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Mutational Frequencies in the Immune System Interacting Proteins NS2A and NS2B in Dengue Virus Isolates

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Background: The genome of the dengue virus (DENV) encodes a total of ten proteins, three of which are structural and seven of which are non-structural (NS). Among the NS proteins, nonstructural protein 2A (NS2A) and NS2B play pivotal roles in the replication and assembly of DENV. This study aimed to determine the frequency of mutations occurring in the NS2A and NS2B regions of DENV within the Punjab Province of Pakistan.

Methods: About 4 mL of blood was collected from DENV patients. RNA was isolated from serum samples and confirmed using an RNA Kit. The RNA was converted to complementary DNA (cDNA) followed by amplification using polymerase chain reaction. The cDNA was subjected to library preparation for whole genome sequencing. We selected 23 samples to undergo whole genome sequencing. Among these, 19 isolates exhibited a significant number of mutations.

Results: In the NS2A, a total of 25 mutations were detected, with 23 being novel in the N-terminal and domains. The most common mutation, I15V, was found in seven genomic isolates, followed by I171A (n = 4), M150R (n = 2), and T34A (n = 2). Within NS2B, there were 20 different mutations, 18 of which were novel. Notably, the N and C-terminal regions exhibited a higher mutation frequency compared to the central two helices of NS2B. Specifically, seven mutations were located in the α1 helix of NS2B. Additionally, four mutations (A10E, V11F, I73F, and K127E) were detected in two isolates, respectively.

Conclusions: The most conserved region was the C-terminal domains extending beyond the 181 amino acids. This study represents the first comprehensive analysis of mutations in the NS2A and NS2B regions, which may help design effective vaccines and antiviral therapies for DENV.

Keywords: NS2A; DENV; mutations; genome; NS2B; virus

Introduction

Dengue fever is a debilitating and potentially life-threatening viral illness, characterized by acute and recurring symptoms, which originates in tropical and subtropical regions. The disease is caused by the dengue virus (DENV), a member of the *Flavivirus* family, and is transmitted to humans through virus-carrying mosquito bites [1], primarily *Aedes aegypti* and *Aedes albopictus*. Over the past few decades, the incidence of dengue fever has increased dramatically, with an estimated 390 million cases occurring annually, making it a significant global health concern [2].

DENV is classified within the *Flavivirus* and *Flaviviridae* family [3]. This virus comprises four distinct serotypes, DENV1-DENV4, each characterized by unique antigenic profile and distinct immunological properties [4]. A complete particle contains a positive sense of 11,000 bp RNA. This RNA genome is translated into a long polyprotein that is subsequently cleaved into ten individual proteins, consisting of three structural proteins (capsid, membrane, and envelope) and seven non-structural (NS) proteins (NS-1, NS2-A, NS-2B, NS-3, NS-4A, NS-4B, and NS-5) [5].

Nonstructural protein 2A (NS2A) possesses both an N-terminus and a C-terminus. The N-terminal region is lo-

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cated within the endoplasmic reticulum (ER) lumen of the host cell, while the C-terminus is located in the cytoplasm [6]. Its functional capabilities include crucial aspects of the virus' life cycle, such as viral RNA synthesis and its interactions with the 3′ untranslated region (UTR), NS3, and NS5. This collaborative interaction leads to the formation of a replication complex for viral replication [7]. Moreover, NS2A plays a pivotal role in DENV assembly [8,9]. NS2A influences the host's immune response. Specifically, the NS2A protein of DENV-2 has been found to inhibit the interferon response—a vital defense mechanism against viruses [10]. Intriguingly, a specific genetic alteration in KUNV NS2A has been linked to reduced interference with the interferon response, thereby attenuating the virus' virulence in mice [11,12]. Similarly, in the context of JEV NS2A, it has been demonstrated that this protein can thwart the activity of the dsRNA-activated protein kinase PKR, thereby subverting a vital aspect of the host's antiviral defense [13].

NS2A possesses five integral transmembrane (amino acids 69 to 209) segments, effectively embedding itself within the ER membrane's lipid bilayer. Moreover, an additional segment, ranging from amino acids 32 to 51, associates with the ER membrane without traversing the lipid bilayer. Interestingly, recent findings have shed light on the structural composition of the initial transmembrane segment (amino acids 69 to 93). This segment consists of two helical components connected by a structural element known as a "helix breaker", facilitated by amino acid P85. Functional investigations have uncovered an unexpected finding: while amino acid P85 was initially deemed pivotal, further analyses demonstrated its lack of significance in viral replication. Instead, a neighboring amino acid R84, which carries a positive charge, has been identified as a crucial factor in two essential stages of the viral lifecycle: RNA synthesis and the assembly/maturation of virions within the host cell [14]. The transmembrane nature of NS2A is attributed to its ability to anchor within the ER membrane.

The NS2B protein is located within the cell membrane and functions as a cofactor for the NS3 protease activity [15]. Previous studies have extensively explored a specific region that consists of 40 essential residues vital for the protease activity of NS3 and proper folding [16,17]. The NS2B sequence contains three hydrophobic regions that may function as transmembrane regions [18]. The secondary structure reveals four helical segments within NS2B, namely, α1 (G-4 to L-19), α2 (L25-M41), α3 (N-90 to G-105), and α4 (P-112-T-125). Functional and structural investigations have demonstrated that NS3 aligns with the C-terminus of α4 and interacts with the region between α2 and α3.

