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STRUCTURAL VARIATIONS AND IMMUNOINFORMATICS ANALYSIS OF Orf6 PROTEIN OF SARS-CoV-2

Deepak Kumar Jha[1], Niti Yashvardhini[2]*, Amit Kumar[3], Manjush Gaurav[3], Kumar Sayrav[4]

1. Department of Zoology, S.M.P. Rajkiya Mahila Mahavidyalaya, Ballia, 277401, Uttar Pradesh, India.

2. Department of Microbiology, Patna Women's College, Patna, 800 001, India.

3. Department of Botany, Patna University, Patna-800 005, India.

4. Department of Chemistry, V.K.S. University, Ara, 802301, India.

ABSTRACT: SARS-CoV-2 (Severe Acute Respiratory Syndrome) is an etiologic agent of novel coronavirus disease (COVID-19), creating an alarming situation globally. Considering its sustained outbreak, the World Health Organization on 11th march 2020 has declared public health emergency around the world. Orf6 is a small accessory protein, encoded by the genome of SARS-CoV-2 and plays an important role in viral life cycle, pathogenesis and virulence. In the present study, 52,496 sequences of Orf6 reported from all over the world were compared to address structural variations in the protein. The results of our study showed a total of 779 mutations submitted in the month of March 2020 to 1st February 2021. Ten frequent mutations were found among the 779 mutations. Subsequently, the nature (deleterious or neutral) of mutations and protein modeling was conducted which revealed these frequent mutations considerably altered the structure of Orf6 protein. Further, physicochemical properties, antigenicity, allergenicity, toxicity and stability of Orf6 protein were estimated to demonstrate the stability of protein. Additionally, we identified 3 T-cell immune epitopes; their MHC restriction as well as MHC cluster analysis has also been performed which showed predicted epitopes can be a promising vaccine candidate to fight against COVID-19 infections.

Keywords: SARS-CoV-2, COVID-19, Orf6. Mutation, Epitope, Vaccine.

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Corresponding Author: Dr. Niti Yashvardhini* Ph.D.

Department of Microbiology, Patna Women's College, Patna, 800 001, India.

Email Address: nitiyashvardhini@gmail.com

1.INTRODUCTION

SARS-CoV-2 is a causative agent of novel coronavirus disease reported from Wuhan city of China in December 2019 [1,2] which has brought the almost entire world to a standstill. COVID-19 is a highly infectious disease that induces acute to severe respiratory distress in the infected individuals [3]. Transmission of SARS-CoV-2 occurs via inhalation or contact with the droplets from infected persons. Generally, the incubation period of COVID-19 infections ranges from 2 to14 days [4]. Coronavirus outbreak has been declared as a pandemic on 11th March 2020 by the World Health Organization (WHO, 2020). As of May 17th, 2021, worldwide 177,926,442 confirmed cases of COVID-19 have been reported to WHO including 3,851,264 casualties [5]. SARS-CoV-2 belongs to Coronaviridae family, it is an enveloped, plus sense, single-stranded RNA virus of ~30 kb length encoding polyproteins of 9860 long chain of amino acids [6]. The genome of coronavirus encodes four structural (spike S, envelope E, membrane M and nucleocapsid N), nine accessory (Orf3a, Orf3b, Orf6, Orf7a, Orf7b, Orf8a, Orf8b and Orf9b) and sixteen different non-structural proteins ranging from NSP1to NSP16 [7,8]. Orf6 is a small accessory protein of 61 amino acids long in SARS-CoV-2 that plays crucial role in viral infection and pathogenesis [9,10]. Previous studies have shown that Orf 6 of SARS-CoV-2 was found to interact with non-structural protein NSP8, involved in the RNA polymerase activity, using the yeast two hybrid systems [9]. It has been observed that RNA viruses exhibit high rate of mutation than DNA viruses. Due to its ability to show high frequency of mutations, SARS-CoV-2 acquires genomic variations that help them to modulate virulence properties in the host and consequently immune evasion of host takes place [11,12,13]. Altogether, we detected a total of 779 point mutations from 52,496 sequences submitted in the month of March 2020 to 1st February 2021 worldwide. Out of 779 mutations 10 mutations were found most frequent. In our present study, we have attempted to identify the recurrent mutations in the Orf6 of SARS-CoV-2. As this protein is involved in the infection and pathogenesis of the virus, therefore, it is essential to understand alterations caused by mutations on the structure and functions of Orf6 proteins. Further, we predicted secondary structure as well as protein dynamics study of ten frequent mutants, considerable alterations was observed in the structure of Orf6 accessory protein. In addition to this, using the predictive tools of immunoinformatics, we tried to design epitope based vaccine candidate to generate long lasting T-cell immune responses against SARS-CoV-2 infections. This study also encompasses physicochemical properties, antigenicity, allergenicity, toxicity of vaccine construct, their MHC restriction as well as MHC cluster analysis has also been performed which revealed predicted epitopes can be a promising vaccine candidate to curb COVID-19 infections. Moreover, this *in silico* investigations further needs validation through *in vitro* and *in vivo* studies.

