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Mutational pattern in Capsid Protein (C) of Dengue Virus isolates from Punjab Province of Pakistan

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ABSTRACT

BACKGROUND & OBJECTIVE: Dengue virus (DENV) genomic study is crucial for understanding how it spreads and presents and becomes dangerous. Three structural and seven non-structural proteins are encoded by the positive-stranded RNA DENV. Capsid protein (C) is one of the structural proteins which helps in the encapsidation of viral RNA. The objective of the study is to identify the DENV serotypes and the most common geographic specific mutations in the C protein circulating in the Punjab province of Pakistan.

METHODOLOGY: It was a cross-sectional study in which about 120 DENV isolates were selected by the procedure of temporal sampling done in the peak season of dengue fever in 2022 from the major tertiary care hospitals for whole genomes sequence analysis. Among these only 23 whole genome sequences were performed after virus isolation, quantification, and cDNA synthesis.

RESULTS: All the samples were found to be of genotypes 1 and 2, in which 44 non-synonymous mutations were detected in the C protein. The most common mutations were; N90S (n=11) and G70S (n=11) followed by V26G (n=5) and R10Q (n=4), in which G70S is novel.

CONCLUSION: Mutations in the current study are of particular interest to the design of DENV vaccine projects in the future. Genomic epidemiology during each outbreak of DENV in different locations is critical for better public health and for designing new policies for future outbreaks.

KEYWORDS: DENV, Genome, Mutations, Pakistan, Structural proteins, Capsid protein

INTRODUCTION

Dengue is an imperative and recurrent viral disease of tropical origins in humans. It is caused by dengue virus (DENV) infection, which manifests itself after 3 to 10 days of an infected female mosquito bite[1] of the Aedes genus, mainly the A. aegypti and A. albopictus. The frequency of dengue has increased exponentially throughout the globe in recent decades. Three hundred ninety million individuals worldwide contract dengue each year [2].

Pakistan has been experiencing an epidemic of dengue fever every year. Khyber Pakhtunkhwa, Punjab, and Sindh are this endemic's three major burden holders. Dengue fever cases were detected to be 22,938 in 2017, more than 3,200 in 2018, 24,547 in 2019, and 3,442 in 2020, according to the National Institute of Health (NIH) in Islamabad [3] . Punjab is the largest province of Pakistan by population-based and second largest by area, located in the central-eastern region of the country. Molecular characterization of DENV local isolates during outbreaks for genomic surveillance and mutation frequency may be useful for better management of the disease in the future.

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DENV is a single-stranded RNA virus of the genus Flavivirus, and it belongs to the family of viruses called Flaviviridae [4]. There are four serotypes of DENV called DEN-1, DEN-2, DEN-3 & DEN-4[5]. Over the years, all four serotypes have been confirmed to be circulating in Pakistan among outbreaks, but overall, serotypes 2 and 3 have been the most prevalent [6].

A mature DENV displays a diameter of approx. 50 nm. The entire genome of DENV is made up of 11000 positive sense bases arranged to form single-stranded RNA (ssRNA) translated into a single polyprotein. This polyprotein is then cleaved by proteases into structural (capsid C, pre-membrane/membrane-prM/M, and envelope E proteins) and non-structural (NS1-NS2ANS2B-NS3-NS4A-NS4B-NS5) proteins [7].

Capsid protein (C) of dengue virus (DENV) has a basic nature with a molecular weight of 12kDa and forms dimers in its structural conformation after getting antiparallel attachment of one monomer to the next one [8]. One monomer of C protein can be of 100 amino acid residues in which 26 amino acids will be basic while 3 will be acidic in nature [9]. Each monomer is made up of 4 helices noted as α1, α2, α3, and α4 and among all these 4, α4 is the longest helix [10]. The N-terminal domain of the monomer has greater density toward positive charges as positively charged amino acids like 8 lysine and arginine are found in the first 22 amino acids at the N-terminal side. The first 3 helices are the main components of the monomer core and form a right-handed structure of the monomer [11]. As C protein forms dimers, these dimers are formed when α2 and α4 helices of one monomer join antiparallel with the α2 and α4 of the next monomer. This dimers formation is required for protein stability and to form mature infectious particles [12,13]. There are some hydrophobic regions in the C protein which is where α2 interact with other α2 through which C remains attached to the endoplasmic reticulum (ER) membrane, which is conserver sequence in flaviviruses. The basic amino acid-rich part of C protein help to remain integrated with viral RNA, and this part is where α4 bind to another α4 helix [9].

