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Role of IgM: IgG ratio in differentiating Primary and Secondary Dengue virus infection in a Tertiary Care Hospital, A Cross Sectional Study

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Abstract

It is reported that secondary dengue causes more severe disease in comparison with primary. Thus to differentiate the two is very crucial. Our aim is to find out cut-off values of IgM:IgG ratio for early detection of secondary dengue which could further help clinicians to prevent the complications.

Methods: A cross-sectional study was conducted over a period of one year involving around 936 suspected cases of dengue. Samples were tested using commercially available capture ELISA method for IgM and IgG. **Real time and nested PCR** was also done to find out the prevalent serotype. IgM:IgG ratio was evaluated by using receiver operating characteristic curve analysis for the differentiation of Primary and Secondary Dengue.

Results: Among the total 91 serologically confirmed dengue patients, forty-seven (51.6%) were found to be Primary and forty-four (48.4%) were secondary dengue infection with male preponderance. Using the WHO diagnostic criteria, dengue fever (DF) patients without warning signs added up to 51.6%, with warning signs 42.9% and severe dengue 5.5% of the total cases. Cut off ratio of **IgM: IgG** ratio=**1.59** found out the best discrimination between primary from secondary infection. 40 out of 91 (44%) patients exhibited ratios of >1.59 whereas rest fifety one (56%) exhibited ratios of <1.59. **DENV 2** was found to be the most prevalent serotype.

Conclusion: Our study recommends the cut-off values for IgM:IgG ratio as **1.59**.Therefore It is being hoped that this will guide the clinicians to early distinguish between primary and secondary dengue. fiurthermore, it can reduce morbidity and mortality because of dengue infections in future.

Keywords: Primary Dengue, Secondary Dengue, IgM : IgG ratio.

1. Introduction

The symptoms of dengue vary, making it difficult to forecast how the illness will develop and affect a person. Dengue has been ranked highest among the re-emerging, serious arbo-viral infectious disease transmitted by *Aedes albopictus* and *Aedes aegypti* mosquitoes. “Dengue Virus”, is a single stranded RNA virus that belongs to the family of flaviviridae and has four serologically related but genetically distinctive virus serotypes, namely, DENV-1, DENV-2,

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DENV-3 and DENV-4. All these can potentially cause severe illness. Recently, scientists have come across a new type of serotype.[1] Primary dengue infection occurs when a previously non-immunized person is infected with any one serotype and subsequent infection with another serotype (secondary infection) or multiple infections with different serotypes more often leads to severe complications like DHF and DSS.[2,3]

Dengue as a whole is endemic across the whole of India and different strains have been responsible for dengue infections in different regions at different times.[4] Both adults and older children in the dengue afflicted areas are likely to be exposed to dengue virus in the past, and this makes them more prone to secondary infections.[5] Numerous virologic and Studies have found that certain immune measurements can help us understand how dengue develops in different people and with different types of infections.[6,7] It's important to be able to tell if someone has had dengue before or if this is their first time, because it can help predict how sick they may become and also tell us more about how the disease spreads.

IgM antibodies can be found in the blood of patients 3-5 days after they get sick. The duration for IgM antibody levels to become elevated is around two weeks, and these antibodies can be measured for about 179 days in primary infections and 139 days in secondary infections. During the initial stage of secondary dengue infections, IgM antibodies can be detected in the bloodstream, high levels of cross-reactive IgG antibodies are detected with prior or simultaneous IgM response.[9] The rapid increase in IgG levels in early days from illness during secondary infection is indicative of dengue when the IgM and IgG ratios are calculated. [8-11]

It has been suggested that methods which discriminate primary and secondary DENV infection may be important from prognostic point of view. Hemagglutination inhibition (HAI) assay was earlier the gold standard test to differentiate between primary dengue and secondary dengue infections. However, due to various practical limitations and requirement of paired sera early diagnosis of disease cannot be made. Now a days, IgM and IgG capture ELISA has replaced HAI assay and has become the most powerful assay for the dengue diagnosis. They are being widely used for diagnosis due to the possibility of automation, simplicity, high sensitivity, and specificity.[12-14]

The objective of this research was to identify the optimal IgM:IgG ratio in an early-stage dengue infection blood sample, enabling differentiation between primary and secondary infections. We also wanted to find out which strain of the dengue virus is most common in this area..

