Molecular Characterization and Serological Analysis of Dengue Viruses in Amritsar District

Rubina Paul[1], Loveena Oberoi[2], Kanwardeep Singh[3], Pushpa Devi[4]

ABSTRACT

Introduction: Dengue is a common mosquito-borne viral disease of humans that in recent years has become a major international public health concern. There are four serotypes of the dengue virus i.e DENV-1, DENV-2, DENV-3, and DENV-4 circulating in Indian population and can cause the full spectrum of disease from a subclinical infection to a mild self-limiting disease, the dengue fever (DF), severe disease that may be fatal, the dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). In the present study, serological analysis, molecular detection and serotyping of dengue virus was done to look for the prevalent strains of dengue virus in our area.

Material and methods: Blood samples were collected from clinically suspected patients of dengue fever selected as per WHO criteria. Samples were screened for the presence of dengue-specific IgM antibodies using MAC-ELISA and dengue NS1 antigen detection was done using Pan Bio (Australia) NS1 ELISA kit, according to the duration of fever as per NVBDCP guidelines. Molecular detection and serotyping was done in 50 NS1 positive samples, by single step multiplex RT-PCR.

Results: Total of 3306 samples were tested for dengue. 60.6% were serological positive for were positive for both seromarkers of dengue infection. Seasonal variation of disease was seen and majority of cases tested positive for dengue were obtained in the month of September and October. Male predominance was seen among patients suffering from dengue. Adult age group had higher burden of disease than the pediatric age. Serotypic characterization observed the predominance of DENV-1 (57%) followed by DENV-3 (40%) and DENV-2 (3%) indicating co-circulation of multiple serotypes.

Conclusion: Laboratory-based active surveillance systems are needed to complement the current passive surveillance and control programs to detect and monitor sudden increases in the numbers of dengue cases or changes in the predominant serotypes.

Keywords: Dengue, NS1 Antigen, RT- PCR.

INTRODUCTION

Dengue is a common mosquito-borne viral disease of humans that in recent years has become a major international public health concern.[1] According to the World Health Organization (WHO), the dengue virus poses a threat to over 2.5 billion people, about 40% of the world's population. The Center for Disease Control and Prevention (CDC) estimates that every year the dengue virus affects over 100 million people, causing undifferentiated febrile illness, dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. These conditions result in approximately 500,000 hospitalizations and 24,000 deaths which mostly occurred in children.[2]

Dengue viruses (DV) belong to the family Flaviviridae, and there are four serotypes of the virus referred to as DENV-1, DENV-2, DENV-3, and DENV-4. Dengue Virus is a positive-stranded encapsulated RNA virus and is composed of three structural protein genes, which encode the nucleocapsid or core (C) protein, a membrane-associated (M) protein, an enveloped (E) glycoprotein, and seven nonstructural (NS) proteins. It is transmitted mainly by *Aedes aegypti* mosquito and also by *Ae. albopictus*. All four serotypes i.e DENV-1 to DENV-4 can cause the full spectrum of disease ranging from a mild self-limiting disease, the dengue fever (DF), to life threatening dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS).[3] In the present study, serological analysis, molecular detection and serotyping of dengue virus was done to look for the prevalent strains of dengue virus in our area. PCR has the added advantage of being able to provide information on circulating serotype, which is essential for virus surveillance.

