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Hyperendemicity and Recrudescence of Dengue Activity Reported from a Tertiary Care Hospital in NCR Region

R. Tomar*, D. Bisht and V. Goel

Department of Microbiology, Santosh Medical College & Hospital, Ghaziabad, Uttar Pradesh, India

**Corresponding author*

Abstract

The objective of this research was to investigate the presence of hyperendemicity and recrudescence activity of dengue by using Real Time RT-PCR for dengue serotyping. 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 four dengue serotypes were found to co-circulate in this region, however no sample harboured more than one serotype. Co-circulation of all four dengue serotypes in the studied region emphasises the need of molecular monitoring of circulating DENV serotypes.

Article Info

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Keywords

Dengue serotypes, Co-circulation, Hyperendemicity, Recrudescence, Concurrent Infection.

Introduction

Dengue virus is the most common arbovirus in India. In recent decades dengue has emerged as a notable public health problem in terms of morbidity and mortality associated with it[1,2]. Dengue illnesses are caused by any of the four serologically related viruses designated as: DEN-1, DEN-2, DEN-3 and DEN-4 which follow the Human Cycle[3]. These four serotypes are genetically similar and share approximately 65% of their genomes[4]. The fifth serotype DEN-5 was recently discovered in October 2013 from Bangkok which follows the sylvatic cycle. The likely cause of emergence of the new serotype could be genetic recombination, natural selection and genetic bottlenecks. DENV-5 has been detected during screening of viral samples taken from a 37 year old farmer admitted in hospital in Sarawak state of Malaysia in the year 2007. The infection in the farmer was initially thought to be an ordinary case of sylvatic dengue caused by DENV-4 which circulates among primates and *Aedes nivalis* mosquitoes in the forests of South East Asia[5].

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[6]. Infection with any one serotype confers lifelong immunity to that serotype but only 2-3 months immunity to other serotypes. Also, each serotype has its characteristic symptoms. Symptoms range from acute febrile illness to severe manifestations, including bleeding and organ failure resulting in the

DHF or DSS[7,8]. In case of Type 1, the symptom is classic dengue fever; Type 3 causes high grade fever without shock. These two are considered relatively mild serotypes. The severe strains are Type 4, which leads to fever with shock, and Type 2, which causes thrombocytopenia, haemorrhagic fever, organ failure and Dengue shock syndrome (DSS). Globally, Type 2 has been identified as the most common cause of Dengue Haemorrhagic Fever (DHF). Heterotypic secondary DENV infection (with a DENV type distinct from the primary infecting type) is the greatest risk factor for DHF/DSS[9,10,11]. In recent years, co-circulation of multiple serotypes has been reported from different parts of India[12]. High percentage of co-infection with more than one serotype was also observed with increased disease severity[13,14,15]. Epidemiological studies have shown that the emergence of a newer dengue serotype after an interval always leads to a major outbreak; therefore a continuous epidemiological surveillance is needed to monitor the epidemiology of dengue viruses. Human viruses of dengue serotypes 1, 2 and 3 are each classified into five genotypes related to their geographic origin, while serotype 4 viruses are classified into four genotypes.

Epidemiological analysis suggests that different genotypes differ in their epidemic potential and virulence[16,17,18]. Need of the hour is to characterize the circulating serotype of dengue virus in our community and understand the evolutionary process influencing the dengue virus as this is expected to impact on vaccine strategies for future. As the outbreaks of Dengue fever is increasing in India, one state after other is getting affected, thus it is very essential to know more about this disease and prevalence, any change in the viral strain, severity of the disease pattern, early detection of the virus and early management of the disease resulting in good recovery. Thus the present study was taken to detect the occurrence of various dengue serotypes in a tertiary care hospital in Ghaziabad (NCR Region).

Materials and Methods

This prospective study was carried out in the Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh, India. The study was conducted on 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

The patient was informed about the procedure and consent for the same was taken before taking the blood sample. 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 using Dengue Ag+Ab Duo Rapid Test Kit manufactured by SD Biosensor Healthcare Pvt. Ltd. Serashowing hemolysis, icterus, lipaemia or microbial growth were excluded as they may cause false positive/negative interpretation.

