Sero-prevalence of Dengue IgM Antibodies in Patients Suspected of having Dengue Fever

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Authors’ contributions

This work was carried out in collaboration between all authors. Author NH designed the study, wrote the protocol and supervised the whole study. Author FA anchored the field study, gathered the initial data, performed preliminary data analysis and interpreted the data. Authors FK and QF managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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ABSTRACT

Background: Dengue virus infection is increasingly recognized as one of the world's emerging infectious disease. In recent decades, dengue fever has become one of the leading causes of morbidity and mortality in tropical and sub-tropical areas throughout the world. Dengue fever is endemic in Pakistan since 1994. The aim of this study was to determine the trend of sero-prevalence of anti-dengue IgM antibodies in Lahore during 2012 to 2013.

Results: Serum samples were collected from 90 patients visiting Jinnah hospital and Mayo hospital with the history of fever and clinically suspected dengue. The sero-prevalence of dengue virus specific IgM antibodies was determined using enzyme linked immuno-sorbent assay (ELISA) ELISA-DENM0120 kit. The anti-dengue IgM antibodies positivity was found to be 48%. The positive dengue cases were higher in male (76.4%) as compared to female (23.6%) though it was not statistically significant (P>0.05). Among different age groups, the highest positive cases (48.9%) were from age group of 15 to 25 years followed by the age group of 35 to 45 years (17%). Out of

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two hospitals, the highest positive cases were in Jinnah hospital (72.3%) followed by Mayo hospital (27.6%). Age and gender were found to be independent predictors. Fever was the most commonly reported symptom among dengue suspected patients (81.1%) followed by myalgia (72.2%). Thrombocytopenia (PLT<150.0x10^3) was found in 100% patients and leukopenia (TLC<4.0 x10^3) was found in 49% of dengue fever suspected subjects.

**Conclusion:** This study indicates that prevalence of dengue virus infection is increasing in Pakistan especially Lahore. Proper control measure should be provided. IgM capture ELISA has become the most accepted technique for the diagnosis of dengue in developing countries like Pakistan.

**Keywords:** Dengue virus; dengue IgM antibodies; leucopenia; Myalgia; ELISA.

**ABBREVIATIONS**

ADE: Antibody dependent enhancement; Bp: Base pair; DC: Dendritic cell; DENV: Dengue virus; DF: Dengue fever; DHF: Dengue haemorrhagic fever; DSS: Dengue shock syndrome; E: Envelope; ELISA: Enzyme-linked immunosorbent assay; ER: Endoplasmic reticulum; FcR: Fc receptor; hnRNP: Heterogeneous nuclear ribonucleoprotein; HRP: Horseradish peroxidase; NS: Non-structural; OD: Optical density; ORF: Open reading frame; Psi: Per square inch or pounds per square inch; Rpm: Rotations per minute; ssRNA: Single strand RNA; WHO: World health organization.

**1. BACKGROUND**

Dengue is an important mosquito borne viral infection. It occurred sporadically till the 19th century. Presently it is endemic in 112 countries around the world [1]. Over 2.5 billion people of the world are now at risk dengue. During recent decades Dengue is the second most prevalent mosquito-borne infection after malaria. The most endemic regions include Southeast Asia, Latin America, Asia, and the Caribbean [2,3]. In Pakistan, DF has been around for the past 20 years approximately. The first documented report was in 1985 whereby Dengue type 2 virus was isolated in a sero-epidemological study for encephalitis. The first major outbreak was reported in 1994. Another epidemic has been witnessed in Karachi following heavy rain falls in 2006 [4].

The word Dengue is originated from Swahili language “ki denga pepo”, which describes sudden cramp like seizure [5]. The disease was first named as Dengue fever in 1779. There are reports that suggested possible epidemics of dengue like illness in four major continents (Asia, Africa, Australia and North America) as early as 1779 and 1780. In 1906, *Aedes aegypti* mosquito as a vector of Dengue virus was first discovered by Bancroft. In 1907 Dengue was the second disease (after yellow fever) that was shown to be caused by a virus [6]. In 1953 a new form of dengue fever was reported from Thailand and Manila, where children suffered from fever followed by bleeding; the disease was then called as Philippine Fever. Over the past two decades, Dengue infection has been regularly reported from Pakistan [7,8].

