

# Epidemiological profile and laboratory characteristics of dengue virus infection during 2011 outbreak in rawalpindi, Islamabad Pakistan

## Abstract

A total of 215 serum samples of clinically suspected dengue patients who presented to the tertiary care hospitals over a period of July-November 2011 were analyzed for the detection of dengue NS1 antigen, dengue IgM and IgG antibodies and 180 (83.7%) samples were found positive by one or more than one testing parameters. Of the 180 samples, 98 (54.4%) were positive to NS1, 168 (93.3%) were positive to IgM and 93 (51.6%) cases were positive to IgG only. Similarly, 93 (51.6%) were positive to both NS1 and IgM, 48 (26.6%) to NS1 and IgG, and 83 (46.1%) were positive to both IgM and IgG and 48(26%) were positive for NS1, IgM and IgG.

A randomly selected subset of 50 serology positive samples were tested by, one step TaqMan real time fourplex RT-PCR assay. A total of 35/50 (70%) samples were found positive by PCR. Positive rates of dengue serotypes were 15/35 (42.8%), 11/35 (31.4%) and 9/35 (25.7%) for DENV-1, DENV-2 and DENV-3 respectively. No DENV-4 serotype was detected.

**Keywords:** Dengue, Epidemic, Pakistan, NS1 antigen, IgM and IgG antibodies, ELI, Dengue virus, Diagnosis

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**Abbreviations:** DHF, Dengue Hemorrhagic Fever; DSS, Dengue Shock Syndrome; NS1, Non Structural Proteins; HFH, Holy Family Hospital; PIMS, Pakistan Institute of Medical Sciences; PCR, Polymerase Chain Reaction

## Introduction

Dengue Virus (DENV) belongs to the genus *flavivirus* and family *flaviviridae*. It is an enveloped positive sense single stranded 11000 nucleotides long RNA virus that caused emerging mosquito borne tropical disease afflicting humanity.<sup>1</sup> There are four different serotypes of dengue virus; DENV-1, DENV-2, DENV-3 and DENV-4 that are responsible for dengue viral infection in different regions of the world and their prevalence vary temporally.<sup>2</sup> The infection caused by only one serotype is usually a mild and self limiting. The more severe form of dengue infection is dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) responsible for high morbidity and mortality in various parts of the world.<sup>3</sup> Globally, more than 100 million cases of dengue virus infection with 25000 estimated deaths have been reported annually and there are more than 100 countries where disease is endemic.<sup>4</sup> Similarly, more than 1.8 billion of world population is at risk for dengue virus infection that lives in member states of World Health Organization, South East Asia and Western Pacific Zone and bears 75% of global disease burden of dengue virus infection.<sup>5,6</sup> The incidence of dengue infection has been increasing worldwide, mostly in tropical and subtropical regions.<sup>1</sup>

The dengue virus is transmitted to humans by mosquito (*Aedes aegypti* and *Aedes albopictus*) bite. *Aedes aegypti*, a principal mosquito vector that breed in artificial as well as the natural water. Similarly, *Aedes albopictus* also called, "Asian Tiger" mosquito, is an epidemiologically important vector in tropical and subtropical countries of South East Asia for the transmission of many viral pathogens including dengue virus.<sup>7</sup>

Dengue has a worldwide history of about 200-400 years, mostly causing infection in tropical and sub-tropical areas. First epidemic of Dengue was occurred in Asia, Africa and North America in 1779-1780 and since then, it is occurred sporadically and has continued to date.<sup>8</sup> In recent years dengue has become a major international health problem with an expanded geographic distribution and a potential to cause major health and economic burdens. Moreover, Pakistan is a dengue endemic country from many years and has experienced a number of dengue fever outbreaks since 1994 during the monsoon and post monsoon season.<sup>9</sup> Some published reports on dengue have been shown that the infection in Pakistan has been documented from 1968 and 1978.<sup>1</sup> The dengue virus infection is perceived as a serious contributor of hospitalization and death amongst human in Pakistan.<sup>10</sup>

Dengue is increasingly becoming a regular epidemic in Pakistan. To date, dengue virus infection has caused several outbreaks in Pakistan. During 2011, Pakistan had worst strike of dengue due to devastating floods as a result of heavy rainfall in monsoon period that provide suitable breeding sites for the dengue virus vectors. During this epidemic, more than 20,000 dengue virus confirmed cases along with >300 deaths were reported and Lahore (capital of Punjab province) was the epicenter with maximum number of cases.<sup>11</sup> The 2011 outbreak in Lahore and almost all cities of Punjab had affected and hospitals were crowded with sufferers of dengue fever that had hit the community severely and strained to the maximum the health care infrastructure and services in the province. In reality, already frail health system of the country is not equipped to handle a sudden upsurge of the disease.

