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ISOLATION OF RARE DEN 4 SEROTYPE FROM A TERTIARY CARE HOSPITAL IN NCR REGION

Microbiology

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ABSTRACT

The objective of this research was to investigate serotype diversity pattern of dengue virus by using Real Time RT-PCR. A total of 246 clinically suspected cases of Dengue were selected based on WHO 2009 Dengue case classification. Acute phase blood samples were tested for NS1 antigen, IgM and IgG antibody and samples positive by one of the parameters: NS1 Ag and/or IgM Ab/IgG Ab were further subjected to Real time RT-PCR. Of the 246 clinically suspected cases of Dengue, 68 (27.6%) were positive for NS1 Ag and/or IgM Ab/IgG Ab and of these, 30 (44%) samples were positive by Real time RT-PCR. All the 4 Dengue serotypes were found to co-circulate in this region of which 3 cases (10%) were positive for the rare DEN- 4 serotype. Co-circulation of all four dengue serotypes in the studied region emphasises the need of molecular monitoring of circulating DENV serotypes.

KEYWORDS

Dengue Serotypes, Den-4, Co-circulation, Real Time Rt-pcr

INTRODUCTION

Dengue has become a worldwide health issue. It has emerged as a notable public health problem in recent decades in terms of mortality and morbidity associated with it[1,2]. Dengue illnesses are caused by any of the 4 serologically related viruses designated as: DENV-1, DENV-2, DENV-3 and DENV-4 which follow the Human Cycle[3]. These four serotypes are genetically similar and share approximately 65% of their genomes[4]. All the four dengue serotypes have been isolated from India. DENV-5 has not been reported from India yet. Serotype prevalence varies between seasons and places. Immunity is serotype specific and there is no cross protection between the serotypes[5]. Infection with any one serotype confers lifelong immunity to that serotype but only two to three months immunity to other serotypes.

MATERIALS AND METHODS

This prospective study was conducted in the Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh, India. Institute Ethics Committee approval was obtained for the study. Patients of all age groups and both sexes having body temperature of >38.5^{o}C for >24 hour and <10 days of illness who were clinically diagnosed as having Dengue fever fulfilling the WHO case definition from various Outpatient departments, Emergency services and IPD were included in the study during August 2017-August 2018. Febrile patients with duration of illness >10 days and cases with evidence of bacterial or other viral illness based on laboratory testing were excluded from the study. Demographic data, details of clinical history and clinical presentations were collected and recorded on a pre-structured datasheet.

SAMPLE COLLECTION AND PROCESSING:

Before taking the sample, patient was informed about the procedure and consent for the same was taken. A standard protocol was followed for venepuncture and collection of blood sample. 5 ml of venous blood was collected under full aseptic conditions in a sterile plain vial.

NS1 ANTIGEN, IGM ANTIBODY, IGG ANTIBODY TESTING:

All the blood samples were centrifuged at 3000 rpm for 10 minutes. Serum obtained was tested for NS1, IgM and IgG testing. Sera showing hemolysis, icterus, lipaemia or microbial growth were excluded as they may cause false positive/negative interpretation. Dengue Ag+Ab Duo Rapid Test Kit manufactured by SD Biosensor Healthcare Pvt. Ltd.

DENGUE SEROTYPING:

RNA EXTRACTION:

PRINCIPLE:

Geno Sen's® Viral RNA extraction Mini Kit combines the selective binding properties of a silicagel based membrane with the speed of microspin and is ideally suited for simultaneous processing of multiple samples. The sample is first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the Column membrane, and the sample is loaded onto the spin column. The RNA binds to the membrane, and contaminants are efficiently washed away in two washing steps using two different wash buffers. High quality RNA is eluted in a special RNase free buffer, ready for direct use or storage.

PROCEDURE:

All seropositive samples obtained after NS1, IgM and IgG testing were used for RNA extraction using (Geno Sen's® Viral RNA Extraction Mini Kit).

