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OUTBREAK OF DENGUE VIRUS SEROTYPES IN DENGUE HEMORRHAGIC FEVER PATIENTS OF PESHAWAR PAKISTAN

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ВСПЫШКА СЕРОТИПОВ ВИРУСА ДЕГЕ У БОЛЬНЫХ ГЕМОРРАГИЧЕСКОЙ ЛИХОРАДКОЙ ДЕГЕ В ПЕШАВАРЕ, ПАКИСТАН

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Abstract

Background: Dengue is the most critical medical problem in tropical and sub-tropical locales of the world transmitted by the bite of the mosquito Aedes aegypti. Dengue virus (DENV) is categorized into 4 serotypes i.e. DENV1, DENV2, DENV3, and DENV4. The role of dengue virus (DENV) serotypes in the causation of primary Dengue hemorrhagic fever (DHF) among dengue patients are not well studied in Pakistan. This research aimed to determine the association of DENV serotypes with DHF in dengue hemorrhagic fever patients of district Peshawar Pakistan.

Methods: A total of 200 blood samples were collected from DF and DHF patients. All of the samples were considered to be positive by NS1 Elisa. RNA was extracted with an RNA kit (TRIzol, USA) using the manufacturer protocol. The extracted RNA was then converted into cDNA by using Revert AID Reverse transcriptase (Thermofisher scientific) the target region for transcription and Amplification is a c-prM region which was amplified by Regular PCR and by using Nested PCR, the DENV serotypes (1-4) (Thermofisher scientific) was identified by using serotypes specific primers (TS1, TS2, TS3, and TS4) the samples was run through gel electrophoresis and then read the product size.

Results: a total of 164 (82%) samples were considered to be positive by both NS1 Elisa and RT-PCR while 36 (18%) were negative by RT-PCR. the study population consists of 60% male and 40% female. Within positive samples, 40 patients have DHF of which 24 (15%) were male and 16 (10%) were females. Most of the affected patients have an age range between 10-30 years followed by 30-40 years. All of the DHF patients were primary patients and had no previous dengue fever history. By using Serotype-specific primers all the DHF samples were positive for Serotype-2 (DENV2). This shows that DENV2 was primarily associated with DHF

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Conclusion: DENV2 is the dominant serotype in the Dengue outbreak in affected patients of Peshawar.

Keywords: Dengue virus Serotypes, Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF), DENV2, RT-PCR, ELISA

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Резюме

История вопроса: Лихорадка Денге является наиболее серьезной медицинской проблемой в тропических и субтропических регионах мира, передающейся через укус комара *Aedes aegypti*. Вирус денге (DENV) подразделяется на 4 серотипа: DENV1, DENV2, DENV3 и DENV4. В Пакистане недостаточно изучена роль разных серотипов вируса денге в возникновении первичной геморрагической лихорадки денге (ГЛД) среди больных денге. Настоящее исследование было направлено на определение связи между серотипами DENV с DHF у пациентов с геморрагической лихорадкой денге в районе Пешавар, Пакистан.

Методы: у пациентов с ЛД и ГЛД всего было взято 200 образцов крови. Все образцы были признаны положительными с помощью NS1 Elisa. РНК экстрагировали с помощью набора РНК (TRIzol, США) согласно протоколу производителя. Далее выделенную РНК конвертировали в кДНК с помощью обратной транскриптазы Revert AID (Thermofisher Scientific). Целевой областью для транскрипции и амплификации является область c-prM, которую амплифицировали с помощью стандартного протокола ПЦР и с помощью Nested ПЦР идентифицировали серотипы DENV (1-4) (Thermofisher Scientific) с использованием праймеров, специфичных для серотипов (TS1, TS2, TS3 и TS4), после чего образцы анализировались гель-электрофорезом с определением размера продукта.