Dengue virus (DENV) is an RNA virus and a member of the family Flavivirida and genus flavivirus. Virions are 40-50 nm in diameter and spherical in shape, 11kb single-stranded RNA containing a single open reading frame. Dengue virus consists of ten proteins, three of which are structural and seven non-structural: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [9]. It has four antigenically but distinct serotypes, namely DENV1, DENV2, DENV3 and DENV4 [10,11].

The *A. Aegypti* mosquito is the primary vector of Dengue [12]. DENV initially infects human dendritic cells, which migrate to lymph nodes to present viral antigens to T-cells, initiating cellular and humoral immune responses. These activated T cells discharge cytokines and chemical mediators to further activate the immune system [13]. First exposure to DENV infection is known as primary infection and characterized by a slow and low-titer antibody response. Immunoglobulin M (IgM) antibodies are the first isotype to appear within three-to-seven days of infection. Immunoglobulin G (IgG) antibodies are first detectable at low titer during the first week of illness, and slowly increase [14]. Each dengue serotype produces unique immunity to the specific serotype. In contrast, during a secondary infection, IgG antibodies appear first after onset of fever and detectable on day 1. IgM antibody levels are significantly lower throughout a secondary DENV infection [15]. If an individual has a secondary DENV infection
with a heterologous serotype, they are at a great risk for severe disease due to antibody-dependent enhancement (ADE) [16].

Dengue disease in humans may lead to death. Infection with any of the four serotypes of DENV results in similar symptoms but may vary in severity. Primary infection results in dengue fever in older children and adults. Symptoms of dengue fever include fever with severe joint pain, rashes, weakness, headache, nausea and vomiting. Dengue Hemorrhagic Fever (DHF) initially resembles a primary DENV infection and is characterized by the sudden onset of fever for two-to-seven days with no symptoms representative for DHF. Plasma leakage is a clinical symptom representative for DHF. Without early diagnosis, some patients experience a mild or severe shock from blood loss and decreased blood flow. In dengue shock syndrome (DSS), fever and circumoral cyanosis, a rapid and weak pulse, restlessness, abdominal pain are followed by the sudden deterioration of the patient’s condition.

Laboratory diagnosis methods for confirming dengue virus infection may involve detection of the virus, viral nucleic acid, antigens or antibodies, or a combination of these techniques. After the onset of illness, the virus can be detected in serum, plasma, circulating blood cells and other tissues for 4–5 days. During the early stages (first 5-7 days) of the disease, virus isolation, nucleic acid or antigen detection can be used to diagnose the infection. At the end of the acute phase of infection, serology is the method of choice for diagnosis. In primary dengue infection, IgM antibodies appear by days 3–5 with levels peak about two weeks after the onset of symptoms. Anti-dengue serum IgG is generally detectable at low titers at the end of the first week of illness, increasing slowly thereafter, with serum IgG still being detectable after several months. In secondary dengue infection, IgG is the dominant immunoglobulin isotype and may persist for life [17].

An estimated 50 million dengue infections occur worldwide annually. In the last 50 years, incidence has increased 30-fold. Pakistan has experienced a number of dengue fever outbreaks since 1992. The epidemic continued to affect a large number of people in Azad Jammu & Kashmir in 2006 but went largely unreported. Over 21,204 people were reportedly infected in the country in 2010 [18]. In 2011, the massive outbreak in Punjab attracted the attention of the Government of Pakistan, especially the Punjab Government. This latest outbreak had resulted in 18,000 cases nationwide. Punjab had borne the brunt of the infection with 16,000 cases and 350 deaths of which 14,000 cases and 300 deaths were reported from Lahore alone.

