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Research Article

A STUDY ON IDENTIFICATION OF CIRCULATION OF DENGUE VIRUS SEROTYPES IN THE CITY OF HYDERABAD, TELANGANA AS DETERMINED BY CONVENTIONAL REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

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ABSTRACT

The present study was aimed to investigate the circulation of dengue virus (DENV) serotypes in Hyderabad. Hyderabad a city in South India, has so far witnessed several reported outbreaks of dengue. Dengue in Hyderabad from being epidemic is slowly changing towards being endemic and hyper-endemic. Circulating type of the virus is also changing over the years. In the absence of an effective vaccine, dengue prevention to a major extent relies on virological surveillance and development of effective, locally adapted control programmes. In the present study, we tried to identify the between-year non-epidemic serotype of dengue virus circulating in Hyderabad during 2014–15.Acute-phase samples were collected from the patients attending the Gandhi Hospital, Hyderabad, Telangana, India. Dengue diagnosis was done using WHO case definitions. In the present study,119 serum samples were randomly selected and subjected to Dengue NS1 Ag ELISA, IgM ELISA and multiplex nested RT-PCR. Among 119 samples screened, 16 were positive only for NS1 antigen ELISA, 55 were positive for both NS1 antigen and Ig M ELISA and 48 were positive only for Ig M ELISA. Out of the 16 cases positive for only NS1 antigen ELISA, 8 were positive by PCR, among the 55 cases that were positive for both NS1 antigen ELISA and Ig M ELISA, 20 were positive by PCR, and among the 48 cases positive only for Ig M ELISA, 1 was positive by PCR. The sensitivity of NS1 Ag was 97.26 % and sensitivity of multiplex RT-PCR was 40.27 %, while specificity for both was 100 %.Of th119 samples, only 29 samples were found to be positive for dengue virus infection by PCR. RT-PCR could detect the virus in serum samples collected before 8 days of illness. The predominant serotype in the year 2014 and 2015 was DENV 1 serotype followed by DENV 3 serotype. However only 23 cases in 2014 and 96 cases in 2015 were subjected to RT PCR which was not sufficient enough for comparison of the serotypes for both the years. DENV1 serotype was found to have more haemorrhagic manifestations (57.6%) than DENV 3 serotype (33.33%). To the best of knowledge, the current study demonstrated the prevalence of the DENV-1 serotype in the Hyderabad city in Telangana, India. Infection with one dengue virus serotype may provide lifelong Immunity against that particular virus serotype but it may confer only partial and transient protection against subsequent infection by the other three serotypes of the virus and sometimes sequential infection may increase the risk of developing severe dengue infection due to cross reactive T cells. Thus serotyping might be helpful in predicting the severity of infection as well as for further management and prevention of the disease. The implementation of this assay in dengue-endemic areas has the potential to improve both dengue diagnosis and epidemiologic surveillance.

Keywords :-Dengue, Epidemic, Serotypes, Polymerase Chain Reaction.

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INTRODUCTION

Dengue is a disease caused by four antigenically distinct but genetically related virus serotypes that cause dengue: *Dengue virus* (DENV 1–4) [1]. It is transmitted by the *Aedes aegypti* mosquito with clinical presentation of the disease ranging from mild forms, such as dengue fever (DF), to serious and even fatal forms. In the mild form of the disease, clinical manifestations include fever, headache, prostration, arthralgia, retro orbital pain, nausea, rash, itchy skin, and others. Dengue viruses belong to the genus *Flavi virus* within the *Flaviviridae* family. Dengue is the most important arboviral infection with four serotypes (DENV 1–4) that are capable of producing disease ranging from self-limiting dengue fever (DF) to severe life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. Each serotype has unique characteristics and can present with severe manifestations, in a particular population depending upon its interaction with the host response [2]. Epidemiology of dengue is ever changing in India. All the four serotypes have been reported from India. Factors such as crowded cities, unsafe drinking water, inadequate sanitation and large number of refugees facilitate the spread of dengue in different parts of the country, resulting in increased morbidity and mortality [3, 4,5].

