Genomic Variations in the Dengue Virus Non-Structural Protein 4A

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Abstract

Objective: To identify the most common geographic specific mutations in the Dengue Virus Non-Structural Protein 4A circulating in Faisalabad, Pakistan.

Material and Methods: This research was conducted at Institute of Molecular Biology and Biotechnology, The University of Lahore, Aziz Fatimah Hospital and Allied Hospital Faisalabad during the dengue outbreak of 2022. About 120 DENV isolates were selected from the laboratories of tertiary care hospitals of Faisalabad and Lahore for analysis of sequencing of the whole genome. Only 23 samples were sequenced after viral isolation, quantification, and cDNA synthesis.

Results: A total of 88 different types of mutations with different frequencies in all domains have been detected in NS4A proteins. Those mutations which presented with the most frequency were Q19L, I89M, and A93V (n=6) each R76K (n=5), V2I (n=4), and G38E (n=3).

Conclusion: Future DENV vaccination development research will be especially profited by the mutations found in the current study. Genomic epidemiology during each DENV outbreak in various regions is essential for improving public health and creating new regulations for outbreaks in the future.

Keywords: DENV; genome; mutations; Pakistan; Non-Structural proteins; NS4A.

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Introduction

Dengue is a viral ailment propagated by mosquitoes that is characterized by its acute and recurring nature. It is brought about by the dengue virus (DENV) and is commonly seen in tropical regions. In the last five to six decades, the incidence of dengue has risen by a factor of thirty. Presently, around 390 million individuals globally acquire dengue fever every year.[1] National Institute of Health (NIH) Islamabad, reported the number of dengue fever in Pakistan to be 22,938 in 2017, over 3,200 cases in 2018, 24,547 patients in 2019, and 3,442 cases in 2020.[2]

Dengue viruses (DENV), belonging to the Flaviviridae family and Flavivirus genus,[3] induce a dengue infection that manifests as high fever, joint and muscle pain, vomiting, exhaustion, myalgia, skin rash, hemorrhagic episodes, abdominal discomfort, and circulatory shock. DENV viruses are enveloped RNA viruses with a single-stranded genome. The open reading frame (ORF) of the DENV genome, which spans approximately 11 kilobases and is flanked by the 5' and 3' untranslated regions, is encoded. The open reading frame (ORF) encodes a solitary polyprotein, which is further segmented into 7 non-structural proteins (NS-1, NS-2, NS-2B, NS-3, NS-4, NS-4B, and NS-5) and 3 structural proteins (C: capsid, M: membrane, and E: envelope).[4]

NS4A is an integral membrane protein of DENV and play many roles in its replication and relations with its

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host. It is a 127aa protein with 16kDa molecular weight. Among the seven domains the upper three domains (aa3-11, 14-31, and 41-45) as shown in Figure 1 are present on the membrane surface. Three domains are membrane embedded and one is cytosol domain (aa80-96). The 2K sequence NS4A is present at C-terminus. Through protease cleavage this 2K is separate from NS4A serving as a signal peptide to translocate NS4B to ER lumen of cell[5].

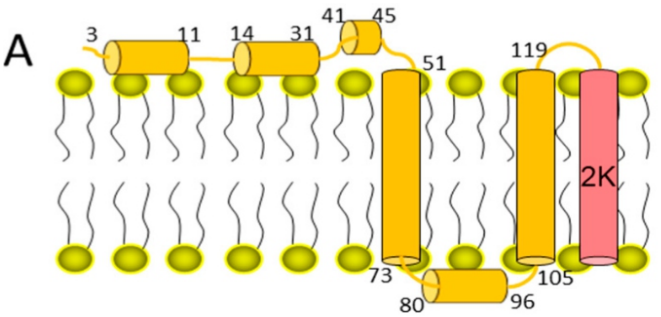


Fig-1: *Domain organization of NS4A.*

The first two domains are present at the membrane surface and one above the surface. Three are embedded in the membrane and one is inside systole. Out of the transmembrane domains (TMDs), it was found that the first TMD, which is made up of 48 amino acids, helps make an amphipathic coil that helps oligomerization happen[6]. The NS4A protein is an important part of the replication complex that is attached to the endoplasmic reticulum membrane[7]. If the amino acid sequences in NS4A are changed it loses its function.

