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A Study on Dengue Serotyping Using Reverse Transcription PCR From Clinical Samples in a Tertiary Care Hospital

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ABSTRACT

Dengue is one of the most common arthropod-borne viral disease of public health importance. Dengue (DEN) virus comprises of four serotypes (DEN1, DEN2, DEN3 and DEN4). Serological assay for detection of Non-Structural protein1 Antigen (NS1Ag), Immunoglobulins-IGM and IGG using Enzyme-linked immunosorbent assay (ELISA) is one of the routinely used diagnostic method. However, with the molecular methodology by Reverse Transcription Polymerase chain reaction (RT-PCR) detection of the serotypes can be performed for epidemiological purposes. A prospective study was conducted. In Department of Clinical Microbiology, at Osmania Medical College, Hyderabad, Telangana from July 2022 to December 2022. Blood samples were collected from clinically suspected dengue patients during study period. Out of 184 Ns_1 Ag positive samples, 110 were subjected to RT-PCR. Among which 48 samples serotyping results were positive. Out of them, majority 22 (45%) belonged to DEN2, followed by DEN3 (18.75%), DEN1(12.5%). Coinfection with multiple serotypes was detected in the present study. Male preponderance (54.16%) was seen among dengue cases. As relatively few studies have been conducted for the detection of dengue serotypes by RT-PCR. Hence this study is carried out to identify the prevalent serotypes of dengue virus in our area and their association with the clinical severity. Therefore, effective surveillance of serotypes will be useful in planning the preventive and therapeutic strategies in dengue fever.

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Key Words
Dengue, serotype, non-structural protein 1 antigen, enzyme-linked immunosorbent assay, reverse transcription PCR

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INTRODUCTION

Dengue is one of the most important arboviral disease worldwide, more prevalent in the tropical and sub-tropical regions. Dengue viruses belongs to family Flaviviridae, genus Flavivirus and is transmitted between humans by arthropod vectors Aedes aegypti mosquito. This mosquito is endemic in the most areas except the 1000 meters above sea level areas[1].

Dengue viruses occur as four antigenically distinct serotypes (DEN1, DEN2, DEN3 and DEN 4) respectively. DEN1 was firstly found in Hawaii in 1944, DEN2 in Papua New Guinea in the same year. DEN3 and DEN4 were found in Philippines in 1956[1]. Infection with the above-mentioned serotypes generally leads to a mild, self-limiting febrile illness, dengue fever[2]. Further, a severe disease characterized by hemorrhage and shock, known as dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS)[3]. Recovery from infection, provides lifelong immunity against that particular serotype. However, cross-immunity to the other serotypes after recovery is only partial and temporary.

Subsequently, people getting infected by one serotype and later get infected with another serotype have a greater risk of developing severe life-threatening dengue infection[4]. An increase in morbidity and mortality rate of dengue virus infection can also be associated with delayed management and missed diagnosis. An early identification of serotype in dengue positive patients is of great help in management of the disease, its proper surveillance and efficient control of spread of disease. Hence, a rapid and accurate laboratory diagnostic methodology is of utmost importance[1]. Serological assays for Ns1 antigen (early phase) IGM antibody (after 5 days) and IgG antibody by using rapid methods and ELISA is one of the most commonly used methods. But these methods, cannot differentiate between the four dengue virus serotypes[1].

Very few studies have been conducted on the Dengue virus serotypes and genotypes circulating in our region and their correlation to the severity of the disease. Similarly, studies on the prevalence of co-infection with multiple serotypes is also less studied. Therefore, diagnostic method that is sensitive and specific is of utmost importance to be clinically and epidemiologically beneficial. Detection of the dengue virus and typing of its serotypes by everse Transcriptase polymerase chain reaction (RT-PCR) is the need of the hrs from the serum samples[2]. Hence, this study is conducted to find out the circulating serotypes of dengue virus in our region which will be helpful to allow timely diagnosis, management and also useful in epidemiologic studies.