In the absence of an effective vaccine, dengue prevention to a major extent relies on virological surveillance, and development of effective, locally adapted control programmes. Hence, continuous monitoring of circulating type of dengue virus is important in a given population to develop such efficient local control programmes. Most studies highlight the circulating serotypes during epidemics, however, between epidemics; there can be significant variation for which public health systems are normally unprepared. In the present study, we tried to identify the between- year non-epidemic serotype of dengue virus circulating in Hyderabad in the year June 2014-November 2015.

Till date diagnosis is mainly by dengue IgM capture ELISA even in tertiary care hospitals and Infectious Disease Surveillance Programme (IDSP) reference laboratories. But IgM appears only after 3-5 days of illness in primary infection and persist for 2-3 months, whereas in secondary infections it is not always positive and dengue IgG persists for many years. Dengue IgG and IgM antibodies in human sera cross-react with other flaviviruses[17]. The detection of dengue specific secretary NS1 (non-structural protein 1), a highly conserved glycoprotein represents a new approach to the diagnosis of acute DV infection, in recent times.

NS1 is highly specific marker for diagnosis of dengue from day 1 of the fever, No need of repeating the test for rising titers. It remains circulating in patient's blood for longer period than does viral RNA and is reported to be detectable even up to 14th day of illness. Although the most effective method to diagnose dengue in the acute phase of the illness recommended by the WHO is detection of DENV RNA, widespread use of dengue molecular diagnostics has been hampered by lack of validated tests and testing capability, perceptions that molecular diagnostics are cost prohibitive compared to immunoassays and lack of recommendations for their use. Molecular diagnosis based on reverse transcription (RT)-PCR, such as one-step or nested RT-PCR, nucleic acid sequence-based amplification (NASBA), or real-time RT-PCR and isothermal amplification methods like transcription mediated amplification (TMA), nucleic acid sequence based amplification (NASBA) and strand displacement amplification (SDA), has gradually replaced the virus isolation methods as the new standard for the detection of dengue virus in acute-phase serum samples. Serotype differentiation has been possible only by testing immune responses by neutralization tests or type-specific reverse transcription (RT)-PCR. Loop-mediated isothermal amplification is a novel method for amplifying DNA and RNA (LAMP and RT-LAMP respectively) with high specificity, sensitivity, and simplicity [6].

RT-PCR has been developed for the diagnosis of several diseases, and during the last years it has been revolutionizing the laboratorial diagnosis of infectious diseases. This method is rapid, sensitive, simple, and if correctly standardized, it can be used for genome detection in human clinical samples, biopsies, autopsy tissue or mosquitoes [7].

A number of RT-PCR procedures that detect and identify dengue serotypes in clinical specimens have been reported [8-13]. These PCR methods vary somewhat in terms of the amplified gene regions of the genome, in the way they detect RT-PCR products, and the virus typing methods. According to the World Health Organization, PCR is a powerful method to be used for dengue diagnosis.

In the present study, the increase of dengue cases accompanied by severe forms of disease and the detection of two dengue serotypes in the city show that it is necessary to study the clinical, molecular, and epidemiological characteristics of dengue cases during a major dengue outbreak in Hyderabad, Telangana, India.

MATERIALS AND METHODS

Approval of the Institute's ethical committee was obtained to carry out the study.

Settings:

Study Place: Department of Microbiology, Gandhi Medical College, Secunderabad

Study design: Prospective Cross-sectional Descriptive study

Study period: 18 months (June 2014-November 2015)

Inclusion criteria:

The patients of all age groups and both the sexes, having temperature>38.5° C for >24 hr and ≤10 days of illness who were clinically diagnosed as having Dengue fever admitted in Medical and Pediatric Wards of Gandhi Hospital

Exclusion criteria

Febrile patients with duration of illness >10 days and Immunocompromised.

