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Research Article

Genomic Variation in Dengue Virus Non-Structural Protein 1 (NS1)

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Abstract: To understand in an improved way how the dengue virus (DENV) spreads, presents, and becomes hazardous, researching its genetic makeup is necessary. The positive sense RNA of DENV encodes three structural proteins and seven non-structural proteins. One of the non-structural proteins that aids in the replication of viral RNA is the non-structural protein 1 (NS1). The objective was to identify the most frequently repeated mutations in the NS1 protein in DENV RNA isolated from dengue patients in the province Punjab, Pakistan. Selection of 120 DENV isolates was done from laboratories of tertiary care hospitals of Punjab for analysis of sequencing of the whole genome. Only 23 samples were sequenced after viral isolation, quantification and cDNA synthesis. A total of 133 different types of mutations were detected along the entire length of NS.1. The most common mutations with the highest frequency were, K324R and K347R (n=7), D278N (n=6), K174R, and F178S (n=4), found at c-terminal of NS.1 protein. Mutations K347R, K174R, and F178S are novel. Future DENV vaccination development research will be especially profited by the mutations found in the current study. During each DENV outbreak in different places, studying genomic variations is crucial for strengthening societal health and developing new policies for future outbreaks.

Keywords: Dengue Virus, Genomic Variation, Mutations, Nonstructural protein 1, Pakistan.

1. INTRODUCTION

Dengue fever, caused by dengue virus (DENV), is a common and grave infectious disease. Humans contract this disease by bites of Aedes female mosquitos, primarily *Aedes aegypti* and *Aedes albopictus* [1]. During the last few decades, dengue has become an epidemic all across the world, affecting 390 million people every year [2].

Dengue fever has been rapidly spreading in Pakistan, with Sindh, Punjab, and Khyber Pakhtunkhwa being the major affected provinces. Dengue fever cases discovered in Pakistan in 2017 were 22,938 as compared to more than 3,200 cases in 2018, 24,547, and 3,442 patients in 2019 and 2020 respectively, according to the National Institute of Health (NIH) Islamabad [3]. For better disease management in the future, molecular characterization of regional extracted DENV during outbreaks for different new mutations which will affect the function of the dengue virus may be helpful.

Dengue infection is brought on by dengue viruses (DENV), which are members of the Flaviviridae family and genus Flavivirus. High fever, muscle and joint pain, vomiting, fatigue, myalgia, skin rash, haemorrhagic episodes, abdominal pain, and circulatory shock are among the signs and symptoms of dengue infection [4]. The

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DENV viruses are encapsulated, single-stranded RNA viruses. There are four DENV serotypes from 1 to 5[5]. All four serotypes have caused outbreaks in Pakistan over the years, but generally, serotypes 2 and 3 have been more common [6]. The DENV genome is an 11-kilobase open reading frame (ORF), which is surrounded by the 5' and 3' untranslated portions and is encrypted. The ORF only encodes one polyprotein. This polyprotein is made up of three structural proteins (C: capsid, M: membrane, and E: envelope) and seven non-structural proteins (NS-1, NS-2A, NS-2B, NS-3, NS-4A, NS4B, and NS-5) [7]. These proteins are essential for the virus's formation and reproduction. While the non-structural proteins have a variety of roles in processes including viral RNA replication and immune evasion, the structural proteins are responsible for creating the viral particle.

The N-linked non-structural glycoprotein 1 (NS1) is 48 kilodaltons of pliability and excellent preservation. It has a membrane-associated form and a soluble, secreted variant. NS.1 is a 352-residue polypeptide with 46 to 55 kDa molecular weight. The soluble NS1 protein is composed of two identical subunits. Each subunit has three separate structural domains as shown in Figure 1. The first, referred to as the "-roll" dimerization domain, is composed of residues 1–29 and consists of two -hairpins (β) stabilized by a disulfide (Cystein4–Cystein15). The four -hairpins intertwine to make a -sheet. Subdomain / (amino acids 38 - 151) has a four-stranded -sheet, two -helices, and a distal tip (residueS.108-128); it is part of the second wing domain (amino acids 30-180) that also contains two glycosylation sites (Asparagine 130, Asparagine 175) and an internal disulfide (C55-C143). The third domain with 18 β-strands (residues 181–352). A conserved tip region of 79 residues (residues278–352) has four β-ladder, three β-sheet, and three disulfides [8]. There is an important role of NS1 protein in DENV replication. NS1 is the protein that can immediately affect the pathogenesis and is related to the severity of the disorder, due to the fact it can additionally cause vascular leaking in the lung, liver, and small intestine. By activating CD4 and CD8 T cells, NS.1 produces drastically excessive stages of each TNF-alpha and IL -6 within side the blood and additionally induces the launch of inflammatory cytokines that can cause vascular leaking resulting in excessive disorder [9]. Vascular leaking is a serious concern of dengue patients as it can cause death [10]. In evaluation to be worried about the pathogenesis of the disorder, it additionally works therapeutically. In addition to small molecule drugs, antibody remedy is nicely favoured due to its specificity towards disorder. Till now, numerous structural proteins targeting antibodies have been fashioned toward DENV infection. These are termed "neutralizing antibodies" and worried about stopping viral attachment with host mobiliary however it's far useless for all 4 DENV serotypes because of the chance of ADE (antibody-based enhancement) [11]. However, in evaluation to neutralizing antibodies, anti-NS.1 Antibodies can offer exceptional healing mechanisms through now no longer simply lowering the viraemic segment but additionally lowering the important segment. Also, it isn't related to the chance of ADE because it isn't a structural protein [9].