Since neither a drug nor vaccine exists for dengue fever and prevention through vector control aided with society contribution is the only choice to manage dengue infection. Dengue infection is mostly clinically diagnosed through symptoms. However, diagnosing dengue infection through symptoms is not reliable, and it needs laboratory studies to confirm the presence of dengue virus.<sup>8</sup> Laboratory

diagnostic methods may involve detection of the virus, viral nucleic acid, antigen, antibodies or combination of these techniques by some specialized laboratories. In developing countries, most of laboratories<sup>9</sup> used the serological methods for the detection of Non structural Proteins (NS1) antigen, IgM and IgG antibody by ELISA.<sup>9,12</sup> NS1 antigen can be detected in the blood of infected patient from day one of infection. IgM antibody appear in the patient blood after the 4-5 days and IgG antibody appear after 10-14 days post infection in case of primary infection but it can be detected within 1-2 days after onset of infection in case of secondary dengue virus infection.

In present study, we focused on serological investigations for the early diagnosis of dengue fever from suspected patients referred from tertiary care hospitals of two major cities; Islamabad and Rawalpindi during July to November 2011. Early diagnosis in case of dengue virus infection is important to reduce the morbidity and mortality.

Therefore, there is a great demand for the rapid detection and differentiation of dengue virus infection in the acute phase of illness in order to provide timely clinical treatment and etiologic investigation and disease control. Our data demonstrated that the combine used of three parameters (NS1 antigen, IgM and IgG) at the same time increases the accuracy of dengue diagnosis.

## Materials and methods

### Study design

This study was conducted to investigate the dengue suspected cases referred from two major tertiary care hospitals; Pakistan institute of Medical Sciences (PIMS) and Holy Family Hospital (HFH) from Islamabad and Rawalpindi respectively during July- November 2011. Both hospitals are center for undergraduate and postgraduate teaching and are providing the health facilities to a large community. All the referred patients presented with the symptoms of dengue fever diagnosed clinically by the clinician.

### Patient serum samples

Blood sample collected from suspected dengue virus cases attending the outpatients department of two hospitals during the outbreak period (July-November 2011) were referred to diagnostic laboratory at department of virology National Institute of Health Islamabad Pakistan for confirmation of dengue virus. A total of 215 blood samples (3-4ml) were referred along with the available demographic data but the timing of sampling was not available in most cases. However, all patients experienced dengue fever as diagnosed by the clinician. Serum was separated from samples after centrifugation at 3000rpm for 5 minutes and were stored at 4-8°C until used for testing. All 215 serum samples were tested for the detection of NS1 antigen, IgM and IgG antibody by ELISA method.

### Dengue NS1 antigen detection ELISA

All serum samples were tested for the detection of NS1 antigen using Platelia TM Dengue NS1 antigen ELISA (Biorad Laboratories, Marnes-la-Coquette, France). Test procedure was followed by the instructions provided by the manufacturer and results were interpreted as Positive, Negative and Equivocal (Borderline).

### Anti Dengue virus IgM and IgG ELISA

Dengue virus specific IgM and IgG antibodies in serum samples were detected using Dengue IgM & IgG Capture ELISA (Panbio diagnostic, Brisbane, Australia). Test protocol was followed as provided by manufacturer. Test results were expressed in terms of Panbio units as recommended by the manufacturer.

The recommendations for the interpretation of results with respect to the primary and secondary dengue infection are as follows:

11 panbio units in case of IgM antibody detection was indicative of active primary infection

22 panbio units in case of IgG antibody detection were indicative of active secondary infection.

Panbio units 9-11 in case of IgM and 18-22 in case of IgG antibodies are indicative of suspected for the primary or secondary dengue virus infection.