Результаты: в общей сложности 164 (82%) образца были признаны положительными как с помощью NS1 Elisa, так и с помощью RT-PCR, тогда как 36 (18%) были отрицательными по результатам RT-PCR. Исследуемая популяция состоит из 60% мужчин и 40% женщин. Среди положительных образцов у 40 пациентов была ГЛД: 24 (15%) мужчин и 16 (10%) женщин. Большинство заболевших пациентов имеют возрастной диапазон от 10 до 30

лет, и далее лица в возрасте 30-40 лет. Все пациенты с ГЛД были первичными пациентами и ранее не имели истории лихорадки денге. Благодаря использованию серотип-специфических праймеров все образцы ГЛД оказались положительными по серотипу 2 (DENV2). Это показывает, что DENV2 был в первую очередь связан с ГЛД.

Вывод: DENV2 является доминирующим серотипом при вспышке лихорадки Денге у больных в Пешаваре.

Ключевые слова: серотипы вируса денге, лихорадка денге (ЛД), геморрагическая лихорадка денге (ГЛД), DENV2, RT-PCR, ИФА.

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1 Introduction

Dengue is the most critical medical problem in tropical and sub-tropical locales of the world transmitted by the bite of the mosquito Aedes *aegypti* [16, 15]. Dengue virus (DENV) is the main causative agent of dengue infection. DENV belongs to the genus Flavivirus within the family *Flaviviridae*. The virion of DENV is enveloped which contains positive-sense single-stranded RNA with a length of 11 kilobase pair [1]. On the basis of differences in the nucleotide sequence, DENV is categorized into 4 serotypes i.e. DENV1, DENV2, DENV3, and DENV4, and ten genotypes. All of these four serotypes circulate globally with usually high prevalence in the most endemic countries for dengue viruses [18].

Dengue infection is normally asymptomatic with a mild flu-like syndrome also known as Dengue fever (DF), however, its clinical manifestation can extend from a self-restricting Dengue fever (DF) to extreme dengue hemorrhagic fever (DHF) and genuine dengue shock disorder (DSS) [8,2]. DF is described by early elevation in body temperature followed by late, cephalalgia, muscle pain (myalgia), pain in the eye, and rashes on the skin [7]. Convalesce from the infection will occur within 1-2 weeks but prolonged asthenia for an extended period usually is occurring. Platelets and white blood cell count diminish habitually perceive [6]. Under certain conditions, DF leads to a DHF followed by a transitory increment in the permeability of blood vessels bringing about seepage of plasma with hyperthermia, drain, haemoconcentration, and thrombocytopenia which can prompt DSS [16].

Transformation of DF to DHF normally occurs in patients with previous DF history due to the phenomenon of antibody-dependent enhancement. During ADE antibodies in a patient, the body is already circulating against the previous DENV serotype which binds to the epitopic region of the heterologous DENV serotype and hence eases the virus entry to the cells bearing “FC” receptor, similar mechanism also occurs during primary infection in newborns of dengue immune mother [9, 3].

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The expansion of dengue virus serotypes has increased the risk of severe infection including many secondary infections, resulting in an increase in dengue disease epidemics [14]. The overall worldwide incidence of dengue disease is 50-100 million and 250,000-500,000 cases of DHF annually. The mortality rate of DHF/DSS is 5-10%. Both males and females are affected by dengue disease however males are more affected as compared to females. [5].

Since there is no particular treatment for dengue infection precautionary procedures rely on vector control and individual assurance measures, which are hard to authorize and keep up, with and are costly. The most excellent strategy for anticipation is the production of a safe and effective vaccine which works again all the DENV infection-causing serotypes. Such an improvement is an earnest need, specifically for kids living in endemic regions [12]. In July 2017 dengue epidemics occurred in Khyber Pakhtunkhwa province which affects more than 15 districts of the province while more than 500 DENV-positive cases were reported as per government sources there were around 75,000 suspected cases, more than 24,382 cased found confirmed/positive and death toll due to Dengue is 73 till date. The majority of the positive cases were reported in Peshawar city [2].

The serotypes responsible for such cases were not yet analyzed. Therefore, this study aims to determine DENV serotypes in DHF patients attending the tertiary care hospital of Peshawar city.

