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Stability of Envelope Proteins of SARS-CoV-2 Variants Could Be a Choice of New Drug Target

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Abstract: Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) has emerged as one of the worst viral pandemics during the past few years. As reported by the World Health Organization, around 77,56 49 520 cases and 70 51 720 deaths were reported from all over the world, which includes approximately 3500 patients reported during the recent past only. Currently, circulating variants of SARS-CoV-2 are KP.3, JN.1, BA.2.86 and KP.2. Mass vaccinations have been provided since the end of December 2020, which led to 5.47 billion people vaccinated till date. However, the disease continues in small foci all over the world. Development of an effective drug target and mutation independent vaccine thus becomes essential research priorities. Owing to the unavailability of a specific drug molecule, the present study has focused on the development of an effective drug target to treat COVID-19. In-house primers were designed for four essential structural genes viz., Spike protein, ORF1ab, Nucleocapsid gene, and Envelope gene. Samples of different waves were amplified using these primers employing the Polymerase Chain Reaction (PCR) assay. A total number of 86 SARS-CoV-2 RT PCR positive samples were studied, and results showed the most frequent appearance (80.2%) of Envelope (E) protein in all the samples. This suggests that during transmission across numbers of human hosts, it is the Envelope protein that was the most stable one. The most severe Delta variant showed the presence of E proteins in all the samples assayed. Blocking E protein as a new drug target to intervene intracellular replication of virus could be an effective drug development strategy.

Introduction

The Severe Acute Respiratory Syndrome Coronavirus-2 arose as a pandemic virus in late 2019, causing millions of morbidity and mortality worldwide (Zhou et al., 2020). Structurally, the virus is 60-140 nm in size and has a RNA genome. The genome is 27-32kb and contains four structural, 16 non-structural, and 14 Open reading frame genes (Lu et al., 2002; Brian and Baric, 2005). The four structural proteins include Spike protein

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(S), Membrane protein (M), Envelope protein (E), and Nucleocapsid (N) protein. The structural proteins are responsible for binding and entry of the virus into host cells, specifically the Spike protein (Li et al., 2003; 2005; Li, 2016). The S protein consists of 2 subunits which are S1 and S2, with S1 having the domain (Receptor - binding domain, RBD) for binding with the host receptor, Angiotensin Converting Enzyme 2 (Wang et al., 2020). The Spike protein, which has undergone a number of mutations, has been focused on the development of a vaccine. Nevertheless, we also need to select a suitable drug target against the virus, which has succeeded in entering into host cells. Our present studies have focussed on the most stable protein, Envelope Protein, which has shown its consistent presence in a number of assays we have performed. Although other studies have also focussed on E protein as the most non-mutated protein (Rahman et al., 2021), their studies pertain to available sequences of E protein, and no wet lab studies have been done. Zhou et al. 2023 have also reviewed the sequences of SARS-CoV-2 to develop a suitable drug target. Other studies have focussed on all the SARS-CoV-2 proteins for developing therapeutic targets (Yan et al., 2022). Of the PCR assays performed on 76 COVID-19-positive samples during the course of our study, E protein was consistently present in even clinical isolates, and realizing the fact that the E protein has a 98% conserved sequence (Rahman et al., 2021), it could be the most abundantly available intracellular viral target to trap through suitable drug molecule. Other studies have also deposited genomic sequences of SARS-CoV-2 in the database (Meredith et al., 2020), and it will make it easy to understand the inter-sample variations. However, the reported mutations in different samples are very few (Harvey et al., 2021).

Materials and Methods

The nasopharyngeal swab samples of the patients referring to Sharda Hospital, Greater Noida, U.P., were taken for the study. The samples which were tested positive using the COVID-19 RT-PCR kit (m/s Trivitron Healthcare, India) were collected and maintained at -80 °C until use. The RNA was extracted using a QIA viral RNA extraction (m/s Qiagen, CA) kit following the manufactures instructions. 60µl of RNA was extracted, and of this, 5µl was taken for the amplification process. A one-step RT-PCR kit (m/s Ambion, USA) was used employing the manufacturer's protocol (Reaction Mix: 12.5µl; Enzyme mix of Reverse Transcriptase and Taq Polymerase: 1µl, $MgSO_4$:0.5µl, nuclease-free water: 4µl and respective forward and reverse primers: 0.5µl each). In-house primers were designed for four genes: S gene, N gene, ORF1ab, and E gene. These were synthesized commercially by Eurofins, India. The PCR cycling conditions were as follows: reverse transcription, 55°C for 20 minutes, amplification for 50 cycles, each cycle of 95°C for 3 minutes, 95°C for 15 seconds, and 58°C for 30 seconds, and final extension 4°C for 30 seconds. After amplification, gel electrophoresis was performed using 2% agarose gel and the image was capture by Gel Documentation System USA.