1.1 Aims and Objectives

Our aim is to find out cut-off values of IgM:IgG ratio for early detection of secondary dengue which could further help clinicians to prevent the complications.

2. Materials and Methods:

A cross-sectional study was conducted in the Department of Microbiology in a tertiary care teaching hospital over a period of 12 months from January 2017 to December 2017. Subjects were recruited from patients presenting in inpatient and outpatient departments of the hospital with a history of acute febrile illness and written informed consent was taken. All the relevant demographical, laboratory, clinical as well as serological data were collected on the day the patient attended the health facility.

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2.1 Ethical Statement

Ethical clearance was obtained from Institutional Ethical Committee vide letter no. SRHU/HIMS/ETHICS/2018/94 dated 16/07/2018.

2.2 Inclusion Criteria

The study recruited patients from a tertiary-care hospital with exceptional care, where those who had a fever persisting for 2-7 days and exhibited two or more symptoms like eye pain, skin rash, joint pain, headache, muscle pain, or bleeding were enrolled. Patients who had a fever for more than 7 days, had a history of urinary tract infection, had signs of pneumonia or abscess, or had clear reasons for their fever were not included.

2.3 Procedure

Blood samples from 936 patients with Acute Febrile Illness and as per the inclusion criteria of the study were collected and subjected to microbiological tests. Five ml blood was collected aseptically, and serum was separated by centrifuging the vacutainers at 1,600Xg for 10 minutes. The testing of NS1 antigen or IgM antibody revealed that dengue was present in 234 of the 936 samples.Special containers known as cryovials were utilized to store a total of 91 random samples at -20˚C. These samples were taken as 10% of the overall sample size. They were stored with the intention of utilizing them later for IgG serology and PCR tests, as all the samples could not be processed because of feasibility and financial issues.

SD BIOLINE Dengue Duo NS1 + Ab combo kit was used to determine NS_1, IgM and IgG result (Sensitivity 92.4%, Specificity 98.4% of NS1&Sensitivity 94.2%, Specificity 96.4% of Ab). IgM and IgG index values are calculated by using Panbio Dengue IgM (Sensitivity 94.7%, Specificity 100%)and IgG Capture ELISA(Sensitivity 85.7%, specificity 100%)respectively. As per the manufacturer's guidelines, we erformed all the tests and deciphered the results.

2.3.1 Criteria for Primary and Secondary infection

Primary DENV infection was defined as either NS_1 Ag $^{+}IgM^{-}$ IgG^{-}or IgM^{+} IgG^{-}. Whereas secondary DENV infection was defined as a IgM^{-} IgG^{+} or IgM^{+} IgG^{+}.

2.4 Sample Size

Sample size calculated by using the standard formula for estimation of sample size i.e.

$$N=Z^2 \times P \times Q / M^2$$

where N is the required sample size, Z is the confidence level at 95% (standard value of 1.96), P is the expected prevalence of Dengue (9.6%) based on the hospital IDSP 2015 report, Q is (1-P) and M is the relative margin of error at 20% (taken as a standard value of 1.92), the required total sample size came out to be 904.3 [(1.96)2 x (9.6) x (90.4)/ (1.92)2 = 904.3]. Figure 1 represents the flowchart depicting patient selection process.

