Original Research Article

Molecular detection of dengue virus serotypes prevalent in central Kerala and its correlation with disease severity

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ABSTRACT

Background: Dengue is the most important mosquito-borne viral disease in the world. In 2017, a concerning increase in dengue cases with high mortality was seen in India with Kerala topping the list. Changing pattern of circulating dengue virus serotype and co infection with multiple serotypes contribute to the increasing trend in severity and increased mortality of dengue fever. The present study focused to find the dengue serotypes prevalent in central Kerala and prevalence of co infection with multiple serotypes here with an attempt to correlate these with clinical severity of dengue

Materials and Methods: This descriptive study was done in the Microbiology department of Jubilee Mission Medical College, Thrissur for a period of 18 months. Blood samples of clinically suspected dengue fever cases which were positive for NS1 antigen and having fever not more than 5 days were subjected to molecular methods to detect dengue virus RNA followed by multiplex RTPCR to find its serotype. Demographic features, clinical details and lab parameters were also collected from each patient.

Results: A total of 108 samples positive for Dengue NS1 antigen were subjected to RT-PCR. Among them 61(56.5%) were positive by RT-PCR. All the 4 Dengue serotypes were found with DENV-2 with 30 cases (49.2%) being the predominant serotype followed by DENV-1 with 20 cases (32.8%), DENV-3 with 12 cases (19.6%) and DENV-4 with 2 cases (3.27%). Three cases (4.9%) of co infection with DENV-1,2, DENV-1, 4 and DENV-2,3 were also found. Two among the 108 patients (1.85%) died in the present study. Significant correlation between severity of dengue infection and serotypes could not be found due to very few severe cases encountered during the study.

Interpretation and Conclusion: The dengue serotypes predominant in central Kerala is DENV-2. The prevalence of co infection with multiple serotypes is 4.9% which proves that this area has become hyper endemic to the disease, which increases the risk of emergence of more severe cases.

1. Introduction

Dengue is the most important arthropod-borne viral disease of public health significance. The viral illness is caused by one of the four closely related but antigenically distinct serotypes of the dengue virus DENV; DENV-1 to DENV-4 which is transmitted by Aedes mosquitoes. Infections can result in a wide spectrum of disease severity ranging from an influenza-like illness to the life threatening dengue hemorrhagic fever/dengue shock syndrome. Recovery from infection by one provides lifelong immunity against that particular serotype. However, cross-immunity to the other serotypes after recovery is only partial and temporary. Subsequent infections by other serotypes increase the risk of developing severe dengue.

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In 2017, an alarming increase in dengue cases was seen in India with an increased rate of mortality. Kerala was at the top of the list which was having the highest number reported from India. 19994 cases of dengue were reported in Kerala and 37 death occurred.[1] The increasing trend in the mortality rate is due to the complicated forms of the disease.

However, there are relatively few studies on serotypes and genotypes of the virus circulating in central Kerala and on their correlation with the severity of the disease. Also information on the prevalence of co infection with multiple serotypes and its role in disease severity is scarce. The factors associated with the recent trends in increasing mortality in dengue fever are yet to be explored. So this study is carried out to find out the circulating serotypes of dengue virus in our geographic area and association with clinical severity which would be useful in planning preventive and therapeutic strategies in dengue fever.

2. Materials and Methods

This descriptive study was conducted in the Department of Microbiology, Jubilee Mission Medical College and Research Institute, Thrissur which is an 1800 bedded tertiary care teaching hospital from July 2018 to December 2020. Institutional ethics committee approval was obtained prior to the study.

The study included all cases of clinically suspected dengue fever with onset of fever not more than 5 days which were positive by Dengue NS1 antigen testing by ELISA.

Sample collection: Four ml of blood sample was collected from all Dengue NS1 antigen positive patients, serum separated, labelled and stored in eppendorf tubes at -80ºC. Details on demographic features, history and clinical examination of these patients were obtained directly from the patients and from the clinical records. Various laboratory parameters were also monitored and recorded.

