Original Article

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Microbiology Section

Differentiating Secondary Dengue Using IgG/IgM Antibody Ratio: A Cross-sectional Study

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ABSTRACT

Introduction: Dengue is a viral infection with diverse clinical manifestations. Infection in most of the patients is subclinical. It may also present as undifferentiated fever, severe dengue like Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). Secondary dengue infection predisposes to severe dengue, a serious threat to the community as they increase morbidities and mortality. The diagnosis of secondary infection is either by Polymerase Chain Reaction (PCR) to detect the serotype or dengue IgG/IgM antibody ratio. A ratio of more than 1.1 is evidence of secondary dengue.

Aim: To find out the prevalence of secondary dengue by measuring the dengue anti-IgG/IgM ratio by Enzyme Linked Immunosorbent Assay (ELISA) test.

Materials and Methods: This cross-sectional observational study was conducted at Government Kilpauk Medical College, Chennai for a period of six months from July 2021 to December 2021. Sample size was 450 calculated at 95% confidence level, (CI 28.3-38.3) with 33.3% as prevalence. Blood samples were collected and Nonstructural Protein 1 (NS1) antigen assay was done. The IgG and IgM antibodies assays were done for NS1 antigen positive individuals. The IgG/IgM ratio was calculated in patients who were positive for both IgG and IgM. Statistical analysis was done with Statistical Package for Social Sciences (SPSS) version 20.0. Percentage, mean and standard deviation were calculated.

Results: Total of 450 patients were included, out of which 194 were NS1 antigen positive, 106 were males and 88 females with the mean age 25±14.85 years. NS1 antigen was positive in 89.17%. Two-tailed Fisher's-exact test showed a significant proportion of patients among NS1 positive were positive for both IgG and IgM. An IgG/IgM ratio of >1.1 was found in 62.04%. The association between IgG/IgM ratio of >1.1 and thrombocytopenia was significant [p-value=0.00504, Odd's ratio (OD) =3.316].

Conclusion: Dengue IgG/IgM antibody ratio of more than 1.1 on the third day of symptom onset and the platelet count assist in the early diagnosis of secondary dengue thereby helps clinicians to initiate the appropriate treatment at the right time, reduce mortality rate and decrease the economic burden of the country.

Keywords: Antibody ratio, NS 1 antigen, Platelet count, Serology, Severe dengue

INTRODUCTION

Dengue is a viral infection with diverse clinical manifestations. The causative agent of Dengue Fever (DF) is flavivirus and is transmitted by the bite of Aedes aegypti and Aedes albopictus to a certain extent. Around 390 million dengue infections occur in a year worldwide [1]. Disease burden in Asia is 70% though globally 129 countries are at risk of dengue infection [2]. Infection in most of the patients is subclinical [3]. It may also present as undifferentiated fever, severe dengue like DHF and DSS. Secondary dengue infection predisposes to severe dengue, a serious threat to the community as they increase morbidities and mortality [4,5]. The probable prevalence of secondary dengue according to a recent study is 33.7% [6]. In a couple of studies, the overall prevalence of secondary dengue has been shown to be 46.9% [7,8].

There are five serotypes of dengue based on their antigenicity. The first four serotypes are DENV-1, DENV-2, DENV-3 and DENV-4. Primary dengue is caused by any one of these serotypes. DENV-5 was reported in a farmer on October 2013 from Malaysia [9]. Primary infection protects the individual from reinfection with the same serotype but does not confer immunity against the other serotypes. Infection with another serotype in persons who had primary dengue earlier leads to secondary dengue due to dysregulation in the immune mechanisms [10]. There is an upsurge in secondary dengue worldwide. The pathogenesis of secondary dengue leading to complications is not clear. Antibody dependent enhancement is the widely accepted hypothesis [11]. The spectrum of dengue infection includes Dengue Fever (DF) , a flu like illness with headaches and myalgias, to DHF and DSS, which is severe and at times fatal disease. DHF is characterised by appearance of haemorrhagic rash or heamorrhagic manifestations in addition to classical DF. DSS is characterised by presence of hypotension and altered mental status [12]. The 'T' lymphocytes, cytokines and complement system are also implicated in the pathogenesis of secondary dengue [13].