C protein is the first encoded protein by viral RNA, after which prM protein will be encoded. These two proteins are attached to each other by a signal peptide which is hydrophobic in nature and this signal is responsible for the translocation of membrane protein (prM) into the ER lumen [14]. The main function of capsid is to protect viral RNA via the formation of nucleocapsids around the viral RNA, which is the main step in DENV assembly [15]. Mutation in dimer formation may lead to impaired protein formation and effects protein stability. Therefore, it is of prime importance while studying the genome of DENV to ascertain if there are any nonsynonymous mutations affecting these sites in the structure of C protein. This will help future researchers to study the effect of such mutations on the function of envelope protein, which will help in the development of vaccines and pharmaceutical measures to tackle this epidemic.

METHODOLOGY

It was a cross-sectional study, and a temporal sampling technique was used. This research was conducted in IMBB/UOL and major tertiary care hospitals of Lahore, Sargodha, Sialkot, and Faisalabad, Punjab, Pakistan, in the peak season of the dengue outbreak of the year 2022. Ethical approval for the current study was taken from the departmental Bioethical, Biosafety and Biosecurity under the reference number, Ref_CRiMM/22/Research/143. Informed written consent was taken from each of the DENV patient along with sociodemographic data. Confirmed patients of dengue infection with up to 7 days from onset of symptoms like fever, retro-orbital pain, headache, body pains, haemorrhagic manifestations with low platelet count (<100,000/UL) and low white blood cell count (<4000/UL) were enrolled in the study. They were confirmed of dengue infection by either a positive polymerase chain reaction (PCR) test for DENV or non-structural protein 1 (NS1) antigen positive or showed positive immunoglobulin M (IgM) antibodies for DENV. They were of both genders and were of more than 13 years in age. Patients suffering from comorbidities like hepatitis, chronic liver disease, typhoid fever, or malaria were excluded from the study. Patients with dengue shock syndrome (DSS) were also excluded. Written consent was taken from these patients. Detailed clinical history, findings of clinical examination, and laboratory and other diagnostic test results were recorded on a pre-tested performa.

About 4ml of blood was collected from the confirmed dengue patients. This blood sample was then centrifuged for 10 minutes at 4000 RPM. The serum separated was then put into Eppendorf tubes, labeled, and stored at minus 80 degrees. About 120 samples were selected from different areas of Punjab. The DENV RNA was extracted and purified directly from the serum of DENV-confirmed patients using GeneJET viral DNA/RNA purification kit (Cat no: K0821) following the manufacturer's protocol. A cDNA copy of the isolated RNA was synthesized using Revert Aid First Strand cDNA Synthesis kit (Cat no: K1622).

The RevertAid cDNA (Thermo Scientific) Synthesis Kit is efficient for to cDNA first strand up to 13 kb using templates of virus RNA. The kit maintains activity at 42-50°C at, effectively protecting RNA templates from degradation. Oligonucleotide primers will be prepared using the previously available information of the alignment of full-length DENV. The cDNA produced was amplified using PCR with Ampliseq Custom Panel having 2 pools each of 92 primer pairs and 196 amplicons. Quantification by gel electrophoresis using a size standard of 100bp and Qubit Assay Qubit dsDNA HS assay kit (Cat no: Q32851). The gel purification step discarded the excess products. The quantified and standardized samples will be used to prepare the library for sequencing. Out of 120 samples, 23 were selected to perform whole gene sequencing based on their quantification and gel purification results.