Viral RNA isolation: RNA was extracted from the serum samples using MN kit viral RNA extraction columns as per manufacturer's instruction (TRIzol method). Approximately, 150 μl serum samples were used for extraction of viral RNA following the manufacturer's protocol. After the steps for lysis, the samples were passed through columns where silica membrane technology was used to extract the viral RNA. The extract was stored at -70 °C until further processing.

cDNA synthesis and PCR amplification: Dengue RNA detection was done by an in-house RT-PCR that amplifies a 654 bp Core-Pre-membrane (C-PrM) coding region of RNA. Two μl of heat inactivated RNA was added to the master mix. An initial reverse transcription for 45min at 45°C was followed with a denaturation step at 95°C for 2 min, and 40 PCR cycles with denaturation at 95°C for 30 sec, primer annealing at 55°C for 1 min and extension at 68°C for 1 min. A final extension follows at 65°C for 5 min and finally termination at 4°C. PCR was done along with positive and negative controls. Amplified products were subjected to 1% agarose gel electrophoresis and observed under a gel documentation system.

Serotype identification: The dengue serotype detection was done in second round multiplex nested PCR with 1μl of 1:50 diluted positive first PCR product using forward primers and type specific primers. Briefly, the protocol was as follows: an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension follows at 72°C for 2 min and finally termination at 4°C. Detection: The PCR products were analyzed by electrophoresis with 1% agarose gels in TBE buffer. The gels were stained with ethidium bromide (75 μl in 500ml distilled water) and PCR products were visualized with UV light using gel documentation system. Because of the position of priming with each of the dengue virus type-specific primers, the size of the resulting DNA band was characteristic foreach dengue virus type (DENV1→482bp, DENV2→119bp, DENV3→290bp, DENV4→389bp).

The results of PCR and details of patients collected were compiled and statistically analysed.

3. Results

During the study period a total of 1375 blood samples were received from clinically dengue suspected cases, of which 123 came to be NS1 antigen positive by ELISA. Out of the 123 samples, 108 were enrolled in this study satisfying the inclusion and exclusion criteria. Seventy four (68.5%) of them were males and 34 (31.5%) were females. Sixty seven(62%) cases belonged to the 20-50 years of age group (Table 3). The geographical distribution and various comorbidities of these patients are shown in Table 3.
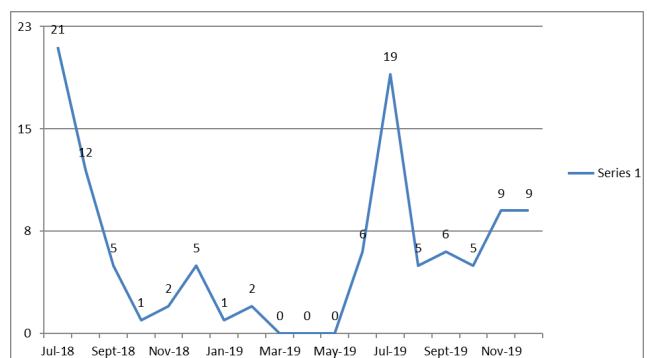

Fig. 1: Month wise distribution of NS1 positive cases

Seasonality of transmission of dengue with increased activity in the monsoon season (June-August) was observed and the highest number of cases was recorded in the month of July both in 2018 and 2019.(Figure 1)

Among the 61 RT PCR positive samples subjected to multiplex RT PCR for serotyping, 20(32.8%) were

Table 1: Primers used for dengue viral RNA detection

Primer name	Sequence (5'→3')	Amplicon size (bp)	Location (with respect to the ref. sequence)	Reference sequence (GenBank accession no)	Reference (publication)
D1F	TCAATATGCTGAACGCGCG AGAAACCG		132–159	NC_001477	2
DencomR2	GCNCCTTCD GMNGACATCC	654	783–765	NC_001477	3

Table 2: Primers used for dengue viral RNA serotype detection

Primer name	Sequence (5'→3')	Amplicon size (bp)	Location (with respect to the ref. sequence)	Reference sequence (GenBank accession no)	Reference (publication)
nTS1	CTGGTTCCGT CTCAGTGATCCGGGGG	489	620–595	NC_001477	4
nTS2	AACGCCACA AGGGCCATGAACA	123	254–233	AY858096	
nTS3	TGCTGGTAAC ATCATCATGA GACAGAGCG	296	427–399	NC_001475	
nDen4	CTCTGTTGTC TTAAACAAGAGAGGTC	395	527–502	NC_002640	5

Table 3: Demographic characteristics of NS1 positive study group

Parameters	Frequency (%)
Age (years)	
<20	17(15.7)
20-50	67(62)
>50	24(22.2)
Geographical Distribution	
Thrissur	87(80.5)
Palakkad	15(13.88)
Idukki	4(4.3)
Ernakulam	1(1.1)
Kottayam	1(1.1)
Comorbidities	
Hypertension	18(16.6)
Diabetes mellitus	13(12)
Dyslipidemia	4(4.3)
Chronic liver disease	2(2.1)
Others	8(7.4)

