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Dengue detection and serotyping using multiplex real time polymerase chain reaction: Study from a tertiary care centre in Eastern India

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Abstract

Introduction: Dengue is an arboviral infection transmitted through the bite of *Aedes* mosquitoes having four distinct serotypes DENV 1-4. Secondary infections often lead to increased disease severity. The study was carried out with an aim to analyze dominant serotype in circulation and to find an association between serotype and hematological parameters.
Material & Methods: A total of 163 Dengue Positive samples were subjected to serotype-specific Real-time Polymerase chain reaction to identify the serotype. Hematological parameters *viz*; WBC counts, Hematocrit value, platelets, lymphocyte % were also studied concurrently for these patients.
Results: The mean age of the participants was 27.2 years with male preponderance.NS1 antigen positive (74.8%), IgM (38%) and both NS1 & IgM positives (12.8%) were detected. The predominant serotype identified was DENV-2(48.4%), DENV-4(43.5%), and DENV-3 (7.9%). Statistically significant difference in platelet counts was observed in DENV-2 & DENV-4.White blood cell counts showed statistically significant differences within DENV-2 and DENV-3 serotypes.
Conclusion: Cocirculation of multiple serotypes of DENV was observed in our study. The application of molecular testing will increase DENV diagnostics, especially in secondary infections which carry more chances of adverse outcomes. We recommend the inclusion of serotype testing in the Dengue diagnostic algorithm to be able to reduce morbidity and mortality.

Keywords: Dengue virus, serotyping, real-time reverse transcriptase polymerase chain reaction, thrombocytopenia, antibody dependent enhancement

Introduction

The earliest documented history about the appearance of the Dengue virus (DENV) can be traced to 992 AD in a Chinese medical encyclopedia (Holmes, 2003; Gubler, 1998). By the end of the 19th century & early 20th century, the virus had widely spread in the tropics &subtropical regions

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causing intermittent epidemics in Asia, especially the South-East Asian region (Holmes, 2003; Monath, 1994). The credit for uncovering the Dengue virus goes to two scientists Ren Kimura & Susumu Hotta in 1943 while studying blood samples from an epidemic in Nagasaki, Japan (Hotta, 1952). The virus was later isolated independently by Albert B Sabin and Walter Schlesinger a year later (Dey, 2017). Since then the Virus has caused major epidemics in almost entire South-East Asian countries, the African subcontinent & Latin America (NCVBDC, 2022; WHO, 2022; CDC, 2022). DENV is an Arbovirus (Arthropod-borne virus) belonging to Family *Flaviviridae* and is transmitted through the bite of Infected Mosquitoes. The primary vector is *Aedes aegypti* & *Aedes albopictus.* DENV has 04 distinct serotypes DENV-1, DENV-2, DENV-3 & DENV-4 (Murugesan *et al*, 2019). Recovery from one particular serotype provides lifelong immunity against that particular serotype but the individual remains susceptible to further DENV infections from other serotypes due to almost nonexistent cross-immunity between serotypes and subsequent infections have an increased risk of developing into severe dengue by a phenomenon known as Antibody-dependent enhancement (Mady *et al.*, 1991; Gupta *et al.*, 2012). From what was considered as an emerging disease just under two decades back, the disease has now shown a dramatic rise in incidence and possess an alarming impact on human health and the economic burden associated with the disease. WHO estimates the annual disease burden at roughly 390 million cases worldwide of which about a quarter manifest clinically. The majority of the disease burden is seen in Asia, accounting for 70% of the total cases (Bhatt *et al.*, 2013). As per data available from the National Vector Borne Disease control program (NVBDCP), India reported a total of 1,23,106 cases of DENV infections and 90 deaths directly attributable to the infection (NCVBDC, 2022). All four serotypes are known to be in circulation in India and it has been observed that amongst four, DENV-2 is the dominant serotype in circulation (Alagarasu *et al*, 2021). Spatial variations in the circulating serotypes are seen and hence it necessitates having a true picture of the dominant serotype in a particular region at any given point of time to help understand the disease physiology and improve patient outcomes (Kumaria, 2010; Gupta *et al*, 2006). ELISA & Rapid tests are widely available which help in simultaneous detection of NS1 antigen, IgM & IgG antibodies, however, they are not able to characterize the serotype. Various Multiplex based formats of Real time Reverse transcriptase Polymerase chain reaction are commercially available which along with detection of DEN virus also characterizes the infecting serotype. This study aimed to analyze the dominant serotype in circulation in this region and to find an association between the serotype and various blood parameters *viz*; White blood cell count (WBC), Hematocrit (Hct), Lymphocytes, Platelets.