2 **Materials and methods**

Patients

This study was conducted in Different Dengue affected areas and in the Khyber teaching hospital of district Peshawar, Khyber Pakhtunkhwa Pakistan. A total of 200 Dengue Positive Samples of various Localities of district Peshawar were included in this study as shown in **figure 1.**

Sample collection

A total of 200 blood samples were collected from susceptible patients of the study area which138 samples were collected from Khyber teaching hospital (KTH) and 62 were collected from the various study areas. Patients with signs and symptoms such as cephalalgia, myalgia, temperature, arthralgia, hyperthermia, skin rash, pain in the abdomen, nausea, vomiting, and retro orbicular pain were selected for blood sample collection. Former knowledgeable approval was taken from each infected individual in the study. A well-prepared questionnaire was used to gather medical plus demographic data of every patient in the study population. The patient's basic history including the person's name, sex, gender, disease, signs and symptoms, and previous history of DENV, etc. was collected in the form of questionnaires.

3mL of blood was collected in a sterilized syringe from patients with Dengue Fever, DHF, and DSS. After sample collection serum was separated from blood at 2500 rpm for 10 minutes and was kept at -20°C for further analysis.

Serological assay

Enzyme-linked immune sorbent assay "ELISA" was used to detect IgG (Anti DENV-IgG kit and IgM [(Anti DENV-IgG kit and IgM kit (Scientific Diagnostic Korea) against DENV in the blood sera of Dengue Patients as per manufacturer protocol.

RNA extraction

RNA was extracted using 100µl of serum sample with an RNA kit (TRIzol, USA) using the manufacturer protocol.

DENV Serotyping

The extracted RNA was then converted into cDNA by using Revert AID Reverse transcriptase (Thermofisher scientific) the target region for transcription and Amplification is a c-prM region which was amplified by Regular PCR and by using Nested PCR the DENV serotypes (1-4) (Thermofisher scientific) was identified by using serotypes specific primers as shown in **Table 1**

Gel Electrophoresis

The amplified DNA of nested PCR was run in 2% agarose gel prepared in 100 ml of 0.5 x buffers (Tris-Cl, Boric Acid EDTA) and 15 µl ethidium bromides. 3µl loading dye was mixed with nested PCR products and run in Agarose gel, 100 bp (Fermentas, USA) DNA ladder marker (10 µl) was also laded parallel to the samples and was run for 30 minutes. DNA bands were become visible and compared by DNA ladder marker, and then visualized under UV trans illuminator (Cell Bioscience, Taiwan).

Statistical Analysis

The two-way ANOVA is performed to analyze the effect of the category of infection and serotype for Gender and age groups. The ANOVA revealed that there is not a statistically significant interaction between the effect of the category of infection and serotype p=0.077. The other two-way ANOVA is performed to analyze the effect of Positivity and serotype for Gender. The ANOVA revealed that there is not a statistically significant interaction between the effect of Positivity and serotype p=0.27.

3 **Results**

Diagnosis of Dengue virus infection by NS1 Elisa and real-time PCR in the study population

A total of 200 blood samples were collected from DF/DHF patients. Two types of diagnostic procedures were performed for the detection of the virus i.e. NS1 Elisa and real-time PCR and compared the result. All of the samples were considered to be positive by NS1 Elisa and a total of 164 blood samples were positive by both NS1 Elisa and real-time RT-PCR. The use of NS1 ELISA is arguably an appropriate and reliable method for early diagnosis in a laboratory setting where the facility of real-time PCR is not available. However, for well-equipped laboratories, the use of real-time PCR is a rapid, sensitive, and suitable diagnostic test that cannot only detect viral RNA but also specify the viral serotypes at the same time **table2.**

Prevalence of serotypes among dengue-infected patients of the study population of DF patients

For the detection of viral RNA, all the serums were run through RT PCR. A total of 164(18%) samples were considered to be DENV positive by RT-PCR while 36(18%) samples were RNA negative. To find out DENV serotypes all the positive samples were analyzed through regular PCR by using serotypes-specific primers. Only the DENV2 serotype was detected among positive samples. The prevalence of the DENV2 serotype was highest in males (n=100) as compared to females (n=64) **table 3**

Prevalence of serotypes among dengue hemorrhagic fever (DHF) patients of the study

Population

Similarly, among positive samples, 40 blood samples were collected from patients with DHF including 24 males and 16 females. Among DHF patient's prevalence of DENV2 is highest in males as compared to female patients. **Figure 2.**

