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RESEARCH ARTICLE
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MOLECULAR EPIDEMIOLOGY OF DENGUE VIRUS SEROTYPES IN MALAKAND DIVISION, KHYBER PAKHTUNKHWA, PAKISTAN.

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Abstract

Background: Dengue is a widely spread mosquitoes transmitted viral infection pose a serious public health concern. The causative agent of dengue fever-dengue virus (DENV) belong to family flaviviridae consist of various four serotypes (DENV-1-4).

Aims: The current study aimed at molecular characterization, clinical features, and dengue virus serotypes in symptomatic patients.

Methodology: A total of 1382 suspects were diagnosed from April 2022 to October 2023 in Malakand Division, KP, Pakistan. The entire cases were serologically screened through NS1 (nonstructural protein-1) followed by IgM (Immunoglobulin-M) and IgG (Immunoglobulin-G) antibodies followed by molecular characterization through real-time PCR.

Results: The prevalence of dengue virus infection was 22.5% (311/1382) where male population were more infected 61.1% (191/311) as compare to female gender. Majority of the patients were from middle age group (15-30yrs) 44.7% (139/311) while rural residence population was more

vulnerable to infection 67.8%(211/311). Most of the cases reported from district swat 39.5% (123/311), whereas during the month of September the highest number of cases 43.7% (136/311) were observed. Leukocytopenia 63.7% (198/311) and decreased number of platelets counts 76.2% (237/311) were significantly correlated with the infection. Molecular characterization revealed the entire four serotypes among which DENV-3 54.1% (168/240) was the most dominating serotype followed by DENV-2 28.3% (68/240).

Conclusion: This study concluded that dengue virus consistently prevailing during the COVID-19 pandemic however majority of the cases were under reported. Majority of the cases were of DENV-3 and DENV-2 serotypes however all the serotypes are present here.

Keywords: Dengue, Outbreak, DENV, real-time PCR, RNA Virus, Pakistan.

Introduction

The dengue virus (DENV), which is largely spread by the Aedes aegypti mosquito, is one of the most significant arboviruses (arthropod-borne viruses), causing dengue infection pose a significant public health concern [1]. Dengue virus is spherical shaped with icosahedral symmetry, enveloped virus with positive sense single stranded RNA genome of 11Kb [2] with ten genes encoding seven non-structural and three proteins [3]. Four dengue virus serotypes with 65% genomic similarity have been identified based on antigenic differences: DENV-1, DENV-2, DENV-3, and DENV4. A fifth serotype, DENV-5, was recently found in Malaysia and is often found in non-human primates[4, 5]. There are several genotypes within each serotype[6], that can be identified phylogenetically using the envelope (E) gene's sequence variation [7, 8]. These serotypes can infect various target cells and provide a varying immunogenic effect, leading to an elevated cytokine response that influences the severity of the disease. Furthermore, the antibody-dependent enhancement (ADE) mechanism of secondary infection with a heterologous serotype may trigger a faster immune response than original infection[5]. Although most of the infection are asymptomatic (40-80%) however, certain serotypes can cause can cause the severe forms of the disease known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)[9, 10]. Common signs and symptoms of dengue fever include headaches, nausea, vomiting, abdominal discomfort and arthralgia. Furthermore, Gum bleeding, fever, anaemia, bloody stools, and plasma leakage that results in respiratory failure are all signs of DHF. Additional indications of DSS include low blood pressure, injury and damage to the vascular system[11].

An estimated 100 million cases of dengue fever (>80% asymptomatic/mild) are recorded annually worldwide, with highest numbers in Southeast Asia [12]. More than 7.5 million dengue cases and more than 3,000 dengue-related fatalities have been reported all over the world since the start of 2024 (DWW, 2024). The World Health Organisation (WHO) states that dengue fever was initially detected in Pakistan in 1994, and that since then, there has been an increase in the number of cases with laboratory confirmation[13]. Dengue is now endemic in Pakistan; outbreaks were documented in 2010, 2017, 2019, 2020, and 2021 [13, 14]. Dengue cases occur sporadically all year long, but during the monsoon season (July to September), the frequency peaks[15]. The dengue outbreak in 2022 occurred during the post-monsoon season (August–October), with Sindh, Punjab, and Khyber Pakhtunkhwa suffering the greatest losses [13].