Material and Methods

The study was carried out at a tertiary care centre cum teaching hospital and its subsidiary diagnostic laboratory in Eastern Uttar Pradesh between July 2021 to January 2022 when the region sees a cyclical surge in DENV cases. A total of 163 non-repeat DENV positive samples obtained from both laboratories were studied. The Inclusion criteria were all consenting individuals who were found to be positive by Rapid lateral flow assay using Dengue Day1 test commercially manufactured by J. Mitra & Co Pvt Ltd which simultaneously detects NS1 antigen, IgM & IgG antibodies. Patients unwilling for subjecting their samples to RT PCR and Patients detected to be non-Reactive by rapid screening test were excluded from the study.

For PCR & Serotyping Hi-PCR Dengue Serotyping Probe PCR Kit (MBPCR137)HI Media Laboratories Pvt. Ltd, India was used which is designed to detect the polyprotein gene of Dengue serotypes 1,2,3 & 4 in FAM, JOE, Texas Red, Cy5 channels respectively with Internal Control in Cy5.5 channel in a single tube reaction. RNA extraction from serum sample was done using TruPCR viral RNA Extraction kit based on spin-column principle as per manufacturer's instructions. The total reaction volume was 25ul/reaction, which comprised of RT buffer-5ul, 10X SolutionH-2.5ul, M-MuLV RT-1ul, DENV 1-4 Primer Probe mix-4ul, IC Primer Probe mix-1ul, IC BDNA-1ul and Molecular grade water -5.5 ul for each reaction.

The PCR program was set as per the kit manufacturer's instructions using a 2-step amplification process. The protocol followed was Reverse Transcription at 50 ^{o}C for 15minutes, Initial denaturation at 95 ^{o}C for 2minutes 30 seconds followed by Denaturation at 95 ^{o}C for 15 seconds and annealing/extension at 60 ^{o}C for 1minute.The cycle was repeated 45times.The interpretation was done at Ct value <= 40 was considered Detected and > 40 or N/A as Not detected. The other parameter under study were WBC count, Hct levels, Lymphocyte count and Platelet count. Whole blood samples taken from these patients were processed by an automated hematology analyzer

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machine, Mindray BC-5150 (Shenzhen, China). The results obtained were tabulated using MS-Excel 2010 software and data was statistically analyzed. Analysis was be performed using R software version 4.0.2. The one-sample Kolmogorov-Smirnov test was employed to determine whether the data sets differed from a normal distribution or not. Normally distributed data was analyzed using parametric tests and non-normally distributed data was analyzed using non-parametric tests. Descriptive statistics was calculated for qualitative and categorical variables. Graphical representation of the variable shown to understand the results clearly and to measure the association for the categorical dataset will be analyzed using the Chi-Square test. Independent T-test or student t-test was applied to measure the mean difference between the two groups. ANOVA (analysis of variance) test was applied to measure the mean difference for more than two groups. The correlation will be estimated to measure the strength of the relationship between two or more quantitative variables. If p-value <0.05, is considered as statistically significant and if p-value >0.05, then it was considered to be statistically insignificant.