While NS2A and NS2B do not directly form a complex like NS2B-NS3, evidence suggests that they may interact or influence each other's functions within the replication complex. These interactions might impact processes such as viral RNA replication, virion assembly, and the overall efficiency of the viral lifecycle. However, the precise nature and significance of NS2A-NS2B interactions in DENV are still under investigation. New findings could shed light on their roles in viral replication and pathogenesis. These interactions represent promising targets for antiviral drug development, as disrupting essential viral protein complexes like NS2B-NS3 could potentially hinder viral replication and control the infection. For instance, a single study identified regions at the N-terminal and C-terminal regions of NS2A within protein transmembrane segment (Protein Trans-membrane Segment (pTMS) 1) and pTMS2 that are responsible for cytopathic effect (CPE), suggesting unforeseen connections between different pTMSs of NS2A and NS2B.

The incidence of DENV fever in Punjab is significant, and a molecular analysis of DENV strains in this region could provide valuable insights for future control strategies by examining their mutation rates. This study aimed to investigate the mutation frequency in NS2A and NS2B through genome sequencing from Punjab. The study found 25 mutations in NS2A and 20 in NS2B in genomic isolates from Punjab Province, with the majority of these mutations in low frequencies.

Material and Methods

Sample Collection

The study enrolled individuals with confirmed dengue infection experiencing symptoms accompanied by low platelet and low white blood cell (WBC) count. DENV infection was confirmed through polymerase chain reaction (PCR), NS1 antigen, and DENV-specific immunoglobulin M antibodies.

Specimen Collection

Approximately 4 mL of blood was extracted from the patients infected with DENV. The blood samples were then subjected to centrifugation at 2000 ×g for 10 min. Following centrifugation, the serum, which had been separated from the blood cells, was preserved at –80 °C.

RNA Isolation

The DENV RNA was isolated from serum samples and confirmed using an RNA Kit (GeneJET, ThermoFisher, Waltham, MA, USA). The serum containing the virus was subjected to centrifugation at 6000 ×g for a few minutes to remove particles and cells. The supernatant was transferred to a new tube and combined with a binding solution (350 µL). The tubes were thoroughly mixed for 15 s to ensure effective particle lysis. Subsequently, 350 µL ethanol (96–100%) was added, and the mixture was vortexed for 15 s.

Ethanol was added to the viral RNA for binding to the purification column membrane. A 2 mL tube was inserted with an RNA spin column, which was then loaded with the

prepared sample. The column was centrifuged at 6000 ×g for 1 min, after which the effluent was discarded, and the tube was retained.

Next, the spin column was placed in the initial tube, and 500 µL of Wash Buffer I was added, followed by centrifugation at 12,000 ×g for 1 min. The liquid was then discarded, and the tubes were retained. The washing step was repeated using Wash Buffer II/III (500 µL). The spin column was then subjected to a final centrifugation at maximum speed for 2 min to remove any residual ethanol. Subsequently, the spin column was transferred to a new 1.5 mL microcentrifuge tube.

Approximately 50 µL of elution buffer was added to the spin column membrane to elute the purified viral RNA, and incubated for 1 min. The column was centrifuged at 6000 ×g for 1 min to transfer the purified RNA into the microcentrifuge tube.

Synthesis of cDNA from RNA

The DENV RNA was converted to complementary DNA (cDNA) using the Thermo Scientific Revert-Aid cDNA Synthesis Kit (Launch Diagnostics, Lakeview West, Galleon Boulevard, Dartford, Kent, England). Using viral RNA templates, the Kit efficiently produces cDNA strands of up to 13 kb. This Kit remains effective at temperatures between 42 °C and 50 °C, ensuring the preservation of the RNA templates.

PCR Amplification

The primers were designed based on existing information regarding the alignment of the complete DENV sequence. The generated cDNA was subjected to amplification through polymerase chain reaction (PCR) utilizing an AmpliSeq Custom Panel containing two sets of 92 primer pairs and a total of 196 amplicons.

Quantification and Library Preparation

DNA quantification was performed using gel electrophoresis, incorporating a 100 bp size standard and using the Qubit dsDNA High Sensitivity Assay Kit. The gel purification phase aimed to remove any surplus products. The quantified samples were then used for the library preparation stage in readiness for sequencing. From the initial pool of 120 samples, a subset of 23 samples was selected for whole genome sequencing based on the gel purification results.

Whole Genome Sequencing (WGS)

The normalized library was loaded into the Ion Chef System (Applied Biosystem 9600, ThermoFisher, Waltham, MA, USA) utilizing the Ion 510 chip. The prepared chip was inserted into the Ion XL 5 sequencer to initiate the sequencing process. The acquired data were then uploaded to the Torrent Suite Server 4.10. at LabGenetix (Ion GeneStudio™ S5 System, Applied Biosystems, Waltham, MA, USA). The sequencing results were extracted in FASTQ file format.

The quality of FASTQ was accessed using FastQC (v0.11.8, Babraham Bioinformatics, Babraham, UK) to identify and remove the poor-quality sequence data, including adapter sequences, which can interfere with downstream sequence analyses. To further improve the quality of sequencing reads, the Trimmomatic tool v0.32 was employed to discard all reads with a low-quality score (Q <30) [19].