2. MATERIALS AND METHODS

During the 2022 dengue epidemic, the data from the Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan and Tertiary care hospitals of Punjab was collected. Ethical clearance was taken from the institute. Patients were selected from hospitals' dengue wards using a temporal sampling strategy, and their informed permission was documented. Dengue infection was verified by a positive PCR test for DENV, a positive NS1antigen test, or a positive IgM antibody test. Performa contained the patient's complete medical record, including their medical history, physical examination results, and all laboratory and other diagnostic tests. Patients from both genders above the age of 13 who have been diagnosed with dengue fever were included in the study. Patients with dengue shock syndrome, infective and chronic liver disease, and any other infection like typhoid fever, and malaria were excluded.

Within 7 days of the commencement of symptoms, dengue patients' blood was drawn, centrifuged, and preserved. The DENV RNA was extracted and purified from the serum of confirmed patients using a GeneJET viral DNA/RNA purification kit. The virus-containing serum was centrifuged at maximum speed to remove particles and cells, followed by adding binding

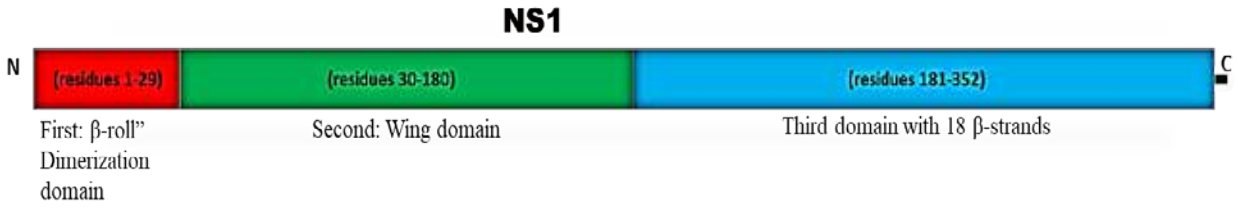

Fig. 1. Domain organization of NS.1 protein of DENV.

solution and 96-100% ethanol to ensure proper lysis. A spin column was then placed in the column, centrifuged, washed, and eluted. The purified viral RNA was then transferred to a new microcentrifuge tube and centrifuged at 6,000 × g for 1 minute to elute the purified viral RNA. Using RTq-PCR and gel electrophoresis, we determined how much RNA had been extracted. Selected samples were sequenced using DENV WGS on an Ion 510 chip. After loading the prepared chip into the Ion XL 5 sequencer, the data was transferred to Torrent Suite Server 4.10. The mutation frequencies were computed and summarised using EpiData Analysis, a software programme created by the WHO. Data quality was ensured by an Excel examination. To isolate RNA, we employed a viral RNA purification kit (K0821, Cat. no.). Quantification of the extracted RNA was performed by polymerase chain reaction and gel electrophoresis. Then, we used the Ion 510 chip to sequence DENV WGS data from a subset of the samples. The data was transferred to Torrent Suite Server 4.10 when the chip was loaded onto the Ion XL 5 sequencer. The mutation frequencies were computed and summarised using EpiData Analysis, a software programme created by the WHO. Data quality was ensured by an Excel examination. Alpha folds 2 and PyMOL tool was used to create a 3-D picture of NS1 protein.

