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Dengue virus serotypes and epidemiological features of dengue fever in Faisalabad, Pakistan

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Abstract. Dengue infection has become a major public health threat in Pakistan, causing several outbreaks from 1994 to 2012. This cross-sectional study was carried out to determine viral serotypes responsible for major outbreak of dengue fever in Faisalabad, Pakistan during 2011. Total 50 patients (37 males and 13 females) reported within 2-4 days of onset of fever, positive for dengue NS1 antigen, were recruited for this study. The median age of the patients was 29 years. Laboratory findings indicate thrombocytopenia in 92.0%, decreased hemoglobin in 48.0%, and leukopenia in 38.0% patients at the time of admission. Liver enzymes were elevated in 100.0% patients. ALT levels were 7.0 times higher, while AST levels were 7.3 times higher than the normal values in dengue patients. Molecular epidemiology of the dengue fever revealed co-circulation of three different DENV serotypes in Faisalabad population. Among them 82.4% were positive for DENV2; 5.8% with DENV3; and 11.7% were reported with DENV4. Co-circulation of three different DENV serotypes in the city is an alarming situation, which could result in a more severe outbreak of DF (DHF/DSS) in the future.

INTRODUCTION

Dengue has emerged as a serious tropical mosquito-borne infectious disease, caused by enveloped single-stranded RNA virus known as dengue virus (DENV) belonging to family *Flaviviridae*. *Aedes aegypti* (*A. aegypti*) and *A. albopictus* mosquitoes are the most significant vectors for the dengue infection (Heymann, 2009; Wiwanitkit, 2010). The disease is caused by any one of the four DENV serotypes (DENV1 to 4), which can manifest as subclinical dengue fever (DF), classical DF, to severe dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (Endy *et al.*, 2002; WHO, 2009).

Primary infection with the DENV provides lifelong serotype specific immunity, while the heterologous secondary infection is associated with the more severe form of the disease (DHF/DSS) (WHO, 2011). The pathophysiology of this phenomenon may be explained by the theory of Antibody Dependent Enhancement (ADE) (Halstead, 2003), which has severely hindered in the development of effective dengue vaccine against all four serotypes (Whitehead & Subbarao, 2017). However, it is noteworthy that the risk of severe disease decreases during third and fourth infections (post-secondary exposure) to either of the two remaining serotypes due to development of protective immunity (Olkowski *et al.*, 2013).

In Pakistan, the disease was mainly limited to port city of Karachi before 2011 (Wesolowski *et al.*, 2015). Devastating floods in the province of Punjab in 2011 not only destroyed property and claimed uncountable lives but also created breeding sites for the proliferation of dengue vector, resulting in the worst outbreak in that year. More than 15,000 cases were recorded from Lahore (Punjab)

alone, which was potentially disastrous for the country's health-care system (Rai, 2011). Furthermore, mobile phone based mobility data predicted extensive travelling between the dengue endemic city (Karachi) and other major metropolitan cities (Lahore, Faisalabad etc.) of Pakistan during this period that might have contributed significantly in the introduction of dengue virus into the previously naïve population (Wesolowski *et al.*, 2015).

Few local studies had reported clinco-epidemiological features of dengue fever, however not a single study is available regarding DENV serotypes in Faisalabad from 2011 outbreak. Current study reports brief disease epidemiology, as well as, DENV serotypes circulating in Faisalabad during 2011.

MATERIALS AND METHODS

Ethical clearance

Ethical clearance was taken from institutional Ethical Review Committee (ERC) of Punjab Medical College (www.pmc.edu.pk), Faisalabad before starting the study. The study participants/legal guardians were briefed about the benefits and risks of participating in the study. Written informed consent was taken from all those agreed to participate in the research study.

Study setting and patient data

Faisalabad, the second largest city in the province of Punjab and the third largest city of Pakistan, located between latitudes 31.41°N and longitude 73.11°E, having a population of more than 2.6 million, is considered as an industrial hub of Pakistan. This six months descriptive cross-sectional study was carried out in Allied Hospital and National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad. All clinically confirmed cases of dengue fever who were admitted to the High Dependency Unit (HDU) of the hospital were screened for NS1 antigen. Total 50 dengue patients, irrespective of age and gender, positive for serological tests (e.g. positive for NS1 antigen) were recruited for further study.