Co-circulation of numerous dengue serotypes, with frequencies ranging from low (5–30%) to high (40–50%), increases the risk of co-infection [16, 17]. All four dengue serotypes are frequently found in Pakistan, and in recent years, co-circulation of multiple DENV serotypes has been documented alongside co-infection cases[18]. A significant prevalence of dengue co-infection (27%) with DENV-1, DENV-2, and DENV-3 was recorded during the 2011 dengue outbreak in Pakistan, and a similar frequency of co-infection (13.8%) with DENV-2 and DENV-3 was reported [19]. Serotype shift and co-circulation of various serotypes have been documented in previous investigations [20] may increase the risk of contracting dengue; therefore, monitoring is necessary to identify serotypes and stop future outbreaks. Henceforth, the current study was designed with the aim to investigate the various DENV serotypes circulating in Pakistan during 2022-2023.

Methodology Description of study

The Malakand division, which makes up 29,800 km²—or 40% of the Khyber-Pakhtunkhwa Province of Pakistan—is a highly populated region. Six districts were included in the study: Shangla, Dir Upper, Dir Lower, Buner, Swat, and Malakand. It shares borders with Mohmand Agencies and Bajaur in the southwest, and Afghanistan to the north and northwest. it is borders Gilgit-Baltistan in the east, which is adjacent to China's Xinjiang Province. It is connected to the heavily populated region of Khyber Pakhtunkhwa in the south, which includes the districts of Swabi, Mardan, Peshawar and Charsadda (Figure 1).

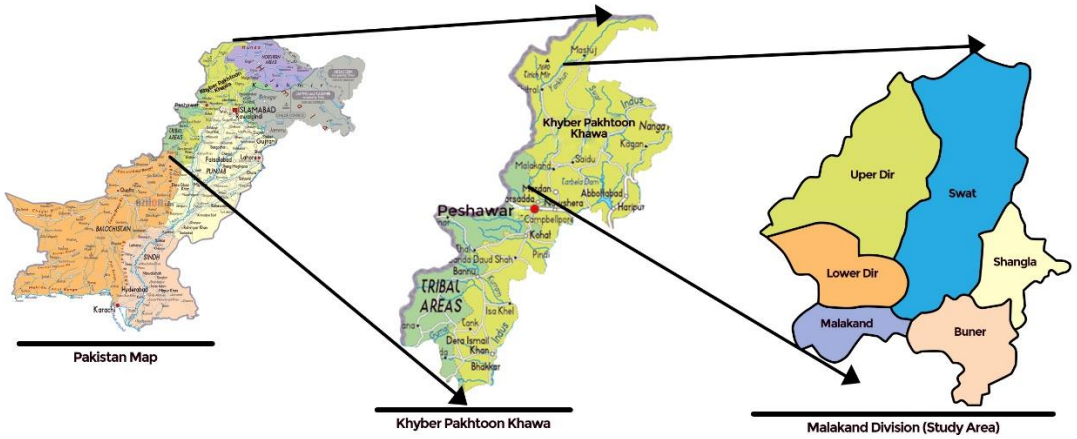

Figure 1: Map of the Studied Area

Data collection

The data of the infected patients were collected from 07 hospitals from April 2022 to November 2023. Ethical approval was taken from the Department of Biosciences, COMSATS University, Islamabad.

Sample size and testing criteria

In this study, 1382 suspected cases of dengue were investigated. Following the onset of clinical signs and symptoms such as skin rashes, body aches, nausea, vomiting, fever, bleeding nose and bleeding gums, all the patients underwent analysis. Various blood parameters including packed cell volume (PCV), neutrophils, hemoglobin, red blood cells (RBCs), white blood cells (WBCs) and platelets were examined. Testing for dengue virus infection, with serological markers such NS1, IgM, IgG, antibodies followed by presence of dengue virus RNA were performed.

Collection and Detection of NS1 Antigen

A 5ml of blood was collected from each patient in EDTA tubes and serum was obtained by sample centrifugation at 3000 rpm for 8 minutes. The serum samples were separated with early dengue NS1 detection by Enzyme-Linked Immunosorbent Assay (ELISA) (Panbio, Brisbane, Australia) as per the manufacturer's instructions[21].