The equalized library is loaded on Ion Chef System for automated template preparation and chip loading. We used Ion 510 chip for the DENV WGS sequencing. The prepared chip was then loaded on Ion XL 5 sequencer for sequencing, and data was uploaded on Torrent Suite Server 4.10. Phylogenetic tree of current isolates was generated with a comparison with previous infections using bioinformatic approaches. MAFT and MEGA software was used for evolutionary analysis. Simple statistics was performed using WHO developed software package, EpiData Analysis for mutations frequencies calculation and summary [16]. The data was analyzed in excel sheet to remove any errors.

RESULTS

A total of 120 serum samples were put through Viral RNA extraction, PCR quantification, and gel purification steps, after which only 23 samples were found suitable to be sequenced. Finally, out of these 23 samples, 4 samples failed to be sequenced fully, and 19 showed numerous mutations in DENV structural and non-structural proteins.

Among the sign and symptoms frequency and percentage of high-grade fever are very high (97%), followed by body aches (74%), vomiting (40%), fatigue (34%), abdominal pain (31.5%), bleeding (21%) and pleural effusion (15%). Besides the low frequency and percentages of symptoms diarrhoea, ascites, cough, oral bleeding, bleeding from the nose, vagina, vomit, stool, and urine were also observed (Figure-I).

Among the 19 samples Sequenced Sample 13, 16, 18, 26, 27, and 30 were genotype 2, and the remaining were genotype 1.

There were a total of 44 non-synonymous mutations of C protein. The highest number of common mutations in C protein has been found in sample 20 (S20) (n=6) followed by samples S14 and S15. The highest frequency was detected in substitution G70S and N90S (n=11) followed by V26G (n=5), R10Q (n=4), and K9R (n=3). To the best of our knowledge, mutation G70S, V26G, R10Q, K9R is a novel mutation reported in this study (Table-I).

The α4 helix of C protein is made up of residue 74 to 96, and plays a very important role in its function. Several mutations like K74N, N75D, S75P, I78S, G83V, M95I, M95L, M95R,

N96K and N96T with the least mutation frequency (n=1) were observed (Table-II) in this region.

Phylogenetic analysis:

Phylogenetic analysis of all samples was performed to observe the relationship among genomic isolates collected from different cities. All the samples were found as genotype 1 and 2. From an evolutionary point of view, sample 13 (S13) is at more distance from all the samples that originated from the root node and represent a separate branch (Figure 2). Samples 10 and 30 are more closely related as they originate from the same node. Samples 15, 16, 18, 26, and 27 make a separate cluster of closely related isolates. The third at the bottom is the largest cluster forming a group of 11 isolates (Samples 11, 12, 14, 17, 19, 20, 21, 23, and 24) that are closely related and originated from the third internode in the three (figure-III).

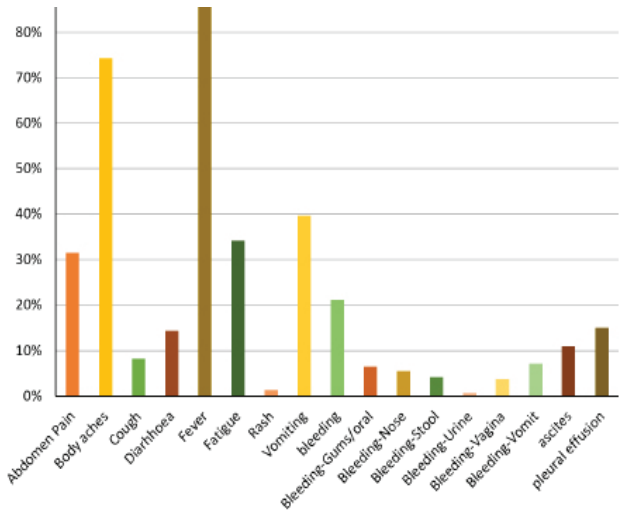

Figure-I: Distribution of signs and symptoms in dengue fever patients.