MATERIAL AND METHODS

The study was carried out in Microbiology department of Government Medical College Amritsar from the period January 2016 to June 2017. Clinically suspected patients of dengue fever along with presence of headache, myalgia, retro orbital pain, rash and haemorrhagic manifestations selected as per WHO criteria,[4] presenting to the emergency, outpatient and indoor services of our institute were included in our study. Demographic details and clinical history with along with the relevant clinical investigations (haematological and biochemical) was recorded were in laboratory request forms. 5 ml of blood sample was collected aseptically by venipuncture in a plain vacutainer and allowed to clot at room temperature and then centrifuged at 2000 rpm for 10 mins. The sera separated after centrifugation was aliquoted into sterile 1.5ml storage vials. As per NVBDCP (National Vector Borne Disease Control Program), if duration of fever in patients was more than 5 days the samples were screened for the presence of dengue-specific IgM antibodies by IgM antibody capture enzyme-linked immunosorbent

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assay (MAC-ELISA) using kit prepared by the National Institute of Virology, Pune, India, strictly following the manufacturer's protocol. In case of, acute dengue fever cases (fever duration ≤ 5 days) detection of dengue NS1 antigen in the acute sera, was done using Pan Bio (Australia) NS1 ELISA kit. Molecular detection and serotyping was done in 50 NS1 positive samples, by single step multiplex RT-PCR using the following primers as described by Lanciotti et al.[5]

RESULTS

During the study period, total of 3306 samples were tested for dengue. Out of total sample tested 60.6% (n=2005) were positive for both seromarkers of dengue. 59% (n= 1181) of samples were tested positive for NS1 antigen whereas, 41% (n=824) samples showed positivity for the presence of IgM antibody. [Table 1]

Majority of cases tested for dengue were obtained in year 2016 in the month of August, September and October with a highest peak in September and October followed by decline in positive cases in November. [Fig 1]

Seropositivity for dengue was high in 21-30 years of age group 837/2005 (42%) followed by 31-40 years of age 549/2005 (27%). Percentage positivities in male and female patients suffering from dengue fever were 64% (n=1283) and 36% (n=722) [Fig 2] and the affected male: female ratio of patients suffering from dengue in this study was found to be 1.8:1.

Dengue serology was positive in 59% cases (n= 1183) in samples from urban areas and 41% positive (n=822) in samples coming from rural areas.

A total of 50 acute phase samples, which were dengue NS1 antigen positive were subjected to one step multiplex RT-PCR to detect the presence dengue serotypes using four serotype specific primers. Out of 50 samples, 35 sample (70%) were tested positive for dengue virus RNA in our study. NS1 positive samples. DENV-1 serotype was found in 20 samples, DENV-2 serotype was found in 1 sample and DENV-3 serotype was found in 14 samples. DENV-4 serotype was not found in any sample. Viral RNA was not detected in 15 samples. [Table 2]

Test performed	Positive	Percentage
NS 1 antigen	1181	59%
IGM antibody	824	41%
Total positive	2005	100%

Table-1: Percentage positivities of NS1 antigen and IGM antibodies

Serotype detected	No. of Samples	Percentage
DENV-1	20	57%
DENV-2	1	3%
DENV-3	14	40%
DENV-4	0	0%
Total	35	100%

Table-2: Percentage of various serotypes detected

Clinical Manifestations	DENV-1	DENV-2	DENV-3	Total
Rash	5	1	6	12
Headache	17	1	13	31
Myalgia	20	1	13	34
Arthralgia	16	1	11	28
Retro-orbital pain	4	0	5	9
Haemorrhagic manifestations	10	1	4	15
Conjunctival haemorrhage	3	0	2	5
Nausea and Vomitting	11	1	8	20
Pain Abdomen	10	1	7	18
ARDS	2	1	0	3
Altered sensorium	0	0	0	0
Decreased urine output	3	0	2	5

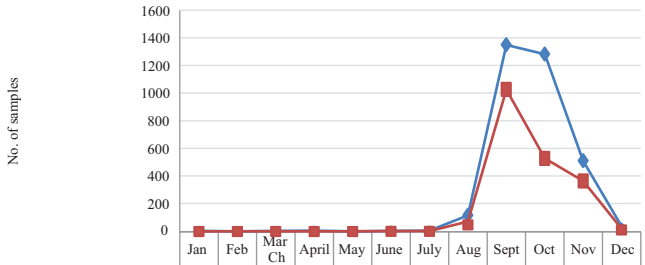
Table-3: Clinical manifestations among dengue serotypes