Material and Methods

This research was conducted at Institute of Molecular Biology and Biotechnology, The University of Lahore, Aziz Fatimah Hospital and Allied Hospital Faisalabad during the dengue outbreak of 2022. After taking the IRB approval Ref: No. IEC/272-23 dated 12-09-2023, the temporal sampling method was applied while selecting patients from the dengue wards of both hospitals, and they were con-sented in writing. It was determined that the patients had dengue infection based on the results of a positive polymerase chain reaction (PCR) test for DENV, a positive NS1-antigen test, or positive IgM antibodies for DENV. On Performa, the results of clinical exami-nations, laboratory tests, and other diagnostic proce-dures were documented along with the clinical history and examination findings. Confirmed patients with dengue fever older than 13 years and of both sexes. The study did not include participants with comorbidities like hepatitis, chronic liver illness, typhoid fever, or malaria. Additionally excluded were patients who had dengue shock syndrome (DSS).

120 Blood samples were collected from the dengue patients within 7 days of the onset of symptoms and centrifuged and stored. The GeneJET viral DNA/RNA purification kit (Cat no. K0821) was used to get viral RNA directly from the serum of DENV-positive patients. The extracted RNA was quantified by performing PCR and gel electrophoresis. DENV WGS sequencing was carried out on selected samples on an Ion 510 chip. Data was uploaded to Torrent Suite Server 4.10 once the prepared chip was put onto the Ion XL 5 sequencer for sequencing. EpiData Analysis, a software program developed by the WHO, was used to calculate and summarize the mutation frequencies. Excel analysis was used to check the data for flaws. The viral DNA/RNA purification kit (Cat no. K0821) was used to extract RNA from the samples. After extraction, the RNA was quantified using PCR and gel electrophoresis. DENV WGS sequencing was then performed on selected samples using an Ion 510 chip. Once the chip was prepared and placed onto the Ion XL 5 sequencer, data was uploaded to Torrent Suite Server 4.10 for analysis. To calculate and summarize the mutation frequencies, EpiData Analysis, a software program developed by the WHO, was used. Excel analysis was used to check the data for flaws.

Table 1: *Frequency of mutations in NS4A protein of DENV.*

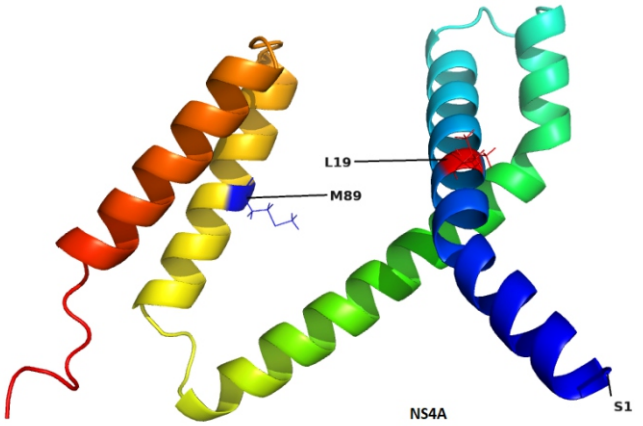
*MUT	*S.14	S.15	S.16	S.17	S.18	S.19	S20	S21	S22	S23	S24	S31	*Freq
V2I	P			P					P			P	4
L8S	P												1
G11E									P				1
Q19L	P			P					P	P	P	P	6
V29I										P			1
G38E	P								P	P			3
G38R										P			1