2. MATERIALS AND METHODS

2.1. Identification of Orf6 mutants

The full length protein sequence of Orf6 protein of SARS-CoV-2 was downloaded from NCBI virus database, submitted from different countries till 1st February, 2021. There were nearly 52,496 sequences released from different countries since the onset of this pandemic. For the mutation studies, a reference sequence of Orf6 protein of Wuhan virus was also downloaded with accession number YP_009724394. To perform multiple sequence alignment of Wuhan type Orf6 protein sequence with those of other isolates, Clustal Omega, an online tool which performs alignment based on HMM profile seeded guide trees [14]. Jalview tool was used to visualize these aligned sequences for analysis of conserved as well as non-conserved sequences. The non-synonymous amino acid variants were analyzed using Protein Variation Effect Analyzer known as PROVEAN v1.1.3 with cutoff predicted score of -2.50 [15] to detect the effect of mutation on the Orf6 protein.

2.2. Protein properties and structure prediction

The 3D models of the Orf6 wild type as well as mutated protein were built using Chimera [16]. Chimera is user friendly structure predicting software which predicts models on the basis of results of homology modeling. Ramachandran plot was prepared using Swissmodel online server. Prediction of the secondary structure of Orf6 protein was done using CFSSP (Chou and Fasman Secondary Structure Prediction) online software [17]. The prediction was done for both wild type as well as mutated protein to detect the variation occurring in the secondary structure of Orf6 protein. The physicochemical properties which includes molecular weight, extinction coefficient, amino acid composition, instability index, estimated half life, aliphatic index and average of hydrophobicity (GRAVY) was calculated using Protparam tool of Expasy online program. Protscale tool of expasy was used for preparing hydropathy plot of Orf6 protein [18].

2.3. T-cell epitope prediction and MHC class I allele identification

Tepitool server of IEDB [19] was used to identify the T-cell epitopes alongwith the detection of MHC allele showing highest affinity for the T-cell epitope. This program provides information on the binding of HLA allele with both type I and type II MHC molecules. MHC cluster 2.0 online tool was used for the cluster analysis of MHC class I and MHC class II alleles which might interact with the epitopes leading to immune responses. This online server predicts epitopes and the allele binding in a phylogenetic way in form of clusters also in the way of heatmap [20]. The antigenicity of the Orf6 protein was estimated using Vaxijen v2.0 server which predicts antigens according to the auto cross-covariance (ACC) transformation of the protein sequences [21]. To detect whether the Orf6 protein was allergenic, AllerTOP server was used which evaluates protein allergenicity on auto cross variance (ACC method) that explains residues based on hydrophobicity, size, flexibility and other parameters [22].

3. RESULTS AND DISCUSSION

3.1. Identification of Orf6 mutants and detection of non synonymous mutants

Altogether 52,496 full length sequences of Orf6, 61 amino acids length were submitted from all across the world from onset of this disease till 1st February, 2021 (Supplementary Table 1). These sequences were downloaded alongwith a reference sequence of Wuhan type virus from NCBI virus database. The multiple sequence alignment was performed and the variations in the isolates were detected using Jalview. Amongst these point mutations, I33T, L4P, I26M, D53G, W27L, D61G, K42N, P57L, I14T and N39K were the most frequently occurring mutations and hence used for further characterization in this study (Figure 1). Out of the ten frequent mutations, only three were neutral (I26M, K42N and N39K) and rest were deleterious for the Orf6 protein at 2.5 cutoff values of PROVEAN score (Table 1).