3. RESULTS AND DISCUSSION

The sequencing of the entire genome was performed on a total of 120 serum samples that were chosen for the investigation. RNA from DENV was extracted from them, and after that, they went through the stages of PCR quantification and gel purification. After that, it was determined that just 23 of the samples were adequate to go through the sequencing process. Only 19 of the samples were able to have their complete sequences determined, whereas the remaining 4 samples could not. A total of 133 different types of mutations have been detected along the entire length of NS.1 as depicted in Table 1. The most common mutations with the highest frequency were, K324R and K347R (n=7), D278N (n=6), K174R, and F178S (n=4). The highest frequency mutations were most commonly detected at the C-terminal of NS.1 proteins. K324R and D278N have been reported and three (K347R, K174R, and F178S) are novel.

Nine mutations have been detected in the first β-roll" dimerization domain with a single frequency each. The second domain harbored 74 mutations with various frequencies from 1 to 4. Position D136 has six different amino acid substitutions in different genomic isolates. The third C-terminal domain contains 49 mutations with frequencies from 1 to 7, among which K324R and K347R were the most common (n=7) as shown in Figure 2.

In Table 2, the effect of Different Mutations is shown on the protein function. In DENV1 serotypes, mutation D278N repeated in 6 samples, K122Q in 2 samples, and I124K in 2 samples affects the protein function. Similarly, in DENV2 serotypes Mutation I135T and D136V were repeated in 2 samples each affecting protein function.

We are taking the mutation D278N into further consideration because of its high frequency and its ability to affect NS1 protein function. In this mutation aspartic acid at position 278 is replated by Asparagine as shown in Figures 3 and 4. A domain with an unidentified function's surface contains this mutant residue. In the employed structure, the residue was not discovered to be in touch with any other domains whose function is known. Even so, this mutation may have an impact on interactions with other molecules or domains.

Urgent action is required in Pakistan to improve the examination of Dengue markers, laboratory capacities for improved case management and identification, and warning indicators for sensitization in order to reduce the seasonality of

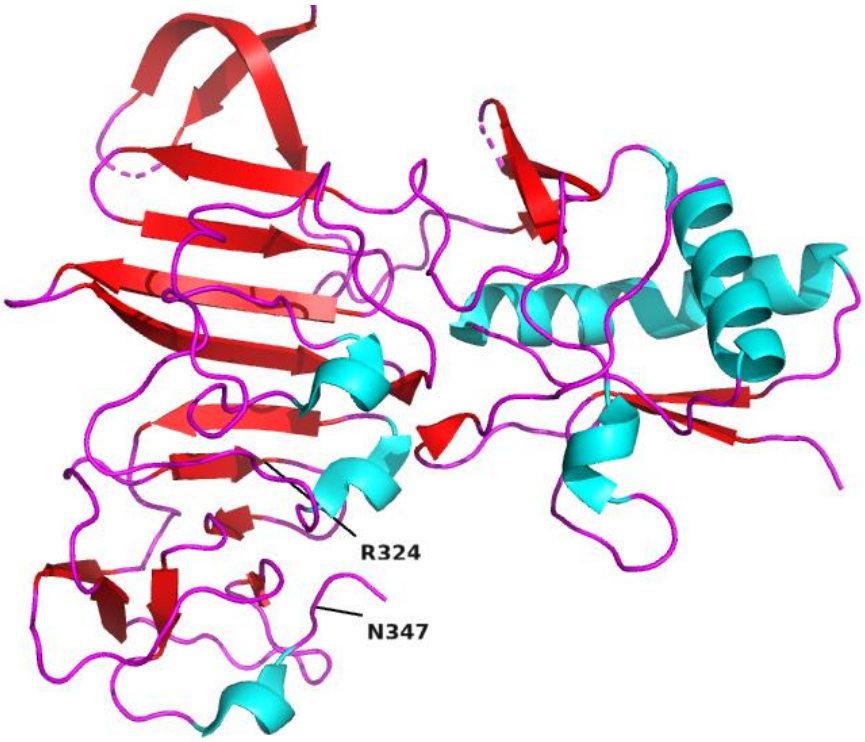

Fig. 2. Structure of DENV NS.1 protein and location of most common mutations at position number 324 and 347.

Fig. 3. Schematic structures of the original Aspartic acid (left) into an Asparagine (right) amino acid at position 278.