Detection of Anti-DENV IgM and IgG Antibodies

Screening of anti-DENV IgG was performed through Dengue Indirect IgG ELISA (Panbio, Queensland, Australia) while Dengue-virus specific IgM antibodies were detected through immunoglobulin M (IgM) capture ELISA (Dengue Fever Virus IgM Capture ELISA Focus Diagnostics, CA, USA) as per manufacturer guidelines.

RNA Extraction and cDNA Preparation

A total of 140 µL of serum sample was used for the extraction of Viral RNA by using QIA viral Mini kit (Qiagen, Germany) according to manufacturer's instructions. By using Moloney Murine

Leukemia Virus (M-MLV) Reverse Transcriptase 4µg of total RNA was reverse transcribed into cDNA (New England Bio labs (UK) Ltd) through RT-PCR.

Molecular Characterization of Dengue Serotypes

Through nested (Two-step) PCR qualitative detection and serotyping of DENV was done. Dengue primers were used to amplify the genome of virus (cDNA) as reported in the previous study by [22]. In first round universal primers D1 and D2 were used for amplification the cDNA to confirm the presence of DENV. In second round of two-step PCR, the four-type specific (TS) primers were used in place of reverse (D2) primer of round first. Each serotype was amplified by using 2ul PCR product of the round first as template. Same thermocycler conditions were used for each round except annealing temperature which was specific for each primer (Table 1).

Table 1: Primers used for serotyping of dengue virus

Sr. No	Primer name	Primer sequence	Band size
1	**Dengue**	**F 3'** TCAATATGCTGAAACGCGCGAGAAACCG-5' **R 3'**TTGCACCAACAGTCAATGTCTTCAGGTTC-5'	**511**
2	**DENV 1 (TS1)**	CGTCTCAGTGATCCGGGGG	**482**
3	**DENV 2 (TS2)**	CGCCACAAGGGCCATGAACAG	**119**
4	**DENV 3 (TS3)**	TAACATCATCATGAGACAGAGC	**290**
5	**DENV 4 (TS4)**	CTCTGTTGTCTTAAACAAGAGA	**392**

Statistical Analysis

IBM-SPSS version 20 was used to perform statistical analysis on the data. The monovariate analysis was used to describe the study sample. The Student's t-test was used to examine the differences between mean peripheral blood parameters in the acute and critical stages.

Results

Prevalence of Dengue Infection

A total of 22.5% (311/1382) patients were found positive on NS1 among which 17.3% (240/1382) were molecularly confirmed through PCR as shown in the Table 2.

Table 2: Prevalence of Dengue Virus Infection

Diagnostics Assay	Positive/Total	Percentage
NS1	311/1382	22.5%
IgM	187/311	60.12%
IgG	124/311	39.87%
RT-PCR	240/311	17.3%
Total	311/1382	22.5%

District Wise Prevalence of Dengue Virus Infection

The District wise prevalence of dengue infection was higher in District Swat (29.5%) followed by District Shangla and Malakand (24.3% and 21.7%) respectively as shown in the figure 2.

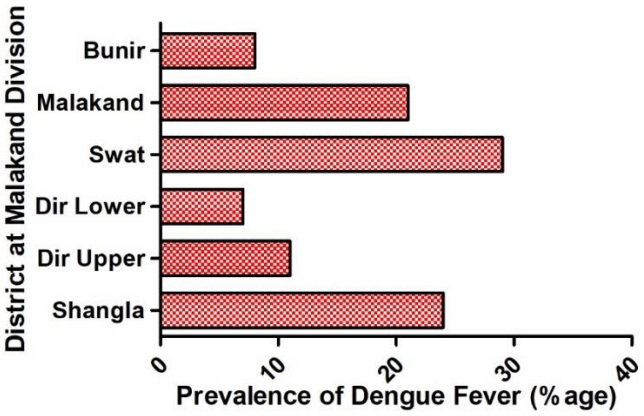

Figure 2: District Wise Prevalence of Dengue Virus Infection

Area-wise distribution of dengue virus infection

The current study observed that majority of the patients were from rural residence (58.7%) as compared to urban area as shown in the Table 4.