The sample multiplexing was improved by incorporating adapter sequences. The high-quality WGS sequencing reads of DENV were mapped to reference genomes (NC_001477 and NC_001274) using Burrows-Wheeler Aligner (BWA) version 0.6 [20]. Within numerous sequence analysis pipelines, the need to remove PCR duplicates is a common step to address the presence of multiple PCR products arising from the binding of the same template on the flow cell. These duplicates can potentially result in false positive variant calls. All duplicate PCR reads were eliminated using Picard Tools (v2.21.6, SE Minneapolis, Minneapolis, MN, USA) [21]. Small insertions and deletions (Indels) were removed during read mapping using "RealignerTargetCreator (v3.8.0, Broad Institute, Cambridge, UK)" and "InDelRealigner (v3.8.0, Broad Institute, Cambridge, UK)". Mutation calling was performed using the Genome Analysis Toolkit (GATK) (v3.8.0, Broad Institute, Cambridge, UK). The HaplotypeCaller tool in GATK (v3.3.0, Broad Institute, Cambridge, UK) realigns the entire genome sequence reads through the local de-novo assembly of haplotypes in regions exhibiting variations, enhancing the accuracy of mutation calling.

Modeling of Protein Structures

The 3D structure of DENV NS2A and NS2B has not been fully resolved and was not available in the Protein Data Bank (PDB). However, the AlphaFold2. Collaboratory tool was used to model the structures of these proteins. The modeling process was initiated by selecting "Runtime" -> "Run all", and the modeled structures were downloaded in PDB format [22].

Visualization of Protein Structure and Mutation Insertion

The PDB structures of NS2A and NS2B proteins were visualized in Chimera [23]. Subsequently, specific sites were targeted using the mutagenesis option in Chimera. Mutations were precisely introduced at the designated positions. The high-quality protein structures were then downloaded for detailed analysis [24].

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Table 1. Mutations and their frequencies in NS2A of DENV.

S. No	MUT	*S11	S12	S13	S14	S16	S17	S18	S19	S22	S23	S26	Freq
1.	V5>I					Y							1
2.	I15>V	Y	Y		Y		Y		Y	Y	Y		7
3.	M31>I										Y		1
4.	M33>V										Y		1
5.	T34>A				Y				Y				2
6.	T36>K								Y				1
7.	G35>E				Y								1
8.	L41>F				Y								1
9.	T42>A					Y							1
10.	T44>I				Y								1
11.	Q47>K				Y								1
12.	T73>A					Y							1
13.	Y74>H					Y							1
14.	M136>I					Y							1
15.	V137>V					Y							1
16.	Q144>H											Y	1
17.	V147>A											Y	1
18.	M150>R					Y						Y	2
19.	H162>D				Y								1
20.	I171>A			Y		Y		Y				Y	4
21.	I171>V				Y								1
22.	I174>I				Y								1
23.	V175>A				Y								1
24.	S176>P				Y								1
25.	L181>P							Y					1

I171A (n = 4), M150R (n = 2), T34A (n = 2), *S: genomic sample. NS2A, nonstructural protein 2A; DENV, dengue virus.

Statistical Analysis

Statistical analysis was conducted using the EpiData (V.2.2.3.187, Epicentre, Jens Lauritsen, Denmark) Analysis Software developed by the World Health Organization (WHO) to compute and provide an overview of mutation frequencies [23]. The data were analyzed in an Excel spreadsheet to identify and rectify any errors.

Results

RNA isolation and PCR quantification were carried out on 120 serum samples. Out of these, 23 samples were eligible for sequencing. Ultimately, complete sequencing could not be achieved for four out of the 23 samples, while 19 samples displayed numerous mutations in both the structural and NS of DENV.

The genome sequences (BioProject ID: PRJNA943555) were submitted to National Center for Biotechnology Information (NCBI). According to the WGS analysis: samples S13, 16, 18, 26, and 30 were identified as DENV2 serotypes, and the remaining samples were identified as DENV1 serotypes. The DENV1 serotype was predominant in the current study.

Among the WGS isolates, S16 was the cosmopolitan genotype in DENV2, while the other four isolates (S13, S18, S26, and S30) belonged to the Asian I genotype. Furthermore, samples S10, 11, 12, 15, 19, 20, 24, 27, and 31 were classified as genotype IV, whereas S14, 17, 21, 22, and 23 were identified as genotype III.

Mutations NS2A Protein

DENV has a positive-sense RNA genome of approximately 11 kb (Fig. 1). The impact of mutations on virus severity can vary widely, depending on the specific virus, the mutation, and the host's immune response. Mutations can change viruses' properties, such as their transmissibility, replication efficiency, immune evasion abilities, and virulence. A total of 25 non-synonymous mutations were detected in the NS2A protein across all domains, of which 23 were novel, while two (L41>F and Q47>K) have been reported in earlier studies. Among the non-structural proteins of DENV, NS2B was the most conserved protein, with only 20 mutations, while NS2A harbored 25 mutations (Tables 1,2).

A total of twelve mutations have been identified in the N-terminal domain (domains pTMS1-pTMS4, amino acids 1 to 118), while thirteen mutations were found in the C-

Table 2. Mutations and their frequencies in NS2B of DENV.