Results

A total of 163 DENV positive samples were studied. 105 (64.4%) samples were from male participants and 58 (35.6%) samples were obtained from females. The mean age profile of the patients was 27.2 years (Range 1year-77 years). Majority of the Patients were outpatients (n=138; 84.7%), inpatients accounted for (n=25; 15.3%) of our study population. NS1 positive was detected in n=122(74.8%) and non-reactive in n=41(25.2%), IgM was detected in n=62(38%) of the samples and IgG reactive was detected in only 1(0.6%) sample. Both NS1antigen & IgM reactive was detected in n= 21(12.88%) of the samples. PCR was able to detect all 163 samples tested by rapid assay. The descriptive statistics have been summarized in Table 1.

Table 1: Descriptive statistics of demographical and laboratory parameters

Parameter	N	Minimum	Maximum	Mean ± SD
Age	163	.9	77.0	27.3 ± 16.05
Platelet count (Lakhs/cmm)	163	.14	5.98	1.37 ± 0.89
Hematocrit (%)	163	21.3	54.3	38.8 ± 6.19
White blood cells(/cmm)	163	2200	88000	7063.93 ± 8177.61
Lymphocyte %	163	6	80	33.66 ± 15.11

Of the 163 DENV positive samples we did not find any DENV-1(n=0), DENV-2 was the predominant serotype accounting for 48.4% of the total (n= 79), DENV-3 accounted for 7.97% (n=13) & DENV-4 was detected in 43.5% (n= 71) of the total cases. The frequency distribution of Dengue Virus Serotypes has been summarized in figure 1. An association study between the serotype obtained and Hematological parameters including WBCs, Platelets, Hematocrit and Lymphocytes has been summarized in Table 2. Post hoc analysis of mean difference of serotypes within groups with respect to different variables is depicted in Table 3.

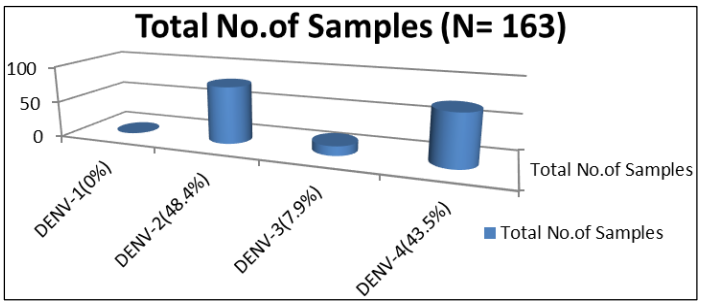

Fig 1: Frequency distribution of Dengue Virus Serotypes detected

Table 2: Comparison of mean difference between serotypes with respect to different variables

Variables Name	DENV	N	Mean ± SD	p-value
Age	2.00	79	28.45 ± 15.61	.649
	3.00	13	27.39 ± 12.42	
	4.00	71	26 ± 17.17	

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	Total	163	27.3 ± 16.05	
Platelet count (Lakhs/cmm)	2.00	79	1.2 ± 0.61	.037
	3.00	13	1.28 ± 1	
	4.00	71	1.57 ± 1.09	
	Total	163	1.37 ± 0.89	
Hematocrit (%)	2.00	79	39.53 ± 5.81	.344
	3.00	13	37.84 ± 7.94	
	4.00	71	38.17 ± 6.25	
	Total	163	38.8 ± 6.19	
White blood cells (/cmm)	2.00	79	6105.32 ± 3307.27	.043
	3.00	13	12207.7 ± 22896.6	
	4.00	71	7188.74 ± 6836.45	
	Total	163	7063.93 ± 8177.61	
Lymphocyte %	2.00	79	34.06 ± 15.65	.893
	3.00	13	34.62 ± 14.19	
	4.00	71	33.03 ± 14.85	
	Total	163	33.66 ± 15.11	

(p-value <0.05, statistically significant)

Table 3: Post hoc analysis of mean difference of serotypes within groups with respect to different variables