Table No 4: Area-wise distribution of dengue virus infection

Suspected cases	Confirmed cases	Negative cases	Male patients	Female patients
1382	311 (22.5%)	1071 (77.5%)	191 (61.2%)	120 (38.6%)
Areas	Rural areas	211 (67.8%)	Urban areas	100 (32.1%)

Age and Gender Wise distribution of Dengue infection

Dengue virus infection was higher in male population (61.3%) as compared to female population while the age wise prevalence of dengue virus infection showed that 15-30 years of the population were more infected (44.7%) followed by age group 30-45 (20.3%) as compare to other age groups as shown in the Table 5.

Table No 5: Age and Gender Wise distribution of Dengue infection

Variable	**Groups**	**Frequency**	**t-value**	**P-value**	**95% Confidence Interval**		**Chi-square-X^2**
Age	1-15 Years	37/311 (11.9%)	-11.613	.001	-.382	-.271	0.538
	15-30 Years	139/311 (44.7%)					
	30-45 Years	63/311 (20.3%)					
	45-60 years	51/311 (16.4%)					
	>60 years	21/311 (6.8%)					
Gender	Male	191/311 (61.2%)	.208	.835	-.139	.171	
	Female	120/311 (38.6%)					

Clinical Characteristics of Dengue Virus Infected Patients

Evaluation of clinical parameters observed that fever was the most consistently reported (100%) followed by body ache (94.2%) and nausea and vomiting (83%) as shown in the Table 6.

Table 6: Clinical Characteristics of Dengue Virus Infected Patients

Characteristics	Infected/Total (%)	t-value	P-value	95% CI	Chi-square-X^2
Fever	311/311 (100%)	3.221	.475	.76---1.17	4.31
Vomiting/nausea	217/311	-.261	.001	-.89---2.12	2.18
Body aches	287/311	4.789	.002	-.96---1.72	3.11
Skin rashes	104/311	7.123	.001	.96----3.45	.127
Nose and/or gum bleeding	39/311	-.324	.192	.89----4.11	1.02
Enlarged liver	189/311	-2.451	0.08	-.91---1.64	.188

Monthly distribution of dengue fever

Monthly wise distribution of dengue virus infection showed that most of the cases were reported in September 136/311(43.7%) followed by August 111/311(35.6%) and October 47/311(15.1%), July 07/311(2.3%), June 06/311 (2.3%), May 02/311, April 01/311 and November 01/311 as shown in the figure 3.

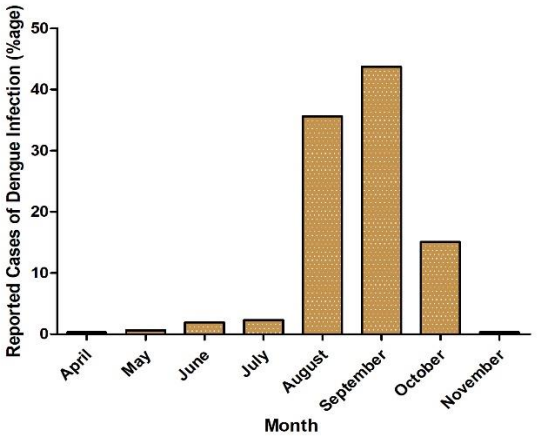

Figure 3: Monthly distribution of dengue fever

Hematological Analysis of Dengue Infected Patients

In the current study, the entire patients were divided in DF (Dengue Fever) and DHF (Dengue Hemorrhagic Fever). Thrombocytopenia (platelet<69,000 cells/mm^3), Lymphocytosis and Neutrocytosis were significantly correlated (P<0.05) with dengue virus infection as shown in the Table 7.