Sample collection

Blood samples were collected from the patients reported within 2-4 days of onset of dengue symptoms as per WHO classification and case definition (WHO, 2009). A patient was considered as a confirmed case of dengue fever (DF) if presented with fever of 2–7 days duration and having two or more of the following symptoms: headache, retro-orbital pain, myalgia/arthralgia, rash, hemorrhagic manifestations (petechiae and positive tourniquet test) and, leukopenia. Blood samples were collected from the patients reported only within 2-4 days of onset of disease as there is successive decline in viremia with the concomitant increase in antibody titer after 4-5 days. Serum was separated and divided into two aliquots. Aliquot-1 was immediately stored at -80°C for RNA extraction and serological analysis, while aliquot-2 was used for serodiagnosis and NS1 antigen testing.

NS1 testing and serotype analysis

NS1 testing was carried out by *OnSite* Dengue Ag Rapid Test-Cassette (CTK Biotech, Inc, USA; Catalog # R0063C) as per manufacturer instructions. The kit have high relative sensitivity of 95.6% and specificity of 95.5%. Serotyping was performed by PCR on the serum samples in two steps at National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad. In the first step, cDNA was synthesized followed by standard PCR, while in the second step nested multiplex PCR was performed using amplified PCR products in the first step as described by Lanciotti *et al.* (1992). Briefly, dengue virus RNA was converted to cDNA by using reverse transcriptase (RT) enzyme and dengue virus downstream consensus primer D2. Subsequently, *Taq polymerase* amplification was used on the resulting cDNA with upstream dengue virus consensus primer D1. The correctly sized DNA products of primers D1 and D2 was obtained having molecular weight of 511 base pairs (bp) each. In the second step, nested multiplex PCR was performed using amplified product of consensus primer D1 and D2 (511 base pairs). Four set of primers (TS1, TS2, TS3 and TS4) specific to four dengue virus serotypes

Table 1. Primers for DENV serotyping (Lanciotti *et al.*, 1992)

Primer	Sequence	Genome position	Amplicon size (bp)	Serotype
D1	5'- TCAATATGCTGAAACGCGCGAGAAACCG -3'	134-161	511	
D2	5'- TTGCACCAACAGTCAATGTCTTCAGGTTC -3'	616-644	511	
TS1	5'- CGTCTCAGTGATCCGGGGG -3'	568-586	482 (D1 and TS1)	DENV1
TS2	5'- CGCCACAAGGGCCATGAACAG -3'	232-252	119 (D1 and TS2)	DENV2
TS3	5'- TAACATCATCATGAGACAGAGC -3'	400-421	290 (D1 and TS3)	DENV3
TS4	5'- CTCTGTTGTCTTAAACAAGAGA -3'	506-527	392 (D1 and TS4)	DENV4

were used to detect DENV serotypes. Agarose gel electrophoresis was used to detect dengue virus serotype on the basis of molecular weight of the amplified products in the presence of serotype specific primers (Table 1).

Laboratory investigations

Five ml blood samples were collected from all the study participants aseptically in vacutainers. One ml patient's blood was used to determine the concentration of hemoglobin (Hb) per ml of the blood, total leukocyte count, differential leukocyte count, red blood cell count, platelet count and hematocrit using hematology analyzer (Sysmax, Japan). Serum was used to determine liver enzymes as described by Hørder and Rej (1983). Platelet count of less than of 150,000 cells/mm^3 was taken as decreased. Hemoglobin level (g/dL) of 13.0-18.0 in males, 12.0-16.0 in females and 11.2-16.5 in children was considered as normal in the dengue patients. Level of leukocytes less than 4500 cells/mm^3 was taken as decreased.

Statistical analysis

Descriptive analysis was done for all variables. Mean±SD was calculated for all quantitative variables like age and platelet count. Frequency and percentages were calculated for all qualitative variables like gender, presence of virus, type of virus and symptoms of the disease. Disease attack rates were calculated by dividing number of positive cases by total population at risk.

RESULTS

Patient characteristics

Among 50 patients, 37 (74.0%) were males and 13 (26.0%) were females, while the median age of the patients was 29 years. The patient's age ranges between 4 to 80 years, including 4 (8.0%) children (up to 16 years of age) and 46 (92.0%) adults. There were 24 (52.2%) young adults (18 to 30 years old) among 46 adult dengue patients. Distribution of dengue patients into different age groups indicated high frequency of dengue fever in younger population. There were 25 (50.0%) young adults belonging to the age group of 16 to 30 years, followed by 10 (20.0%) participants belonging to age group of 31 to 45 years. Least number of dengue patients were reported from the age group of <15 years. However, attack rates shows that the maximum number of dengue patients from the age group of 46 to 60 years. Nevertheless, the attack rates were lowest for the age group of <15 years. Overall, the data indicate higher prevalence of male patients reported with dengue fever than the female patients and similar trend was seen within each age group. Highest frequency of male patients (38.0%) were reported from the age group of 16 to 30 years of age, followed by highest prevalence (14.0%) in the age group of 31 to 45 years of age. Likewise, highest number of females (12.0%) were reported from the age group of 16 to 30 years.