The following are the case definitions as per WHO classification 1997 which were applied for the study[14].

Suspected clinical case of dengue fever is defined as acute febrile illness with 2 or more of the following: headache, retro orbital pain, myalgia, arthralgia, rash, haemorrhagic manifestations & leucopoenia.

Probable case of dengue is a case compatible with clinical description and with positive IgM antibody test in acute serum specimen.

Confirmed case of dengue fever is defined as a case compatible with clinical description and laboratory confirmed either by detection of nucleic acid detection in the serum or NS1 antigen detection by validated immunoassay or fourfold rise in IgM or IgG antibodies in paired samples collected in acute and convalescent stages.

Materials

From June 2014 to November 2015, 1026 clinically suspected dengue patients attending Gandhi Hospital serum samples were referred to Microbiology laboratory for Dengue diagnosis. Serum samples were screened for dengue NS1 antigen and IgM antibodies by ELISA(Group A)

Out of 1026 clinically suspected cases, a subset of 330 cases, who could be followed up and who gave consent were included in the study(Group B), detailed clinical and epidemiological and laboratory data were recorded using structured proforma. IgG ELISA was also done for these samples.

Out of 330 cases in Group B, serum samples from randomly selected 119 patients (Group C) were subjected to nested multiplex RT PCR for detection and serotyping of Dengue virus.

Methods

Dengue NS1 antigen detection was done by sandwich ELISA (Panbio, Australia).Dengue IgM antibody detection was done by MAC ELISA supplied by Division of Arbovirus diagnostics(NIV, Pune),dengue IgG ELISA was done by indirect ELISA (Novatech immunodiagnostica, GmbH, Germany) . All the tests were performed according to the manufacturer's instructions.

Dengue viral RNA detection and serotyping.

From 119 patients (Group C), serum samples were subjected to multiplex nested reverse transcription PCR.

Molecular method by multiplex nested RT- PCR

RNA extraction was done by conventional method-Trizol-Chloroform-Isopropyl Alcohol Method. The amplified PCR product was subjected to 2% agarose gel electrophoresis. The amplified bands were detected by gel doc system. Well characterized serum samples showing NS1 positivity and RT PCR positivity were used as controls.

RESULTS

Serological profile of clinically suspected cases included in the study group (n=330)

Out of 330 samples, NS1 antigen was positive in 75 cases (22.7%), IgM ELISA positive in 118 cases (35.7%) and IgG was positive in 281 cases ((85.1%). Though the percentage of IgG positive samples was high, they were not considered due to their persistence lifelong and also as paired sera was not collected from the patients for confirmation of Dengue infection (Table 1).

Among the 330 clinically suspected cases (group B), 72 cases were confirmed cases. Among 119 cases (group C) that were selected randomly, in 71 NS1 Ag positive cases, RT PCR positivity was 39.4%. In 103 IgM positive cases, RT PCR positivity was 20.38 % (Table 4).

Thus sensitivity of NS1 Ag was 97.26 % and sensitivity of multiplex RT PCR was 40.27 %, while specificity for both was 100 %(Table 5).

The predominant serotype in the year 2014 and 2015 was DENV 1 serotype followed by DENV 3 serotype (Table 6). However only 23 cases in 2014 and 96 cases in 2015 were subjected to RT PCR which was not sufficient enough for comparison of the serotypes for both the years.

DENV1 serotype was found to have more haemorrhagic manifestations (57.6%) than DENV 3 serotype (33.33%) (Table 7).