Table 7: Hematological Analysis of Dengue Infected Patients

Characteristic	Fever Stage	T-value	P-value	95% Confidence Interval	R-square
					0.226
Leukocytosis	DF = 223	-.716	.475	-.249----.117	
	DHF =88				
Thrombocytopenia	DF = 223	-.637	.001	-.225----.116	
	DHF =88				
Lymphocytosis	DF = 223	5.667	.002	.567----1.175	
	DHF =88				
Neutrocytosis	DF = 223	5.834	.001	.553----1.122	
	DHF =88				
Erythrocytes	DF = 223	-1.311	.192	-.254----.052	
	DHF =88				
Hemoglobin	DF = 223	6.113	0.08	-.225----.116	
	DHF =88				
PCV	DF = 223	4.761	0.06	.567----1.175	
	DHF =88				

Molecular Characterization of Dengue Virus Serotypes

Molecular characterization of Dengue virus serotypes showed that DENV-3 was more prevalent 168/311 (54.1%) followed by DENV-2 68/240 (28.3%), DENV-1 70/311 (22.5%) and DENV-4 05/240 (2%) as shown in the Figure 4.

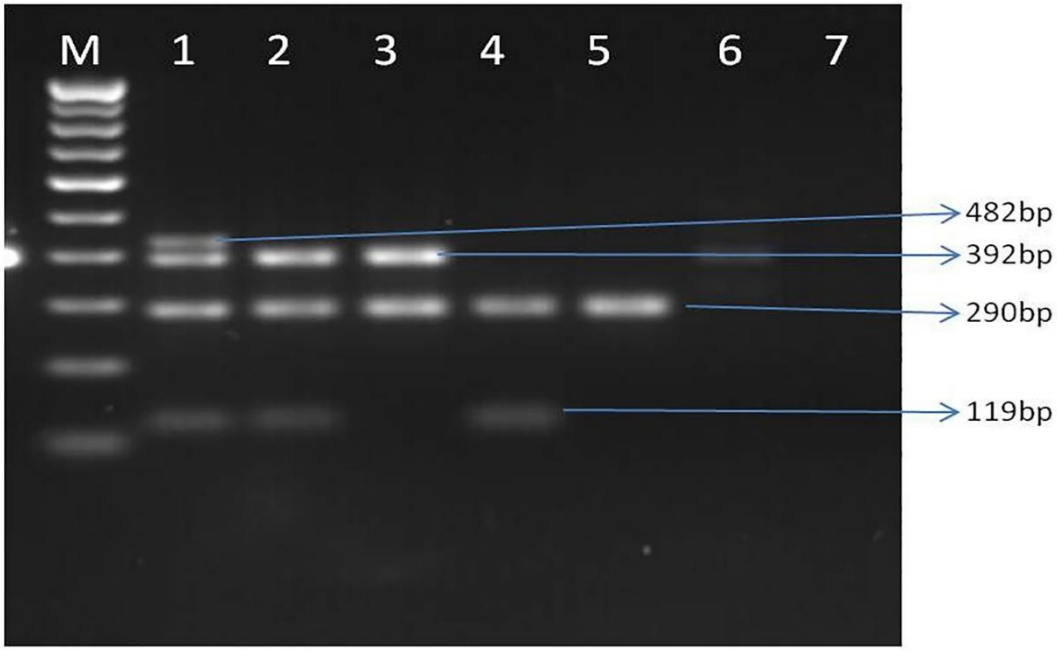

Figure 4: Gel Electrophoresis of Dengue Virus Serotypes: Dengue Virus Serotype I= 482bp: Dengue Virus Serotype II= 119bp: Dengue Virus Serotype III=290bp: Dengue Virus Serotype IV= 392bp

Discussion

Dengue infection is one of the most common viral infection that is reemerging and emerging which kills millions of people worldwide each year [23]. It is thought that tainted dengue vector eggs on tyres in Karachi's harbour may have carried the dengue virus into Pakistan where first case of dengue has been documented during 1994 [20, 24]. Soon after in 1995, the dengue virus infection burden subsequently expanded to the province of Baluchistan [25]. While dengue is a risk in over 128 countries, Asia accounts for around 70% of reported cases [26, 27]. Over the last decade, there have been multiple epidemics throughout the nation that have killed portions of thousands of people and infected thousands more [28-32]. Similarly, the current study also observed the prevalence of dengue infection in Malakand division of Khyber Pakhtunkhwa, Pakistan. The hot, humid summers in Pakistan encourage the spread of Aedes species, which are known to be vectors for the dengue virus [33]. In 2011, a severe epidemic killed almost fifty thousand people in Punjab, a populous province bordering KP. The WHO's data indicates that in 2013, there was a significant dengue infection outbreak in the district swat in the province of Khyber Pakhtunkhwa, with 33 fatalities and an estimated 8546 cases in the region.