S. No.	MUT	S13	S14	S16	S17	S18	S26	S27	S31	Freq
1.	P3A		P							1
2.	I8V			P		P	P			3
3.	M9I			P						1
4.	A10E	P					P			2
5.	V11F	P					P			2
6.	M13V			P						1
7.	V14G			P						1
8.	S15R			P						1
9.	D63H						P			1
10.	Q64N						P			1
11.	I73F			P			P			2
12.	T96A	P								1
13.	I106V								P	1
14.	V109V								P	1
15.	L118F								P	1
16.	V120L								P	1
17.	W121R								P	1
18.	Y122D								P	1
19.	V126E	P								1
20.	K127E	P		P						2

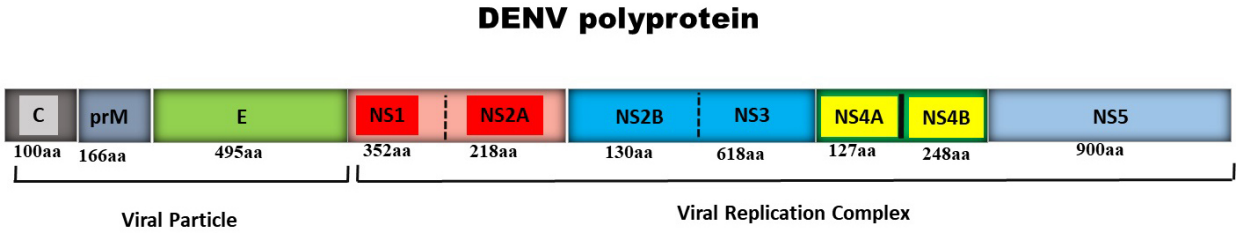

Fig. 1. DENV polyprotein representation. The protein arrangement of the DENV genome is characterized by the presence of structural and non-structural proteins, each with a specific number of amino acids. The viral structural protein is located at the N-terminal end of the genome, while the C-terminal end comprises the viral replication complex.

terminal domain (domains pTMS5-pTMS8), as shown in Table 1. The most common mutation was I15V in 7 genomic isolates, followed by I171A (n = 4), M150R (n = 2), and T34A (n = 2). Notably, the most conserved region was the C-terminal domains (Table 1), extending beyond the 181 amino acids.

In NS2A, there are 8 predicted transmembrane segments pTMS designated as pTMS1-pTMS8 (Fig. 2). Among these, pTMS1 carries two mutations (I15>V, V5>I), with I15>V observed in 7 genomic isolates. Notably, specific residues in two proteins, NS2A-L181>F and NS2B-I114>T, play crucial roles in virus assembly and cytopathic effects. In this study, we identified a novel mutation, L181>P, in NS2A from a single genomic isolate (S18) (Table 1), potentially affecting interactions with NS2B. Interestingly, the cytosolic region of NS2A, containing amino acids 210 to 218 at the protein's C-terminus, emerged as the most conserved region, where no mutation was identified.

The pTMS2 domain harbored nine non-synonymous mutations. Mutation T34>A was detected in two genomic isolates. Position T34 has two different substitutions. The remaining mutations were detected in a single isolate (Table 1). The pTMS1 (3–24 aa) and pTMS2 (32–51 aa) regions are present outside the endoplasmic reticulum lumen and do not exhibit membrane-associated activity. Five of these mutations were detected in the N-terminal region of pTMS2, and four were found in the C-terminal region.

In the pTMS3 region of NS2A, there were two helices along with a helix breaker (amino acids R84 and P85). The residue at position R84 plays a crucial role in RNA synthesis and virion assembly, whereas P85 is not significantly involved in these processes. Two mutations, T73>A and Y74>H, have been identified within the pTMS3 segment spanning amino acids 69 to 93. This domain exhibits higher conservation compared to the first one. Notably, the most conserved domain was found in pTMS4 (100 to 118 aa), where no mutations were observed. Within pTMS5 (120–140 aa), two mutations were identified: M136>I and V137>V.

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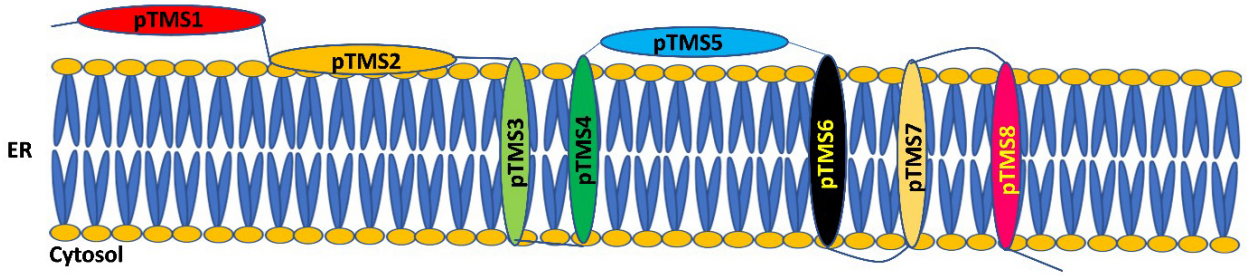

Fig. 2. NS2A membrane topology. pTMS2 is located within the ER lumen, whereas pTMS>3, pTMS>4, pTMS>6, pTMS>7, and pTMS>8 span the ER membrane. The specific regions are as follows: pTMS1 (3–24 aa), pTMS2 (32–51 aa), pTMS3 (69–93 aa), pTMS4 (100–118 aa), pTMS5 (120–140 aa), pTMS6 (143–163 aa), pTMS7 (165–186 aa), and pTMS8 (189-209 aa). pTMS, Protein Trans-membrane Segment; ER, endoplasmic reticulum.

The pTMS6 region of NS2A exhibited four mutations (Q144>H, V147>A, M150>R, and H162>D). A compensatory mutation, T154>P, identified in the pTMS6 region of DENV-2, plays a crucial role in restoring virus assembly disrupted by the I59N mutation. While the current study did not identify mutations in these residues, it did detect two mutations (V147>A and M150>R) surrounding the compensatory region, suggesting a potential influence. Additionally, certain mutations at positions 47 and 44 were found to be associated with the compensatory mutation T154>P in NS2A of DENV-2, although their precise function remains unknown.