Table 1. Serological profile of clinically suspected cases (n=330)

Parameter	No of cases positive by respective ELISAs
NS1 Ag only	8
NS1 Ag + IgM	23

NS1 Ag + IgG	8
NS1 Ag + IgM + IgG	36
IgM + IgG	41
IgM only	18
IgG only	196
Total	330

Table 2. Duration of illness in dengue confirmed cases at the time of admission (n= 72)

Duration of illness at the time of admission	No of confirmed dengue cases(n=72)%
1-4 day	34(47.22%)
5-7 day	25(34.72 %)
>8 day	13(18.05%)
Total	72

Table 3. Clinical Classification of dengue confirmed cases (WHO guidelines) (n=72)

Grade	No of confirmed cases (n=72) (%)
DF	49(68.05%)
DHF grade 1	9(12.5%)
DHF grade 2	6(8.3%)
DHF grade 3	5(6.9%)
DHF grade 4	3(4.16%)
Total	72

Table 4. Relationship between dengue serology parameters and multiplex RT PCR (n=119)

Positive by	No of cases screened for PCR	No of positive cases detected by PCR
NS1Ag only	16	8(50%)
NS1Ag + IgM	55	20(36.36%)
IgM only	48	1(2.08%)
Total	119	29(24.3%)

Table 5. Efficacy of the NS1 antigen and RT PCR assays used in the diagnosis of dengue fever

Test	Sensitivity	Specificity	Positive predictive value	Negative predictive value
NS1 Ag	97.26%	100%	100%	97.91%
Multiplex nested RT PCR(RT PCR)	40.27%	100%	100%	52.22%

Table 6. Distribution of Dengue serotype among dengue cases positive by multiplex RT PCR

Dengue virus serotype	No of positive cases (n= 29)	
	2014	2015
DEN 1	2	24
DEN 3	1	2
Total	3	26

Table 7. Association of haemorrhagic manifestation with serotype isolated

Serotype	Total no of cases positive for the serotype	No of cases showing hemorrhagic manifestations (%)
DENV 1	26	15 (57.6%)
DENV 3	3	1 (33.33%)
Total	29	16(55.17%)

Table 8. Comparison of RT PCR positive samples among Confirmed dengue cases with Sample collection post fever onset

Sample collection post fever onset	Anitha Chakravarthi et al (Delhi)(2006)	Nishat Hussain et al (Delhi) (2014)	Swathi et al (Telangana) (2011-2012)	Present study (Telangana) (2014-2015)
Day1	5	3	0	0
Day2	4	12	0	1
Day 3	14	23	3	10
Day 4	22	7	6	6
Day 5	21	5	5	8
Day 6	6	1	3	2
Day 7	3		3	1
Day 8	1		2	1
Day 9			1	0
Day 10			0	0

DISCUSSION

Dengue virus infection is a major, growing public health problem with an estimated 2.5 billion people at risk of infection. Dengue viruses cause a range of well-described clinical illness. Infection ranges from an asymptomatic infection to a self-limiting febrile illness, DF, to severe dengue, a clinical syndrome that typically presents with capillary permeability and can lead to DF and DHF. Dengue epidemics can have a significant economic and health toll in any country. Globally dengue transmission has expanded in recent years and all the four dengue serotypes (DENV-4) are now circulating in Asia. Development of effective surveillance and disease prevention programmes for any disease depend upon the variation in the epidemiology.

In the present study, higher prevalence of DF cases was seen (68.05%). These finding were similar to previous findings [15, 16,17]. Some studies showed contrary results and reported more prevalence of DHF than DF cases reported [18, 19, 20].

In the present study, NS1 antigen only was positive in 2.12 %, IgM only positive in 6%, IgG only in 59.3%. This study was in agreement with other previous studies[15]. Whereas some earlier studies reported 29.6% positive by NS1 antigen only, 53.3 % by IgM only and 2.8 % by IgG only [21]. Earlier studies reported, a total of 190 serum samples were tested positive by either one or a combination of the four methods whereas, only 59 serum samples were tested positive by all four methods. 34.1% were laboratory-confirmed acute dengue. The overall test sensitivity was 91.6%, 40.5%, 48.4% and 58.9% for dengue NS1 antigen-capture ELISA, virus isolation, conventional RT-PCR and real-time RT-PCR respectively [22].