G38V			P					P		2
D50E								P		1
R76K	P		P			P	P		P	5
V88S								P		1
I89M	P		P		P	P	P		P	6
I89S								P		1
I89V		P								1
A90E								P		1
A93R								P		1
A93V	P		P		P	P	P		P	6
A106G								P	P	2
V2I	P		P			P			P	4
L8S	P									1
G11E						P				1
Q19L	P		P			P	P	P	P	6
G38E	P					P	P			3
G38R							P			1
G38V			P					P		2
D50E								P		1
T51R								P		1
I52K								P		1
I52M						P				1
I52V						P				1
E53G						P				1
E53W								P		1
L55G								P		1
L55M			P							1
M56W								P		1
L57N								P		1
A59W								P		1
I61K								P		1
A62H								P		1
V63F								P		1
T65Q								P		1
G66H								P		1
V68S								P		1
T69P								P		1
F71N								P		1
F72K								P		1
L73T								P		1
S74N								P		1
R76K	P		P			P	P		P	5
I89M	P		P		P	P	P		P	6
I89S								P		1
A93V	P		P		P	P	P		P	6
L95T								P		1
A106G								P	P	2
R127A								P		1
R127G		P								1

Results

After quantification 23 of 120 blood samples were sequenced. A total of 88 different types of mutations with different frequencies in all domains have been detected in NS4A proteins. Those mutations which presented with the most frequency were Q19L, I89M, and A93V (n=6) each R76K (n=5), V2I (n=4), and G38E (n=3). A total of 21 mutations T51R, I52K, I52M, I52V, E53G, E53W, L55G, L55M, M56W, L57N, A59W I61K, A62H, V63F, T65Q, G66H, V68S, T69P, F71N, F72K, and L73T have been detected in the fourth domain of NS4A with different frequencies as shown in **(Table-1).**

The fifth cytosol domain has 18 mutations with different frequencies. A total of 13 mutations have been detected in the sixth membrane embedded domain with 1 and 2 frequencies each. The last K domain has five mutations



at C-terminal regions.

Figure 2. Structure of DENV NS4A protein and location of most common mutations.

In figure 2 Mutation which are very common have been labeled with red (L19) and blue (M89). The NS4A contain three alpha helix inter wingle with each other. Two mutations at position L8S and G11E, have been detected in first surface domain of NS4A. Similar to the first domain, the second domain also harbored two mutations at positions Q19L and V29I respectively while the third smallest domain among all has no mutation.

In **(Table-2)** the effect of Different Mutations is shown on the protein function. In DENV1 serotypes, mutation G38E repeated in 3 samples and A106G in 2 samples affects the protein function. Similarly, in DENV2 sero-types Mutation E122G and R127G were repeated in 1 sample each is affecting protein function.

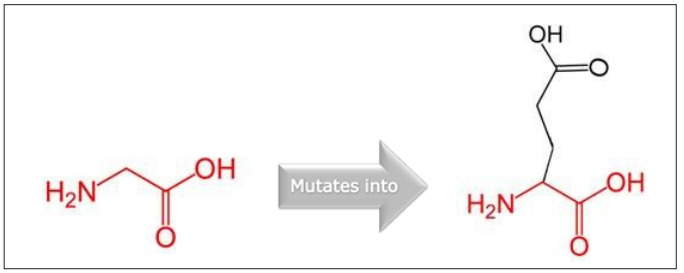


Figure 3: *Schematic structures of the original Glycine (left) into a Glutamic acid (right) amino acid at position 38*

We are taking the mutation G38E into further consideration because of its high frequency and its ability to affect NS4A protein function.

Discussion

In our study a total of 88 different types of mutations with different frequencies in all domains have been detected in NS4A proteins. NS4A protein has seven