Dependent Variable	DENV		Mean Difference (I-J)	95% CI_LL	95% CI_UL	p-value
Age	2.00	3.00	1.06	-10.33	12.46	0.97
		4.00	2.45	-3.78	8.68	0.62
	3.00	2.00	-1.06	-12.46	10.33	0.97
		4.00	1.39	-10.10	12.88	0.96
	4.00	2.00	-2.45	-8.68	3.78	0.62
		3.00	-1.39	-12.88	10.10	0.96
PLT	2.00	3.00	-0.08	-0.70	0.55	0.96
		4.00	-.36729*	-0.71	-0.03	0.03
	3.00	2.00	0.08	-0.55	0.70	0.96
		4.00	-0.29	-0.92	0.33	0.51
	4.00	2.00	.36729*	0.03	0.71	0.03
		3.00	0.29	-0.33	0.92	0.51
HCT	2.00	3.00	1.69	-2.69	6.06	0.63
		4.00	1.36	-1.03	3.75	0.37
	3.00	2.00	-1.69	-6.06	2.69	0.63
		4.00	-0.33	-4.74	4.09	0.98
	4.00	2.00	-1.36	-3.75	1.03	0.37
		3.00	0.33	-4.09	4.74	0.98
WBC	2.00	3.00	-6102.376*	-11815.32	-389.43	0.03
		4.00	-1083.42	-4204.85	2038.02	0.69
	3.00	2.00	6102.376*	389.43	11815.32	0.03
		4.00	5018.96	-739.29	10777.21	0.10
	4.00	2.00	1083.42	-2038.02	4204.85	0.69
		3.00	-5018.96	-10777.21	739.29	0.10
Lymph %	2.00	3.00	-0.56	-11.32	10.19	0.99
		4.00	1.02	-4.86	6.90	0.91
	3.00	2.00	0.56	-10.19	11.32	0.99
		4.00	1.59	-9.26	12.43	0.94
	4.00	2.00	-1.02	-6.90	4.86	0.91
		3.00	-1.59	-12.43	9.26	0.94

(p-value <0.05, statistically significant)

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Discussion

The term Arbovirus derives its name from the Arthropod-Borne Virus which collectively possess a major challenge to public health because of frequent epidemics occurring in different parts of the world. The last two decades have witnessed a phenomenal rise in Arboviral Infections of which DENV is a part. DENV belongs to the *Flaviviridae* family and possesses single-stranded linear & monopartite RNA (Sukhralia *et al.*, 2019). DENV genome consists of Envelope, Membrane & Capsid as the 03 structural components and the 07 Nonstructural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B & NS5 (Briton, 2015). As described earlier DENV has 04 distinct serotypes which have 60-80% homology (Sim, 2016). Clinically the disease is classified into the Hemorrhagic fever group and WHO in 2009 revised its classification guidelines from the earlier guideline issued in 1997. The present guideline classifies patients into 3 major groups *viz*; Dengue without Warning signs, Dengue with Warning signs and Severe Dengue to facilitate better triage of patients, especially during a disease outbreak and also to provide uniformity in case management (Horstick *et al*., 2014). Dengue without warning signs includes the presence of Fever and a minimum of 2 other symptoms/signs *viz*; nausea, rash, aches/pain, positive tourniquet test, and leucopenia with Laboratory confirmation of Dengue either by Enzyme-linked Immunosorbent assay, Molecular conformation or more commonly adopted lateral flow Point of care (POC) assays. These POCs are only an aid to diagnosis and the findings of such assay require confirmation by either of the 1st two methods mentioned.