Aim and objectives:

- To determine dengue virus serotypes in Ns1 antigen positive clinical samples
- To identify prevalent dengue virus serotypes in our geographical area using RT-PCR
- To correlate the serotypes identified to the severity of the disease

MATERIALS AND METHODS

The present prospective study was conducted in Department of Clinical Microbiology, Osmania medical college, Hyderabad, Telangana for a period of six months from July 2022 to December 2022. Five milliliter of blood samples from clinically suspected dengue patients were taken by venipuncture without anticoagulant and centrifuged to collect serum. Hundred dengue NS1 positive serum samples during the study period were further stored at-80°C for serotyping by reverse transcription-polymerase chain reaction.

Dengue Serotyping-RT PCR: The QIAamp Viral RNA kit was used according to the manufacturer's instructions. Each clinical sample was incubated with lysis buffer for 10 min in a total volume of 140 µ1. After viral particle lysis the samples were mixed with ethanol and applied to the spin column provided, centrifuged and washed twice with buffers AW1 and AW2. Then, using 60 mL of extraction buffer, RNA was extracted from the columns and kept at-80°C for further use. After extraction quantification and quality check of RNA was done for further processing. The first complimentary DNA (cDNA) strand was synthesized by commercially available DSS Takara First Strand Synthesis kit according to the manufacturer's instructions. Target viral RNA was converted to CDNA prior to enzymatic DNA amplification by use of reverse transcriptase (RT) and the dengue virus downstream consensus primer, homologous to the genomic RNA of the four serotypes.

Dengue virus consensus primers were designed from sequences obtained from NCBI data Bank. The type-specific oligonucleotide primers were designed to anneal specifically to each of their respective genomes. The designed oligonucleotides were procured from the Regene Biologics Pvt, Ltd. Hyderabad[5]. Target cDNA was amplified with the dengue virus type-specific primers. The samples were subjected to 20 cycles of denaturation (94°C, 30s), primer annealing (55°C, 1 min) and primer extension (72°C, 2 min). The resulting amplified PCR product was analyzed by electrophoresis on a 4% composite agarose gel. The size of resulting DNA band was compared with the dengue virus type specific primers band.

Ethics statement: This study was approved by the Institutional Ethics Committee, Osmania medical college, Hyderabad, Telangana, India (IEC/OMC/2023/M.No(02)/Acad-3).

RESULTS

Out of 184 dengue NS_1Ag positive samples,110 were subjected to RT-PCR. Among which serotypes of 48 samples were identified.

DISCUSSIONS

Dengue is one of the important viral infections, the overall epidemiology of which has changed greatly in the course of the last 20 years, dengue became major public health problem worldwide. Dengue illnesses are caused by any of the four serotypes as DENV-1, DENV-2, DENV-3 and DENV-4. Co-circulation of all kinds of dengue virus serotypes has been reported from various parts of the world including India[6]. As the number of dengue virus infection cases are increasing the disease is a serious health issue in tropical areas. Therefore, it is important to have a reliable method to identify the serotypes for clinical diagnosis and epidemiological purposes[7]. RT-PCR provides precision and speed in serotype specific diagnosis of multiple circulating dengue viruses and details about co-circulation of various subtypes[6]. In this study the serum samples that were collected within five days of illness and that were positive for NS1 Ag by ELISA were included. For dengue virus accurate detection by PCR, serum sample should be procured during the febrile period, ideally before the fifth day of illness the period marked by virus replication and also absence of virus-specific neutralizing antibodies. However, dengue viral RNA can be detected even after the fifth day of disease by PCR, if sample are stored and extracted correctly[8]. In this study among 110 NS1Ag positive samples subjected to RT-PCR, serotypes of 48 (43.63%) samples could be identified. Retrieving of virus from dengue positive sera might fail if the samples contained anti-dengue antibodies or potency of serum is lost due to improper or long-time storage[9]. Out of 48 isolated serotypes, 37 cases have single serotype infection. Majority 22 (45%) belongs to DEN2 followed by DEN3. Co-infection is seen in 11 (22.91%) samples, with both DEN1 and DEN2 in 5 (10.41%) samples and DEN2 and DEN3 in 6 (12.5%) respectively. In contrary, DEN3 dominated the outbreak with 26 of 48 seropositive samples and coinfection in 9 (19%) cases, most common serotype combination observed being DEN1 and DEN3 in Preethi Bharaj study[6]. Of 48 patient samples in which dengue serotypes was detected male to female ratio is 1.26. young adults and middle age people are affected more than children and old age. This scenario may be seen due more outdoor activity and movement. which is in accordance with other studies[6,10]. In our study there is no much difference in distribution of cases in urban and rural settings. Peak season for dengue cases was October in present study. It is in accordance with Anju Dinkar et al study[10]. In all 48 patients in whom dengue