Laboratory diagnosis of dengue virus infection includes virus isolation, PCR, NS1 antigen detection or detection of specific IgM or IgG antibodies [14]. NS1 antigen assay is 90% sensitive during the pyrexial period of primary dengue but in subsequent infections it is only 60-80% sensitive [15]. In primary and secondary infection during the first 5 days NS1 antigen is detectable in serum. Dengue specific IgM and IgG antibodies are detectable on the 4th and 7th day of symptom onset, respectively. On the contrary, in secondary dengue IgG is the first antibody detectable in high titre early on from 3rd day of symptom onset and IgM is either negative or in low titre [16,17].

The diagnosis of secondary infection is either by PCR to detect the serotype or dengue IgG/IgM antibody ratio. A ratio of more than 1.1 is evidence of secondary dengue. According to a study conducted in Indonesia where the ratio was measured within three days of onset of disease, the best ratio was found to be 1.14 [18]. As the number of patients with severe dengue requiring hospitalisation is increasing every year following monsoon a comprehensive study was undertaken to find out the prevalence of secondary dengue. Early diagnosis of secondary dengue helps in triage of patients and better management

of severe dengue. This will in turn reduce the length of hospital stay in patients and the economic burden of the country. Keeping this in mind, the aim of this study was to find out the prevalence of secondary dengue infection by measuring the dengue IgG/IgM antibody ratio on the 3rd day of symptom onset by ELISA in patients hospitalised at a tertiary care centre with laboratory confirmed DF.

MATERIALS AND METHODS

This was a cross-sectional, observational study carried out at Government Kilpauk Medical College, Chennai, Tamil Nadu, India. The study was conducted for a period of six months from July 2021 to December 2021. The Institutional Ethics Committee approved the study vide Protocol ID.No.285/2019,5-12-2019, and written informed consent was obtained from the subjects.

Sample size calculation: The calculated sample size was 363 based on the formula given below. Sample size was calculated at 95% Confidence Level, (CI 28.3-38.3) with 38% as prevalence. Actual number of samples screened for dengue was 450 since the prevalence varied from 38% to 57% in a systematic review and meta-analysis [19].

$n=z^2 \times P(1-P)/e^2$

$1.96^2 \times 0.38 \times (1-0.38)/0.05^2$

n=363

Inclusion criteria:

- Patients presented features suggestive of DF, headache, retro-orbital pain, rashes, arthralgia.
- Dengue with warning signs-hypotension, bleeding tendencies

Exclusion criteria:

- Fever of insidious onset
- Fever of other origin

Procedure

A total of 450 patients presented with clinical features of dengue in a tertiary care hospital were recruited for the study after obtaining written informed consent. All the patients were tested for dengue NS 1 antigen by ELISA test on the day of admission. The NS 1 antigen ELISA kits used was Panbio diagnostics manufactured by Abbott Diagnostics Korea Inc. Republic of Korea. Out of the 450 tested, 194 patients were NS 1 positive and they were enrolled for further study. Those 194 subjects were tested for the presence of IgM and IgG antibodies on the 3rd day of symptom onset. Under strict aseptic precautions blood samples were collected from these patients, centrifuged at 1000 RPM for five minutes and sera were stored at -20°C samples were tested for dengue IgG and IgM antibodies by ELISA test using the dengue IgM and IgG Microlisa kits procured from J.Mitra. Co., Ltd., New Delhi. The principle of the ELISA test is an enzyme immunoassay based on "MAC Capture ELISA.