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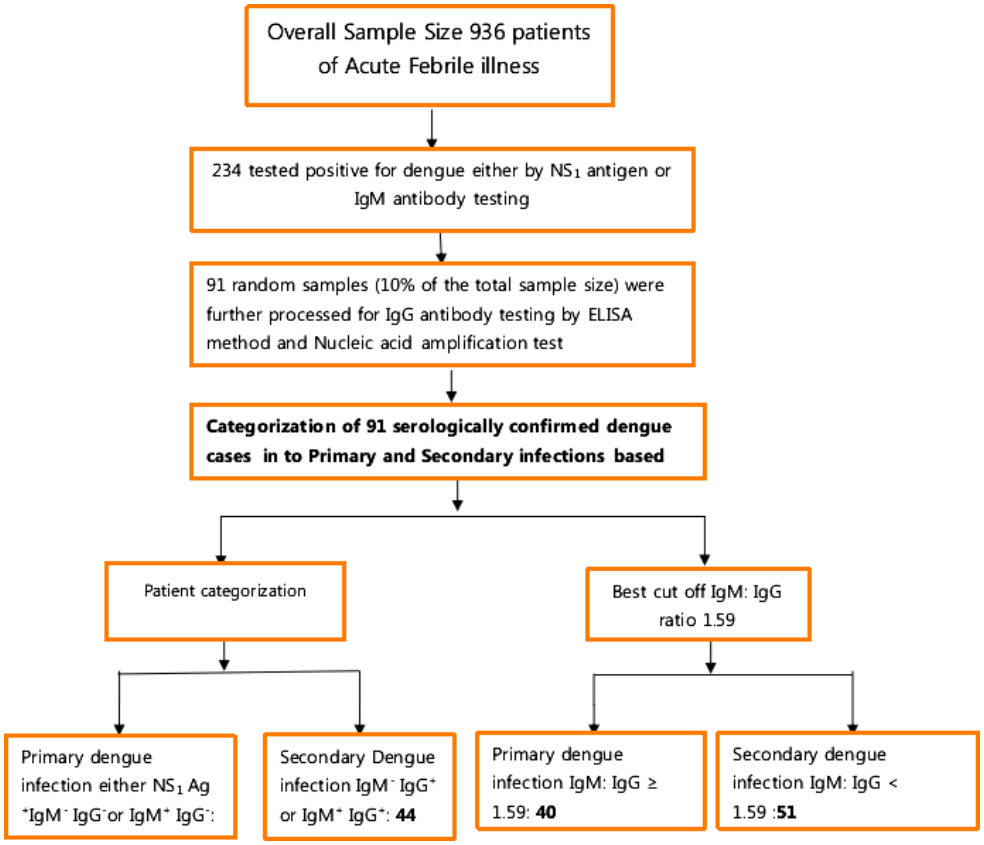

Figure 1: Flowchart depicting patient selection process

2.5 RT-PCR

Using a kit called QIAmp Viral RNA Mini kit produced by the German company QIAGEN, the doctors extracted the viral RNA from the serum samples, following the instructions that came with the kit. Real time PCRwas carried out and two step nested PCR was done on samples with high viral load of dengue virus for serotype detection. Extracted viral nucleic acid were first converted to cDNA by reverse transcription and then amplified in a thermocycler. The primer sequences described by Lanciotti et al. were used.[16]

Amplified products were separated by agarose gel electrophoresis through 1.6% agarose gel in Tris-acetate-EDTA (TAE) buffer. Electrophoresis was carried out at 100-150 Volts for 45 min or until the bands were resolved. Gel was screened under UV light on electronic UV transilluminator gel documentation system for the presence of511 bp bands of DNA.

After the first round, only those samples were processed for serotyping which showed 511 bp bands. The forward primer and the serotype-specific reverse primers primer TS1, TS2, TS3 and TS4 for DENV-1, DENV-2, DENV-3 and DENV-4 respectively targeting C-preM gene junction were used for detection. Amplification products were separated by gel electrophoresis as described above**.** With the help of gel documentation system, the serotype-specific bands of each dengue serotype were visualized. Bands specific for DENV-1, DENV-2, DENV-3 and DENV-4 of respective base pairs482bp, 119bp, 290bp and 392bp were tested. Including samples that yielded both known positive and negative results, we took precautions to avoid obtaining incorrect outcomes during the test.

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2.6 Data Management & Statistical Analysis

Recorded data was analyzed using statistical software **(**SPSS version 20 .0). The demographic profile, clinical features and laboratory investigations were described by descriptive statistics. The categorical variables were compared by Pearson Chi Square and Fisher exact probability test**.** The independent sample unpaired t-tests was used to assess the statistical difference between the continuous variables conforming to normal distribution whereas the distribution which was skewed were compared using the Mann–Whitney U-test. Sensitivity, specificity, likelihood ratio, accuracy and cut-off point for IgM:IgG ratio were determined by Receiver Operator Characteristic (ROC) curve.