Overall age-wise prevalence of DENV serotype among patients of the study population

To determine the prevalence of DENV serotypes and their correlation with primary DHF different age groups were created such as ≤10 years, 11-20, 21-30, 31-40, 41-50, and ≥51 years. The entire 164 (82%) sample was positive for the DENV2 serotype .of the 164 patients 40 were suffering from DHF. The numbers of male patients are more than female patients. All of the age groups from ≤10 years to ≥51 years were infected with the DENV2 serotype. Most males and females belonging to age groups 11-30 followed by 31-40 were affected by DENV2.

Similarly among DHF patients 2(5%) have ages ≤10 years while other infected DHF patients were showing the following results i.e. 10(25%), 9(22%), 8(20%), 6(15%) 3(8%), 2(5%) in the age group 11-20 year, 21-30 years 31-40 years, 41-50 years, ≥50 years respectively.

Out of total 40 most of DHF positive patients were male (60%) while (40%) were female. **table 4 and figure 3**

Clinical features of dengue-infected individuals

Common symptoms in both DF: DHF patients observed during the study period in the following ratio i.e.

Fever (100%), headache (92%:95%), body and joint pain (92%:100%), nausea (85%:70%), vomiting (56%:80%), retro-orbital pain (69%:62%), fatigue (93%:97%), skin rash (15%:100%). while 12% DHF patients have pleural effusion and ascites. **Figure 4**

4 **Discussion**

During the last decades, epidemics of DHF have occurred in Pakistan, India, Bangladesh, China, Srilanka, and Maldives. All four serotypes of Dengue cause similar types of infections but DENV2 and DENV3 are more often associated with severe and fatal DHF [13].

In the current study, we analyzed the association of the DENV serotype which is responsible for the most severe DHF in the recent epidemic of dengue in the district of Peshawar Pakistan. According to the present study, most of the DF cases and few DHF and DSS cases were reported in the months of October and November followed by December. It was reported earlier that the ratio of Male patients was higher in all age groups as compared to Females because Females mostly stayed inside the house as well as properly covered their bodies and hence were less prone to vector bite [8, 15].

Demographic data analysis showed that the infection rate in males was double the rate in females, with a male-to-female ratio of 2:1, an observation that corresponds with previous results from studies of the KPK province [7]. In the present study, a total of 200 blood samples were collected from susceptible patients in the study area. The ratio of male patients including DF and DHF were more as compared to female patients, similarly, patients in the age groups between 10-30

years followed by 30-40 years of age people were mostly affected by DHF. Most of the male which have DHF either are students or servants who were exposed to different environments. Another reason is that most of the people in affected localities belong to middle-class or poor families which can travel by local transport or walk similarly during summer the males and children sleep without wearing shirts congruent and therefore are the easy target of vector bite [10, 11].

Lack of awareness about vector prevalence is also a factor people in the affected area mostly use room cooler therefore the water remain present for many days without changing it. During 2013 and 2015, in severe dengue outbreaks in district Mansehra and Swat district of KP Pakistan, the majority of deaths were reported due to DHF where DENV variants analyzed were closely related to strains previously detected from Lahore and Karachi [16]. In previous Dengue epidemics in Pakistan from 1994-2017, the most prevalent serotype was DENV2. Similarly, during the 2015 dengue epidemics, dengue cases were reported continuously from adjacent districts including Kohat, Mansehra, and Malakand, also sporadic cases were reported from districts Shangla, Lower Dir, and Upper Dir which is nearby to the district Swat [19]. All four serotypes (DENV1, DENV2, DENV3, and DENV4) cause DF but the most prevalent worldwide serotypes are DENV2 and DENV3.

