Pakistan J. Zool., vol. 49(3), pp 1119-1122, 2017. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2017.49.3.sc4

Short Communication

Prevalence and Molecular Detection of Dengue Virus in 2013 Outbreak in KPK and Punjab, Pakistan

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ABSTRACT

Dengue Virus is transmitted to humans by *Aedes* ***aegypti*** and *Aedes albopictus* having five serotypes 1-5 and infecting about 100 million people each year. To address dengue outbreak 2013 in Pakistan we aimed to conduct a comprehensive study at molecular level to determine the main causative serotype of 2013 DENV outbreak in Pakistan. Overall 703 serologically positive suspected patients from different major health centers of Pakistan were registered in present study of which 214 were females and 489 were male. Overall weighted prevalence of PCR positivity was 38% (268). Of the total 268 PCR positive subjects, 0.74%, 56.71%, 15.67% were positive for serotype 1, 2 and 3 respectively. Only 72 suspected patients had concurrent infection with serotype 2 and 3. Higher incidence of RNA positivity by PCR was found in males (71%) as compared to the females (29%). The higher incidence of PCR positivity was found in age group of 16-25 rather than teenage (≤16) and older age group (≥50). From the result of this febrile illness study we concluded that males and young people are more susceptible to Dengue virus infection. Moreover serotype-2 was the main causative serotype of the 2013 outbreak of dengue fever.

Article Information
Received 21 September 2016
Revised 12 December 2016
Accepted 09 February 2017
Available online 26 May 2017

Authors' Contributions
MS, IA and SA analyze the samples, MI conceived the idea of study and ZF, SZ and UA helped in result analysis and writing the manuscript.

Key words
***Aedes aegypti*, *Aedes albopictus*, Prevelance, PCR, Serotyping**

Dengue virus is mosquito-borne human pathogen that causes severe and widespread disease in tropical and subtropical regions of the world (WHO, 2015; Guzman and Kouri, 2002). The five dengue virus serotypes DEN 1-5 are transmitted to man by *Aedes aegypti* and *Aedes albopictus* mosquitoes (Mustafa *et al*., 2015). The occurrence of dengue cases is dependent upon the activities of the vector involved (mosquitoes) in different areas and seasons. Normally, the rate increases during or after the rainy season (WHO, 2012). Disease symptoms range from uncomplicated fever to more serious and potentially fatal dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Hastlead, 2007). A primary dengue infection results in life-long immunity to the homologous infecting virus and the development of more severe disease is associated with secondary infection with a different virus serotype (Halstead, 1998).

Dengue fever in Pakistan was first reported in 1982 from Punjab and since then at least eight small outbreaks of dengue fever had been reported from Pakistan (Hayes et al., 1982; Chan *et al*., 1995; Paul *et al*., 1998; Khan *et al*., 2007; Humayun *et al*., 2010; Fatima *et al*., 2011). A large epidemic of dengue fever hit Punjab in the year 2011 in which the number of reported cases was more than 50,000 patients in Lahore alone, by the end of November 2011 (Rai, 2011). In the year 2013, another major outbreak of dengue fever was reported from Pakistan, especially in Khyber Pakthunkhwa (KPK) province. According to the WHO (2013), the number of cases was 8,546, including 33 reported deaths in Swat district, with the prevalent serotypes being DENV 1, 2 and 3. A study showed that out of a total 6000 reported cases in Swat, 68.73% patients were male (Khan and Khan, 2015). Apart from the KPK province, random cases were also reported from Punjab, Sindh and Balochistan provinces. In Punjab during 2013, 2165 dengue cases were witnessed by health departments and 50% of them were reported from Rawalpindi city alone (Waseem *et al*., 2014).

Timely and correct diagnosis can help in patient management before the appearance of warning signs of severe and life-threatening complications like dengue hemorrhagic fever (DHF). Although there are many sensitive serological techniques which have been employed for early diagnosis of Dengue virus, the most widely used

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0030-9923/2017/0003-1119 $ 9.00/0

method is nested PCR because it is economical and specific for serotyping analysis for better disease management (Lanciotti *et al*., 1992).

The present study was planned to assess the utility of concomitant and simultaneous detection in suspected dengue patients' samples in 2013 outbreak in Pakistan, especially in Punjab and Khyber Pakhtunkhawah (KPK) provinces, in order to attain faster and sensitive diagnosis.

Materials and methods

A total of 703 suspected blood serum samples were collected on the basis of demographic characteristics, dengue specific symptoms, area, hematological and biochemical features and duration of onset of the ailment, from September to end of December 2013, at the Division of Molecular Virology, CEMB University of the Punjab Lahore for the molecular characterization and detection of different serotypes of DENV from Pakistan. An informed agreement was taken from all hospitals and enrolled patients to conduct the present study.