Anti-human IgM antibodies are coated onto microtiter wells in the 96 wells microtiter plate. Specimens and controls are added to the microtiter wells and incubated. If the specimen has dengue antibodies it will bind to the anti-IgM. After washing to remove unbound antibodies the Horseradish peroxidase conjugated dengue antigen (DEN 1-4) is added to each well. The conjugate will bind to dengue specific IgM antibodies complexed with anti-human IgM antibodies. In the next step chromogenic substrate is added in all the wells. Blue colour will develop in proportion to the amount of antibodies present in the samples. The reaction will be stopped by stop solution. Using an ELISA reader OD of the controls, calibrators and samples are detected at wavelength 450 nm.

Test validity as given in the kit insert:

Negative Control (NC) OD <0.3

Positive Control (PC) OD >1.0

Mean Calibrator OD ≥0.35

Cut-off value ≥1.5×NCOD

Ratio of PC OD/cut-off >1.1

Calculation of results:

Cut-off value= mean OD of calibrator × calibration factor

Sample OD ratio= Sample OD/Cut-off value

Dengue IgM units= Sample OD ratio ×10

Interpretation of results:

Dengue IgM units

<9 units- Negative for dengue

9-11 units- Equivocal

>11 units-Positive

Dengue IgG antibodies:

The microwells are coated with antihuman IgG antibodies and hence the principle, validity, calculation and interpretation are similar to the above and the results are given in IgG units.

IgG/IgM ratio:

The IgG/IgM ratio was calculated for all the samples and the cut-off value for secondary dengue was a ratio of >1.1. An IgG/IgM ratio of >1.10 was diagnostic of secondary dengue [20]. Platelet count was done for all the samples using a six part auto-analyser.

Operational Definition for Primary and Secondary Dengue

Primary dengue: Subjects who are IgM negative/IgG negative or IgM positive/IgG negative on the sample tested on the 3rd day of symptom onset.

Secondary dengue: Subjects who are IgM negative/IgG positive or IgM positive/IgG positive on the sample tested on the 3rd day of symptom onset.

STATISTICAL ANALYSIS

The data were entered into a Microsoft Excel spread sheet and then analysed with SPSS 20.0 version. Percentage, mean and standard deviation were calculated. Chi-square test was done to find out significance of dengue in genders at a p-value 0.05. Fisher's-exact test was deployed to find out odds ratio and significance at a p-value <0.05.

RESULTS

All patients hospitalised with clinical manifestations of dengue were tested for NS1 antigen on the day of admission. The results of NS 1 antigen assay are tabulated in [Table/Fig-1].

NS1 antigen	Frequency (n)	Percentage (%)
Positive	194	43.11
Negative	256	56.89
Total	450	100

[Table/Fig-1]: NS 1 antigen positivity in patients with clinical features of dengue n=450.
Among the 450 patients with clinical manifestations of dengue, NS1 antigen was positive in 43.11%

Number of males affected with dengue was compared with the number of females affected and the results are tabulated in [Table/Fig-2]. In this study, there was only a slight increase in the percentage (around 9.5%) of males affected when compared with diseased females.

Gender	Dengue positive (n)	(%)
Male	106	54.64%
Female	88	45.36%
Total	194	100%

[Table/Fig-2]: Gender distribution of dengue n=194.
Chi-square (3.3402); The p-value (0.067606); Not significant at p <0.05

To find out the mean age of the individuals affected with DF in the geographical location where the study was undertaken,

dengue in different age groups has tabulated in [Table/Fig-3]. Majority of the subjects lied in 16-25 years (41.76%) with mean age of 25±14.85 years.

Age in years	Frequency (n)	Percentages (%)
5-15	27	13.91
16-25	81	41.76
26-35	35	18.04
36-45	23	11.86
46-55	11	5.68
56-65	10	5.15
66-75	7	3.60
Total	194	100

[Table/Fig-3]: Age distribution of dengue n=194.

Patients presented with various symptoms and the clinical features at the time of hospitalisation are shown in [Table/Fig-4]. Fever was present in all the patients and the next predominant symptom was headache with retro-orbital pain (85%). Bleeding tendencies were present in 12.9% of the patients.