The aim of this cross-sectional study was to study the trend of sero-prevalence of anti-dengue IgM antibodies in the patients suspected of having dengue fever. Moreover, the regular cross sectional, age stratified, serological surveys are the surveillance tools for monitoring the impact of dengue prevention and control. This study also helps to plan for potential epidemics. Most countries throughout the world have poor surveillance capabilities for the detection of both i.e., dengue and the vector Aedes mosquito population. So there is an increased need of such studies. Due to relative ease and low cost, stratified sero-prevalence surveys are a useful tool in the surveillance of directly transmitted diseases.

2. MATERIALS AND METHODS

In this study, dengue IgM ELISA technique was performed. All the reagents of ELISA kit DENV0120 were stored at -20ºC.

2.1 Study Population

Serum samples of 90 patients suspected of having dengue fever were collected from Jinnah hospital and Mayo hospital, Lahore. Dengue IgM antibody test ELISA was performed at Al-Razi Health Care Center, Lahore. Blood samples were collected from patients after obtaining their signed consent. Patient selection was based on a medical history obtained by questionnaire.

2.2 Inclusion Criteria

Patients above 15 years of age with characteristic dengue fever symptoms like fever, headache, arthralgia, low complete blood count, low platelet count, rashes and bruises were selected for this study.

2.3 Exclusion Criteria

Patients below 15 years of age and with any other viral, bacterial and parasitical infection were not selected for this study.
2.4 Sample Collection

Three to five ml of blood was collected aseptically from anticubital fossa. Anticoagulant or preservatives were not added. Blood was allowed to clot and centrifuged at 3000 rpm for 15 minutes. The serum was collected with micropipettes and stored at -20°C till assayed. Hemolyzed, lipemic or bacterial contaminated sera were not used.

2.5 ELISA (Enzyme Linked Immuno-sorbent Assay)

2.5.1 Procedure

All the 90 samples were diluted as 1+99 with IgM sample diluent. Immediately 100 µl of diluted serum (1:100) was added into wells coated with dengue virus antigens. The plate was incubated at 37°C for 60 minutes. Then, the plate was washed three times with diluted wash buffer. Then 100µl of dengue virus anti-IgM conjugate was added to the wells. Again the plate was incubated at 37°C for 30 minutes. The plate was then washed again three times with diluted wash buffer. One hundred µl of TMB substrate solution was pipetted and added to each well. Incubation was done at 25-30°C for 15 minutes. Finally, 100 µl of stop solution was added and observed the change in color pattern.

2.6 Measurement and Interpretation of Results

ELISA Microwell Plate Reader was used to measure the absorbance of all wells at 450 nm and recorded the absorbance values for each control and patient sample in the distribution and identification plan. Samples were considered POSITIVE if the absorbance value was higher than the cut-off. Samples were considered NEGATIVE if the absorbance value was lower than the cut-off. Results are calculated by this formula:

\[
\frac{(\text{Patient (mean) absorbance value} \times 10)}{\text{Cut-off}} = \frac{\text{NovaTec-Units = NTU}}{10 \text{ NTU}}
\]

Cut-Off: 10 NTU
Negative: < 9 NTU
Positive: >11 NTU

2.7 Statistical Analysis of Results

The collected data was analyzed using Statistical Package for Social Science version 16 (SPSS v.16). Chi-square statistics and Levene’s test for equality of variances with p<0.05 were considered statistically significant.

3. RESULTS

This cross-sectional study was performed to determine the IgM sero-prevalence in dengue suspected patients. A total of 90 patients suspected of having Dengue fever were included in this study. Blood samples were collected from Jinnah Hospital and Mayo Hospital, Lahore. A total of 69 (76.66%) samples were collected from Jinnah Hospital and 21 (23.33%) samples were collected from Mayo Hospital, Lahore (Fig. 1). Samples were collected from September to November, 2012. Maximum number of subjects suspected of having dengue fever was found in October. Serum samples of 32 suspected subjects were collected in September, 2012. In October 47 subjects were found to be suspected for DF. In November, the frequency of suspected patients was declined and only 11 subjects suspected of having DF were found. Results are illustrated in Fig. 2.