Table 2: *Predictions About Mutations on NS4A protein Function.*

Serotype	Mutation	Frequency	Median Sequence Conservation	Sequences Represented At This Position	Protein Function	Prediction Score
DENV1	Q19L	6	3.04	18	Tolerated	0.24
	I89M	6	3.04	33	Tolerated	0.52
	A93V	6	3.04	33	Tolerated	0.34
	R76K	5	3.04	33	Tolerated	1.00
	V2I	4	3.67	33	Tolerated	1.00
	G38E	3	3.04	33	Affect protein function	0.00
	A106G	2	3.04	33	Affect protein function	0.01
DENV2	E122G	1	2.99	29	Affect protein function	0.00
	R127G	1	2.99	29	Affect protein function	0.00

domains. Out of which first two domains are present at the membrane surface and one above the surface. Three are embedded in membrane and one inside systole. In a study it was found that the N-terminal 49 residues of NS4A are found in the cytoplasm, where the N-terminal NS3–4A cleavage site is where the viral protease processes them[6]. Out of the trans membrane domains (TMDs), it was found that the first TMD, which is made up of 48 amino acids, helps make an amphipathic coil that helps oligomerization happen[7]. The NS4A protein is an important part of the replication complex that is attached to the endoplasmic reticulum membrane[8]. If the amino acid sequences in NS4A are changed it loses its function.

Membrane associated NS4A is linked to another protein NS4B by 23aa long conserved signal peptide. Previous study showed that mutations in NS4A (Leu48Ala, Thr54Ala, and Leu60Ala) influences the NS4A interactions with NS4B, abolishing the viral replication while mutations Phe71Ala and Gly75Ala has no effect on interaction of NS4A-NS4B on replication, highlighting the importance of mutations on NS4A-NS4B interaction and replication[9]. Therefore, blocking the interaction of NS4A-NS4B is a good antiviral strategy. In clinical setup dengue virus is diagnosed with clinical presentation but there are different parameters liver function test, serum proteins and viral markers for its diagnosis.[10,11]

Little hydrophobic protein NS4A (16 kDa) is ineffectively caught on, and it is still hazy what part it plays within the viral replication cycle. Kunjin infection (KUNV) NS4A is watched to limit to the assumed destinations of RNA replication and polyprotein preparing and interaction between NS4A and NS.[1] is essential for RNA to replicate proposing that flavivirus NS4A is included in a few steps of viral RNA intensification, conceivably by securing replicase components to intracellular layers[12]. Four anticipated transmembrane portions (pTMSs) make up the minor necessary layer protein known as NS4A. Even though pTMS4, too known as the 2k part, isn't a component of the developed NS4A, when it is cleaved from the developed NS4A and it will acts as a signal peptide for the NS4B localization within the ER12. The first 48 N-terminal set of NS4A serve a basic part in viral RNA's replication, as appeared by the L6E and M10E transformations which crush the both hydrophilic as well as hydrophobic nature of the N-terminal of NS4A. Moreover, when embedded as single changes, these changes had a comparable effect, NS4A interatomic positively with exceedingly bent liposomes, as which has been already illustrated. These two-point changes radically disable this interaction, as appeared by CD spectroscopy[13,14]. NS4A has also been directly linked to the formation of DENV virus replication organelles (vRO) using a method that doesn't depend on replication[15]. It is likely that the placement of NS4A, NS4B, or the NS4A-2K-4B precursor within the membrane leaflet makes it easier for the membrane to bend, which is needed for vRO formation[16].

Knowledge about certain areas of NS4A protein might help in ascertaining the sites where drugs can works against it. A study conducted showed that Helix α4 and the PEPEKQR sequence are good places for drugs to target in NS4A because they are needed for NS4A – 2K cleavage and NS4A–NS4B association, respectively[15].

Conclusion:

The sequencing techniques used in the molecular investigation of DENV's whole genome offer a clear picture of the diversity of the virus in terms of mutations that arise in the many DENV genotype targets. During the course of this analysis, we found that the DENV NS4A protein included a number of different alterations. The results of experiments reveal that mutations can have an effect not only on a virus's ability to replicate but also on its severity, its ability to penetrate a host cell, and its ability to disseminate. On the basis of this genetic heterogeneity, diagnostic procedures and markers can be developed, which may in the future lead to improvements in the treatment of DENV fever.

Conflict of Interest: *None*

Funding Source: *None*

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Authors Contribution

SM: Conceptualization of Project
RS: Data Collection
SJ: Literature Search
FNT: Statistical Analysis
ZM: Drafting, Revision
AZB: Writing of Manuscript