In the current study, DENV2 was the predominant serotype found circulating in the dengue outbreak in Pakistan which is consistent with the previous studies. RTPCR analysis shows that Out of 200 positive cases, 119 samples were positive for DENV2. While Peoples from previously affected areas of KP Province (Malakand, Kohat, Charsadda, Swat, Dir) are attached to the Peshawar district similarly district Charsadda and Kohat are nearby to the Peshawar both districts were affected by DENV2 and DENV3 during 2013, and 2015 epidemics it was possible that dengue vectors extension of current epidemics has linked to previously affected districts. In the current study, it is analyzed that such comparison between dengue serotypes of the previously affected area and the current study area shows that the

serotypes were not completely eradicated from the KP region, and also there is no preventive measurement for mosquito control hence the serotypes were still circulated in the region hence affect peoples of Peshawar which is capital of province city and a central region which connect peoples of the surrounded area particularly previously affected regions in every aspect.

It was previously known that secondary DENV infection happening in the region which is endemic to DENV or areas affected by more than one serotype at a time which corresponds to a major public health risk and is also associated with the progression of disease from DF to DHF and DSS [2]. In the current study, we found DENV2 to be the predominant serotype involved in the dengue outbreaks in Peshawar in contrast to Suleiman *et al* [16]. Moreover, all patients with DHF were associated with DENV2 followed by DENV3, Which supports that DENV2 is also primarily associated with fatal DHF [18].

The change in temperature and humidity (ecological conditions) plays a significant role in the survival/breeding of vector mosquitoes and their population density. A more recent study [9], has shown that higher temperature (>25˚C) produces a large number of mosquitoes with frequent blood-feeding nature. Also, it is documented that a 1˚C increase in temperature (above average) may increase the risk of dengue transmission by 1.95 times [9, 4].

In our study, there was seen a peak in DF and DHF hospitalization during September-October and November which according to our study may be direct to the overcrowding population, and the increase in temperature and humidity. Peshawar is mostly affected because of increased population, trading (tires, room coolers, etc.), and high-temperature peoples mostly use room coolers and also sanitation problems hence environment is suitable for vector breeding. Dengue cases also continue to be reported from many neighboring areas like Charsadda, Mardan, Sawabi, and Khyber agencies where data collected from dengue-positive patients were dengue was spreading from Peshawar. The affected area also has

problems of sanitary due to which water from sewages, rain, etc accumulated and continuous use of the room cooler was also done.

5 **Conclusion**

DENV2 was responsible for the Dengue outbreak in Peshawar. According to the questionnaire and Patients History, all the samples were taken from the patients of affected regions of district Peshawar. The result shows that the dominant serotype was DENV2 and also primarily associated with DHF in Dengue epidemics in Peshawar

Acknowledgment

We thank all the doctors and nursing staff of the concerned hospital to provide access to the patients and helping us with data collection. The results organized during this research are the private views of the author.

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TABLES

Table 1. Primers used in the study.

D1	**Forward Primer** 3'TTGCACCAACAGTCAATGTCTTCAGGTTC'5 511 bp
D2	**Reverse primer** 5'TCAATATGCTGAAACGCGCGAGAAACCG'3
	Serotypes Specific primers
TS1	5' CGTCTCAGTGATCCGGGGG 3' 482bp
TS2	5' CGCCACAAGGCCATGAACA 3' 119bp
TS3	5' TAACATCATCATGAGACAGAGC 3' 290bp
TS4	5' CTCTGTTGTCTTAAACAAGAGA 3' 392bp

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Table 2. Diagnosis of Dengue virus infection by NS1 Elisa and real time PCR in study population.

	$NS1^+$
PCR^+	164(82%)
PCR^-	36(18%)
Total	200(100%)

Table 3. Prevalence of DENV serotypes among dengue infected patients of study population.

Gender	Total patients	DENV2 detected	DENV2 non detected
Male	120	100	21
Female	80	64	15
Total	200	164	36

Prevalence of serotypes among dengue hemorrhagic fever (DHF) patients of study Population

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Table 4. Age wise prevalence of DENV2 serotype in DF and DHF patients.

Age groups	gender	DF	DHF
≤10	Male	2	1
	Female	2	1
11-20	Male	24	6
	Female	16	4
21-30	Male	25	5
	Female	17	4
31-40	Male	15	5
	Female	10	3
41-50	Male	17	4
	Female	11	2
≥ 51	Male	17	3
	Female	8	2
Total	Male	100	24
	Female	64	16
		164	40

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FIGURES

Figure 1. Prevalence of dengue fever in various districts of study area.

