence could be the probable causes. Prevalence of both Ae. aegypti and Ae. albopictus mosquitoes, which are the main vectors of the virus, across the state have been documented.

Previous studies from other parts of the world have shown that these vectors can get simultaneously infected with more than one serotype of dengue virus. Mainly, the feeding behavior of these mosquitoes, characterized by the multiple blood-meals in a single gonotrophic cycle, enables them to acquire different serotypes of virus during each feeding in a disease hyperendemic area. Vector surveillance studies also confirmed the presence of both species of mosquitoes in the locality, and entomological investigations showed very high vector indices (house/breteau/container indices) within the hospital premises. However, further studies are essential to prove that co-infection of mosquito vectors were a contributing factor for the concurrent infection in this outbreak.

Co-circulation of multiple serotypes of dengue virus is considered as the most common factor contributing to emergence of severe and fatal dengue in endemic areas. Coupled with a high vector index, this could lead to concurrent infection of the same patient with multiple serotypes of the virus, which might alter clinical expression of the disease. In the present outbreak the disease severity was mild and there were no case fatality, in spite of the combined
Fig. 5—Phylogenetic tree of CprM nucleotide sequences of Ernakulam and other Indian Dengue strains. Scale bar represents amino acid substitutions per site. Sequences identified in the current study (▲) are indicated by arrows. The tree was reconstructed by Neighbor-Joining method, with 10,000 bootstrap replications. Bootstrap values >50% are shown at the nodes. GenBank accession number of strains, followed by strain name is given for Indian strains. Genotypes identified in each of the serotypes are also indicated.
infections and also the presence of unique genetic changes in the circulating virus. Only one patient, who had a combined infection with Den-2 and Den-3, and negative for serum anti-dengue IgG antibody, developed hemorrhagic fever; but recovery was uneventful.

In conclusion, the results of the study revealed that multiple serotypes of dengue virus are co-circulating in certain areas of the state, such as Ernakulam, making these places hyperendemic to infection. The viral strains circulating in these parts are genetically similar to the ones characterized from other parts of the country. Genetic alterations observed in these viruses indicate that these strains have been circulating in the area for longer periods, undergoing micro-evolutionary changes. The incidences of combined infection with multiple serotypes, and emergence of newer strains with genetic variations could alter the disease profile in future dengue outbreaks in Kerala.

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