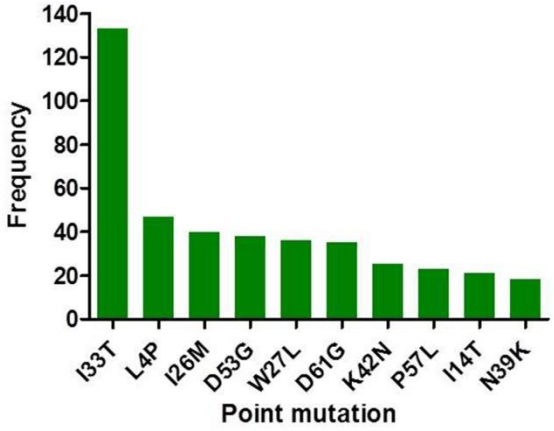

Figure 1. Frequency of different point mutations found in Orf6 protein since the outset of this pandemic till 1st February 2021.

Table 1: List of 10 nonsynonymous as well as frequent amino acid substitutions in Orf6 protein (cutoff= -2.5)

Variant	PROVEAN score	Prediction (cutoff= -2.5)
I33T	-4.714	Deleterious
L4P	-6.500	Deleterious
I26M	-1.500	Neutral
D53G	-6.750	Deleterious
W27L	-6.000	Deleterious
D61G	-6.679	Deleterious
K42N	0.679	Neutral
P57L	-9.036	Deleterious
I14T	-3.643	Deleterious
N39K	0.464	Neutral

3.2. Estimation of physicochemical properties and hydropathy index of Orf6 accessory protein

The estimation of physicochemical properties of Orf6 protein revealed that Orf6 protein is 61 amino acids long with a molecular weight 7272.54, aliphatic index 130.98, instability index 31.16, and GRAVY score of 0.233 (Table 2). The hydropathy plot showed N-terminal amino acid to be more hydrophobic as compared to the C-terminal end of Orf6 protein (Figure 2A). The 3D protein models of Orf6 wild type as well as mutated proteins were built using Chimera online modeling software. The models of both wild type and mutated Orf6 proteins are shown in Figure 2B. The Ramachandran plot of Orf6 protein is shown in Figure 2C. To study the impact of mutation on secondary structure of Orf6 protein, CFSSP method was used to visualize the secondary structure of wild type as well as mutated protein. Only one mutation P57L showed significant alteration in secondary structure upon mutation, whereas rest showed no significant change in either formation loss of helix or sheet structure (Figure 2D). The replacement of proline by leucine resulted in formation of helix at position 54, 55, 56 and 57. Leucine being a non polar amino acid favors the formation of alpha helix rather than turn; hence its addition resulted in helix formation.