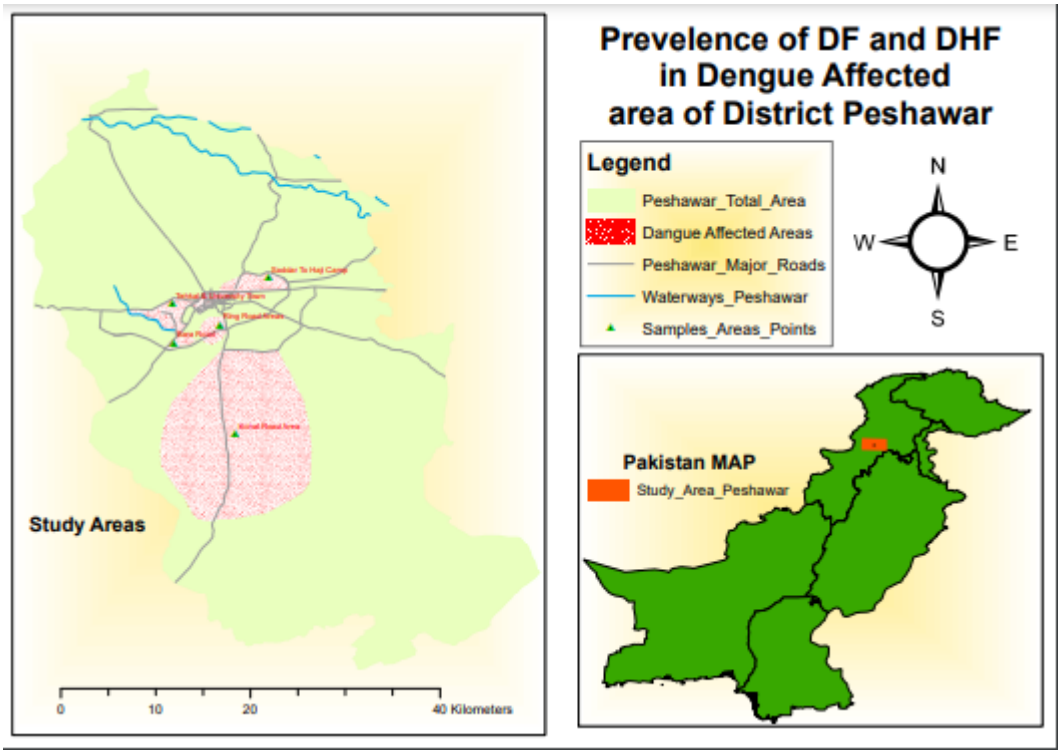

Figure 2. Prevalence of serotypes among dengue hemorrhagic fever (DHF) patients of study.

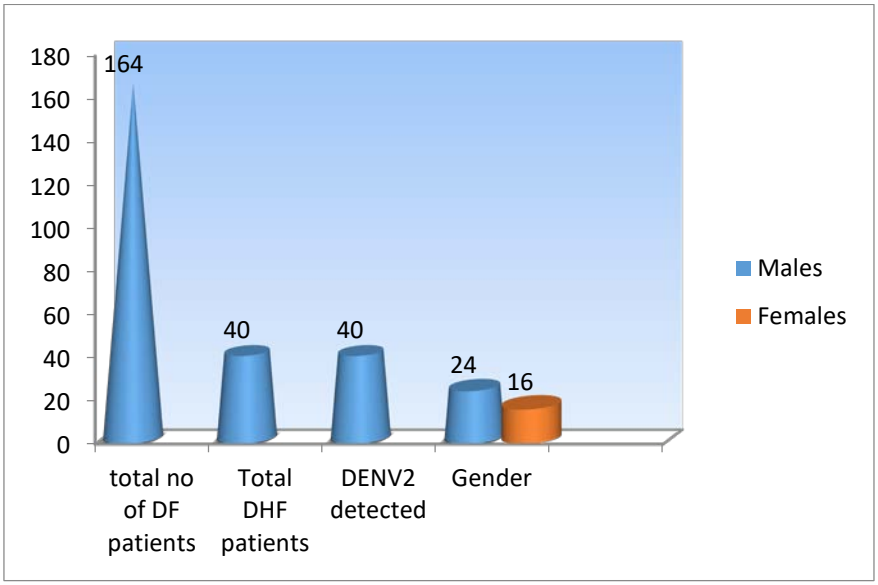

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Figure 3. Age wise prevalence of DENV2 serotype in DF and DHF patients.