Viral nuclear RNA was isolated from 150 µl of blood serum by using FavorPrep™ Viral Nucleic Acid Extraction Kit I according to the kit protocol. Extracted RNA was stored at -70°C for supplementary experimental analysis.

C-prM gene junction was used to design precise DENV specific degenerative primers according to the primer sequences published by Fatima *et al*. (2011). The amplicon sizes were 411,403 453 and 401 bp for DENV1, DENV 2, DENV3 and DENV4, respectively.

cDNA synthesis was carried out by incubation at 37 °C for 50 min with 5 µl of RNA and 200 U of M-MLV reverse transcriptase enzymes. The M-MLV was heat inactivated at 95°C for 5 min.

For nested PCR the serotype-specific primers were divided into 4 sets to detect 4 different DENV serotypes. Nested PCR was performed for serotyping analysis of PCR product obtained from RT-PCR using serotype-specific primers. Nested PCR was carried out by using 2 µl amplified RT-PCR product with 8 µl of PCR mix containing 1.2µl of $MgCl_2$ (from 25 mM stock), 1µl of 10 × PCR buffer, 1µl of each 10 pM primer (forward and reverse), 1µl of 250 µM dNTPs, 2.5µl of dH_2O and 0.3µl of *Taq*-DNA polymerase enzyme (Invitrogen, USA). The rest of the procedure was similar to that defined in the RT-PCR. The Nested PCR products were separated on 2% agarose gel for serotype-specific DNA fragments. Ethidium bromide was used to stain the gel, which was assessed under a UV trans-illuminator. A 100-bp DNA marker (Invitrogen, USA) was run in one well as DNA size standard and the DENV serotype/s was determined by comparing the DENV specific PCR band with the DNA marker.

SPSS v.17 software was used for statistical analysis. In order determine the statistical association, the Chi Square test was applied among age, gender and dengue RNA positive PCR data. All data are presented as number of subjects and percentage. Results were considered significant having P-values less than 0.05.

Results

Table I shows the characteristics of the study population (n = 703 cases) that contributed acute serum samples to the test panel. The panel (n = 703) was enrolled consecutively. Prior to the test sample being collected, the median period of illness was 3 days (range: 2-5). Of the 703 suspected samples, 38% (268) were found to be RNA positive by RT-PCR. The majority of the samples belonged to Dengue serotype 2 (56.71%), while 0.74% and 15.67% were found positive for serotypes 1 and 3, respectively. Only 72 (26.86%) of suspected patients had concurrent infection with serotype 2 and 3. Higher incidence of RNA positivity by PCR was found in males (38.85%, n=100) compared with females (36.44%, n=78).

A higher prevalence of DENV (45.71%) was found in the 16-25 year age group, than in the teenage group (≤16) and older age group (≥50). The median age of dengue fever patients was 30 years, with the range extending from 4 to 95 years. Males and young people were more susceptible to DENV infection, moreover, DENV2 was the foremost causative serotype of the current dengue endemic along with Serotype 3, which has persisted at a low rate in Pakistan for last three decades. Serotype 1 was also detected in the current study in 2 patients from Lahore (Table I).

The number of patients enrolled for the current study in September, October, November and December were 141, 271, 109 and 81, respectively. The epidemic data show that most of the suspected dengue fever cases were enrolled in the third and fourth weeks of October. Most of the suspected individuals fell in the 26-35 year age group and the male to female ratio was 2.3:1. The dengue fever specific symptoms were hemorrhagic rash, headache, fever and myalagia. Almost all the patients had a significant reduction in platelet count.

Out of the total of 703 serum samples of suspected dengue patients collected from different hospitals in different districts (Rawalpindi: n=446, 63.4%; Lahore: n=241, 34.28; Faisalabad: n=2, 0.28%; Multan: n=3, 0.42%; Swat: n=11, 1.56%). 268 were found to be positive for dengue RNA by PCR. The highest incidence of PCR positivity (n=184) was reported from the district of Rawalpindi. Of these 184 PCR positive subjects, 107 and 34 were found positive for serotypes 2 and 3, respectively, and 43 subjects had concurrent infection with serotypes 2 and 3.

Table I.- Total Serum samples collected from patients of different hospitals examined by dengue-specific RT-PCR and serotyping with gender and age groups.