AMPLIFICATION:

PRINCIPLE:

Amplification was based on Taqman principle. The forward and reverse primers used were *DEN TYF 1* and *DEN TYR 1* respectively. The reporter dyes used were: *DEN TYP1 FAM, DEN TYP2 JOE, DEN TYP3 ROX, DEN TYP4 CY5*. During PCR, forward and reverse primers hybridize to a specific sequence product. A TaqMan probe, which is contained in the same reaction mixture and which consists of an oligonucleotide labeled with a 5'-reporter dye and a downstream, 3'-quencher dye, hybridizes to a target sequence within the PCR product. A Taq polymerase which possesses 5' - 3' exonuclease activity cleaves the probe. The reporter dye and quencher dye are separated upon cleavage, resulting in an increase in fluorescence for the reporter. Thus, the increase in fluorescence is directly proportional to the target amplification during PCR. The Specific Master mix contains reagents and enzymes for the specific amplification of Dengue Typing 1/2/3/4 and for the direct detection of the specific amplicon in fluorescence channel Cycling : *Green (TYPE 1), Yellow (TYPE 2), Orange (TYPE 3) & Red (TYPE 4)* of Rotor Gene 6000.

PROCEDURE:

Extracted RNA was subjected to amplification using (Geno-Sen's Dengue Typing 1/2/3/4 Real Time PCR Kit) for Rotor Gene™ 6000 (Corbett Research).

THERMAL PROFILE:

Thermal profile of the assay was defined and caliberated in the PCR computer program in the following way:

STEP	DURATION AND TEMPERATURE
RNA Extraction (cDNA Synthesis)	First hold 50°C for 15 minutes
Denaturation	Second hold 95°C at 10 minutes

Cycling	95°C for 15 seconds; followed by 55°C for 20 seconds and defining the Data acquiring channel i.e FAM and JOE, ROX and Cy5
Extension	72°C for 15 seconds (Setting the Number of Cycles to 45 cycles in the cycling profile)

PCR was run and the Interpretation & Analysis of the generated data was done. Data analysis is performed with the RotorGene™ software according to the manufacturer's instructions.

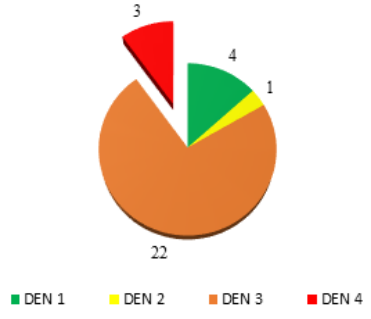
DATA INTERPRETATION & ANALYSIS:

If a signal is detected in fluorescence channel Cycling A. GREEN: The result of the analysis is positive: The sample contains Dengue typing 1 RNA. Cycling A.YELLOW: The result of the analysis is positive: The sample contains Dengue type 2 RNA. Cycling A.ORANGE: The result of the analysis is positive: The sample contains Dengue type 3 RNA. Cycling A.RED: The result of the analysis is positive: The sample contains Dengue Type 4 RNA. No signal is detected in fluorescence channel Cycling A: Green, Yellow, Orange & Red: The sample does not contain Dengue RNA or there are chances of inhibition in the sample.

OBSERVATION AND RESULTS

A total of 246 clinically suspected cases of Dengue were selected based on WHO 2009. Dengue case classification. Of the total 246 samples of Dengue suspected cases, 68(28%) were seropositive by atleast one component (NS1, IgM, IgG). 36 (53%) samples out of these 68 samples were NS1 only positive. 7 (10.2%) samples were positive for both NS1 and IgM. 5 (7.3%) samples were positive for both NS1 and IgG and 2 (3%) samples were positive for all the three parameters i.e. NS1, IgM and IgG. 18 (26.4%) samples were only IgM positive. None of the samples were IgG positive only or IgM and IgG both positive. Of the total 68 seropositive samples, 30 (44%) samples were positive by Real time RT-PCR and 38 (56%) samples were negative. Of the 30 PCR positive samples, serotype 4 was positive in 3 patients (10%) [Figure 1]. Most prevelant serotype was serotype 3 in 22 patients (73.33%) followed by serotype 1 in 4 patients (13.33%), and serotype 2 in 1 patient (3.33%) respectively.