Symptoms	Frequency (n)	Percentages (%)
Fever	194	100
Headache and retro-orbital pain	165	85
Arthralgia	163	84
Cough, sore throat	96	49.5
Rashes	75	38.7
Hypotension	61	31.4
Bleeding tendencies	25	12.9

[Table/Fig-4]: Clinical features among NS 1 positive subjects N=194.

The dengue IgG and IgM antibodies ELISA test was done on the 3rd day of symptom onset and based on the isotype of antibody detected data was stratified as only IgM positive and both IgG, IgM positive. It is depicted in [Table/Fig-5].

Antibody isotype	Positive (n)	Negative (n)
Only IgM	65	108
Both IgG and IgM	108	65
Total	173	173

[Table/Fig-5]: Antibody Isotype in dengue antibody positives n=173.
Statistical test two-tailed Fisher's-exact was applied to find out the significance of isotypes of dengue antibodies in patients with DF; Two-tailed p-value <0.001

The IgG antibody index was divided by IgM antibody index in patients who were positive for both isotypes and the IgG/IgM ratio was calculated. Number of patients with a ratio of more than 1.1 and less than 1.1 are given below in [Table/Fig-6].

Ratio	Frequency (n)	Percentages (%)
<1.1	41	37.96%
>1.1	67	62.04%

[Table/Fig-6]: Dengue IgG/IgM antibody ratio in IgG, IgM antibodies positives N=108.

Among the patients who were IgG and IgM positive the proportion of patients who presented with IgG/IgM ratio of more than 1.1 was higher compared to ratio of less than 1.1.

Platelet count was done on the 3rd day of symptom onset for all the 108 patients who were both IgG and IgM positive and the association between platelet count and IgG/IgM ratio is given in [Table/Fig-7].

Two tailed Fisher's-exact test was deployed to find out whether there was any association between IgG/IgM ratio and platelet count. A significant proportion of patients with an IgG/IgM ratio of more than 1.1 had a platelet count of less than 30,000 and the odd's ratio was 3.316.

Ratio	Platelet count <30000	Platelet count >30000	Total
>1.1	44	23	67
<1.1	15	26	41
Total	59	49	108

[Table/Fig-7]: Association between dengue IgG/IgM ratio and platelet count n=108.
p-value= 0.00504; Significant at 0.05

DISCUSSION

Dengue is a vector borne viral disease with protean clinical manifestations caused by the bite of infected Aedes mosquitoes. When it presents as undifferentiated fever it is underdiagnosed or misdiagnosed [21]. Global burden of dengue has been steadily increasing every year. In 2008, it was reported by WHO that approximately 2.5 billion people across the world were at risk of dengue and that the disease was endemic in more than 100 countries. From 2010 to 2016 the number of dengue cases recorded globally increased from 2.2 billion to 3.34 billion. There was a sharp increase of cases in 2019 and 2020, with outbreaks across countries in the western Pacific region, Africa, and the Americas [22].

A total of 450 patients with suspected dengue were enrolled in our study. All these patients underwent NS1 antigen assay by ELISA test on the day of admission. Prevalence of NS1 antigen in this study was 43.11%. Since the study was conducted during postmonsoon season others could have had fever due to viral infections like influenza, influenza like illness, other viral infections, leptospirosis, malaria, enteric fever etc. A systematic review and meta-analysis revealed that among the clinically suspected DF patients, the prevalence of laboratory-confirmed dengue infection was 38%. The study also showed 57% as seroprevalence of dengue in a pooled data based on 7 different studies undertaken in India. The seroprevalence of dengue in the geographical area where our study was conducted is in between the prevalence rates shown in the above systematic review and meta-analysis study done in India [19].