For the primers and their sequences, which were designed in-house, the GISAID (Global Initiative on Sharing All Influenza Data; https://gisaid.org/), Germany and NCBI, USA was searched for the reference SARS-CoV-2 genomes, Indian Strain. The genome reported first from India was from the state of Karnataka and was selected as a reference genome (ID: OM073843) for the study. Using the NCBI primer designing software and Primer 3 plus software, the primers for Genes Spike (S), Nucleocapsid (N), ORF1ab, and Envelope (E) were deduced (Table 1).

Results

There were 76 positive SARS-CoV-2 samples collected from 2020 to 2023. Each sample was individually amplified for all four genes, and the amplicon sizes were analyzed by Agarose gel electrophoresis. The presence or absence of bands for few samples are shown in Figures 1 to 7 and summarized in Table 2.

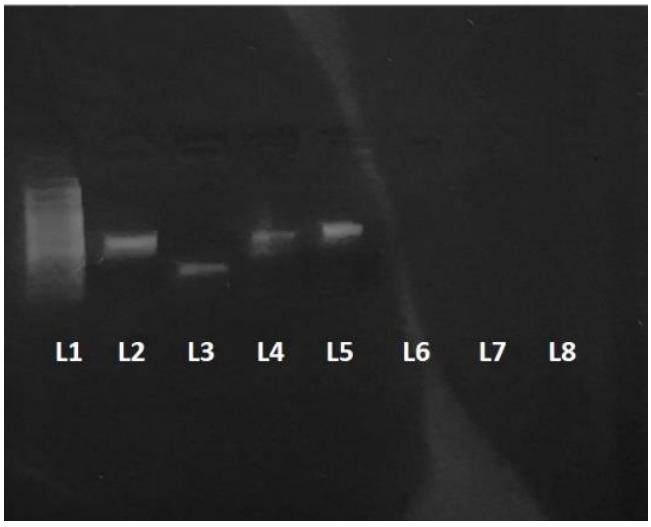

Figure 1. Agarose Gel showing bands for SARS-COV-2 genes (sample no. ACVI/COVID/04; L1: DNA ladder; L2: amplified with primer for S gene; L3: amplified with primer for E gene; L4: amplified with primer for N gene; L5: amplified with primer for ORF1ab gene).

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Table 1. In-house primers designed for the amplification of SARS-CoV-2 positive samples.

Sl. No.	Target Gene	Primer type	Primer sequence 5' to 3'	Expected size in base pair
1.	Nucleocapsid (N)	Forward Primer	GGTTCACCGCTCTCACTCAA	519
		Reverse Primer	CAAGCAGCAGCAAAGCAAGA	
2.	ORF1ab (ORF)	Forward Primer	GCCGCTGTTGATGCACTATG	587
		Reverse Primer	CTCCAAGCAGGGTTACGTGT	
3.	Spike (S)	Forward Primer	CTGCACTGTTAGCGGGTACA	546
		Reverse Primer	GTGCTGACTGAGGGAAGGAC	
4.	Envelope (E)	Forward Primer	TCGTTTCGGAAGAGACAGGT	216
		Reverse Primer	AGACCAGAAGATCAGGAACTCT	

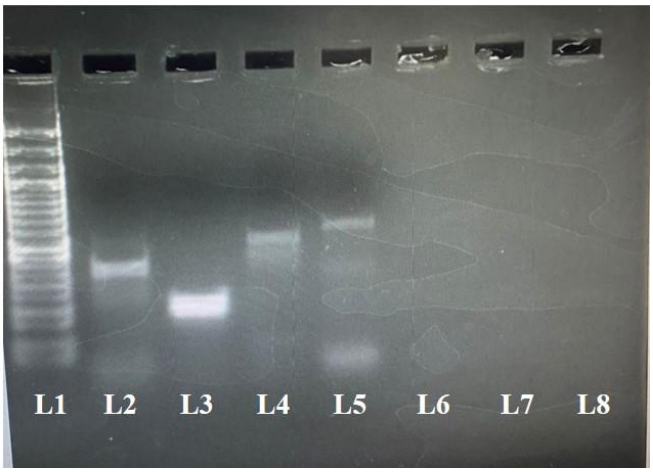

Figure 2. Agarose Gel showing bands for SARS-COV-2 genes (sample no. ACVI/COVID/09; L1: DNA ladder; L2: amplified with primer for S gene; L3: amplified with primer for E gene; L4: amplified with primer for N gene; L5: amplified with primer for ORF1ab gene).