### RNA extraction and real time RT-PCR

Viral RNA was extracted from 140ul serum sample using Qiaamp Viral RNA extraction kit according to the manufacturer's protocol with the exception of elution volume which was 40ul. A one step real time TaqMan RT-PCR for detecting and typing dengue viruses was carried out according to Barbara et al. Briefly, singleplex reaction mixture of 25ul was run for each DENV serotype (DENV1-4) in ABI7500 real time thermo cycler using the Super Script III Platinum one-step qRT-PCR kit (Invitrogen). Amplifications for each serotype was carried out in 25ul reaction mixture containing 5ul RNA, 12.5ul of 2X reaction mixture, 0.5ul enzyme mix, 2uM of each primer and 1uM of TaqMan probe. The cycling conditions were as follows: RT step 50°C at 10 minutes, initial denaturation at 95°C for 5 minutes, and 45 cycles at 95°C for 15 s and at 60°C for 60 s. The data was analyzed using software SDS version 1.4.

## Results

A total of 215 serum samples of clinically suspected dengue patients who presented to the tertiary care hospitals over a period of July-November 2011 were analyzed for the detection of dengue NS1 antigen, dengue IgM and IgG antibodies and 180(83.7%) samples were found positive by one or more than one testing parameters (Figure 1). Of the 180 samples, 98(54.4%) were positive to NS1, 168(93.3%) were positive to IgM and 93(51.6%) cases were positive to IgG only. Similarly, 93 (51.6%) were positive to both NS1 and IgM, 48(26.6%) to NS1 and IgG, and 83 (46.1%) were positive to both IgM and IgG and 48(26%) were positive for NS1, IgM and IgG. Majority of the dengue suspected cases 113(52.5%) were from Holy Family Teaching Hospital Rawalpindi followed by PIMS Islamabad 102(47.4%).

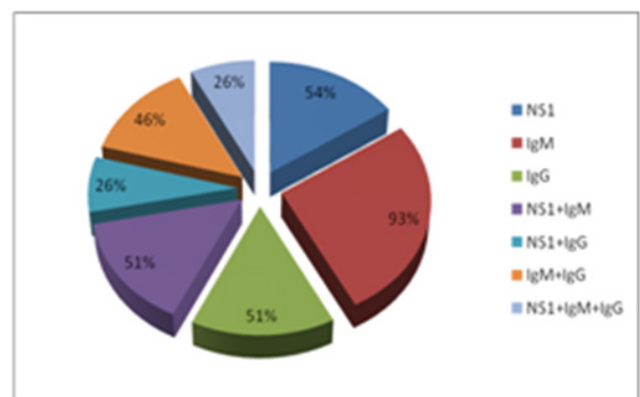


Figure 1 Distribution of Dengue positive cases by Serology during 2011.

Additionally, one step TaqMan real time fourplex RT-PCR assay was conducted for only 50 samples that were found positive on serology (Table 1). A total of 35/50 (70%) samples were found positive by PCR. Positive rates of dengue serotypes were 15/35

(42.8%), 11/35 (31.4%) and 9/35 (25.7%) for DENV-1, DENV-2 and DENV-3 respectively. No DENV-4 serotype was detected.

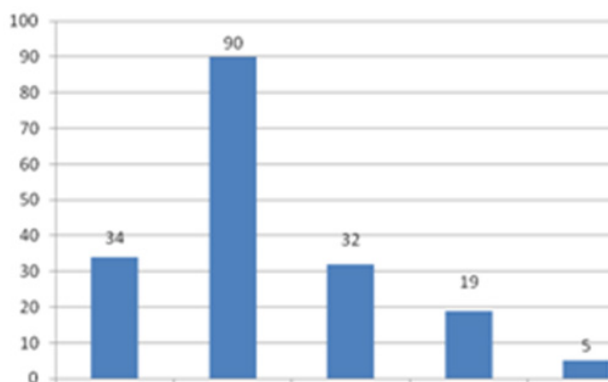
**Table 1** Distribution of Dengue Suspected Cases Tested by qRT-PCR

N=50	PCR+	PCR-	Total
NSI+	25	3	28
NSI-	10	12	22
Total	35	15	50
IgM+	35	15	50
IgM-	0	0	0
Total	35	15	50
IgG+	22	10	32
IgG-	13	5	18
Total	35	15	50

The positivity of dengue cases was higher in male 151/180 (83%) as compared to female 29/180 (16%) with 6:1 male to female ratio. Age wise distribution of positive dengue fever cases showed the highly affected age group were individuals who were 16-30 year old (50%) followed by those who were 31-45 year old (17%) (Figure 2). The highest number of suspected and ELSA positive samples were collected in the month of August to November, which is a period that was preceded by intense precipitation and increased humidity. The details of reported cases along with weather indices are summarized in (Figure 3). The distribution of serologically diagnosed cases into primary and secondary dengue infection is shown in Table 2. The positive IgM plus negative IgG (IgM+ plus IgG-) results showed that 48.3% of those patients had a recent primary dengue infection, while the positive IgG plus either positive or negative IgM (IgG+ plus IgM+/-) results indicated that 51.7% had dengue for at least a second time (recent secondary infections). The majority of the cases (58%) were primary dengue cases followed by secondary infection (41%).