There was a marginal increase in the prevalence of dengue in males compared to females and it was not statistically significant. Some of the studies have shown a higher prevalence of dengue in males than females probably due to the outdoor activities of males and the difference in the biological effects of dengue virus in both sexes [23]. As the males and females are equally engaged in outdoor activities in recent day gender difference is not much in the prevalence of dengue. There is a higher prevalence in males than in females according to some of the studies carried out in hospitals at India [24]. Geographical area and literacy rate may also determine the difference in prevalence of dengue among male and female. Mean age of DF in our study is 25 years. Recent studies have shown a shift in the mean age from 34 years to 27.2 years. The serotype causing outbreaks varies from time to time. Different serotypes may have a predilection for certain age groups and this area needs further research to prove this [25,26]. A major proportion of the patients who were NS1 positive were dengue antibody positive on the 3rd day of symptom onset. Both IgM and IgG antibody ELISA tests were carried out. In this study, prevalence of primary dengue was less compared to secondary dengue.

Fever was the presenting symptom in all the patients who were NS1 antigen positive. The other predominant symptoms present were headache, retro-orbital pain, arthralgia, rashes and sore throat in this study. Bleeding tendencies like purpuric spots, epistaxis was present in 12% of the patients. After an intrinsic incubation period of 3-7 days, it presents as fever of sudden onset associated with headache, retro-orbital pain, fatigue, arthralgia and skin rashes It also mimics influenza and the patients may present with running nose, sore throat and cough [27]. In these patients, it is a challenge to differentiate dengue from fever due to causes other than dengue infection [28]. Severe dengue occurs in patients with secondary dengue infections or infection with virulent strains [29]. In these patients, there is plasma leakage and the platelet count is low. This in

turn leads to DHF and DSS which are fatal unless early intervention is done [30]. However, early diagnosis and proper management at the appropriate time can reduce the case fatality rate to below 1% [31].

As per various studies an IgG/IgM ratio of more than 1.1 is a diagnostic marker in secondary dengue. The ratio was found to be different on different stages of illness [32,33]. An IgG/ IgM ratio of 1.10 and above was found to be the best cut-off value to differentiate secondary dengue from primary dengue in a study carried out at Indonesia [34]. Unlike the above studies wherein the ratio was calculated at different periods of illness, the ratio was calculated only once during hospitalisation in our study. In our study, a dengue IgG/IgM ratio of more than 1.1 was fixed as cut-off value in the diagnosis of secondary dengue based on the available data and above-mentioned reference studies and the prevalence of secondary dengue in this study was 62.04%.

Dengue infection with another serotype in patients who had prior infection with a serotype unrelated to the secondary infection predisposes to severe dengue in the form of DHF and DSS in some of the individuals. Due to the antibody dependant enhancement vascular endothelium is affected and this provokes increased capillary permeability, plasma leak, thrombocytopenia and bleeding tendencies. This is termed as DHF [35-37]. In the DSS, because of the plasma leak patients develop pleural effusion, pericardial effusion, ascites, pulmonary oedema and hypotension [38]. Complete blood count shows leukopenia, thrombocytopenia and a raised haematocrit in these patients. Liver function test reveals increased aspartate aminotransferase [39,40].

In our study, complete blood count was done for all the 108 patients who were IgG and IgM positive. The platelet count was drastically reduced in patients whose IgG/IgM ratio was more than 1.1. Hence, the clinicians have to take utmost care while treating DF in patients who present with increased IgG/IgM ratio as there is a propensity for developing DHF and DSS. A platelet count of less than 50,000 is associated with increased mortality as per studies [41,42]. As per the above studies a low platelet count in patients with DF has a strong association with complications of dengue and severe dengue. This study plays major role in the early identification of severe dengue.

Limitation(s)

In this study, the dengue IgG and IgM-ELISA test was done only once on the 3rd day of symptom onset and the IgG/IgM ratio was calculated once unlike in other studies in which the ratio was calculated during the various days of hospitalisation.

CONCLUSION(S)

This novel study was simple, needs less expertise and cost-effective in the diagnosis of secondary dengue. Dengue IgG/IgM antibody ratio of more than 1.1 on the third day of symptom onset and the platelet count assist in the early diagnosis of secondary dengue thereby helps clinicians to initiate the appropriate treatment at the right time. This will save the life of patients, bring down the mortality rate and this in turn reduce the economic burden of the country.

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