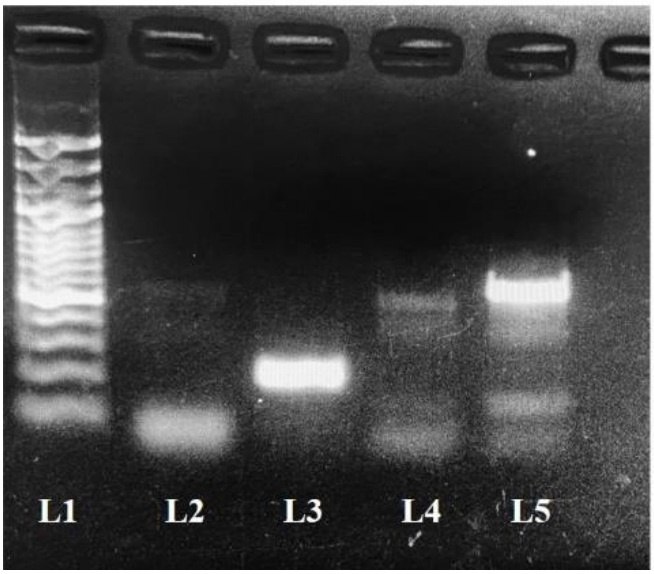

Figure 3. Agarose Gel showing bands for SARS-COV-2 genes (sample no. ACVI/COVID/10; L1: DNA ladder; L2: amplified with primer for S gene; L3: amplified with primer for E gene; L4: amplified with primer for N gene; L5: amplified with primer for ORF1ab gene).

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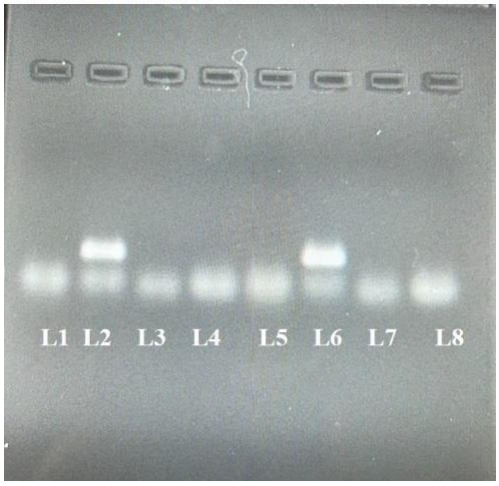

Figure 4. Agarose Gel showing bands for SARS-COV-2 genes (sample no. ACVI/COVID/25: L1: amplified with primer for S gene; L2: amplified with primer for E gene; L3: amplified with primer for N gene; L4: amplified with primer for ORF1ab gene; sample no. ACVI/COVID/26: L5: amplified with primer for S gene; L6: amplified with primer for E gene; L7: amplified with primer for N gene; L8: amplified with primer for ORF1ab gene).

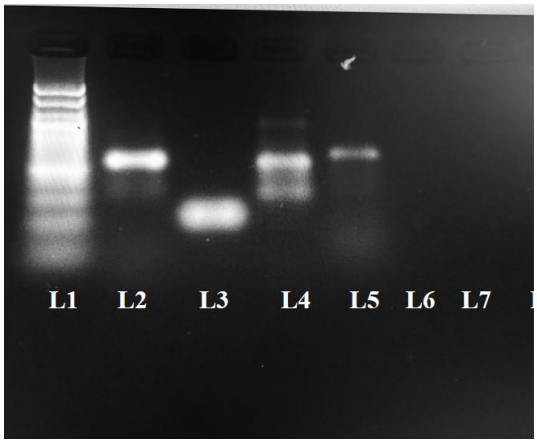

Figure 5. Agarose Gel showing bands for SARS-COV-2 genes (sample no. ACVI/COVID/30: L1: DNA ladder; L2: amplified with primer for S gene; L3: amplified with primer for E gene; L4: amplified with primer for N gene; L5: amplified with primer for ORF1ab gene).

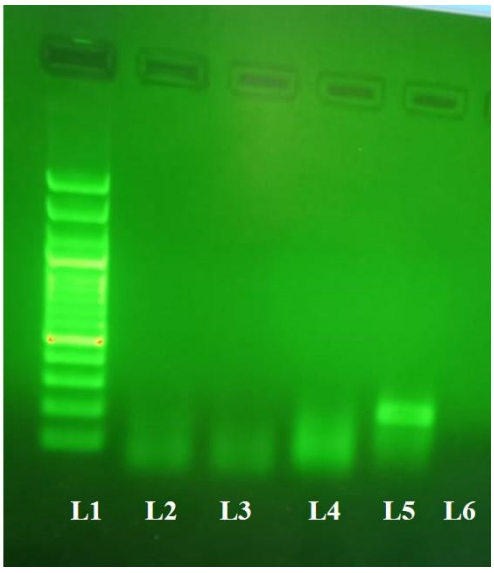

Figure 6. Agarose Gel showing bands for SARS-COV-2 genes (sample no. ACVI/COVID/45: L1: DNA ladder; L2: amplified with primer for Orf1ab gene; L3: amplified with primer for S gene; L4: amplified with primer for N gene; L5: amplified with primer for E gene).