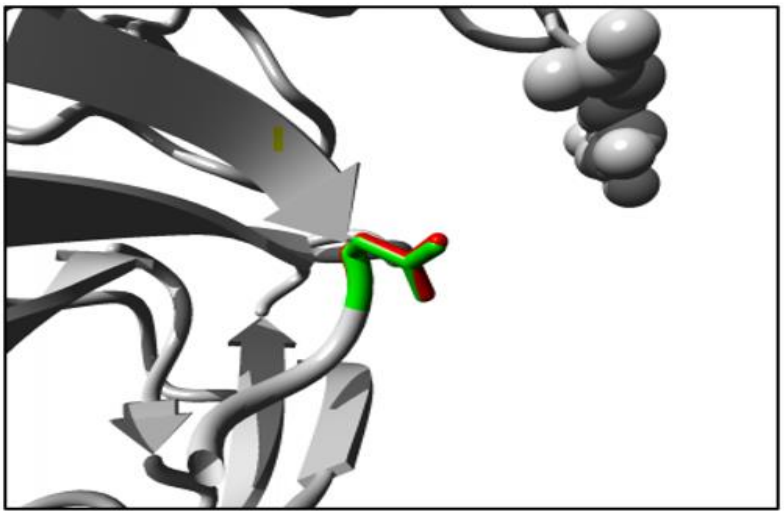

Fig. 4. Close-up of the mutation D278N. The protein is colored grey, and the side chains of both the wild-type and the mutant residue are shown and colored green and red respectively.

Table 1. Mutations in the NS.1 protein and their frequency.

MUT	S.11	S.12	S.13	S.14	S.15	S.16	S.17	S21	S22	S23	S24	S26	S30	S31	Freq
W8G							P								1
W8R				P											1
K9E				P											1
N10K			P												1
N10T			P												1
K11Q			P												1
K11T			P												1
E12G			P												1
E12K			P												1
K33R	P														1
D37N							P								1
R41G				P											1
G47E				P											1
K48R	P														1
C55Y	P														1
M67I				P											1
E74K				P											1
L75F							P								1
H77Y	P			P			P								3
S80T						P									1
N82I				P											1
N82Y							P								1
T87I							P								1
V88A							P								1
M89I						P									1
G91R							P								1
D92E				P		P									2
V93A	P			P			P								3
S94N	P			P			P								3
G95R				P											1
I96V	P			P			P								3
A98T	P			P			P								3
Q99H							P								1
G100E							P								1
K101N							P								1
K101R							P								1
K102E							P								1
K102R							P								1
K102X	P			P			P								3
S.103P						P									1
I104V							P								1
R105W						P									1
P106L				P											1
S.117T				P											1

MUT	S.11	S.12	S.13	S.14	S.15	S.16	S.17	S21	S22	S23	S24	S26	S30	S31	Freq
T117A						P									1
W118G				P											1
G119E				P											1
A121P	P														1
A121T						P									1
K122Q				P			P								2
I123F	P														1
I123T	P														1
M123L						P									1
I124K	P			P											2
I124V	P														1
D127G							P								1
S.128L						P									1
V128R				P											1
V128T	P			P			P								3
N130D							P								1
Q131H						P									1
L134R			P												1
L134V			P												1
I135F			P												1
I135L			P									P			2
I135M			P												1
I135S			P									P			2
I135T			P												1
I135V			P			P									2
D136A			P			P									2
D136E			P												1
D136G			P									P			2
D136H			P			P									2
D136N												P			1
D136V			P												1
G137A			P												1
N139D	P			P			P								3
N146D	P			P			P								3
I162V	P			P			P								3
K174R			P			P						P	P		4
V177I						P						P	P		3
V177T			P												1
F178S			P			P						P	P		4
D190E						P									1
D190G						P									1
D190N						P									1
N191H						P									1
F217L												P			1
N222S			P			P						P			3

MUT	S.11	S.12	S.13	S.14	S.15	S.16	S.17	S21	S22	S23	S24	S26	S30	S31	Freq
S239G								P		P					2
E240G									P						1
I246V								P							1
L247F			P			P						P			3
G248E										P					1
G249R	P														1
I264T			P			P						P			3
T265A			P			P						P			3
G266V	P														1
H269R	P			P											1
G271D										P					1
K272R			P			P						P			3
E274G	P														1
F277L	P					P		P							3
D278I								P							1
D278K								P							1
D278N	P			P			P	P	P	P					6
C280R										P					1
D281E			P			P						P			3
E281G								P		P					2
N293H			P												1
N293K			P												1
N293S			P												1
R294K			P												1
R294S			P												1
V303A							P								1
K306E						P									1
K306X						P									1
T307I	P			P			P	P	P	P					6
I308T				P											1
T309A						P									1
E310K	P														1
C313S								P							1
C313Y	P														1
P319T				P											1
K324R	P	P		P			P	P	P	P					7
G328E						P									1
G328R	P														1
E340D						P									1
E340G						P									1
N344S						P									1
V346A										P					1
K347R	P	P		P			P	P	P	P					7

Table 2. Predictions About Mutations on NS1 protein Function.