Table 4: Prevalence of dengue virus serotypes and co infection

Serotypes	PCR positives	DENV 1	DENV 2	DENV 3	DENV 4	Co Infection
Total Samples	61(56.5%)	20(32.8%)	30(49.2%)	12(19.6%)	2(3.27%)	3(4.9%) DENV 1, 4 DENV 1, 2 DENV 2, 3

identified as DENV-1, 30(49.2%) as DENV-2, 12(19.6%) as DENV-3 and 2(3.27%) as DENV-4. Three (4.9%) cases were co infected with two serotypes; DENV-1,2 DENV 1,4 and DENV 2,3. (Table 4)

Clinical features like fever, vomiting, sore throat, headache, rash, retro orbital pain, abdominal pain, lethargy, arthralgia and myalgia were noted and percentage of patients with the clinical feature in each serotype was calculated separately. (Table 5)

Laboratory parameters including hematological parameters and biochemical parameters were noted and median value of all samples belonging to each serotype was calculated separately as shown in Table 6.

4. Discussion

Dengue fever, a mosquito-borne viral infection, causes significant morbidity and has become endemic in the Indian subcontinent. The incidence of dengue fever has been increasing in Kerala state, since 2001. From 1997–2002 dengue cases had a restricted geographical distribution in Kerala. But since 2003, after a major outbreak, all districts of the state continue to have disease outbreaks during the pre monsoon- 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. By 2017, 19994 cases of dengue were reported in Kerala and 37 death occurred. By 2019 cases had declined to 4652 and death cases were 16.[1] Kerala has an abundant Aedes albopictus population, an established vector for dengue fever in Asia, and restricted prevalence of Aedes aegypti in urban situations.[4]

Dengue viruses occur as four antigenically distinct serotypes. Infection with any of them generally leads to a mild, self-limiting febrile illness (dengue fever). However, a more severe form of the disease, involving vascular and hemostatic abnormalities (dengue hemorrhagic fever-dengue shock syndrome), occurs when infected with another serotype and it is responsible for a high mortality rate. Serotyping is necessary so that appropriate prevention, treatment, and control measures can be initiated and accurate epidemiologic data can be maintained.

The present study aimed at molecular detection of Dengue virus followed by identification of the serotypes of the virus prevalent. We also attempted to find out whether there was any correlation between severity of the illness and the virus serotypes or co infection with multiple serotypes.

A total of 1375 samples were received from clinically dengue suspected cases during the study period, of which 123 came to be NS1 antigen positive. Out of the 123 samples, considering cases with onset of fever not more than 5 days, 108 were enrolled in this study. Among them 74(68.5%) samples were from males and 34(31.5%) from females. Thus majority of samples were from males.

Greater male exposures to dengue-carrying mosquitoes during daytime hours either at the workplace or while travelling to and from work might be the reason for this.[6]

Majority of patients were in the age group between 20-50 years (n=67; 62%). Another study conducted in India shows a higher recurrence of the disease in age group above 20 years.[7] In the various studies reporting epidemics, it was seen that children <15 years of age were quite severely affected, but majority of infection occurred in active adults in the age group of 21–60 years.[8]

In the present study samples were obtained from patients coming from different parts of central Kerala including the districts of Thrissur, Palakkad, Ernakulam, Idukki, Kottayam. Majority of the samples 87(80.5%) were from Thrissur followed by Palakkad with 15(13.88%) samples, Idukki with 4(4.3%) samples and Ernakulam and Kottayam with one sample each(1.1%). Majority of the cases were from Thrissur district as the study centre is located here though it caters to neighbouring districts also.

Majority of the patients were from urban areas (n=74;68.5%) than from rural areas (n=34;31.5%). Studies related to entomological analysis of dengue done in Ernakulam district in 2019 also showed similar results.[9] The increase in vector density due to the developmental activities, population growth, environmental and ecological changes in the urban areas might be the reason in contributing to disease burden.[9] The density of dengue vectors in the urban areas of the district would give an insight into the extent of the transmission potential of the diseases.