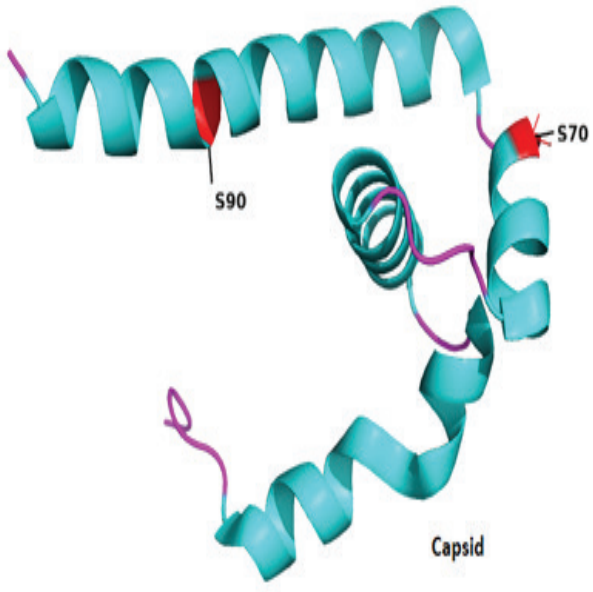

Figure-II: Capsid structure and location of most common mutations. Mutations N90S and G70S have been highlighted in red. Both mutations are present in the helix region.

Table-I: Frequency of most common non-synonymous mutations in Capsid protein.

MUT	S 10	S 11	S 12	S 14	S 15	S 16	S 17	S 18	S 19	S 20	S 21	S 22	S 23	S 26	S 27	FREQ
G70S	P	P	P	P	P	-	P	-	P	P	P	P	P	-	-	11
N90S	P	P	P	P	P	-	P	-	P	P	P	P	P	-	-	11
V26G	-	-	P	P	P	-	-	-	-	P	-	-	-	-	P	5
R10Q	-	-	-	P	P	-	-	-	-	P	-	-	-	-	P	4
K9R	-	-	-	-	-	P	-	P	-		-	-	-	P	-	3
L46M	-	-	-	P	P	-	-	-	-	P	-	-	-	-	-	3
I59T	-	-	-	-	-	P	-	-	-	P	-	-	-	-	-	2

***MUT: Mutation, S: sample, Freq: Frequency, P: present**

Table-II: Frequency of least common non-synonymous mutations in Capsid protein.

MUT	S 10	S 11	S 12	-S 14	S 15	-S 16	S 17	S 18	S 19	S 20	S 21	S 22	S 23	S 26	S 27	FREQ
N3T	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
Q4K	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
T8A	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
L16P	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	1
A19E	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
N21S	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
V23M	-	-	-	-	-	-	-		-	P	-	-	-	-	-	1
G36D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	1
L37C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	1
L38S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	1
S39Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P	1
K45E	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
L57P	-	-	-	-	-	-	-	-	-	-	-	-	-	P	-	1
T58A	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	1
T58K	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	1
T58P	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	1
I59F	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	1
I59N	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	1
A67D	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	1
R68G	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	1
K73T	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
K74N	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	1
N75D	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	1
S75P	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	1
I78S	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	1
G83V	-	-	-	-	-	-	-	-	-	-	P	-	-	-	-	1
M95I	-	-	-	-	-		-	-	-	P	-	-	-	-	-	1
M95L	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
M95R	-	-	-	-	-	--	-	-	-	P	-	-	-	-	-	1
N96K	-	-	-	-	-		-	-	-	P	-	-	-	-	-	1
N96T	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
R97G	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
R98G	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
R98K	-	-	-	-	-	-	-	-	-	P	-	-	-	-	-	1
K99R	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
R100G	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
R100K	-	-	-	-	-	-	-	P	-	-	-	-	-	-	-	1