The C-terminal Domain 7 (pTMS7) exhibited six mutations: I171>A, I171>V, I174>I, V175>A, S176>P, and L181>P. The I171>A mutation was identified in four genomic isolates. Domain 8 and the C-terminal residues do not harbor any mutations.

Flexibility Analysis of L181P and L181F

Protein flexibility plays a crucial role in protein function, as alterations in flexibility can significantly affect a protein's ability to perform biological tasks. Proteins exhibit dynamic behavior, continuously undergoing conformational changes crucial for their interactions with other molecules, such as ligands, substrates, and other proteins.

The residue at position L181 has a compensatory role in virus assembly. The mutation L181F, as discussed previously, compensates for defects in DENV assembly. Our study compares the flexibility effect of L181F and L181P on NS2A. The earlier mutation demonstrated an increase in NS2A flexibility, while the latter mutations exhibited a decrease in NS2A flexibility. This flexibility might have an advantage in virus assembly. Analyzing the interactions involving the mutated residue in the current study (L181P), the number of interactions in the wild type (L181) was higher than in the mutant (Fig. 3). Similarly, in the investigation of the L181F mutant in the previous study, it was noted to have more hydrogen bonding interactions than the wild type (Fig. 4).

Mutations in NS2B

The NS2B exhibited 20 different mutations, including 18 novel mutations. Notably, the I8V mutation was detected in three genomic isolates (S16, S18, S26). Four mutations, A10E, V11F, I73F, and K127E, were detected in two isolates each (Table 2). The secondary structure of NS2B is characterized by four helical regions: α-1 (G>4 to L>19), α-2 (L>25 to M>41), α-3 (N>90 to G>105), and α4 (P>112 to T>125). The N- and C-terminal regions of NS2B showed a higher frequency of mutations compared to the central two helices. The residue locations are shown in Fig. 5. Seven of these mutations were detected in the α1 helix. The α2 helix and the majority of the long loop region remained conserved in our study. The long loop region (aa45–aa94) harbored only three mutations with single and double genomes. The α3 helix harbored three mutations (T96>A, I10>6, T96>A) at both terminals, while the loop region between α3 and α4 exhibited one synonymous mutation (V109V). The α4 helix displayed six mutations, except K127E, which had a single frequency (Fig. 5).

Discussion

As of 2022, Pakistan has reported a total of 62 fatalities among 25,932 confirmed cases of DENV according to the World Health Organization (WHO). Notably, around 74% of these cases emerged in September alone. Swift improvements of vector monitoring, laboratory capacities, early case detection, and identification of warning signs are crucial to minimize the impact and seasonal occurrence of DENV. During outbreaks, investigating the genetic composition of DENV strains isolated locally in Punjab and analyzing mutation occurrence can provide valuable insights for improved disease management. Understanding DENV's proteomic facets will also enhance our understanding of mutation trends associated with higher frequency and severity of the disease. Despite technological advancements, the precise role of protein information in DENV infection and its link to DENV-related illnesses remain unclear. A previous study confirmed the presence