3. Results

Among the 91 serologically confirmed dengue cases, 51.6 % (47) were of primary dengue infection and 48.4% (44) of secondary dengue infection. Mean age and sex distribution among primary and secondary dengue is shown in Table 1. The study group presented commonly with headache (79.12%), lethargy (76.92 %), fever (65.93%) and other symptoms. Headache, lethargy, hemorrhagic manifestations were significantly associated with the disease (P value<0.05) (Table 1). Clinical outcome in both the groups has been compared in (Table 2).As per the WHO 2009 classification patients were grouped into "Dengue fever without warning signs, Dengue fever with warning signs and Severe dengue." (Figure 2).

Best Cut Off of IgM:IgG ratio was found to be 1.59 with sensitivity of 85.11 %, specificity of 100 %, accuracy level 92.3 %, positive (-) and negative likelihood ratio (0.15) as compared to other ratios(Table 3). It had shown good performance by the ROC curve as the area under ROC curve was 0.982 (Figure 3). With the best cut-off ratio of 1.59, 44% (40 cases) were thus classified as primary dengue and 56% (51 cases) as secondary dengue.

In the present study, DENV 2 was found to be the most prevalent serotype detected both in primary and secondary dengue patients than DENV 1 and was isolated in patients who have been classified as Dengue fever with warning signs and severe dengue (Table 4).

Table 1: Comparison of demographic, clinical and laboratory parameters in Primary and Secondary Dengue infection (n=91)

Parameters		**Primary dengue n = 47**	**Secondary dengue n = 44**	**Total n = 91**	**P value**
Male (M)	**n (%)**	31(66)	31 (70.5)	62 (68.1)	**0.645**
Female (F)	**n (%)**	16 (34)	13 (29.5)	29 (31.9)	
Age (years)	**Mean ± SD (Median)**	29.79(18.33) (27)	35.45(19.99) (32.50)	32.53(19.26) (29)	**0.181**
Headache	**n (%)**	41 (87.23)	31 (70.45)	72 (79.12)	<0.05

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Lethargy	**n (%)**	32 (68.1)	38 (86.36)	70 (76.92)	<0.05
Fever > 99.5 ° F	**n (%)**	31 (65.95)	29(65.90)	60(65.93)	0.996
Aches & Pains	**n (%)**	28 (59.6)	27 (61.4)	55 (60.4)	0.862
Gastrointestinal symptoms	**n (%)**	21(44.7)	21(47.7)	42(46.2)	0.771
Hemorrhagic manifestation	**n (%)**	19 (40.42)	27 (61.36)	46 (50.54)	<0.05
Retro-orbital pain	**n (%)**	11 (23.4)	11 (25)	22 (24.2)	0.859
Hemoglobin (g /dl)	**Mean ± SD (Median)**	13.2 ±2.94 13.9	13.5 ±3.34 (14.1)	13.39 ±3.12 (14.03)	0.606
Total leucocyte count ($x10^3/mm^3$)	**Mean ± SD (Median)**	9.21 ± 6.57 (7.37)	9.34 ±5.59 (7.61)	9.28 ±6.08 (7.43)	0.589
Leucopenia ($x10^3/ mm^3$)	**Mean ± SD (Median)**	2.89 ± 0.749 (2.79)	3.72 ±0.552 (4)	3.33 ±0.76 (3.39)	<0.05
Platelet count ($x10^3/ mm^3$)	**Mean ± SD (Median)**	54.1 ± 52.3 (30)	53.9 ±63.8 (31)	54.1 ±57.8 (30)	0.802
AST / ALT	**Mean ± SD (Median)**	2.077 ± 1.17 (1.72)	2.27 ±1.35 (2.06)	2.17 ±1.26 (1.8)	0.341
Alkaline Phosphatase (ALP IU/L)	**Mean ± SD (Median)**	132.78 ±110.4 (92)	172.57 ±124.7 (145)	152.4 ±118.6 (108)	0.083
A/G ratio	**Mean ± SD (Median)**	1.08 ±0.421 (1)	1.03 ±0.280 (0.97)	1.05 ±0.356 (1)	0.616
Blood urea nitrogen (mg/dl)	**Mean ± SD (Median)**	13.2 ±13.7 (9.2)	13.9 ±9.43 (10.3)	13.5 ±11.77 (9.85)	0.266
IgM Index	**Mean ± SD (Median)**	2.21 ±0.78 (2.06)	2.05 ±0.875 (2.08)	2.13 ±0.825 (2.07)	0.349
IgG Index	**Mean ± SD (Median)**	0.906 ± 0.462 (1)	4.66 ±1.917 (3.63)	2.72 ±2.329 (1.775)	<_0.0005
IgM: IgG Index	**Mean ± SD (Median)**	2.99 ± 1.64 (2)	0.532 ± 0 .346 (0.410)	1.806±1.722 (1.17)	<_0.0005