The current study observed that male was more infected as compare to female population. Similarly, the majority of the male patients were reported somewhere else [34-37] while in contrast to these reports more female targets than male targets among the affected patients [38] were also reported. Environmental, cultural and Social factors may have led to the phenomenon of gender specificity in respect to dengue infection may have resulted from many sources. This may be the result of amenia and a lack of concern for women's health issues [39]. Evaluating the age-related differences between dengue in males and females is crucial since both gender and biological-related factors can alter throughout the course of a person's life and vary between nations. The cause(s) of these sex-specific disparities must be determined through additional research in order to target preventive actions and lower the dengue burden in the area [40]. The current study observed that most of the dengue

positive patients were from middle age group (15-30 years) as similarly reported by [34, 41]. Middle-aged persons may have a higher prevalence because they are more exposed to the environment, which increases their likelihood of coming into contact with the vector. Additionally, exposure to various situations while travelling for work or other reasons may raise the chance of infection. In comparison, the other age groups—which primarily consist of children and elderly people—have very little exposure to the outdoors [34].

The most widely observed symptoms among the studied population were fever, enlarged liver and inner bleeding as reported previously [34]. The direct effect of the virus or host immunity on liver cells may be the cause of the liver wounds in dengue fever, which may then become a factor in vascular leakage within the liver [30, 42]. The World Health Organisation states that the clinical presentation that confirms the sign and symptoms may be a crucial component in diagnosing dengue virus infection [22, 43].

This study reported that rural populations were more infected as compare to urban population. Likewise reports have been observed in previous literature [34, 44]. When dengue spread to new locations and from urban to rural areas, the frequency of outbreaks increased thirty times [20]. Furthermore, this study also observed that most of the cases were reported in the month of september followed by august as mentioned in earlier study [34]. The reason for this could be that the post-monsoon season is ideal for mosquito growth, which raises the risk of illnesses carried by vectors. Furthermore, the Malakand Division is mostly comprised of rural areas. One of the main causes of dengue mosquito development and exodus during rainy days in the area may also be the muddy puddles and stagnant water. In comparison to the monsoon season, the post-monsoon season has a higher rate of dengue virus infection, as previously reported[45].

Blood parameter of the patients studied showed thrombocytopenia during secondary dengue infection (observed IgG) which is in consistence to previous study [34]. Similarly, low platelets counts were also observed in most of the cases as mentioned earlier [34, 46]. In some of the dengue virus infected individuals the increased peripheral blood parameter was observed which is similar to the existing published literature [34, 47]. Non-significant association of hemoglobin was observed in dengue patients as already reported in the existing reports [34, 48]. Leukopenia was also associated with dengue infection (<500 cells/mm^3) while neutrophil and WBCs were not associated with dengue infection likewise reported earlier [34, 49].

The current study observed that all serotypes were present in dengue patients from Malakand division. Where other reports also mentioned that the most common serotypes were DENV-1, DENV-2 and DENV-3 [42, 44, 50, 51]. The previous outbreak of dengue virus (2011 & 2013) the entire four serotypes has been reported from Khyber Pakhtunkhwa and Punjab with DENV-2 and DENV-3 were dominating serotypes[31]. The serotypes identification on time could help in management and triaging of the patients, prompt clinical intervention and suitable epidemiological monitoring [52]. Lack of labs with capabilities of serotyping is one of Pakistan's biggest problems. The National Institutes of Health currently acts as the nation's central laboratory, accepting samples from all over the country. Strengthening provincial public health reference labs to conduct real-time nationwide surveillance of dengue serotypes will be crucial in responding to future outbreaks [15].

Conclusion

This study concluded that dengue virus consistently prevailing during the COVID-19 pandemic however majority of the cases were under reported. Majority of the cases were of DENV-3 and DENV-2 serotypes however all the serotypes are present here.

Conflict of interest

The authors declare that there is no conflict of interest.

Funding

No funding was received for this study.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

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