Dengue Serotype	Mild (100,000 - 1.5 lakh/cu mm)	Moderate (50,000 - 100,000/cu mm)	Severe (< 50,000/ cu mm)
DENV-1	2	12	6
DENV-2	0	0	1
DENV-3	5	6	3
Total	7	18	10

Table-4: Thrombocytopenia among dengue serotypes

Lab Findings	DENV-1	DENV-2	DENV-3	Total
Leukopenia	18	1	11	30
Hemoconcentration	10	1	6	17
Hepatic Dysfunction	13	1	7	21
Renal Dysfunction	6	1	3	10

Table-5: Other laboratory findings among dengue serotypes



	Jan	Feb	Mar Ch	April	May	June	July	Aug	Sept	Oct	Nov	Dec
Total samples tested	2	0	2	4	0	3	5	115	1349	1281	509	36
Total samples positive	0	0	0	0	0	1	1	70	1026	527	363	17

Figure-1: Month wise distribution of dengue fever cases (2016)

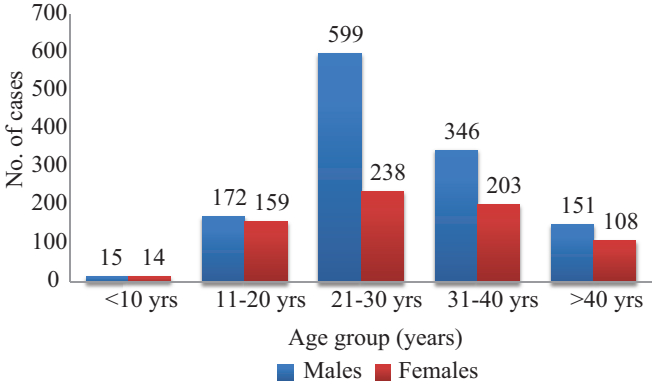


Figure-2: Age and sex distribution of dengue positive cases

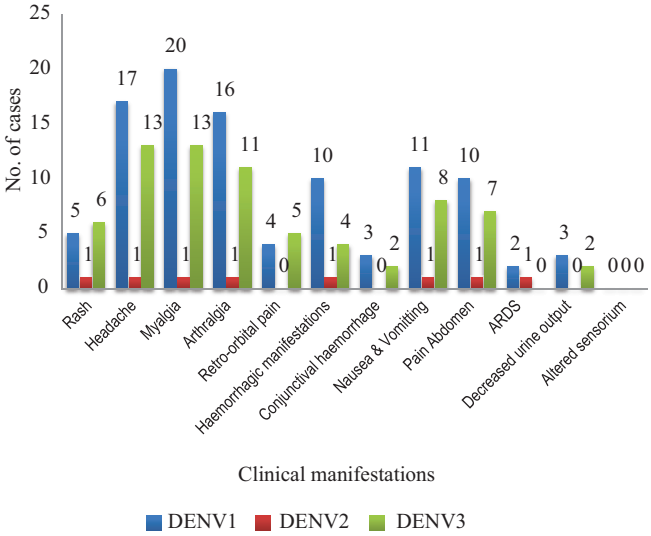


Figure-3: Clinical Manifestations among dengue serotypes

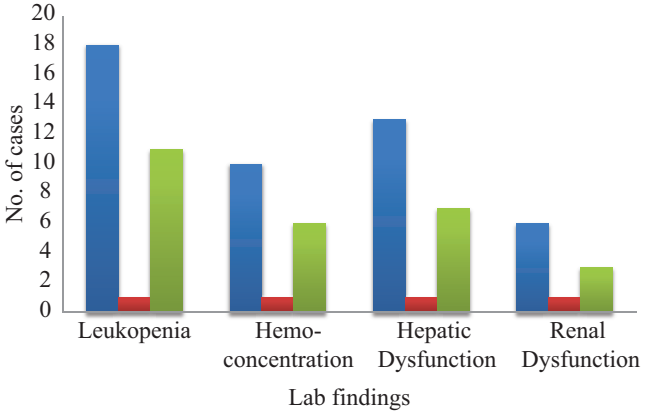


Figure-4: Other Laboratory findings among dengue serotypes

Pic-1: Gel documentation digital image of RT-PCR for dengue virus serotyping showing DENV-1, DENV-2, DENV-3 serotypesa