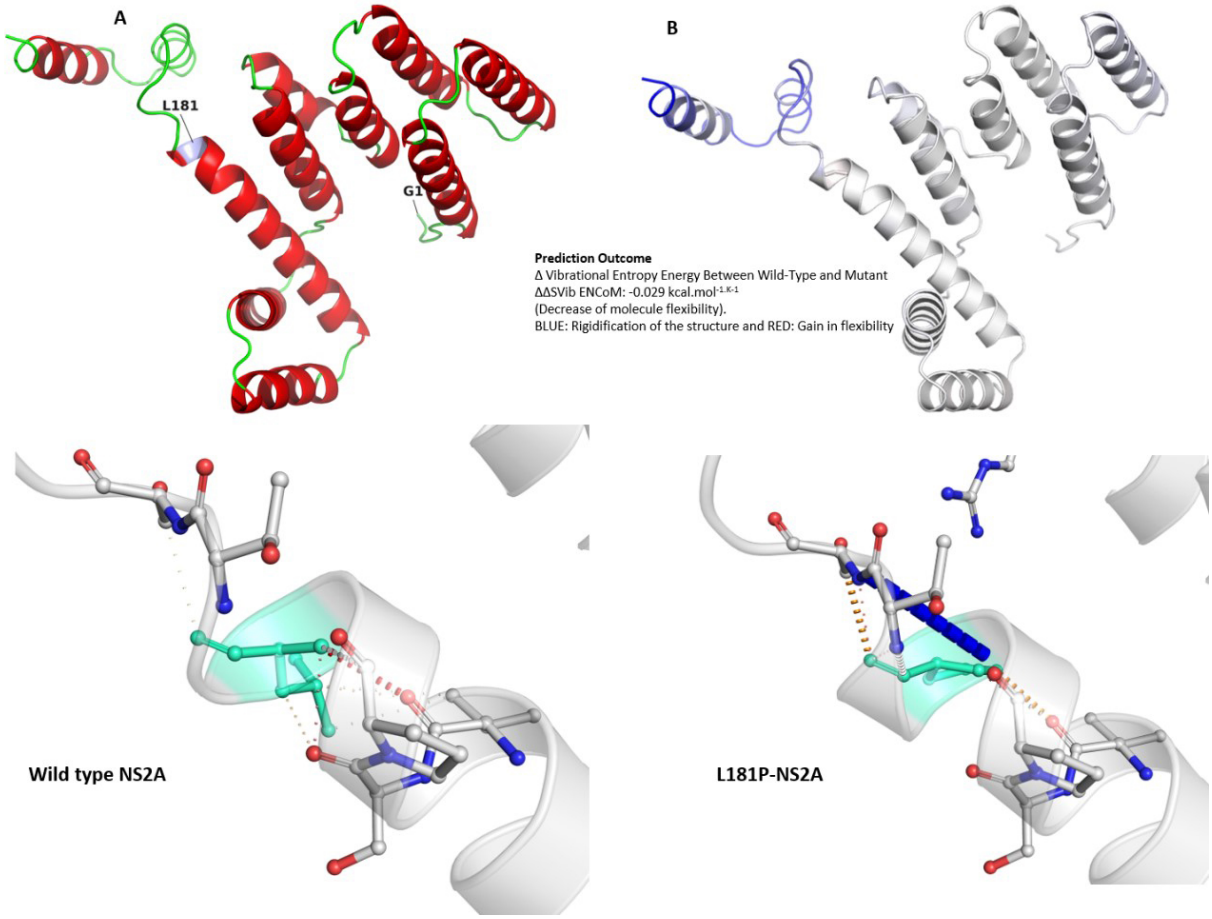

Fig. 3. Location of L181 and the effect of L181P on NS2A structure flexibility and interactions with nearby residues. (A) Modeled structure of NS2A and the location of L181 residue. (B) Flexibility analysis of our study mutation (L181P). Wild type NS2A: interactions of L181 residues with nearby amino acids. L181P interactions residues with nearby amino acids.

of all four DENV serotypes circulating in Punjab, Pakistan, with DENV2 and DENV3 exhibiting the highest prevalence [25]. A recent investigation in Pakistan highlighted DENV-2 as the predominant serotype, with DENV-1 following in frequency [26]. However, genome sequencing was conducted on a limited number of 10 DENV samples across Pakistan, omitting serotype data specifically from the Punjab province. Additionally, there is a lack of mutational data essential for understanding the structural proteins of the virus.

Between residues at positions 69 and 209, there are five TMS that fully span the ER membrane. A previous study found that the first domain (pTMS1) does not exhibit any membrane-associated functions, while pTMS2 is loosely associated with the ER membrane. Among these transmembrane segments, three domains (pTMS3, pTMS6, and pTMS8) extend from the luminal to the cytosolic side of the membrane, while pTMS4 and pTMS7 cross the membrane from the cytosol to the luminal side. Conversely, pTMS5 lacks any membrane-associated properties. The C-terminal amino acids numbered 210 to 218 are positioned within the cytosol [14].

Mutations within the NS2A may significantly affect the behavior of the virus, impacting its replication, virulence, and interactions with the host's immune system. The current study analyzed the mutation frequencies across all eight domains of NS2A, revealing distinct patterns in the N-terminal and C-terminal regions. Notably, the C-terminal domain (pTMS8) and the ER cytosolic fragment remain wild type. Previous research [27] highlighted the critical role of the N-terminal portion of NS2A in both RNA replication and virus-induced cytopathic effect (CPE). Furthermore, the study suggested that interactions within NS2A's Protein Trans-membrane Segments (pTMSs) and between NS2A and NS2B proteins are essential for these processes.

The *Flavivirus* NS2A protein plays a dual role, simultaneously participating in both the assembly and secretion of virions and contributing to the development of virus-induced CPE. A mutation at amino acid 190 (K190>S) of the yellow fever virus did not impact viral RNA synthesis but did hinder the generation of infectious virus particles [8,28]. In the case of West Nile virus, a mutation at amino acid position 59 (I59>N) was found to impede the production of secreted virus particles, with no dis-

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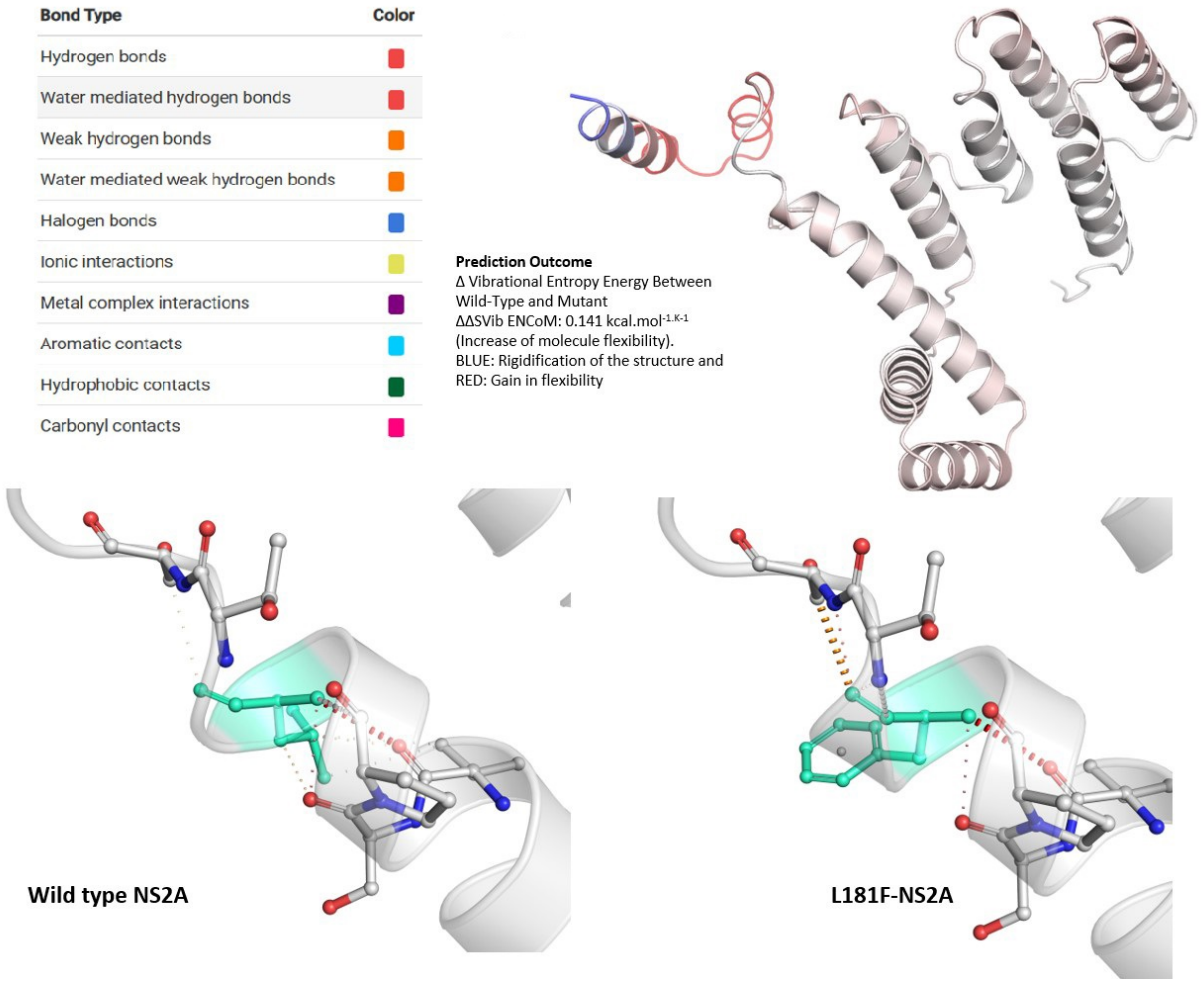