**Table 2** Details of Serologically confirmed primary and secondary dengue virus cases

Age Group	Primary Infection	Secondary Infection	Total
15-Jan	29(16%)	5(2.7%)	34
16-30	4(22%)	49(27%)	90
31-45	21(11%)	11(6%)	32
46-60	13(7%)	6(3.3%)	19
>60	2(1%)	3(1.6%)	5
Total	106 (58%)	74(41%)	180

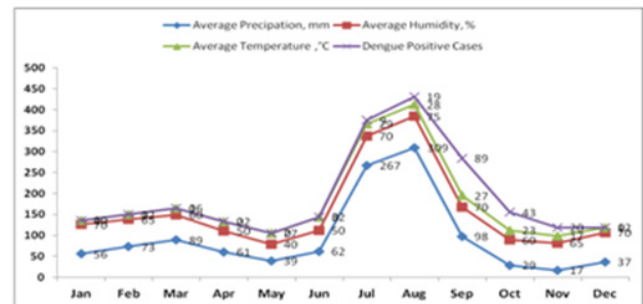


**Figure 2** Age wise distribution of Dengue positive cases.

## Discussion

Dengue virus infection is emerging as a major public health problem in developing countries like Pakistan due to lack of effective and

strong surveillance system, poor diagnostic facilities and insufficient patient management and improper strategies for the vector control measures. It is observed as one of the world's most prevalent vector-borne diseases with a 30 fold increase in disease incidence over last 50 years.<sup>8</sup> In Pakistan, first outbreak of dengue was reported from Karachi in 1994 followed by another outbreak in Quetta in 1995.<sup>13,14</sup> Later an epidemic was noted in 2005 which is extended to a large epidemic in the year of 2006. Dengue virus transmission is expanding in the country since 2006 with rapidly assumed the proportions of epidemic during 2011 that caused thousands of morbidities and mortalities.<sup>15</sup> Currently, dengue virus has spread almost all over Pakistan due to increased number of infected people among the population. The objective of this study is to investigate the dengue suspected cases referred from two tertiary teaching hospitals of metropolitan cities Islamabad and Rawalpindi by serology laboratory assay.



**Figure 3** Month wise Distribution of Dengue Suspected Cases with Climate Indices for Islamabad and Rawalpindi.

Geographically, Pakistan is located in the tropical and sub tropical regions. Islamabad, capital of Pakistan, is located in the Potwar Plateau in northeastern part of the country and has been a part of the crossroads of two provinces (Khyber Pakhtunkhwa and Punjab). Similarly, Rawalpindi is located in Potwar region of the northern Punjab province. Both cities contain plains and mountains without existence of any defined boundary and are recognized as twin cities. The climate parameters of both cities provide ideal conditions for the transmission and growth of the dengue vectors as also indicated by our data. Around 80% of total annual rainfall is received during summer monsoon months (July, August and September).

In 2011, both cities received rainfall higher than the normal during monsoon season as compared to previous years<sup>16</sup> that resulted in devastating flood and developed suitable environment for the dengue vector development. Furthermore, increased urbanization and population in both cities also affected the flushing effect of water in urban areas by some means that result the prevalence of dengue in the areas of high drainage density of both cities. The seasonal trend of dengue virus infection in Pakistan reveals that the vector control measures must be taken before monsoon season to prevent the outbreaks and epidemics of dengue virus infection in the country.

The age distribution of cases agreed with the preceding with the epidemiological back ground that suggest that maximum number of primary dengue infection limited to the children less than 15 year of age and number of secondary dengue cases were maximum in adult population as most individuals in endemic areas are already exposed previously to the dengue virus because the study area (Rawalpindi and Islamabad) has repeatedly experience dengue outbreaks from 2008-2011.<sup>16</sup> Additionally, male predominance was seen that has been reported earlier<sup>10,17</sup> and might be due to the gender bias in seeking healthcare. Secondly, proper covering of body and maximum indoor stay in females could be another reason for this low female predominance.