Laboratory findings

Liver involvement, as indicated by deranged liver enzymes, was observed in various dengue patients. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was raised in all 50 (100.0%) patients. ALT levels were 7.0 times higher, while AST was 7.3 times higher than the normal values in dengue patients. The mean value for ALT was 322, while for AST was 261. Majority of the patients were presented with thrombocytopenia at the time of admission. Thrombocytopenia was observed in 46 (92.0%) patients. Mean thrombocytes level was 100,000 cells mm^{-3}. A decrease in hemoglobin (Hb) level in dengue patients was observed in this study. Total 24 (48.0%) patients were reported with lower Hb level, which include 16 (32.0%) male patients and 8 (16.0%) female patients. Mean value for Hb was 12.8 g/dL among the dengue patients. All of the patients reported with decreased Hb were adults and none of the children was reported with reduced Hb. Leukopenia was also observed in 19 (38.0%) patients. Average leukocyte count was 5900 mm^{-3} in the study participants. Table 2 presents the laboratory finding of the dengue patients reported in Allied Hospital Faisalabad.

Serotype analysis

Results indicate co-circulation of three different DENV serotypes in Faisalabad population: DENV2, DENV3 and DENV4. However, no case of DENV serotype-1 was detected among the population. Although all total 50 patients were positive for NS1 antigen however, only 17 (34.0%) patients were PCR positive. Among them 14 (82.4%) were positive for DENV2; 1 (5.8%) with DENV3; and 2 (11.7%) were reported with DENV4. Figure 1 shows agarose gel analysis of the products of nested multiplex PCR for DENV serotyping Table 3 Summarizes the frequency of different DENV serotypes detected in NS1 positive dengue patients reported in Allied Hospital, Faisalabad.

Table 2. Laboratory findings of dengue patients

Characteristics	N (%)
Thrombocytopenia at presentation	46 (92.0)
Low Hemoglobin	24 (48.0)
Leukopenia	19 (38.0)
Increased ALT	50 (100.0)
Increased AST	50 (100.0)

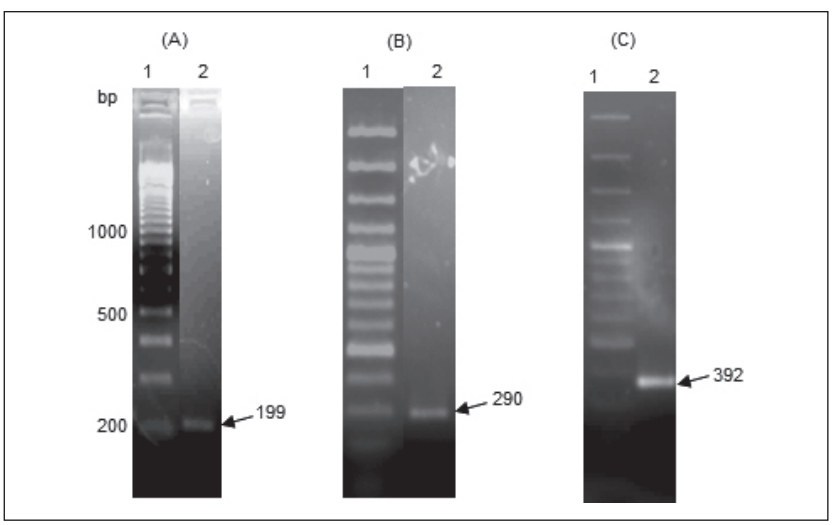

Figure 1. Agarose gel analysis of the products of nested multiplex PCR for dengue virus serotyping.

(A) Amplified products of D1 and TS2 primers for DENV2; (B) amplified products of D1 and TS3 primers for DENV3 (C) amplified products of D1 and TS4 primers for DENV4. Lane-1 DNA marker (SM0323, Fermantas), Lane-2 amplified DNA product.

Table 3. Prevalence of DENV serotypes detected in Faisalabad, Pakistan

DENV serotype	N (%)
Serotype 2	14 (82.4)
Serotype 3	01 (5.8)
Serotype 4	02 (11.7)

DISCUSSION

Studies conducted previously in Asia, using surveillance data, correlated the age of the effected patients with the severity of disease. Recently, study conducted in Pakistan during 2003-2007 noted decline in the median age of the dengue patients and reported age of the affected individuals was 11-25 years (Khan *et al.*, 2010). In contrast to previous studies, dengue attack rates were higher among the age group of 46-60 years in the current study. Similarly, Raza *et al.* (2014) reported higher incidence of dengue fever in older population than in younger population from Faisalabad during 2011-2012.