Figure 1: Number of DEN 4 Cases (n=30)



DISCUSSION

The four distinct serotypes of dengue can cause clinical manifestation ranging from mild self limiting illness to severe DHF and DSS. Severity in dengue viral infection is known to be affected by secondary infection with heterologous antibodies or with certain DENV serotypes and genotypes[6,7]. Hence, circulating DENV serotypes should be closely monitored for prevention of fatal outcomes in secondary infections. In past decades, DENV-4 which is a rare serotype appeared in many areas, including Thailand, Singapore, Indonesia, Brazil, and India[8,9,10]. DEN-4 has recently been reported from North, West, South and Central India. Our study reports 3 cases positive for DEN-4. The changing pattern of dengue serotypes in a geographic location necessitates the continuous molecular surveillance of the circulating serotypes[11] hence the present study laid emphasis on the occurence of various Dengue serotypes. Few serotypes of dengue are more dangerous than the others. Rapid diagnosis and serotyping remains the key for better patient management and prevention of disease spreading in the community. Highly sensitive, specific and rapid real time RT-PCR assay is the most promising tool among all the available molecular diagnostic methods. This will serve a rapid and reliable sim ultaneous dengue virus detection as well serotyping assay in near future.

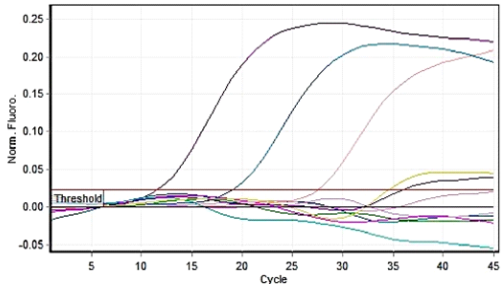
Serotyping showed that of the 68 seropositive samples, 30 samples (44%) were positive by real time RT-PCR and 38 samples (56%) were negative. A study from North India by Prakash O et al (2015)[12] showed 39.1% positivity by RT-PCR. Our study showed the co-circulation of all the four serotypes DEN-1, DEN-2, DEN-3, DEN- 4. A study conducted by Gupta E et al (2006)[13] in the neighbouring State of Delhi also reported all the four dengue serotypes to be co-circulating in the year 2003. However, it was observed that no sample was harbouring more than one serotype indicating absence of concurrent infection. A recent study by Reddy MN et al (2017)[14] reported the co-circulation of all 4 serotypes with samples harbouring more than one serotype of dengue indicating 100% concurrent infection. Another recent study by Racherla RG et al (2018)[15] also reported co-circulation of all the four dengue serotypes.

This study inferred that there is a circulation of multiple serotypes which suggests that the studied region is becoming a hyperendemic province for dengue; therefore, continuous surveillance is suggested for understanding the epidemiology of the diseases and monitoring the changes in the characteristics of circulating DENV strains. Thus the present investigation will assist in designing control strategies for the epidemics. Further this molecular study will also help us to determine the evolutionary pattern of the emerging Dengue virus.

SUMMARY

Of the total 68 seropositive samples, 44% samples were positive by real time RT-PCR. All the four dengue serotypes (DEN 1, DEN 2, DEN 3, DEN 4) were found to co-circulate in the present study suggesting hyperendemicity of dengue in the studied population. However no sample harboured more than one serotype indicating absence of concurrent infection. Most prevelant was Serotype 3 in (73.33%) patients suggesting milder form of dengue this year as compared to the severe form with Serotype 2 in (3.33%) patients which was the least prevelant serotype. Our study reports isolation of DEN 4 which is a rare serotype [Figure 2].

Figure 2: PCR Amplification Graph for Dengue Serotype 4 (Sample Amplifying In CY 5 Channel Showing Serotype 4 Positive Patients)



CONCLUSION

In conclusion, periodic monitoring of circulating DEN viral serotypes is essential for epidemiological purposes and for the patient management as each dengue serotype is associated with different symptoms and severity. Dengue in this region where more than one DENV serotype circulate simultaneously is alarming and is of special significance since such regions are more prone to severe dengue infection. This study will help us to characterize the circulating serotype of dengue virus, to better understand the evolutionary process influencing the dengue virus in our region, to monitor the epidemiology thus expecting it to impact on vaccine strategies for future.

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