Seasonality of transmission of dengue with increased activity in the monsoon season (June-August) was observed and the highest number of cases was recorded in the month of July with 21(19.4%) positive samples in 2018 and 19 (17.59%) samples in 2019. These findings also correlate with that of the previous other studies conducted in Kerala, which reported highest number of cases in monsoon.[4] Thus the correlation between occurrence of dengue and monsoon season is clearly evident in all these studies and is further supported by similar findings from Ludhiana.[8] The temperature plays a significant role in dengue transmission.[10] The most suitable temperature for dengue transmission is 28.7°C.[10] Rainfall provides breeding sites for the mosquitoes to hatch and develop into the adult stage. Both factors have a significant impact to the mosquito population and the dengue transmission dynamics.

In the present study we also analyzed co morbidities present in dengue patients. Hypertension and Diabetes mellitus (16.6% and 12.03%) were the most prevalent co morbidities among the present study group. Similar findings were found in other studies also.[11] Recent studies have raised the proposition that co morbidities like hypertension, diabetes mellitus, stroke etc. may contribute, together with

Table 5: Correlation of clinical features with dengue serotypes in PCR positive patients

Symptoms	PCR positivity NO:	DENV 1NO: (%)	DENV 2NO: (%)	DENV 3NO: (%)	DENV 4NO: (%)
Fever	61 (100)	20 (100)	30 (100)	12 (100)	2 (100)
Vomiting	20 (32.7)	5 (25)	13 (43.33)	3 (25)	-
Sore throat	5 (8.19)	2 (10)	2 (6.66)	1 (8.33)	-
Headache	42 (68.8)	16 (80)	20 (66.66)	5 (41.66)	2 (100)
Rash	4 (6.55)	3 (15)	1 (3.33)	-	-
Retro orbital pain	20 (32.7)	6 (30)	11 (37.9)	4 (33.33)	1 (50)
Abdominal pain	8 (13.11)	2 (10)	6 (20.68)	1 (8.33)	-
Lethargy	21 (34.42)	8 (40)	11 (36.66)	2 (16.66)	1 (50)
Arthralgia	20 (32.7)	6 (30)	11 (36.66)	3 (25)	1 (50)
Myalgia	40 (65.57)	16 (80)	18 (60)	5 (41.66)	2 (100)

Table 6: Correlation of lab parameters with dengue serotypes in PCR positive patients

Lab Parameters	PCR	Type 1	Type 2	Type 3	Type 4
HCT (vol%)	41.22	43.53	42.34	42.42	42.50
Total WBC (cells/μl)	3,652	4,091	3,100	4,505.00	3,595
Platelet (cells/μl)	135,321	145,050	122459	159,333	80,000
Total Bilirubin (mg/dl)	0.95	1.02	0.83	0.77	0.60
Direct Bilirubin (mg/dl)	0.39	0.44	0.32	0.17	0.40
Total Protein (g/dl)	6.57	6.84	6.54	6.38	7.25
Albumin (g/dl)	3.76	3.81	3.54	3.60	3.95
Globulin (g/dl)	3.02	2.97	3.02	3.18	3.10
AST (units/ml)	66.0	58.75	70.75	50.36	79.50
ALT (units/ml)	68.10	89.45	55.76	32.78	56.50
Alkaline Phosphatase (IU/L)	71.40	68.05	73.54	77.43	65.00
Serum Creatinine (mg/dl)	1.00	1.10	0.92	0.85	0.85
Serum Urea (mg/dl)	22.45	25.10	19.13	20.56	19.50

old age, to severe clinical manifestations of dengue.[12] The impact of co morbidities to the severity of disease when infection occurs will be critical to identifying vulnerable populations, to whom effective clinical monitoring practices should be particularly targeted.

Among the 108 NS1 antigen positive samples, 61(56.5%) samples became RTPCR positive for Dengue virus. A similar study conducted in Kerala in 2019, 159 cases became RTPCR positive among the 274 total Dengue NS1 antigen positive cases which was 58%.[13] Another study from southern India in 2016 had a PCR positivity rate of 53%.[12]

Among the 61 RT PCR positive samples subjected to multiplex RT PCR for serotyping, 20(32.8%) were identified as DENV-1, 30(49.2%) as DENV-2, 12(19.6%) as DENV-3 and 2(3.27%) as DENV-4. Three(4.9%) cases were co infected with two serotypes; DENV-1,2, DENV

1,4 and DENV 2,3. So in the present study, all the four serotypes were identified along with co-infections. DENV-2 was found to be the most predominant serotype in circulation followed by DENV-1. Another study conducted in 2010 at Ernakulam district also showed DENV-2(43.2%) as predominant serotype.[3] Genotyping study conducted in Kerala in the year 2010 also had a predominance of DENV-2 serotype (51.72%).[4]

However, a similar study from a tertiary care center in Central Kerala for a period of one year (January to December, 2016) had shown a predominance of DENV-1.