DENV-1 was most common serotype isolated from the patients admitted in our hospital 18/25, whereas DENV-3 serotype was identified in patients attending the OPDs 8/10 Myalgia (20/34), headache (17/31), haemorrhagic manifestations (10/15) and arthralgia (16/28) were seen in higher number of DENV-1 infected patients. Rash (6/12) and retro-orbital pain (5/9) were seen in higher number of DENV-3 infected cases. Acute respiratory distress syndrome (2/3) was seen in DENV-1 infected patients. Dengue haemorrhagic fever was observed in 10/15 cases infected with DENV-1 and 4/15 cases with DENV-3. [Table3 and Fig 3]

Moderate thrombocytopenia was seen in DENV-1 (12/18) and DENV-3 (6/18) respectively. DENV-2 infected case showed severe thrombocytopenia. [Table 4]

Among laboratory findings leukopenia was observed in 18/30 patients and 11/30 patients infected with DENV-1 and DENV-3 serotypes. Hemoconcentration as evidenced by raised hematocrit was seen 10/17 patients infected with DENV-1 and 6 / 17 patients infected with DENV-3 serotypes. Hepatic dysfunction (13/21) and renal dysfunction (6/10) was observed more in DENV-1 infected cases.[Table 5 and Fig 4]

DISCUSSION

Dengue is an important emerging disease of the tropical and subtropical regions today. Dengue infection has been known to be endemic in many parts of India for over two centuries as a benign and self-limited disease. Epidemics of dengue are increasing in frequency. Detection of all four dengue serotypes in India has now rendered India hyperendemic.[6]

In our study, 3306 patients clinically suspected of dengue fever were tested for the seromarkers of dengue fever. In this study, 60.6% (2005/3306) of patients were serologically positive for dengue virus out of total samples tested, in concordance with a study showing 53.2% seropositivity,[7] indicating increased dengue virus activity. The reason may be due to high prevalence of mosquitoes in this region. Studies have proposed that ecological and climatic factors influence the seasonal prevalence of the vector *Aedes aegypti* and Dengue virus.[8]

In the current study, 59% (1181) of total samples (3306) were tested positive for NS1 antigen whereas, 41%(824) samples

showed positivity for the presence of dengue specific IgM antibody. On analysis of data on monthly basis, seasonal variation of disease was seen. The seasonality of transmission of dengue with increased activity in monsoon and post monsoon season in the present study was in accordance with the reported patterns of dengue transmission in other studies.[9] This can be explained by stagnant water sources following heavy rainfall favoring breeding of the mosquito vector resulting in increased post monsoon incidence of dengue, thereby maintaining the vector population throughout the year.[10] Effective control measures and preventive measures should come into full swing during water stagnation periods after the initial bouts of rainfall and at the end of monsoon.[11]

In the present study, seropositivity for dengue fever was high in 21-30 years of age group, (42%) followed by 31-40 years of age, (27%) out of total dengue positive cases. These findings are consistent with other Indian studies.[12,13,14] Gupta et al[13] and Chakravarti and Kumaria[15] also reported maximum cases in the age group 21–30 years. This may be due to the reason that active adults are doing more outdoor work so there are more chances for them to get infected. The higher prevalence of dengue infection was noted among males (64%) than females (36%) The affected male to female ratio was 1.8:1 which correlates well with other studies undertaken in North India[16,13] and South India[12] where male affliction of the disease has been reported.