Serotype	Mutation	Frequency	Median Sequence Conservation	Sequences Represented At This Position	Protein Function	Prediction Score
DENVI	K324R	7	3.00	43	Tolerated	I .00
	K347R	7	3.00	43	Tolerated	1.00
	D278N	6	3.00	43	Affect protein function	0.02
	T3071	6	3.00	43	Tolerated	0.48
	H77Y	3	3.00	44	Tolerated	I .00
	V93A	3	3.00	44	Tolerated	
	S94N	3	3.00	44	Tolerated	0.39
	196V	3	3.00	44	Tolerated	I .00
	A98T	3	3.00	44	Tolerated	0.28
	V128T	3	3.00	44	Tolerated	0.53
	N139D	3	3.00	44	Tolerated	0.92
	N146D	3	3.00	44	Tolerated	0.56
	1162V	3	3.00	44	Tolerated	I .00
	F277L	3	3.00	44	Tolerated	0.55
	D92E	2	3.00	44	Tolerated	0.21
	K122Q	2	3.00	44	Affect protein function	0.04
	1124K	2	3.00	44	Tolerated	0.45
	S239G	2	3.00	43	Affect protein function	0.00
	E281G	2	3.00	43	Affect protein function	0.01
DENV2	K174R	4	2.97	38	Tolerated	0.23
	F178S	4	2.97	38	Tolerated	0.58
	771	3	2.97	38	Tolerated	0.47
	N222S	3	2.98	37	Tolerated	0.79
	L247F	3	2.98	37	Tolerated	0.06
	1264T	3	2.98	37	Tolerated	I .00
	T265A	2	2.98	37	Tolerated	0.76
	K272R	2	2.98	37	Tolerated	0.34
	D281E		2.98	37	Tolerated	0.34
	1135T	2	2.97	38	Affect protein function	0.00
	D136V	2	2.97	38	Affect protein function	0.01

the occurrence of DENV disease. The province of Punjab, which may be found in the middle of Pakistan's eastern half, is the nation's second-largest in terms of both its population and its land area [12]. It may be beneficial to perform molecular characterization of locally extracted DENV from Punjab during an epidemic in order to conduct genomic surveillance and determine the mutation frequency. This would allow for better disease management in the future. When we have a better understanding of the DENV proteome, we will have a better understanding of the mutational

patterns that are responsible for the high incidence and severity of the disease. Despite recent scientific progress, our understanding of the clinical significance of protein information in DENV infection, and its role in the development of DENV-related diseases, remains incomplete. Mutations at position Lys227Arg were detected in the current study and Thr307Ile is absent in the current study, in the NS.1 has also been reported in earlier studies [13]. Previously it was reported that mutations in NS.1 clinical samples, Val236>Ala or Trp68>stop are associated with low NS.1 protein secretion [14]. In another study, it was shown that Thr164Ser mutation inNS.1 is associated with the disease severity in the Americas [15]. The NS.1 monomer structurally consists of three domains. The initial dimerization domain, known as a "roll" domain (residues 1-29), consists of two -hairpins that are held together by a disulfide (Cystein4-Cystein15). The hairpins combine to create a four-stranded sheet. The second wing domain (amino acids 30-180) has two glycosylation sites (asparagine 130, asparagine 175), an internal disulfide (C55-C143), and two subdomains, one of which, subdomain /, has a four-stranded -sheet, two -helices, and a distal tip (residueS.108-128). The third domain (residues 181-352) has 18 -strands. There are four -ladder residues, three -sheet residues, and three disulfides in a conserved tip area of 79 residues (residues 278-352) [16].

4. CONCLUSIONS

During this analysis, we found that the DENV NS1 protein included 133 different types of mutations along the entire length. The most common mutations with the highest frequency were, K324R and K347R (n=7), D278N (n=6), K174R, and F178S (n=4). The highest frequency mutations were most commonly detected at the c-terminal of NS.1 proteins. K324R and D278N have been reported and three (K347R, K174R, and F178S) are novel. 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. Based on this genetic heterogeneity, diagnostic procedures and markers can be developed, which may in the future lead to improvements in the treatment of DENV fever.

5. CONFLICT OF INTERST

The authors declared no conflict of interest.