A three times higher incidence of dengue fever in males (74.0%) as compared to females (26.0%) was noted in this study. These gender specific differences were also noted in a recent study conducted to determine the incidence of dengue infection in six Asian countries (Anker & Arima, 2011). Similar observations were also made in other studies conducted in different regions of Asia (Ooi *et al.*, 2006; Shekhar & Huat, 1992; Yew *et al.*, 2009). In contrast to Asian population, a different trend was seen in other regions of the world. Interestingly, the incidence of dengue infection in North America reported either equal proportions of both females and males or females in higher proportions (Garcia-Rivera & Rigau-Perez, 2003; Gunther *et al.*, 2009; Trravassos da Rosa *et al.*, 2000). This phenomenon of gender specificity in relation to dengue infection in Pakistan might have been contributed by social, cultural (women being covered) and exposure reasons in Pakistan.

All of the 50 NS1 positive dengue patients were screened for alanine transaminase (ALT) and aspartate transaminase (AST) levels. Levels for both of the enzymes were elevated in 100.0% of the patients. The level of ALT was 7 times higher, while AST was 7.3 times higher than the baseline values. Previously, it has been noted that the DENV can infect numerous cell types and cause diverse clinical and pathological effects (Seneviratne *et al.*, 2006). Liver involvement, characterized by hepatomegaly and deranged liver enzymes, is a common finding during dengue infection, particularly in DHF patients. Similarly, hepatomegaly, associated with increased liver enzymes, was significantly higher in DHF patients (Wahid *et al.*, 2000). Various studies has suggested the use of liver enzymes (ALT and AST) as a parameter to evaluate severity the disease in dengue patients. Since grossly elevated liver enzymes are known to be an early warning sign for the severe disease and clinical bleeding (Gulati & Maheshwari, 2007; Raza *et al.*, 2014; Wesolowski *et al.*, 2015).

Dengue virus vector exists in this region even before the partition of British India in 1947 (Barraud, 1934), however, the first outbreak of DF in Pakistan due to DENV1 and DENV2 was reported in 1994 affecting thousands of patients (Rasheed *et al.*, 2013). Decade later, another outbreak due to DENV3 was reported in 2005 in Karachi with a sudden surge in the number of DHF patients. The increase in the disease severity was thought to be contributed by the infection with DENV3, in a population sensitized previously with DENV1 and DENV2, through antibody dependent enhancement (ADE) (Jamil *et al.*, 2007). Another outbreak occurred in 2006, due to DENV2 and DENV3, affecting the population north to south of Pakistan. Total 3500 confirmed cases of DF were reported and 52 deaths were recorded during the outbreak (Khan *et al.*, 2008).

In 2011, Province of Punjab, Pakistan was hit by devastating floods resulting in economic losses and claimed uncountable human lives. Moreover, it had provided excellent breeding sites for the proliferation of vector borne diseases, such as dengue fever, resulting in the worst DENV outbreak in the history of Pakistan. This study undertakes to reveal DENV serotypes circulating in Faisalabad in 2011. Serotype

analysis for the DENV has revealed that the outbreak was caused by three different serotypes: DENV2, DENV3 and DENV4. It has been known that the primary dengue infections are generally asymptomatic and lifelong immunity is conferred by the infection with one of the four serotypes, however, secondary dengue infection with the heterologous serotype is thought to progress to more severe form of disease (DHF/DSS) (Halstead, 1988; WHO., 2009). Therefore, co-circulation of three different DENV serotypes among the population is an alarming sign for a more severe outbreak in the future (WHO, 2009). The situation demands immediate action by the authorities to take appropriate measures for the control vector population in the city.

CONCLUSION

Dengue is epidemic or endemic in virtually every tropical country. It has become a major public health problem in Pakistan as well, causing several endemics from 1994 to 2012. The central place of Faisalabad city amongst health wise underdeveloped surrounding districts makes it a hotspot for health care seekers. Dengue epidemiology in the city revealed that older population is more at risk of contracting DENV infection in Faisalabad, as compared to younger population. Moreover, a higher incidence of dengue fever was reported in males as compared to female. Such gender specific differences might be an interplay of social, cultural (women being covered) and exposure reasons. Liver involvement, characterized by elevated ALT and AST levels, was evident in all patients, which could be used predictor of severe DF. Co-circulation of DENV2, -3 and -4 in Faisalabad is an alarming situation, which could result in outbreak of severe DF among the population. Secondary infections with heterologous serotypes could result in more severe form of DF (DHF/DSS), resulting in greater number of deaths in future.

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