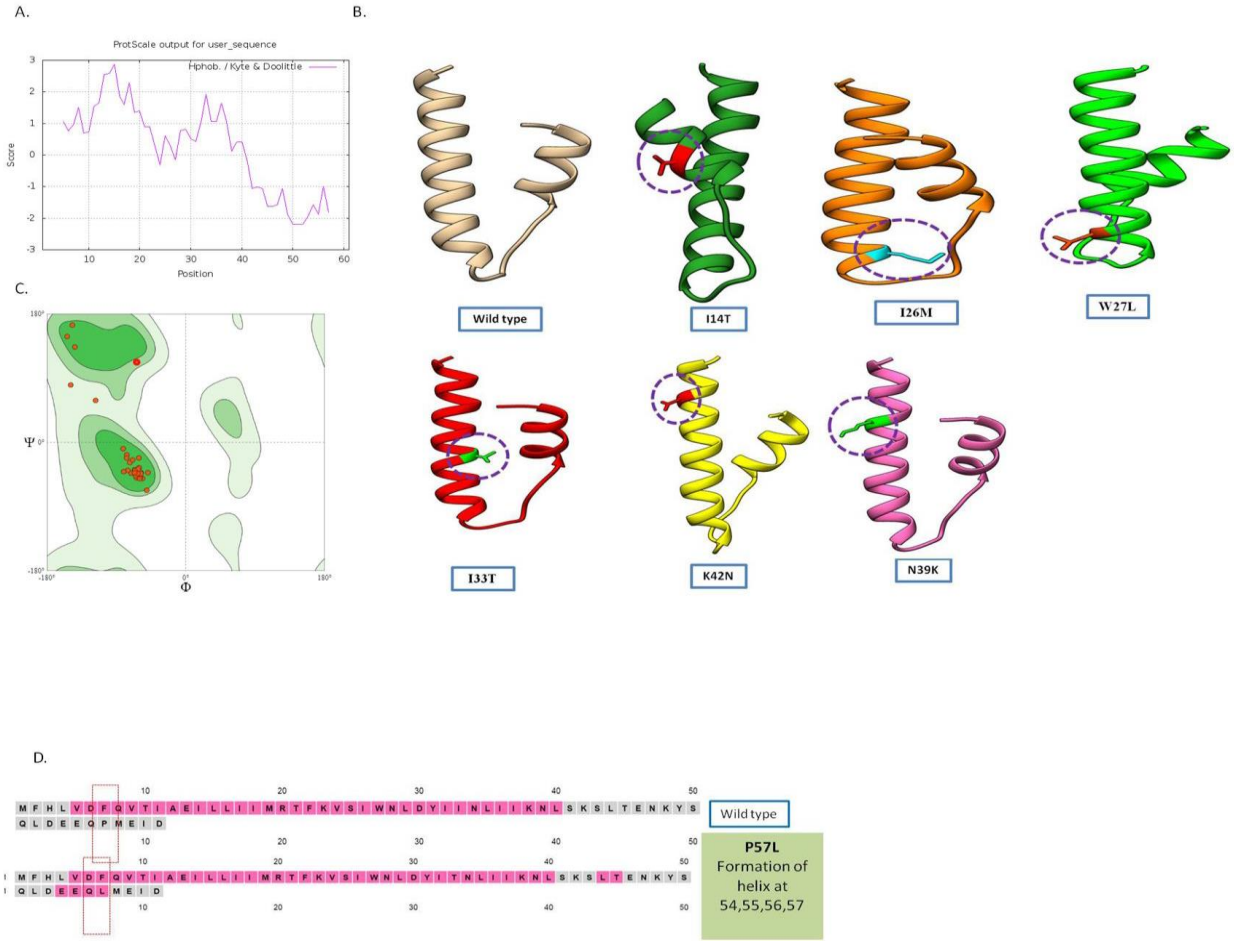

Figure 2. A. Hydropathy plot of wild type Orf6 protein showing hydrophobic amino acid residues, **B.** Protein modeling of wild type and mutant Orf6 protein. Models were prepared by Chimera software, **C.** Ramachandran plot of Orf6 protein, **D.** Secondary structure prediction of Orf6 protein. The mutation location and respective secondary structures are marked with boxes.

Table 2: Physicochemical properties of Orf6 protein (wild type)

Physicochemical properties	Orf6	Amino acid composition	No.	Percent composition (%)
Molecular weight	7272.54	Ala (A)	1	1.6
No. of amino acids	61	Arg (R)	1	1.6
Theoretical pI	4.60	Asn (N)	4	6.6
Instability index	31.16	Asp (D)	4	6.6
No. of negatively charged (Asp+ Glu)	9	Cys (C)	0	0.0
No. of positively charged (Arg+ Lys)	5	Gln (Q)	3	4.9
aliphatic index	130.98	Glu (E)	5	8.2
Grand average of hydropathicity	0.233	Gly (G)	0	0
Estimated half-life (mammalian reticulocytes, in vitro)	30 hours	His (H)	1	1.6
Atomic composition		Ile (I)	10	16.4
C	334	Leu (L)	8	13.1
H	532	Lys (K)	4	6.6
N	78	Met (M)	3	4.9
O	96	Phe (F)	3	4.9
S	3	Pro (P)	1	1.6
Formula	$C_{334}H_{532}N_{78}O_{96}S_3$	Ser (S)	4	6.6
Total number of atoms	1043	Thr (T)	3	4.9
		Trp (W)	1	1.6
		Tyr (Y)	2	3.3
		Val (V)	3	4.9
		Phy (O)	0	0.0
		Sec (U)	0	0.0

3.3. Prediciton of T-cell epitope with its allergenicity and MHC class I immunogenicity

A total of 3 T-cell epitopes were predicted for Orf6 protein which is shown in Table 3 with its sequence and allergenicity. The three epitopes were PRFPRTEIN, EACTERESP and RNAVIRSMF amongst which only PRFPRTEIN epitope was allergenic, other two were non-allergenic. The MHC allele binding was also analysed for these three epitopes as shown in table 3. These T-cell epitopes can bind with MHC alleles and hence can induce cytokine production to reduce the infection of SARS-CoV-2.