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 labelled 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:

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

Data interpretation and analysis

If a signal is detected in fuorescence channel Cycling A. GREEN: The result of the analysis is positive: The sample contains Dengue typing 1RNA. 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 fuorescence channel Cycling A: Green, Yellow, Orange & Red: The sample does not contain Dengue RNA or there are chances of inhibition in the sample.

Results and Discussion

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%). Most prevelant serotype was serotype 3 in 22 patients (73.33%) followed by serotype 1 in 4 patients (13.33%), and serotype 2 in a single patient (3.33%) respectively [Figure 1].

Dengue is one of the important mosquito-borne viral infection of public health concern. The four distinct serotypes of dengue can cause clinical manifestation ranging from mild self limiting illness to severe dengue haemorrhagic fever and dengue shock syndrome. Severity in dengue viral infection is known to be affected by secondary infection with heterologous antibodies or with certain DENV serotypes and genotypes[19,20]. Circulating DENV serotypes should be closely monitored for prevention of fatal outcomes in secondary infections. Also the changing pattern of dengue serotypes in a geographic location necessitates the continuous molecular surveillance of the circulating serotypes[21]. Hence, the study laid emphasis on the occurrence of various Dengue serotypes. Few serotypes of dengue are more dangerous than the others (DEN-2 being the most dangerous of all). 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 simultaneous dengue virus detection as well serotyping assay in near future. Serotyping showed that of the 68 seropositive samples, 30 samples (44%) were