Table 1: Dengue-serotype positivity

Dengue-erotype	Positive samples (n = 48)	Percentage
DEN1	06	12.5
DEN2	22	45
DEN3	09	18.75
DEN4	0	0
Coinfection with DEN1 and DEN2	5	10.41
Coinfection with DEN2 and DEN3	6	12.5

Table 2: Sociodemographic characteristics

Characteristic	Value (n = 48)	Percentage
Gender		
Male	26	54.16
Female	12	25
Age (years)		
≤20	16	33.33
21-40	22	45.83
41-60	08	16.66
≥ 61	02	6.25
Locality		
Urban	23	47.91
Rural	25	52.03

Table 3: Correlation of clinical features with dengue serotypes in PCR positive patients

Clinical features	DEN 1	DEN 2	DEN3	Co- infection with	Co- infection with
Fever	6	22	9	5	6
Headache	4	22	7	3	5
Rash	3	12	2	5	6
Retroorbital pain	5	20	7	2	3
Arthralgia	3	18	4	3	4
Myalgia	6	22	5	5	6

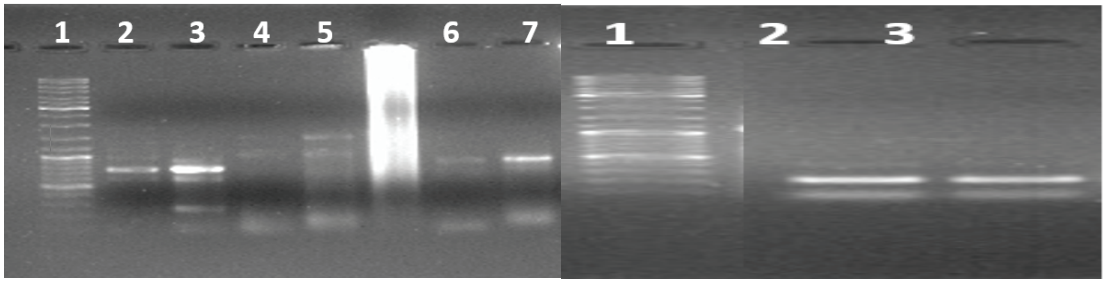

Fig. 1: Agarose gel electrophoresis-showing various serotypes isolated
Lane 1-DNA Ladder, Lane 2 and 3-Dengue Serotype 3, Lane 4 and 5-Dengue Serotype 1, Lane 6 and 7-Dengue Serotype 4 Lane 1- DNA ladder. Lane 2 and 3- Serotype 2

serotypes were identified by PCR, fever was common clinical feature present, followed by Myalgia in 44, Headache In 41 patients. Rash was seen only in 28 cases but t his was predominant clinical feature in co-infection cases. Similar clinical scenario was noted in Bharaj *et al*.[6] study.

CONCLUSION

The climatic conditions in India is very much suitable for dengue virus transmission leading to several epidemic outbreaks. More over circulating DENV undergoes rapid changes in genome as RNA genome is prone to mutational changes. These changes in genome results in emergence of new serotypes. So
it is very essential to characterize serotypes of circulating DENV[11]. It is also important to understand evolutionary processes of infecting dengue virus, as it is expected to impact vaccination strategies[5]. RT-PCR is a sensitive test and even detects dead viruses present in viremic serum samples, co-infection with multiple serotypes may lead to adverse clinical outcome of the disease[12]. The accuracy and speed of the RT-PCR assay make it an appealing test for the diagnosis of dengue for epidemiologic surveillance[2]. Hence the present study gives an insight into the circulating serotypes in our area.

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