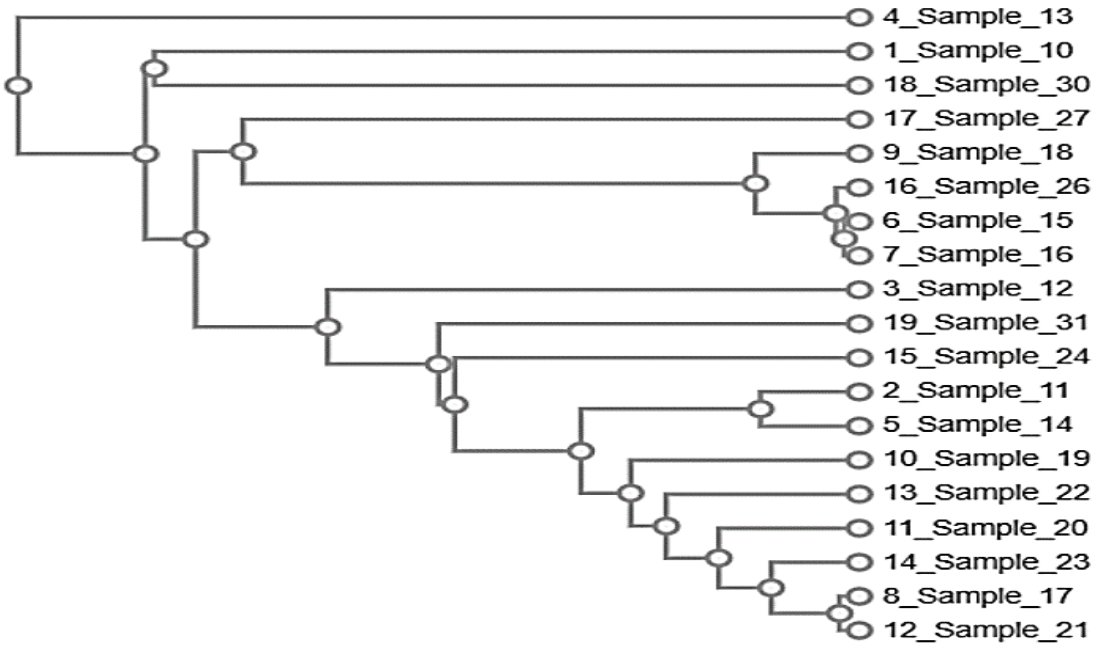

Figure-III: Phylogenetic analysis of DENV genome from Punjab province of Pakistan.

DISCUSSION

According to the WHO report 2022, in Pakistan, a total of 25, 932 confirmed cases and 62 deaths have been reported, among which 74% occurred in the month of September alone [17]. The current increase may be due to the flooding in June 2022. With the current flood, a high-risk health impacts are expected from DENV fever. There is an urgent need of improved vector surveillance, laboratory capacity for better management and cases detection, and warning signs for sensitization may reduce the DENV disease burden seasonality incidence Punjab is the largest province of Pakistan by population-based and second largest by area, located in central-eastern region of the country [18]. Molecular characterization of DENV local isolates in Punjab during outbreaks for genomic surveillance and mutations frequency may be useful for better management of the disease in future. Insight into the proteomic of DENV will further increase our understanding and pattern of mutations involved in high incidence and disease severity. The biological significance of protein information in DENV infection, and consequently in DENV pathogenesis, has not yet been fully understood despite recent technological developments.

In the current project, 23 whole genome sequencing was performed on clinical samples among which four were not completely sequenced, and 19 showed numerous mutations. The DENV C protein showed 44 non-synonymous mutations. The highest frequency was detected in substitution G70S and N90S (n=11) followed by V26G (n=5) and R10Q (n=4). N90S has been reported in previous study [19] while G70S is novel mutation in our current study. However, the effect of these mutations on C binding affinity has not been investigated. Experimental work on the most common mutations in C protein may be conducted for better understanding of the virus pathogenicity. Some other mutations that have been detected in genomes were V26G (n=5) R10Q (n=4), K9R and L46M (n=3) (Table-I). Among these mutations, L46M was reported in the Indian isolates [20], while V26G (n=5) R10Q (n=4), K9R are novel in our study.