The warning signs in DENV include abdominal pain, persistent vomiting, evidence of fluid accumulation, mucosal bleed, lethargy, restlessness, liver enlargement >2cms and Laboratory parameters which includes an increase in Hematocrit concurrent with a rapid decrease in platelet counts. The 3 criteria for severe dengue include Severe Plasma Leakage leading to Shock: Fluid accumulation with Respiratory distress. Severe Hemorrhage as evaluated by Clinician and Severe organ involvement including Liver impairment as evidenced by a marked rise in AST/ALT, Central Nervous system involvement in the form of impaired consciousness &involvement of Heart and other organs (WHO, 2022). Both Innate & adaptive immune responses have a definite role in the body's defense against DENV infection. The innate immune response is rapid but does not sustain for long, it also activates the Complement system which helps in clearing of viraemia. The more sustained and specific action is seen with the adaptive immune response with activation of both Humoral & Cellular Immunity. Infection with DENV induces the production of Neutralizing & Non-Neutralizing antibodies. The Neutralizing antibody against the infecting serotype provides lifelong immunity against that particular serotype but not against the other serotypes. In secondary infection with DENV, the non-neutralizing antibodies form virus-antibody complexes and inhibit bystander B cell activation leading to enhanced viral replication. These antibodies promote the recruitment of mononuclear cells and cause the release of a large number of cytokines. Thus both increased DENV replication, infectivity and suppression of host immune response leads to increased pathogenicity and the phenomenon known as Antibody-dependent enhancement (ADE) (Harapan *et al*, 2020). Various review of literature establishes the fact that the spectrum of disease severity is varied with different serotypes. Whilst Serotype 1 is known to cause more severe disease whilst Serotype 2 & 3 are proposed to be more virulent and serotype 4 usually causes milder symptoms (Umakanth, 2020; Duyen *et al*, 2011). Association of DENV infections with variables *viz* age, Hematocrit, Lymphocyte % showed non-significant association with p values 0.649, 0.344, 0.893 respectively as depicted in Table 2. Mild Thrombocytopenia was observed in our study. However, a statistically significant difference was observed when mean Platelet counts were compared with DENV infection (p value=0.037). Mean WBC counts also showed a significant association with DENV infections (p value=0.043). Post hoc analysis of Platelets & WBC was compared between different serotypes. We observed significant difference between DENV-2 & DENV-4 (p value=0.03). Similar findings were observed in the studies of Yung *et al*. and Perez *et al*. (Yung *et al*., 2015; Perez *et al*., 2006). For white blood cell counts showed statistical significance when comparing between DENV-2 and DENV-3. (P value=0.03). However, studies of Gupta *et al*. (Gupta *et al*., 2021) and Kriensak *et al*. (Limkittikul *et al*., 2005) found no significant association between different serotypes and WBC counts. Knowledge of the recent dynamics of the vector and disease propagation is the need of the hour which would help in improving region-specific standards of care and ultimately lesser mortality. To the best of our knowledge, this is the first study undertaken from this part of the country and in times when the whole world's healthcare delivery mechanisms have been tested to their limits owing to the ongoing COVID-19 pandemic. Our study showed co-circulation of serotypes 2,3 & Serotype-4. DENV-2 was the predominant serotype as has been documented in various published literature (Masyeni *et al*., 2018) [28]. Cocirculation along with concurrent infection with multiple serotypes have been shown to be a well-established fact in all the

outbreaks that have occurred in the past (Kumaria, 2010; Reddy *et al*., 2017; Sharma *et al*., 2018). Correlation between infecting serotype and hematological parameters showed a greater reduction in Platelet counts in DENV-2 infection as compared to DENV-4. No such statistically significant association could be inferenced when comparing DENV-2 with DENV-3 (p value=0.96). Our study has the limitation of less sample size and having a multicentric periodic review of the same would be immensely beneficial in achieving a true picture of the circulating predominant serotype.

Conclusion

Knowledge of infecting serotype helps in facilitating better triage, especially in secondary infection of DENV. With the capacity enhancement of Molecular testing in the country, we recommend the inclusion of serotype testing and history of DENV infection in the case definition & management protocols to prevent morbidity and mortality consequent to dengue infection.

Conflict of interest: None to Declare.

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Ethical approval

This study was approved by the Research Ethics Committee of the Institute. This study was conducted in accordance with the Declaration of Helsinki.

Author's contribution

MR conceptualized the study design, performed Molecular testing and manuscript writing, SSD performed the hematological sample testing and interpretation, SK performed the data entry, manuscript writing, SS contributed to the data analysis and manuscript proofreading, AA & AS performed the statistical analysis, data interpretation and protocol preparation, ANS contributed to the data and performed manuscript review and proofreading.

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