One of the limitations of the study was that the lack of history (Sample time collection, Symptoms) of most of the patients whose blood samples were referred for confirmatory test to NIH therefore it is difficult to compare the diagnostic parameters with reference to onset of disease. It may also be kept in mind that in Pakistan there was no local surveillance system or a notifying body for dengue cases and there were no standard operating procedures according to international guidelines for the health care workers to manage the dengue patients. Furthermore, the increased in dengue reported cases magnified the workload in hospitals.

Early management of patients with dengue infection is essential to ensure a favorable evolution of the disease and prevent the occurrence of severe forms. Until recently an early confirmed diagnosis was only achievable in specialized laboratories. It is very unfortunate that in the Pakistan still most cases of dengue are diagnosed on basis of clinical manifestations. This practice can support but not be considered as confirmatory. The possible confirmatory tests (NS1 Ag, IgM and IgG) are not even thoroughly available in public hospitals. The dengue NS1 antigen ELISA is considered as a promising tool for early dengue detection.<sup>12</sup> NS1 Ag circulates uniformly in all serotypes of dengue virus and it circulates at high level during the first few days of illness.<sup>18</sup> In current study, the detection rate of NS1 antigen was 54.4% with clinical suspicion of dengue in a single serum sample that strongly suggests recent DENV infection. Furthermore, studies claim that in addition to an early diagnosis, NS1 antigen may be an indicator of disease severity.<sup>18,19</sup>

Libraty et al.<sup>19</sup> observed that a very high concentration of NS1 antigen within 72 hours of illness identified patients at risk of developing DHF.<sup>19</sup> In addition, the detection of IgM and IgG antibodies is also considered the main technique for the laboratory diagnosis, to know the immune response model and to differentiate between primary and secondary infections for dengue.<sup>7,20</sup>

The correlation between the IgM and IgG helps the clinician to decide the current or past infection, for monitoring the spread of the epidemic and also for identifying the risk of severe forms of the disease especially in a situation when you have a single serum sample of DENV suspected patients. Therefore, the clinician should review the laboratory results to keep in view the patient's past medical history, geographical location and recent travel history to determine the likelihood that the current acute febrile illness is due to an infection with dengue virus. In our study 58% detection rate for IgM indicates that a segment of the population is at risk for secondary infection, which is associated with severe dengue as evident from other studies.<sup>21</sup> Therefore it is necessary to tell the patients that they have been infected with dengue and should, consequently, protect themselves from infection with subsequent serotypes.

Serotyping results of present study indicate that the multiple serotypes (DENV-1, DENV-2 and DENV-3) of dengue virus are prevalent in the country as indicated by various molecular studies conducted in Pakistan from 2005-2010. All these studies revealed the circulation of three serotypes DENV-1, DENV-2 and DENV-3 but no isolation of DENV-4. It has been shown in a study that viral emergencies or re-emergences may play different roles in the disease epidemiology, especially when many serotypes co-circulate in the community.<sup>22</sup> Furthermore, toll of 30% PCR negative but IgM positive cases indicate that the patients may have chronic infection because IgM antibodies for dengue may remain elevated for 2 to 3 months after the illness.<sup>23</sup> The elevated IgM observed in a sample could be the result of an infection that occurred 2 to 3 months ago. On other hand 6% samples that are NS1 positive but PCR negative may be due to the

some problem of maintenance of cold chain for sample transportation and their storage.

In an endemic country, especially in the context of an epidemic, it seems that the sensitivity of the NS1 alone is too low and that only positive results should be taken into consideration. However, our data reveal that combined NS1, IgM and IgG test results are more significant and appear to be a useful tool for the clinicians to manage the cases during the epidemic and to control the further transmission of the infection when there is fear that misdiagnosed dengue infection evolves towards a DHF or a DSS while these complications are pretty easy to prevent with simple clinical management, etc. Furthermore, these tests can be performed in limited recourses especially in the context of a developing country like Pakistan. Additionally, present study proposed that a highly sensitive molecular technique, Polymerase Chain Reaction (PCR), must be implemented for the screening of ELISA negative samples and if possible the genomic sequencing is another important tool to understand the molecular epidemiology of dengue virus in the country.

## Conclusion

From the present study it can be concluded that for early diagnosis of dengue virus infection, it is important to use the detection of NS1 antigen/viral RNA through real-time PCR, along with IgM and IgG antibodies at the same time which is helpful for the case management and dengue control program as well.

## Acknowledgments

None.

## Conflicts of interest

None.

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