In a previous study [21]; mutations at residues 81 and 88 in the 4th helix of capsid protein (C) may affect the virus pathogenicity and disrupting viral assembly [22]. DENV2 C protein and its single-point mutations (Lys50Ser, Lys54Ser, Lys81Arg, and Ile88Arg) have been evaluated for structural stability and oligomeric states. The study also observed how these substitutions affected the DENV RNA-capsid interaction. These findings emphasize the relevance of the DENV2 C protein's dimer interfaces including α2-α2′ and α4-α4′; mutations in these region of protein secondary structures can impair the structure stability of protein's and diminish RNA-C protein binding affinity.

The secondary structure of the DENV2 C protein is affected by mutations at the α2-α2′ and α4-α4′ dimer interfaces. The wild-type DENV2C and its single-point mutations L50S, L54S, L81N, and I88N were produced and purified in large quantities. The proteins were appropriately folded, as evidenced by Circular dichroism (CD) and tryptophan

fluorescence spectroscopies of their secondary and tertiary structures, respectively. Single-point mutations in the DENV2C protein were found in the 2 helix (L50S and L54S), reproducing mutants previously published by Anoop et al,[21] and the 4 helix (L81N and I88N), replicating mutants previously described by Samsa et al [22]. Another study, discovered that the DENV2C protein accumulates at the boundaries of lipid droplets in the cytoplasm of DENV-infected cells and that the number of lipid droplets per cell increases during DENV infection; these findings suggest a link between viral replication and lipid-droplet metabolism. Additionally, drug therapy targeting the lipid droplet decreased viral replication. Assembling viral particles is hampered by DENV2C mutations at the L50 and L54 residues, which have been characterized as interfering with capsid integration into lipid droplets.

Our study showed mutations like I78S, M95I, M95L, and M95R. In a previous study, using a homology model based on the DENV2C structure [22], Patkar et al, showed that viral assembly was hampered by mutations at acids 81 and 88. The single-point mutations at position Lys78Arg, Met92Arg, and Lys95Arg had only little effect on the activity of C protein. The primary portion of the dimerization surface, which is made up of α2- α2′ and α4- α4′, includes the α2 and α4 helices, two antiparallel alpha helices. The DENV2C protein has an internal hydrophobic area, is the region of interaction with host membranes. The regions that interface include α2- α2′ is a main component of this interfacing region. The side chains of the residues I78, L81, I88, L92, and L95 on one monomer engage hydrophobically with those on the antiparallel monomer to maintain the structure of α4- α 4′. The α4– α4′ region has many basic residues, and it has been suggested that this region interacts with RNA[23-24].

CONCLUSION

Molecular characterization of the DENV whole genome using sequencing approaches demonstrates a good picture of virus variability in terms of mutations emerging in the different targets of DENV genotypes. In the current study, we found that genotype 1 and genotype 2 are the most prevalent isolated in Pakistan. These genotypes were harboring numerous mutations in C protein of DENV. Mutations can have effect on virus severity, entry into a host cell and replication capability may be experimentally confirmed. Diagnostics approaches and markers can be designed based on this genomic variability and can lead to better management of DENV fever in the future.

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CONFLICT OF INTEREST: No known conflict of interest.

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The data that support the findings of this study are available on request from the corresponding author.

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Authors' Contribution:

Saira Mushtaq: Article writing, data collection, methodology, result compilation.
Muhammad Tahir Khan: Supervision and provision of resources during data collection.
Malik Ihsan Ullah Khan: Supervision, the main conceptualization of the project, defining aims and objectives.
Maira Mahmood: Support in providing resources and supervision.
Farhat Humayun: Questionnaire built-up.
Aneeqa Shahid: Statistical analysis.

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