6. REFERENCES

1. S. Gwee, L. Ashley, G.C. Gray, and J. Pang. Animals as potential reservoirs for dengue transmission: A systematic review. *One Health* 12: 100216 (2021).
2. C. Guo, Z. Zhou, Z. Wen, Y. Liu, C. Zeng, D. Xiao, D., M. Ou, Y. Han, S. Huang, D. Liu, and X. Ye. Global Epidemiology of Dengue Outbreaks in 1990-2015: A Systematic Review and Meta-Analysis. *Frontiers in Cellular and Infection Microbiology* 7:317 (2017).
3. M. Khurram, G. Ali, U.A. Awan, and M.S. Afzal. COVID-19 and alarming dengue co-epidemics in the dilapidated healthcare system in Pakistan: Where to focus. *The Journal of Infection* 84(4): 579–613 (2022).
4. A. Murugesan, and M. Manoharan Dengue Virus. *Emerg Reemerging Viral Pathogens, Elsevier Academic Press.* 281–359 (2020).
5. V.D. Dwivedi, I.P. Tripathi, R.C. Tripathi, S. Bharadwaj, and S.K. Mishra. Genomics, proteomics and evolution of dengue virus. *Briefings in Functional Genomics* 16(4): 217–27 (2017).
6. A. Ali, H. Ahmad, M. Idrees, F. Zahir, and I. Ali. Circulating serotypes of dengue virus and their incursion into non-endemic areas of Pakistan; a serious threat. *Virology Journal* 26; 13(1):144 (2016).
7. Q. Li, and C. Kang. Structures and Dynamics of Dengue Virus Nonstructural Membrane Proteins. *Membranes* 12(2): 231 (2022).
8. D.L. Akey, W.C. Brown, S. Dutta, J. Konwerski, J. Jose, T.J. Jurkiw, J. DelProposto, C.M. Ogata, G. Skiniotis, R.J. Kuhn, and J.L. Smith. Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system. *Science* 343(6173): 881–885 (2014).
9. D.R. Glasner, H. Puerta-Guardo, P.R. Beatty, and E. Harris. The Good, the Bad, and the Shocking: The Multiple Roles of Dengue Virus Nonstructural Protein 1 in Protection and Pathogenesis. *Annual Review of Virology* 5(1), 227–253 (2018).
10. C.F. Lin, H.Y. Lei, A.L. Shiau, H.S. Liu, T.M. Yeh, S.H. Chen, C.C. Liu, S.C. Chiu, and Y.S. Lin. Endothelial Cell Apoptosis Induced by Antibodies Against Dengue Virus Nonstructural Protein 1 Via Production of Nitric Oxide. *The Journal of Immunology* 69(2): 657–664 (2002).
11. H.R. Chen, Y.C. Lai, and T.M. Yeh. Dengue virus non-structural protein 1: A pathogenic factor, therapeutic target, and vaccine candidate. *Journal of*

Biomedical Science 25(1): 58–58 (2018).
12. M.S. Rana, M. Usman, M.M. Alam, A. Ikram, M. Salman, R. Faryal, and M. Umair. The outbreak of dengue during the COVID-19 pandemic in Pakistan: The emergence of overlapping crises. *Asian Pacific Journal of Tropical Medicine* 15(2):53 (2022).
13. M. Anoop, A.J. Mathew, B. Jayakumar, A. Issac, S. Nair, R. Abraham, M. G. Anupriya, and E. Sreekumar. Complete genome sequencing and evolutionary analysis of dengue virus serotype 1 isolates from an outbreak in Kerala, South India. *Virus Genes* 45(1): 1–13 (2012).
14. A. Ghosh, S. Sukla, H. Nath, R. Saha, A. De, and S. Biswas. Non-structural protein 1 (NS1) variants from dengue virus clinical samples revealed mutations that influence NS1 production and secretion. *European Journal of Clinical Microbiology and Infectious Diseases* 41(5): 803–814 (2022).
15. K.W.K. Chan, S. Watanabe, J.Y. Jin, J. Pompon, D. Teng, S. Alonso, D. Vijaykrishna, S.B. Halstead, J.K. Marzinek, P.J. Bond, and B. Burla. A T164S mutation in the dengue virus NS1 protein is associated with greater disease severity in mice. *Science Translational Medicine* 11(498): eaat7726 (2019).
16. B.E.K. Tan, M.R. Beard, and N.S. Eyre. Identification of key residues in dengue virus N1 protein that are essential for its secretion. *Viruses* 15(5): 1102 (2023).