In the present study, DENV1 and DENV 3 serotypes were prevalent in the years 2014 and 2015. In previous study in Telangana reported during the year 2011-2012, showed DENV 1 as the predominant serotype in the year 2011 and DENV 3 as the predominant serotype in year 2012.While no study was conducted in the year 2013, DENV 1 serotype was found to be the predominant serotype again in 2014 and 2015 while DENV 3 was isolated only in 3 samples may be because of the herd immunity acquired in the previous years.

In the present study DENV1 was associated with more haemorrhagic manifestations than DENV 3, these findings similar to other previous reports [23]. Some earlier other reports found that more haemorrhagic manifestations in DENV-1 and DENV-3 coinfection. [23, 15]. But some reported more haemorrhagic manifestations with DENV-2 serotype [24].

In the present study, we found the circulation of DENV-1 type in Hyderabad during the non-epidemic period of 2011–12 after complete predominance of DENV-3 in earlier reported outbreaks [15]. Epidemic causing aggressive and more pathogenic strain is always replaced by milder strain of the virus during the non-epidemic period. Earlier similar pattern was observed following massive outbreak by DENV-3 in Hyderabadin the year 2012 following which DENV-1 strain predominated the circulation in the nonepidemic period. Clinical significance of the findings is that most of the people living in Hyderabad are probably immune to DENV-3 and DENV-1 but chances of an outbreak by less prevalent serotypes are still there. Subsequent infection with different serotype of the virus leads to more severe clinical disease due to antibody enhancement phenomenon. Mosquito control programme needs to be more active to prevent any future outbreak.

During present study, highest viral isolation was seen with 3 days duration of illness in this study [Table 8]. These findings were similar to other previous reports [25]. Whereas some reports found highest viral isolation with 4 days of duration of illness [15, 26].

In the present study, sensitivity of RT PCR was 40.27 %, these findings were similar or close to other previous reports.[15,27]. The other contrary reports found that low sensitivity (5.3%) and high sensitivity [28, 29].

The earlier findings found that the overall test sensitivity was 91.6%, 40.5%, 48.4% and 58.9% for dengue NS1 antigen-capture ELISA, virus isolation, conventional RT-PCR and real-time RT-PCR respectively [22]. Further benefits of the DENV multiplex RT-PCR include its single-reaction set-up and a reaction set-up-to –result time that is much shorter than the hemi nested PCR. This study involved a rigorous laboratory evaluation of the DENV multiplex RT-PCR, which was designed using more than 500 DENV sequences from varied geographic locations over the past twenty years.

From these studies we mainly observed with household mosquito elimination programmes on an overdrive, dengue-causing *Aedes* mosquitoes seemingly infect the young employed population which is mostly outdoors. Our finding further corroborates that dengue infection in Hyderabad more commonly affects the age group 11–30 yrs. Maximum cases were seen during the months of September– October in concordance with various other studies and similar to that is seen during epidemic period. This is due to post-monsoon collection of water and increased availability of breeding sites for the *Aedes* mosquito. To our knowledge, this is the prospective clinical study to compare DENV-1 and DENV-3 serotype patients in relation to antibody response pattern and severity of the disease. Our findings contribute to the understanding of clinical differences and immune status related to serotypes DENV-1 and DENV-3 in Hyderabad city.

CONCLUSION

The problem of dengue is huge in our country and needs proper implementation of control measures before any further huge epidemic occurs. All of us have developed immunity to DENV-1 in Hyderabad which is new to the population and may take a form of epidemic strain. Adequate and timely preventive measures should be taken to prevent any future epidemic. Such issues may be of interest to epidemiologists and virologists for planning effective control of the virus or for future vaccine development. Future endeavours should be made to develop improved laboratory- based surveillance systems that can forecast impending dengue epidemics.

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