Note: P value < 0.05 is considered to be significant

Table 2: Comparison of clinical outcome in Primary and Secondary dengue infection (n=91)

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Parameters		Primary	Secondary	Total	P value
Morbidity					
Hospitalization days	**Mean(SD) (Median)**	4.83±2.77 (4)	4.82 ±2.43 (4)	4.82±2.60 (4)	0.793
Platelet recovery days	**Mean(SD) (Median)**	1.81 ±0.821 (2)	2.13 ±0.957 (2)	1.97 ±0.897 (2)	0.193
Acute liver failure	**n (%)**	17 (36.4)	28 (63.6)	45 (49.5)	<0.05
Acute renal failure	**n (%)**	6 (12.8)	13 (29.5)	19 (20.9)	<0.05
Multi-organ Dysfunction	**n (%)**	1 (2.1)	6 (13.6)	7 (7.7)	<0.05
Shock	**n (%)**	2 (4.3)	3 (6.8)	5 (5.5)	0.054
Seizures	**n (%)**	1 (2.1)	-	1 (1.1)	0.331
Encephalopathy	**n (%)**	-	1 (2.3)	1 (1.1)	0.299
Intensive care	**n (%)**	4 (8.5)	6 (13.6)	10 (11)	0.435
Case fatality rate		-	2.27	1.09	-

Note: P value < 0.05 is considered to be significant

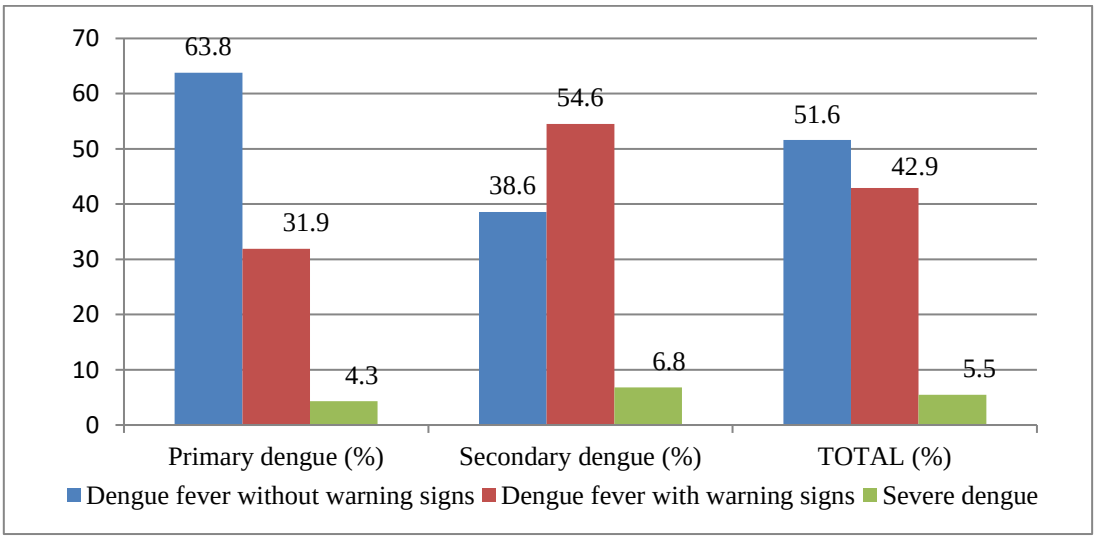

Figure 2: Categorization of Primary and Secondary dengue cases as per WHO classification (n=91)