Seropositivity of dengue fever was high in urban (59%) as compared to the rural population (41%). DF/DHF has been reported as occurring predominantly among urban populations where density of dwellings and short flying distance of the vector create the right conditions for transmission.[17] Rapid unplanned urbanization with unchecked construction activities and poor sanitation facilities contribute to fertile breeding grounds for mosquitoes.[11]

Molecular detection and serotyping by single step multiplex RT-PCR detected viral RNA in 35 samples out of 50 (70%) NS1 positive samples.The studies conducted in Delhi during 2013[18] and 2014,[19] reported, dengue viral RNA, RT-PCR positivity to be 71.16% and 80% respectively in concordance with our study. In the present study, 15 samples, where NS1 antigen was positive but RT-PCR was negative, duration of fever was 5 days, as by this time viremia declines. Only 3 out of 50 sample samples showing presence of dengue viral RNA by RT-PCR were positive for dengue specific IgM antibodies, due to the fact that acute phase samples (fever upto 5 days) were collected during the study in order to get dengue serotypes positive as per the objective of our study and IgM antibody appears after five days of onset of fever. Serotypic characterization observed the predominance of DENV-1 (57%) followed by DENV-3 (40%) and DENV-2 (3%) indicating co-circulation of multiple serotypes. DENV-4 serotype was not reported in our study and no case of concomitant infection with more than one serotype was observed. Thus DENV-1 dominated in most patients followed by DENV-3 in the present study.

In our study, DENV-1 was reported to be causing more hospitalizations among dengue fever affected patients as it was prevalent predominantly among indoor patients (51%) and DENV-3 was found among patients attending OPDs (23%) of our hospitals. This association was found to be statistically significant (p value<0.05). Similar findings were observed in a study done in Cambodia where 44.4% of DENV-1 cases were hospitalized.[20]

The frequency of clinical manifestations was higher in DENV-1 affected patients also leading to increased hospitalizations as observed in this study. Haemorrhagic manifestations like petechiae, purpurae, epistaxis, positive tourniquet test (20 or more petechiae in 2.5 cm^2 area) myalgias and arthralgias were in significantly (p value <0.05) higher in patients infected with DENV-1 whereas as cutaneous manisfestation of rash had a significantly higher prevalence in DENV-3 infected individuals. DHF was also significantly higher in DENV-1 infected patients. In our study, complication of acute respiratory distress syndrome (ARDS) was seen in only 2 cases of DENV-1 infected serotype and single case of DENV-2 serotype.

Patients with a detectable DENV-1 serotype were more prone to present with several clinical and laboratory features. In the present study moderate to severe thrombocytopenia, leukopenia, hemoconcentration as evidenced by raised hematocrit percentage, hepatic dysfunction and renal dysfunction were significantly higher among DENV-1 patients. Dengue virus severity may be influenced by multiple factors such as by the virus serotype, genotype, host and environment factors. Among viral factors nature of infecting dengue serotypes may play an important role in disease severity.[21]

CONCLUSION

Dengue fever is a re-emerging public health problem with two-fifths of world population being at risk of infection. approximately 100 million dengue fever (DF) cases and several hundred thousand cases of dengue hemorrhagic fever(DHF) occur annually.[22] Our study highlights 3 serotypes i.e DENV-1,DENV-2 and DENV-3 with DENV-1 and DENV-3 serotypes predominantly in circulation in our geographical region. Laboratory-based active surveillance systems are needed to complement the current passive surveillance and control programs. Regular sentinel surveillance and sample surveys during interepidemic periods are also necessary to detect and monitor sudden increases in the numbers of dengue cases or changes in the predominant serotypes which usually precede major outbreaks. New molecular diagnostic techniques, such as RT-PCR, are particularly useful in this context, their speed and sensitivity enabling the rapid detection of increased viral circulation or changes in predominant serotypes.

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