Fig. 4. Flexibility and interactions analysis of L181F on NS2A with nearby residues. Flexibility analysis of L181F. Wild type NS2A: interactions of L181 residues with nearby amino acids. L181F interactions residues with nearby amino acids.

cernible impact on RNA replication [26]. The topology of DENV NS2A showed eight predicted transmembrane segments (pTMS1-pTMS8) and five integral transmembrane segments (pTMS3, pTMS4, and pTMS6 to pTMS8) spanning the ER membrane's lipid bilayer [14]. Mutations within the first integral transmembrane segment (residues 69–93) showed that the substitution R84>A has no effect on RNA synthesis but hinders the formation of virions [14]. Analysis of the C-terminal region (pTMS4 to pTMS8) identified specific amino acids and pTMSs that played a role in RNA replication and assembly [29]. Complex interactions within these transmembrane segments imply that a consensus reversion mutation at position L181>F could potentially restore the functionality of multiple mutants in the C-terminal domain of NS2A [30]. Additionally, the mutation A30>P in the N-terminal region had dual effects, diminishing the virulence of the virus in mice and impairing its capacity to suppress the production of type I interferon [12]. Although this mutation did not impact virus production, the West Nile virus A30P mutant virus could not induce a CPE without interferon [30].

We identified 23 new mutations in the entire protein of NS2A, with all mutations in the C-terminal domain being novel. One newly discovered non-synonymous mutation (L181>P) was found in the C-terminal region of NS2A. Previously, the L181>F mutation in NS2A was reported to function as a compensatory mutation for various NS2A mutants with alanine substitutions in the C-terminal region [29]. The L181P mutation exhibited increased flexibility, potentially playing a role in restoring virus assembly. However, flexibility analysis of the L181P mutation demonstrated a decrease in NS2A flexibility. Experimental validation of the compensatory role of the L181P mutation is required to understand its impact on virus assembly and release. It is important to note that certain protein functions, such as membrane transport or structural changes, require significant conformational changes. Increased flexibility can make it easier for a protein to transition between different conformations, which is essential for functions like channel gating, molecular transport, and cell signaling.

In the current study, a mutation (L181P) was identified at position 181 in NS2A, while another mutation (L181F)