Parameters	Total patients	RT-PCR Positive	Serotype 1	Serotype 2	Serotype 3	Concurrent (2 and 3)
Number of Participants	703	268	2	152	42	72
Gender						
Male	489	190	0	100	33	57
Female	214	78	2	52	9	15
Age groups						
0-15 Years	82	24	0	18	1	5
16-25 Years	210	96	0	48	23	25
26-35 Years	228	94	1	55	11	27
36-45 Years	97	28	0	15	3	10
46-55 Years	31	8	0	6	1	1
≥56 Years	55	18	1	10	3	4

None was found positive for serotypes 1 and 4 in Rawalpindi. Serotype 1 was reported only from Lahore (n=2). The highest incidence of concurrent infection (90.9%) with serotypes 2 and 3 was reported from Swat. The overall prevalence of serotype 2 (n=107) was found to be highest in Rawalpindi.

Discussion

Dengue virus infection is endemic in Pakistan and has circulated over the past 2 decades, infecting the population in the post-monsoon season, causing serious epidemics of dengue infection affecting more than 5 million people, 500 of whom died in the post-monsoon outbreak in 2011. More than 20,000 cases of dengue infection were reported in the dengue epidemic of 2011 (Rasheed *et al*., 2013) and 365 of them died, however, the actual figure may be greater than 700.

The foremost aim of the current study was to characterize the serotypes of dengue for the current epidemic in Pakistan. We were able to collect, serologically analyze and serotype a large number of blood serum samples (n=703).

The overall prevalence of dengue virus was 38% in this study compared with the previous epidemiological study conducted in Lahore in 2012, which was 82%. The highest prevalence of DENV was found in the 16-25 year age group (n=96, 35%), rather than in other groups. This incidence of elevated dengue infection in the teenage group is similar to that in other studies directed in different epidemic areas (Fatima *et al*., 2013), but in contrast to the epidemic study piloted in Singapore (Yap *et al*., 2013).

A greater incidence of DENV was noted in males (n=190, 71%) than in females, which is consistent with former studies in subtropical and tropical regions of the world, in which males were more susceptible to dengue infection than females. This difference may relate to differences in daily outdoor activities, restricted intellectual settings and gender specific contact, but this variation by sex was not statistically significantly linked with DENV (p=0.68). Dengue has been noted as a fatal and urban disease and attributed somewhat to the more populated towns than the countryside. This epidemic of DENV infection arose in the post-monsoon season, in concordance with other studies previously conducted in Pakistan and the neighboring country India (Ali *et al*., 2013).

In the present study, we were able to successfully serotype 38% of enrolled subjects, which is far less than in the previous study conducted in Lahore by Fatima *et al*. (2013), in which it was 82%. Serotype 2 DENV dominated the current epidemic with an infection frequency of 57%, compared with serotype 3 with a 16% infection rate. Concurrent infection with both serotypes 2 and 3 was found in 27% of the enrolled subjects from different areas of Pakistan, which is contrary to other studies which reported all four serotypes (Humayun *et al*., 2010). It was established in the previous study that serotypes 2 and 3 were the responsible agents in the outbreak of 2007-2009 in Pakistan (Fatima *et al*., 2011). The dengue fever outbreak in 2008 revealed that serotypes 2, 3 and 4 were found in the population of Lahore, with serotype 4 dominating (Humayun *et al*., 2010). This variation may be due to differences in specificity and sensitivity of the approaches applied for serotyping. We exploited the most extensive and specific nested PCR and sequencing analysis of samples from arbitrarily selected subjects. The conclusions of the present study, which were based on the most sensitive techniques available on the market, clearly revealed that serotype 2 was the main cause of the present dengue epidemic in Pakistan, though viral infection serotype 3 was also found in a low ratio along with concurrent infection with serotype 2. It is anticipated that serotype 3 also has persisted in the Pakistani population at a low infection rate for the last few years in this geographical region of globe.

Serotype 3 has five genetically different assemblies called genotypes. The spread of serotype 2 DENV is alarming for three reasons in the present study. Firstly, serotype 2 is more contagious than other serotypes, as most of the previously studied dengue epidemics were found to be allied with serotype 2. Secondly, there are greater chances of DSS or DHF occurring in patients who have been previously infected with one serotype in the past and infected again with a different serotype. This occurrence is recognized as antigenic sin and in this infection, the patient's immune system fails to react adequately to the resilient infection, resulting in severe consequences of the secondary infection. It is expected that the chances of DHF or DSS will be increased significantly in this region of the world than in the present epidemic if the dengue outbreak next year is caused by a different dengue serotype from DENV2.

Conclusions

It is concluded that serotype 2 is the leading cause of the current dengue rampant in Pakistan, along with Serotype 3, which persisted at a low rate in Pakistan for last three decades. The other Serotype detected in the current study was DENV 1 in 2 patients from Lahore.

Conflict of interest statement

We declare that we have no conflict of interest.

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