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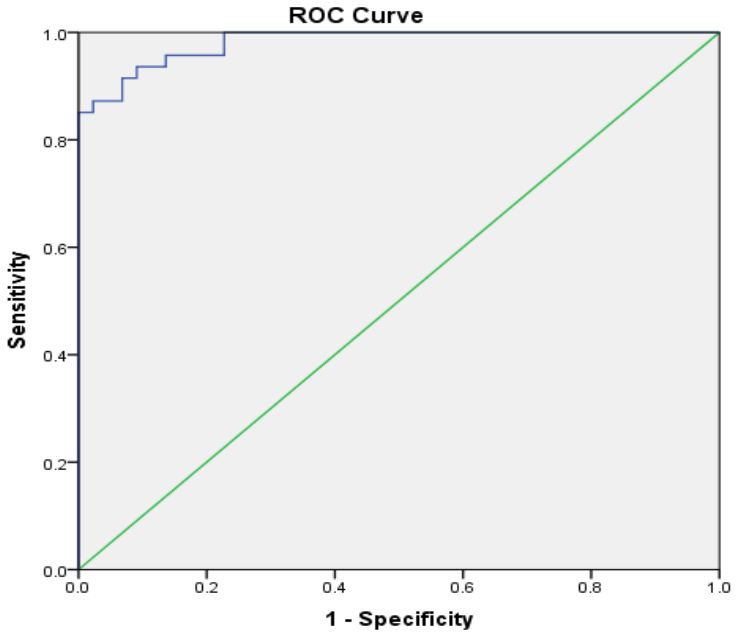

Figure 2: Receiver Operator Characteristic (ROC) curve for determining IgM: IgG cutoff

Area	p value	95% Confidence Interval	
		Lower Bound	Upper Bound
0.982	<_0.0005	0.963	1.000

Table 3: Analytical values of various cut off points of IgM: IgG ratio in differentiating Primary and Secondary infections

IgM:IgG Ratio	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	PLR	NLR
1.09	93.62	90.91	91.67	93.02	92.31	10.3	.07
1.16	91.49	93.18	93.48	91.11	92.3	13.42	.09
1.21	87.23	93.18	93.18	87.23	90.11	12.79	.14
1.40	85.11	97.73	97.56	86.00	91.21	37.45	.15
***1.59**	85.11	100	100	86.27	92.31	-	.15

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1.77	82.98	100	100	84.62	91.21	-	.17
1.87	78.71	100	100	81.48	89.01	-	.21

Table 4: Dengue virus serotype associated with Primary and Secondary dengue infection and serotype distribution according to severity of dengue infection

	No of Samples serotyped	**DENV 1 n (%)**	**DENV 2 n (%)**
Primary Dengue Infection	*22	8 (36.36)	11 (50)
Secondary Dengue Infection	**13	5 (38.46)	7 (53.84)
WHO definition			
Dengue fever without warning signs	21	11 (52.38)	7 (33.33)
Dengue fever with warning signs	11	2 (18.18)	9 (81.8)
Severe dengue	3	--	2(66.6)

4. Discussion

In the Indian subcontinent, the trajectory of dengue fever has undergone a discrete change over decades. It has changed in terms of severity of illness with different prevailing strains and different geographical locations being affected. Its incidence has increased significantly. Dengue is endemic in more than 100 countries and still it has been categorized as a "neglected tropical disease".[17] The delay in diagnosis as well as an appropriate management is substantially associated with morbidity and mortality.

Of the 91 serologically confirmed dengue cases, 51.6 % were of primary dengue infection and 48.4% of secondary dengue infection. Primary to secondary dengue infection ratio was 1.07:1.59% primary dengue, 41% secondary dengue infection and Primary: Secondary ratio of 1.4:1 has been reported [18] which correlates well with the present study. While another study has observed 34.3% primary dengue and 65.7% secondary dengue.[19]

In our study, numbers of males (68.1 %) with dengue illness were considerably higher than females (31.9 %); overall male; female ratio was 2.13:1. Comparable pattern of male predominance has also been found in other studies all across the globe.[20-22] However, an equitable sex distribution has also been documented.[23,24] The main reason for male preponderance in the present study could be that in our Indian society males are considered to