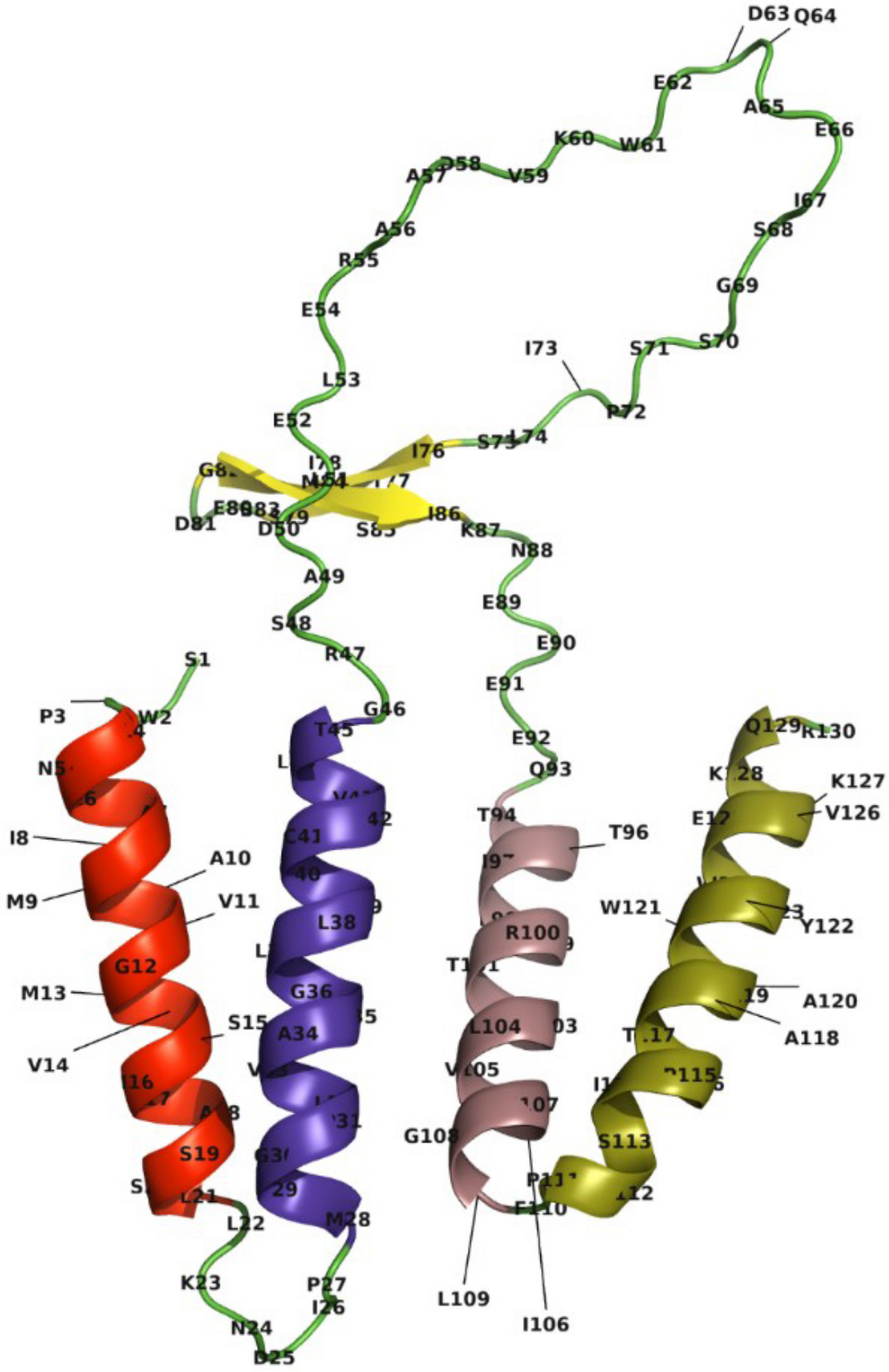

Fig. 5. Structure of NS2B. The structure of the NS2B protein is characterized by the presence of four transmembrane helices. The longest loop that extends outside the membrane is highlighted in green. Mutations within the connector region are shown.

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was found in the pTMS7 region of NS2A, and a third mutation (I114T) was observed in NS2B. These mutations have been shown to enhance infectivity, addressing previous issues related to impaired RNA replication or reduced capacity to induce cellular damage [27]. However, no mutation at position 114 in NS2B was detected in this study. These findings provide valuable insights into the functions of DENV NS2A and its transmembrane segments. Moreover, the identified rescuing mutations shed light on the complex interactions among different pTMSs of NS2A and NS2B.

Exploring the frequency of mutations in NS2B is essential for designing effective antiviral strategies and gaining a deeper understanding of the molecular mechanisms that influence virus replication and pathogenesis. NS2B interacts with the virus NS3 to facilitate polyprotein processing. Here, the mutation frequency in the NS2B was lower than in NS2A. The NS2B plays a vital role in replication and infection processes [31], and variations in mutation frequency may affect its activity. In the NS2B protein, the N-terminal is associated with the viral membrane, and the C-terminal interacts with the NS3 protein to facilitate the proteolytic cleavage activity of polypeptides. Mutations in the immunogenic regions of NS2B that could potentially stimulate an immune response were not detected. Exploring the mutation frequency in the NS2B is important due to its critical role in the replication and assembly of the virus, and it may represent an attractive target for vaccine development.

Conclusions

The current study identified 23 novel mutations in the NS2A protein and 18 in the NS2B protein across various domains. Notably, the pTMS4 and pTMS8 of NS2A were found to be the most conserved domains. The C-terminal regions of NS2A, in particular, exhibit a high degree of conservation, which may provide valuable insights into the interactions among the pTMSs of NS2A, NS3, and NS2B. These interactions, whether within the molecule or between molecules, are thought to play a crucial role in governing virus replication, assembly, and virus-induced CPE. Further studies are needed to unveil the effects of these mutations on virus assembly and release, as well as CPE. These findings enhance our understanding of how to better manage DENV and develop new vaccines and anti-flaviviral drugs.

Availability of Data and Materials

All data included in this study can be obtained from NCBI BioProject (PRJNA943555) (Accession ID: SAMN33731366-SAMN33731384).

Author Contributions

SM and MTK: Methodology, Validation, Formal analysis, Writing-original draft. MTK: Supervision, Methodology, Conceptualization, Validation, Formal analysis. MIUK: Methodology, Validation, Formal analysis. MAA and RS: Methodology, Validation, Formal analysis, Funding. YW: Methodology analysis, Writing original draft. HJ: Data analysis, Writing, review and editing. All authors contributed to important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The present study obtained ethical approval from the Bioethical Committee (IMBB-CRiMMResearch-143) and adhered to the principles outlined in the Declaration of Helsinki. Every participant diagnosed with DENV was required to provide written informed consent before their engagement in this study.

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Conflict of Interest

The authors declare no conflict of interest.

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