To find out the probable number of confirmed dengue positive patients out of these 90 suspected subjects, IgM antibody screening was used in this study. The qualitative immunoenzymatic determination of IgM-class antibodies against Dengue Virus was based on the ELISA (Enzyme-linked Immunosorbent Assay) technique. 47 out of 90 subjects were found to be positive for dengue IgM antibodies. Out of 90 subjects, 53 subjects were found to be non-reactive for dengue IgM antibodies. Frequency distribution of absorbance of Dengue virus by ELISA is shown in Fig. 3. Absorbance of sera is shown on x-axis, and frequency on the y-axis. The average mean of absorbance was 12.59 ± 1.06 along with minimum and maximum values that were 0.71 and 70.55 respectively. Results of ELISA are summarized in Fig. 4.

Gender distribution of the total dengue affected patients (n=47) was 36 (76.4%) males and 11 (23.6%) females. Here male to female ratio was 3:1. Chi-square test for independence and t-test were used to confirm that absorbance value of IgM ELISA was gender biased or not. In chi-square test p-value for the data of this study was 0.964 (Table 1). The p-value for IgM ELISA with reference to gender was found to be 0.621. The p-value in both cases was greater than the standard p-value (0.05). This difference in the p-values of tested data and standard indicated that there was no statistically significant association between gender and dengue fever prevalence.
Out of 90 samples, 69 (76.66%) samples were collected from Jinnah Hospital and 21 (23.33%) samples were collected from Mayo Hospital.

Of these 90 subjects 32 suspected subjects were found in September, 47 in October and 11 were found in November.

Table 1. Gender distribution of dengue patients (n=47)

| Gender | Frequency | Percent | $\chi^2$-test | p-value |
|--------|-----------|---------|---------------|---------|
| Female | 11        | 23.6    |               |         |
| Male   | 36        | 76.4    |               | 0.964   |
| Total  | 47        | 100.0   |               |         |

Age distribution of Dengue IgM positive males and females is given in Fig. 5. The average mean of age was $35.23 \pm 1.57$ along with minimum and maximum values that were 15.00 and 75 respectively. Levene’s Test and Independent sample test were applied on the data to find out the association between DF and age, because in this study decline in the
incidence of DF was observed with an increase in age. In Levene’s test p-value for the data of this study was found to be 0.097. The p-value of the data was greater than standard value (p<0.05). This difference in the p-values of tested data and standard indicated that there was no statistically significant association between age and dengue fever prevalence. Dengue fever is equally prevalent in all age groups. Different patterns in results regarding age groups would be because of environmental effects.

The most common clinical features of dengue include fever, itching, headache, nausea, vomiting and myalgia. In this study, out of 47 dengue positive patients, itching was reported by ten (22.2%) patients, 23 (48.9%) patients had rash, 24 (52.2%) patients experienced nausea, 23 (48.9%) patients had a history of vomiting, 24 (51.1%) patients had arthralgia, 29 (63.3%) patients complained about headaches, 34 (72.2%) patients experienced myalgia, and 38 (81.1%) patients had fever. During this study fever was found to be the most common symptom among dengue infected patients. Results are summarized in Table 2.

Complete blood count (CBC) was performed for all dengue positive patients (n=47). On preliminary screening by lab tests for dengue fever; test for Anemia (Hb<12 g/dl), Leukopenia (TLC <4× 10^3 µl), Thrombocytopenia (PLT <150 x 10^3 µl), HCT >25% were positive for some dengue suspected patients. Tests for Anemia (Hb<12 g/dl) were positive for 68.08% cases while 51.06% cases showed leukopenia (TLC <4× 10^3 µl). All dengue suspected patients showed remarkable decrease in platelet count (PLT <150 x 10^3 µl) and 4.2% cases showed increase in HCT level. Results are summarized in Table 3.