The first serotype isolated in India was DENV-1 in the year 1945 from Kolkata.[14] However, large and severe outbreaks in India have been mostly caused by DENV-2. Till 2006 the predominant serotype circulating in India was DENV-2. After the outbreak of dengue in 2006 replacement of the earlier circulating serotype DENV-2 with DENV-3

was observed.[14] Towards the south, in a study conducted during an outbreak in 2003 in Kanyakumari district in Tamilnadu, DENV-3 was the predominant serotype.[15] Our study findings shows the replacement of DENV 3 back with DENV 2. This study also reveals that all four serotypes were found to be co-circulating in central Kerala as detected by conventional multiplex reverse transcriptase (RT)-PCR. This finding correlates with another genotypic study done in Kerala during 2010.[4]

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.

In our study DENV 2 serotype was prevalent mainly during pre monsoon and monsoon period but DENV 1 serotype was seen circulating throughout the year. Only scarce data is available regarding seasonal distribution of dengue serotypes.

A wide spectrum of mild to severe clinical and hemorrhagic features were analyzed during the study. Among the 61 RTPCR positive samples, fever 61 (100%), headache 42 (68.8%) and myalgia 40 (65.5%) were the most common symptoms and were more observed in DENV-1 than DENV-2. There was an increased incidence of gastrointestinal manifestations, like vomiting in 20(32.7%) and abdominal pain in 8(13.1%) cases and these were more observed in patients infected with DENV-2 than DENV-1. However we could not find any statistical significance in the association between the serotypes involved, their clinical manifestations and the severity of disease.

Two, among the 108 patients included in this observational study, died. Thus the mortality observed in the present study is calculated to be 1.85%. Both these patients were suffering from chronic liver disease. Mortality in these cases could be attributed to associated alcoholic liver disease and related hepatic encephalopathy. Alcohol consumption may be an independent predictor for severity of dengue related hepatic damage.[16] Moreover the finding shows risk of dying is higher in the presence of underlying co morbidities.[17] All others were symptomatically better and stable at the time of discharge.

In our study various laboratory parameters of the enrolled patients were also recorded. The main hematological change found in our study was thrombocytopenia. Both neutropenia (10.24%) and thrombocytopenia (57.3%) were markedly seen in DENV 2, indicating its chances of going to severe manifestations. In previous molecular studies conducted on dengue also, it has been reported that DENV2 infection cause severe forms of dengue.[18] But we could not obtain any significant statistical evidence regarding this association in our study. Of biochemical variables, the most frequent changes occurred in liver function tests such as in serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT). Among the study group, DENV1 serotype infected cases showed maximum(30%) derangement of liver function test.

The study also aimed at correlating dengue virus serotypes with clinical severity. But during the study period very few cases of severe dengue with hemorrhagic manifestations were admitted in the center because of which no significant statistical association was found between the serotypes and clinical manifestations or severity.

It is not entirely understood why some individuals develop more severe disease, although host genetic factors and previous exposure to DENV are all known to affect the outcome. It has been observed that different dengue serotypes, and even strains of the same serotype, differ in their inclination to cause severe disease.[19]

The hyperendemicity with co-infection of two or more serotypes during the same time period has been widely suspected as one of the major causes of disease severity in dengue patients in India.[20] Various studies have tried to establish a connection between causative serotype and disease outcome.[21] But a definite link between distinct serotypes and severe manifestations has not been established yet. Small sample sizes, lack of all the four serotypes, short study durations and inconsistencies in findings are weaknesses of the studies including the present study.

Another drawback of the present study was that many clinically suspected cases which were severe and Dengue antibody positive could not be included since they were referred late to our hospital by which time NS1 antigen became negative and could not be included in our study as per the inclusion criteria.

In conclusion, this study reveals that all four serotypes and co infection with multiple serotypes were found to be circulating in central Kerala which suggests that this could be a hyper endemic province for dengue. DENV-2 was the predominant serotype followed by DENV-1. Thus the results of the present investigation can assist in designing control strategies for future epidemics and to determine the evolutionary pattern of the emerging Dengue virus.

5. Source of Funding

6. Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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