Table 3: T-cell epitopes and MHC restriction of the T-cell epitopes of SARS- CoV-2 Orf6 accessory protein.

	MHC Restriction of CTL Epitope		**Allergenicity**
1.	PRFPRTEIN	HLA-Cw*0401	Allergen
	PRFPRTEIN	H2-Db	
	PRFPRTEIN	H2-Dd	
	PRFPRTEIN	H2-Kb	
	PRFPRTEIN	H2-Kd	
	PRFPRTEIN	H2-Ld	
	PRFPRTEIN	HLA-G	
	PRFPRTEIN	H-2Qa	
	PRFPRTEIN	Mamu-A*01	
2.	EACTERESP	HLA-Cw*0401	Non-allergen
	EACTERESP	H2-Db	
	EACTERESP	H2-Dd	
	EACTERESP	H2-Kb	
	EACTERESP	H2-Kd	
	EACTERESP	H2-Ld	
	EACTERESP	HLA-G	
	EACTERESP	H-2Qa	
	EACTERESP	Mamu-A*01	
3.	RNAVIRSMF	HLA-B*51	Non-allergen
	RNAVIRSMF	HLA-Cw*0401	
	RNAVIRSMF	H2-Db	
	RNAVIRSMF	H2-Dd	
	RNAVIRSMF	H2-Kb	
	RNAVIRSMF	H2-Kd	
	RNAVIRSMF	H2-Ld	
	RNAVIRSMF	HLA-G	

RNAVIRSMF	H-2Qa
RNAVIRSMF	Mamu-A*01

The cluster analysis of MHC class I allele is shown in Figure 3A, 3B while that of class II allele is shown in figure 3C, 3D, where the red zone denotes strong interaction of the HLA allele with the epitopes of Orf6 protein whereas yellow depicts weak interaction. We analyzed the binding ability of all the possible alleles with the Orf6 epitopes. To predict the antigenicity of Orf6 protein VaxiJen v2.0 server was used, which predicts antigenicity based on the ability of vaccine candidate to bind with the B-cell and T-cell receptors and hence can enhance the immune response. This analysis revealed the antigenic nature of Orf6 protein with antigenicity score of 0.6131, at a threshold of 0.4%. A good vaccine candidate needs to be non-allergenic, hence the allergenicity as well as toxicity analysis of Orf6 protein revealed its non-allergenic nature, hence a potent vaccine candidate.