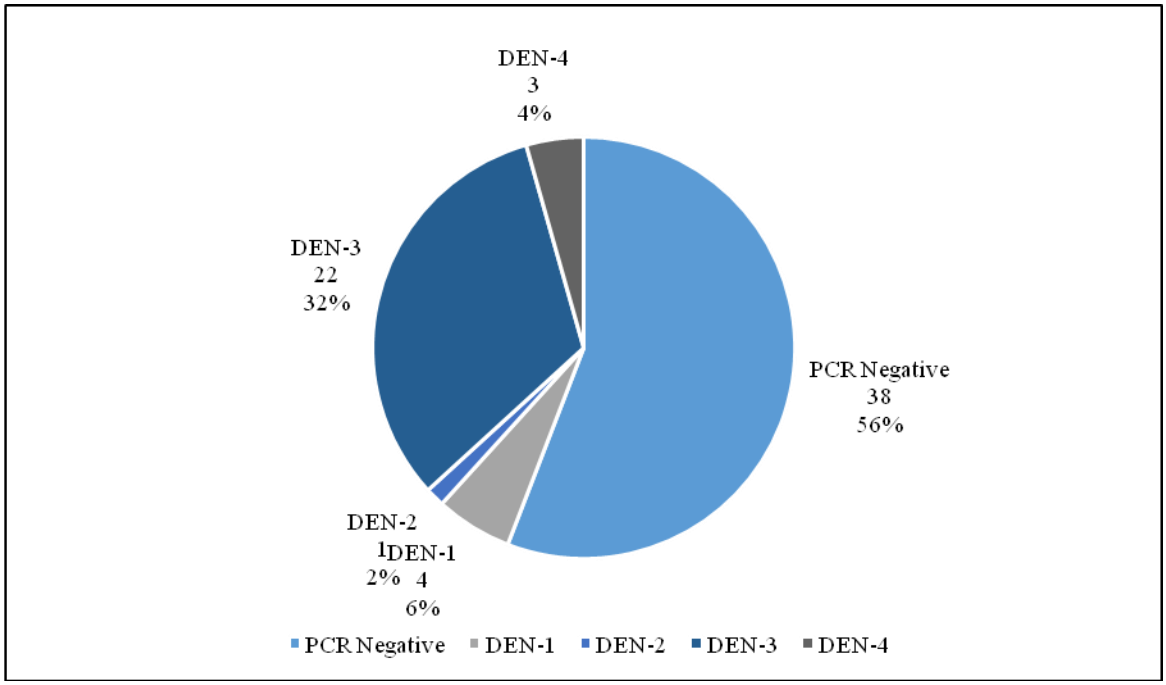
positive by real time RT-PCR and 38 samples (56%) were negative. A study from North India by Prakash, *et al.,* (2015)[22] showed 39.1% positivity by RT-PCR. Our study showed the co-circulation of all the four serotypes DEN-1, DEN-2, DEN-3, DEN-4 in this region. A study conducted by Gupta, *et al.,* (2006)[23] in the neighbouring State of Delhi also reported all the four dengue serotypes to be co-circulating in the year 2003, followed by complete predominance of dengue serotype 3 in the year 2005. However, it was observed that no sample was harbouring more than one serotype indicating absence of concurrent infection. A recent study by Reddy, *et al.,* (2017)[24] reported the co-circulation of all 4 serotypes with samples harbouring more than 1 serotype of dengue indicating 100% concurrent infection. Another recent study by Racherla RG *et al.,* (2018)[25] also reported co-circulation of all the four dengue serotypes. Of the 30 cases positive by real time RT-PCR, Serotype 3 was the most prevalent serotype with 22 cases. Serotype 3 has also been reported to be the prevalent serotype in different studies from various places all over India. A study in Lucknow (North India) by Prakash, *et al.,* (2015)[26] reported DEN-3 to be the prevalent serotype in 2013. Kumar NP *et al.,* (2013)[27]in a study in the state of Kerala (South India) found that DEN-3 was the prevalent serotype in the year 2008. A study in Delhi by Gupta E *et al.,* (2006)[28] showed that all the four dengue serotypes were seen co-circulating in the year 2003, followed by complete predominance of dengue serotype 3 in 2005. Bharaj, *et al.,* (2008)[29], Dash, *et al.,* (2006)[30] also reported DEN-3 to be the most prevalent serotype in their studies in the neighbouring state of Delhi. A study by Khan, *et al.,* (2014)[31] from the North eastern most state of Arunachal Pradesh also stated DEN-3 to be the most prevalent serotype in that year. Study by Muruganandam, *et al.,* (2014)[32] in Port Blair reported DEN-3 to be the prevalent serotype. This data clearly suggests that DEN-3 has been frequently isolated as the most prevalent serotype not only from Northern or Southern India but from all the different regions of the country. A single case of Serotype 2 was detected as the least common serotype (3.33%) in our study. In contrast, however in a study by Mishra G *et al.,* (2014)[33] from the year 2009-2012 in UttarPradesh, DEN-2 was predominantly detected in 56.6% patients. In our study, of the total 36 NS1 antigen positive cases, 20 (55.5%) cases were positive by Real time RT-PCR. Of the total 7 NS1 antigen+IgM antibody cases, 5 (71.4%) cases were positive by Real time RT-PCR and out of a total of 5 NS1 antigen+IgG antibody cases, 3 (60%) cases were positive by Real time RT-PCR. This shows that NS1 antigen+IgM antibody positive cases showed maximum Real time RT-PCR positivity of 71.4%. Therefore, combining the IgM parameter with detection of NS1 indicates more possibility of finding dengue viral RNA genome making it an ideal sample enhancing the chances of dengue serotype detection. This study inferred that there is a circulation of multiple serotypes which suggests that Ghaziabad 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. The return of DEN-3 in our study coincides with the recrudescence of dengue activity in India in the recent years, supporting the idea that increase in dengue activity may be connected with changes in predominant serotypes. 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.

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 prevalent 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 prevalent serotype. The return of DENV-3 in our study coincides with the recrudescence of dengue activity in India in the recent years, supporting the idea that increase in dengue activity may be connected with changes in predominant serotypes. Our study also reports isolation of DEN 4 which is a rare serotype. 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.

Table.1

RNA Extraction (cDNA Synthesis)	First hold 50^{O}C for 15 minutes.
Denaturation	Second hold 95^{O}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).

Fig.1 PCR Positivity and Serotypes Isolated



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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