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be the earning members of the family and spend more time outdoors for making the livelihood, hence are more exposed to insects. Also, differential seeking behavior of medical care for females in whom mild illnesses are generally neglected could account for the same. The highest prevalence of dengue infection was observed in economically productive age group. In the present study, the mean age group in primary dengue infection was 29.79 ± 18.3 years, whereas in secondary dengue infection, the mean age group was 35.45 ± 19.9 years. An adult predominance in dengue fever with mean age of patients being 27.5±11.7 years and 30.7±14.4 years in primary dengue and secondary dengue fever were observed respectively.[25] Fever in the hospital after admission was documented in 65.95 % of patients; however, 100 % of patients had given the history of fever for 4-6 days prior to their hospital visit. Those who were not presenting with fever had visited the hospital for consultation for following complaints: headache aches and pains, lethargy, gastrointestinal symptoms and hemorrhagic manifestations. These findings are in congruence with the report in this same institute in 2013 in which 66.3% patients had fever on presentation in hospital.[26] On stratified analysis, patients with primary dengue were significantly associated with headache, however patients with secondary dengue infection were significantly associated with lethargy and hemorrhagic manifestation (p value <0.05). Our findings of common symptoms are in concordance with the findings of others.[25]

As per the WHO 2009 classification, in the present study 51.6% cases of DF without warning signs, 42.9% of DF with warning signs and 5.5% cases of severe dengue were observed. The difference of proportions between primary and secondary dengue came out to be statistically significant in the first two classes (p values <0.05). Comparable findings have been reported earlier.[27] They have also opined that the new 2009 classification is more competent in triaging severe dengue cases which they felt was missed in 1997 WHO Classification.[27]

In a comparative study in Uttarakhand in 2015 and 2016, case fatality ratio had been reported as 1.25% and 2.06% respectively.[28] These findings are conducive with the results of the present study where the case fatality rate is of 1.09 in severe dengue case.

Antibody capture ELISA was performed on serum samples from dengue patients collected on various days of infection. IgM antibodies were detected in 51.64% of the samples and IgG in 11% whereas both IgM & IgG were detected in 37.36 % of patients. These figures are slightly different than the findings of other workers.[29,30] The mean IgM index, IgG index and IgM:IgG index were also calculated for primary and secondary infection and significantly higher association was seen in IgG index and IgM: IgG index with dengue infection.

Various workers have calculated the IgM:IgG cut off ratio to be in the range of 1.2 to 2.0 depending upon the various diagnostic assays and interpretation protocol(Table 5). The workers in various studies have reported this cut off ratio to be as follows: 1.78[12], 1.8 [31], 1.4 [13], 1.32 [32] and 1.2 [14]. The possible reasons for different ratios in these studies could be the different settings and sero-epidemiologies. In our study, we have found IgM:IgG ratio to 1.59 as the best cut off for differentiating acute primary and acute secondary dengue infection in this region.

In the present study, DENV 2 was found to be the most prevalent serotype detected both in primary and secondary dengue patients and was found associated with severe form of dengue illness whereas DENV 1 was isolated mainly from patients with dengue fever without warning signs. DENV 2 is among the most virulent serotype of dengue infection and this could

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be the reason that in our study differences in clinical profile, lab parameters, days of hospitalization and platelet concentrate transfusion rate were not significantly seen in primary and secondary dengue patients. Soo et al [33] in their meta-analysis study have mentioned that the dengue serotypes involved in causing dengue infection and the interval between the primary and secondary infections affected the severity of dengue infection. Additionally, DENV-2 and DENV-4 are more commonly associated with secondary DENV infection. Therefore, patients who have been exposed to dengue virus previously must be cautious during outbreaks of DENV-2 and DENV-4 infections.[33] Table 5 represents the IgM: IgG cut-off ratios for differentiating Primary and Secondary infections in various studies.

Table 5: IgM: IgG cut-off ratios for differentiating Primary and Secondary infections in various studies

Study	Country	IgM:IgG ratio
Innis et al[12]	Bangkok,Thailand	1.78
Chanama et al[33]	Nonthaburi, Thailand	1.8
Kuno et al[13]	Puerto Rico	1.4
Prince et al[34]	California, USA	1.32
Shu et al[14]	Taiwan,Republic of China	1.2
Our study	**India**	**1.59**

5. Conclusion

On the basis of this thorough study, a first of its kind in this region of Uttarakhand, the cut off value for IgM:IgG ratio is recommended as 1.59. It is hoped that this will guide the clinicians to diagnose dengue more accurately and this would go a long way in reducing the morbidity, mortality and economic burden of this very prevalent, obnoxious and possibly fatal disease especially in the secondary dengue cases.

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