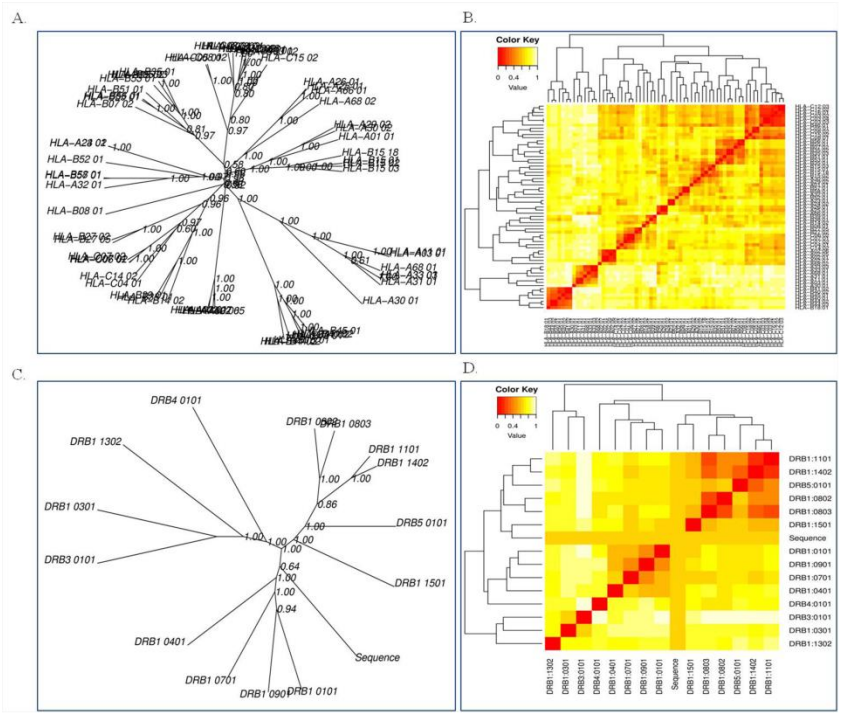

Figure 3. The results of MHC cluster analysis. A. tree map of MHC class I cluster, B. heat map of MHC class I cluster, C. tree map of MHC class II cluster D. heat map of MHC class II cluster.

Severe acute respiratory syndrome (SARS-CoV-2), emerged in China in late December 2019, and has become serious threat to public health around the world. Therefore, an effective, safe and durable antiviral therapeutics is urgently required to fight against COVID-19 infections. Primarily, the novel coronavirus causes respiratory illness and pneumonia with multiorgan disease in humans, whose manifestation includes dyspnea (shortness of breath), dry cough, sore throat and fever. The symptoms of the COVID-19 start within 2 days or it may continue upto ≥ 14 days. SARS-CoV-2 infections may have wide range of symptoms or it may appear to be asymptomatic.

SARS-CoV-2 belongs to the RNA virus groups and has enormous capacity to show high rates of mutation in a very short span of time [23]. It is quite evident from the numerous earlier studies that mutation plays crucial role in the viral evolution and subsequent adaptations [24,25]. Because these traits are supposed to be the key factors for viruses to survive in the host dynamic environment and enabling them to evade the pre-existing immunity of host and acquire drug resistance properties. Rapid spread of SARS-CoV-2 infection relies on many factors such as fidelity of its RNA polymerase, different geographical regions, and population density, as well as poor health or hygiene, and environmental conditions [26]. Mutational investigations of this virus provides better understanding of its epidemiology, pathogenesis and to design appropriate antiviral therapeutic strategies to combat COVID-19 infections. The results of our study showed, 779 point mutations identified from 52,496 sequences of Orf6 protein around the world. Due to these point mutations, significant alterations were observed in the secondary and 3-D structure of Orf6 proteins (Figure 2B and 2D). Kumar *et al.,* (2007) [9] have reported that in addition to IFN-I antagonism, Orf6 could have a role in the viral replication process and also observed Orf6 of SARS-CoV, was found to be co-localize and showed interaction with non-structural protein, NSP8. Epitope based designing of vaccine using computational tools of immunoinformatics gained much attention nowadays for various infectious diseases. Earlier methods of vaccine development include experimental identification of epitopes, and then to find a correlation with coronavirus for developing a potential vaccine construct. However, epitope based vaccine designing, using the predictive tools of immunoinformatics, have shown to be advantageous over the traditional ways of vaccine development because *in silico* methods are seems to be quite specific, and are able to avoid undesirable immune responses, eliciting long lasting immunity, reasonably cheaper and less time consuming [4]. Earlier studies have reported that epitope based vaccine candidate could be a good target against SARS-CoV-2 infections [27,28]. The T-cell epitopes consisted of short fragments of peptide and hence found to be more propitious, that generate prolonged immunogenicity mediated by CD8+ T-cells [15]. Additionally, physicochemical properties, antigenicity, allergenicity, toxicity and stability of Orf6 protein were estimated to demonstrate the stability of protein. Further, we identified 3 T-cell immune epitopes; their MHC restriction as well as MHC cluster analysis has also been performed which showed predicted epitopes can be a promising vaccine candidate to fight against COVID-19 infections [29,30].

4. CONCLUSION

Occurrence of frequent mutations in the Orf6 of SARS-CoV-2 provides a deep understanding of its virulence properties. For the designing of vaccine construct, Orf6 of coronavirus has been chosen as good target because Orf6 shows antagonism with INF-1 and also found interacting with NSP8 protein of SARS-CoV-2 and plays important role in the viral replication cycle. Moreover, our study sheds light on, high efficacy and durability of designed epitopes based vaccine using several

predictive tools of immunoinformatics; further, *in vitro* and *in vivo* studies are mandatory to validate designed vaccine candidate.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The author confirms that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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