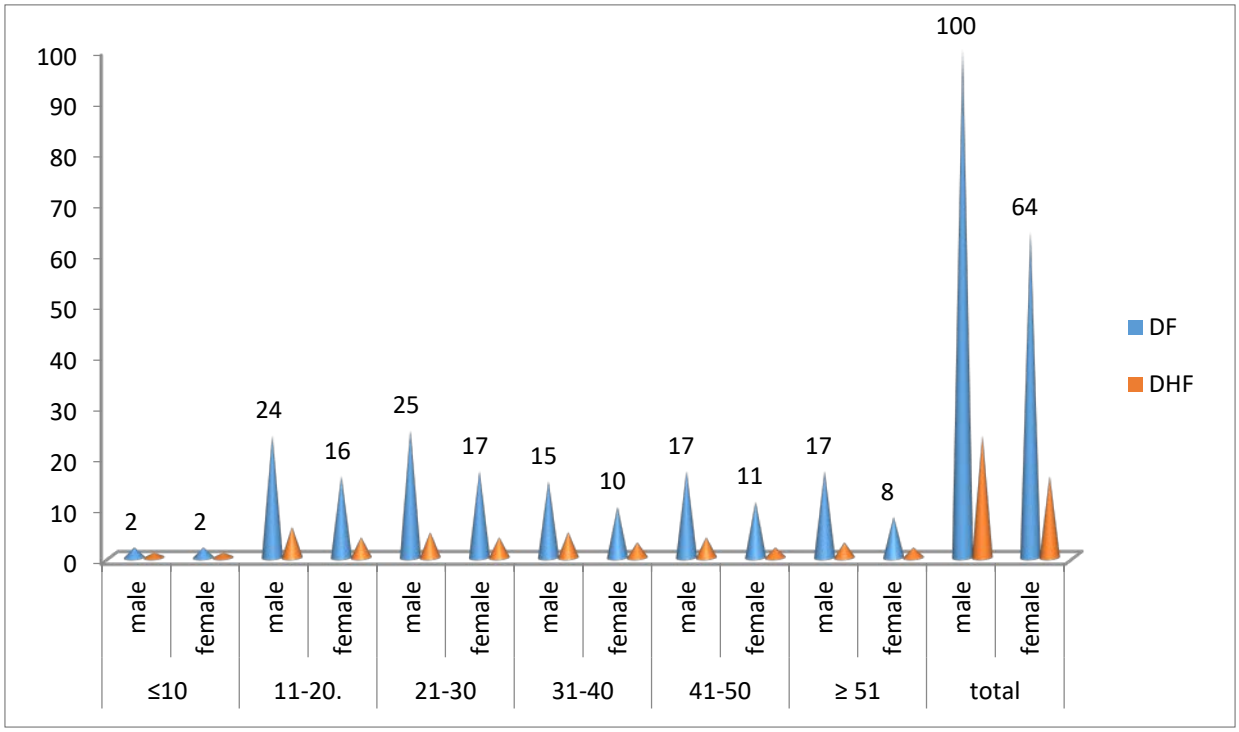

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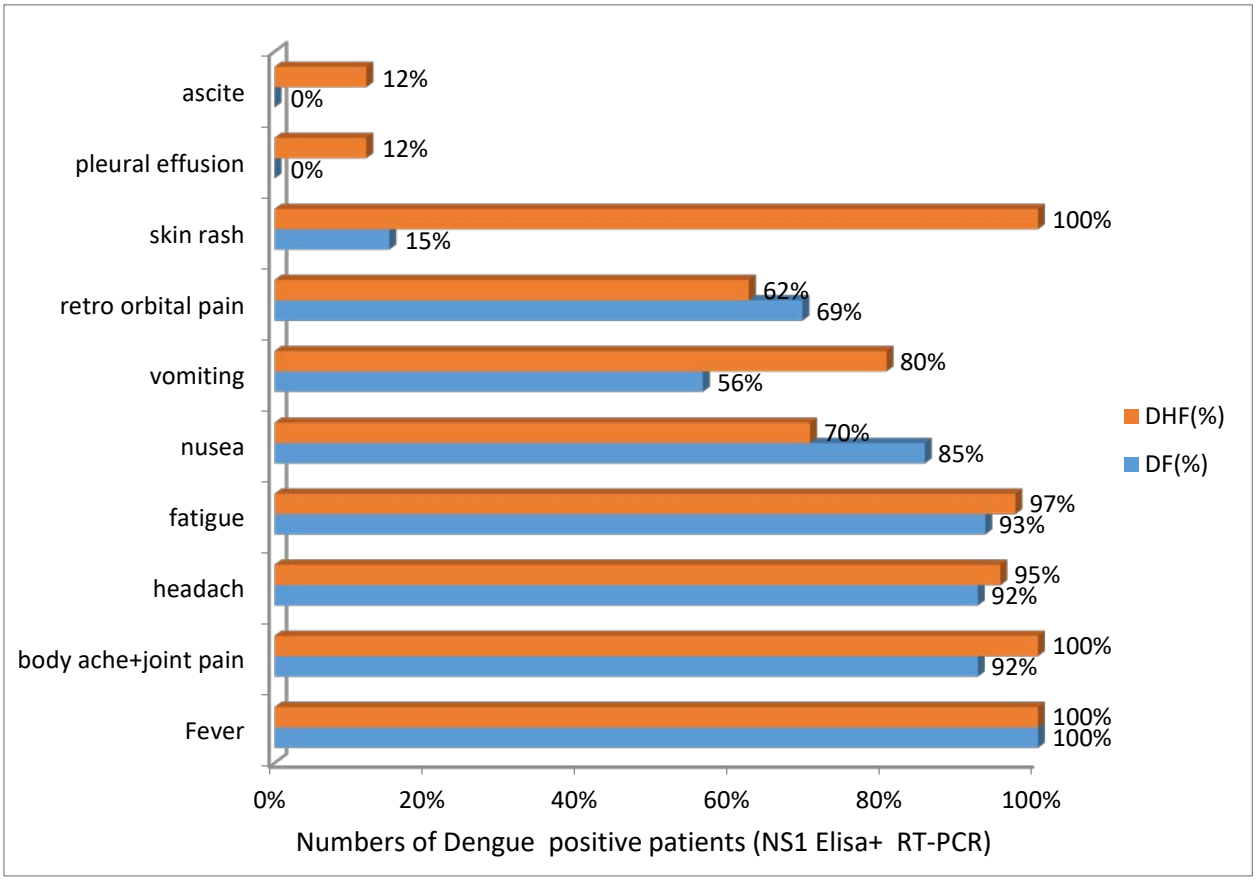
Figure 4. Clinical features of dengue infected individuals.



Notes: Dengue Positive Samples of various Localities of district Peshawar were included in this study as shown in figure.

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TITLE PAGE_METADATA

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Блок 3. Метаданные статьи

PREVALENCE OF DENGUE VIRUS SEROTYPES IN DENGUE HEMORRHAGIC FEVER PATIENTS OF PESHAWAR PAKISTAN

ВСПЫШКА СЕРОТИПОВ ВИРУСА ДЕГЕ У БОЛЬНЫХ ГЕМОРРАГИЧЕСКОЙ ЛИХОРАДКОЙ ДЕГЕ В ПЕШАВАРЕ, ПАКИСТАН

Running head:

OUT BREAK OF DENGUE VIRUS SEROTYPES

ВСПЫШКА СЕРОТИПОВ ВИРУСА ДЕНГЕ

Keywords: Dengue virus Serotypes, Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF), DENV2, RT-PCR, ELISA.

Ключевые слова: серотипы вируса денге, лихорадка денге (ЛД), геморрагическая лихорадка денге (ГЛД), DENV2, RT-PCR, ИФА.

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