**Fig. 3. Frequency distribution of absorbance (NTU) of IgM antibody (n=90)**
Absorbance of sera is shown on x-axis, and frequency on the y-axis. The average mean of absorbance was 12.59 ± 1.06 along with minimum and maximum values that were 0.71 and 70.55 respectively

**Fig. 4. Sero-prevalence of anti-dengue IgM among dengue suspected patients (n=90)**
52% subjects showed positive results and 48% subjects showed negative results
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Fig. 5. Frequency distribution of dengue IgM positive patients (n=47) in different age groups
Most of the affected subjects were found to be in 15 to 25 years of age group followed by 35 to 45 years of age group

Table 2. Distribution of the history of symptoms parameter of DF (n=47)

| Symptoms  | Male (n=36) | Female (n=11) | Total number (n=47) | Percentage % |
|-----------|-------------|---------------|---------------------|--------------|
| Itching   | 8           | 2             | 10                  | 22.2         |
| Rash      | 15          | 8             | 23                  | 48.9         |
| Nausea    | 18          | 6             | 24                  | 52.2         |
| Vomiting  | 19          | 4             | 23                  | 48.9         |
| Arthralgia| 20          | 4             | 24                  | 51.1         |
| Headache  | 23          | 6             | 29                  | 63.3         |
| Myalgia   | 26          | 8             | 34                  | 72.2         |
| Fever     | 31          | 7             | 38                  | 81.1         |

Table 3. Laboratory tests of CBC report for preliminary screening for DF (n=47)

| Hematological features | Frequency n=47 | Percentage % |
|------------------------|----------------|--------------|
| Hemoglobin test        | 32             | 68.08        |
| Leukopenia             | 24             | 51.06        |
| TLC <4x10^9 µl         | 47             | 100          |
| THrombocytopenia       |                |              |
| PLT <150 x 10^3 µl     |                |              |
| Hematocrit level       |                |              |
| HCT >25%               | 2              | 4.2          |

Normal range of Hemoglobin = 12 to 16.8 g/dl
Normal range of TLC = 4.5 x 10^9 to 10 x 10^9 µl
Normal range of Platelets = 150 x 10^9 to 400 x 10^9 µl
Normal level of Hematocrit = 45% for men and 40% for women

4. DISCUSSION

Dengue infection has emerged as a major health concern in Southeast Asia, the pacific and America. In Pakistan, dengue virus circulates throughout the year with a peak incidence in the post monsoon period. In the present study most of the dengue cases were also recorded between September and November with the highest numbers seen in September. Similar pattern of incidence was reported in a study done in Karachi in 2006 [19] and also in a study done by Murugan et al. in [20]. This similar pattern indicates that climate and environmental aspects play a critical role in the distribution and prevalence of both the dengue virus and its vectors (A. aegypti and A. albopictus) [21,22].

The present study indicated gender-related difference in dengue incidence. Here male to female ratio was 3:1. According to chi-square test for independence p =0.964. This value indicated that there was no statistically significant association between gender and dengue fever dominance as p-value was found to be greater than the standard (p<0.05). In a previous study of dengue incidence in Malaysia between 1973
and 1987 the majority of reported cases were also found to be male [23,24]. These results in Asia are in contrast to studies in South America, which have found either equal proportions of male and female dengue cases or a greater proportion of female cases [25]. Weibull model also indicates that the age-specific risks of symptomatic disease in adults both during primary and secondary infection remain almost independent of age [26]. The reason for this male preponderance of dengue in many Asian countries (including Pakistan, Malaysia and Singapore) is might be the exposure differences among males and females, and also the fact that the labour force in Asian countries has more males than females. Women, in these countries, are also less likely to be taken care of at a hospital when ill or are taken at late stages of the disease, when no other options are available.

In the present study the most affected age group was found to be 15 to 25 years of age. Incidence of DF was gradually decreased with increasing age, suggesting DF as age dependent disease, but according to Levene’s Test and Independent sample test the absorbance value was not dependent on age (p=0.621). Guzman et al. also found clinical outcome of severe DEN infection in adults more favorable than in children during a study conducted in 2002 [27,28]. According to a study conducted in Indonesia by Setiati et al. during 2006, they also found a decline in the force of infection [29], resulting in a shift in the age distribution of DHF toward older age groups [30,31]. Rocha and Taui [32] in an epidemiological study conducted in Manaus also reported increased incidence of dengue in >15 years old patients.

In 2004 the most common clinical feature of dengue were studied by Pervin [33]. He reported occurrence of rash in 33% of patients and of myalgia in 84.5% of patients. In the present study 63.3% patients complained about headaches, whereas 51.1% cases of arthralgia were observed. Fever was observed in 81.1% of cases. Nausea was reported in 52.2% cases. While vomiting and rash were seen in 48.9% of patients. The rash was typically macular, often becoming confluent and sparing small islands of normal skin. The rash was not associated with scaling. Myalgia was observed in 34 (72.2%) of patients. Comparing the present study with above described study, there was an increased occurrence of rash reported in patients and a decline was observed regarding myalgia.

Preliminary screening by lab tests for dengue fever included anemia (Hb<12 g/dl), leukopenia (TLC <4×10³ µl), thrombocytopenia (PLT <150×10³ µl), HCT >25%. Tests for anemia (Hb<12 g/dl) were positive for 68.08% cases in the present study while in another study by Banerjee et al. [34], anemia (Hb 6.5-9.5 g/dl) was reported in 11% of patients. As compared to this report [34], percentage of anemia was quite high in the present study. In the present study 51.06% cases of leukopenia (TLC <4×10³ µl) were observed while only 26% cases were observed during another study done by Ratagiri et al. [35] in 2005. Banerjee et al. reported thrombocytopenia (platelets <1,00,000/ cm) in 19% of patients and Ratagiri et al. reported thrombocytopenia in 82% of patients. In the present study all dengue suspected patients (100%) showed remarkable decrease in platelet count (PLT <150×10³ µl) and 4.2% cases showed increase in HCT level.

To-date no study from Pakistan has been published assessing the risk factors associated with developing complications in patients with DF. In Singapore, there are 4,000–5,000 reported cases of dengue fever or dengue hemorrhagic fever every year [36]. In the year 2004, there were seven deaths from dengue hemorrhagic fever every year [36]. In the year 2006, 37% cases of hepatomegaly and 6% cases of spleenomegaly were reported in Karachi. In this study majority of patients had decreased level of hematocrit. Only 2 patients out of 47 showed an increased percentage of hematocrit. Of the patients who had raised hematocrit only one patient developed DHF. There were no cases of hepatomegaly and spleenomegaly reported during this study. Recent studies have shown that in addition to recommended WHO criteria: blood type, race, new clinical and biochemical markers like clinical bleeding, high serum urea, low serum protein, viral load assessment, low lymphocyte proportion, viral serotype testing, and circulating endothelial cell detection tests are associated with development of complications like DHF and DSS [37,38]. The present study showed that severity of DF in the form of risk of developing DHF can be predicted by presence of bleeding, raised hematocrit and raised serum level. To control this threat, effective anti-vector campaigns in high vector density areas have been initiated before and during the monsoon season targeting. More and improved inspection is needed for travelers from epidemic regions as well. Currently, there are no antiviral compounds available against dengue virus and there is a
need to develop antiviral compounds that can target all four serotypes of dengue with same efficiency.

5. CONCLUSION

The study is not representative of actual DF suspected population of the country, but raises the question for the future studies. It also indicates that, in population of Pakistan DF is more prevalent in males and among people of 15 to 25 years of age group. DF/DHF has also economic consequences: diagnostic and ambulatory costs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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