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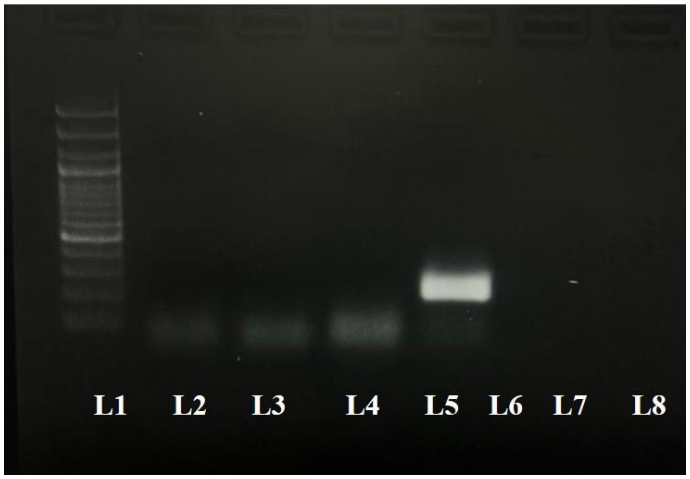

Figure 7. Agarose Gel showing bands for SARS-COV-2 genes (sample no. ACVI/COVID/55: L1: DNA ladder; L2: amplified with primer for ORF1ab gene; L3: amplified with primer for N gene; L4: amplified with primer for S gene; L5: amplified with primer for E gene).

Table 2. Analysis of the bands displayed in the SARS-CoV-2 positive samples.

Sl. No.	Sample code	Stain type	Bands displayed after amplification employing RT-PCR			
			S gene	E gene	N gene	ORF 1ab gene
1.	ACVI/COVID/01	Wuhan	-	✓	✓	-
2.	ACVI/COVID /02	Wuhan	✓	✓	✓	✓
3.	ACVI/COVID/ 03	Wuhan	✓	✓	✓	✓
4.	ACVI/COVID/31	Wuhan	-	✓	-	-
5.	ACVI/COVID/32	Wuhan	-	✓	-	-
6.	ACVI/COVID/33	Wuhan	-	✓	-	-
7.	ACVI/COVID/34	Wuhan	-	✓	-	-
8.	ACVI/COVID/35	Wuhan	-	✓	-	-
9.	ACVI/COVID/36	Wuhan	-	✓	-	-
10.	ACVI/COVID/42	Wuhan	-	✓	-	-
11.	ACVI/COVID/43	Wuhan	-	-	-	-
12.	ACVI/COVID/44	Wuhan	-	✓	-	-
13.	ACVI/COVID/45	Wuhan	-	✓	-	-
14.	ACVI/COVID/46	Wuhan	-	✓	-	-
15.	ACVI/COVID/47	Wuhan	-	✓	-	-
16.	ACVI/COVID/48	Wuhan	-	✓	-	-
17.	ACVI/COVID/49	Wuhan	-	✓	-	-
18.	ACVI/COVID/50	Wuhan	-	✓	-	-
19.	ACVI/COVID/51	Wuhan	-	-	-	-
20.	ACVI/COVID/52	Wuhan	-	✓	-	-
21.	ACVI/COVID/63	Wuhan	-	✓	-	-
22.	ACVI/COVID/64	Wuhan	-	✓	-	-
23.	ACVI/COVID/76	Wuhan	-	✓	-	-
	Total: 23		**2 (8.69%)**	**21 (91.30%)**	**3 (13.04%)**	**2 (8.69%)**
24.	ACVI/COVID/04	Omicron	✓	✓	✓	✓
25.	ACVI/COVID/05	Omicron	✓	✓	✓	✓
26.	ACVI/COVID/06	Omicron	-	-	-	✓
27.	ACVI/COVID/07	Omicron	✓	✓	✓	✓

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28.	ACVI/COVID/08	Omicron	✓	✓		✓
29.	ACVI/COVID/09	Omicron	✓	✓	✓	✓
30.	ACVI/COVID/10	Omicron	✓	✓	✓	✓
31.	ACVI/COVID/11	Omicron	-	-	-	-
32.	ACVI/COVID/12	Omicron	-	-	-	-
33.	ACVI/COVID/13	Omicron	-	-	-	✓
34.	ACVI/COVID/14	Omicron	✓	✓	✓	✓
35.	ACVI/COVID/15	Omicron	-	✓	-	-
36.	ACVI/COVID/16	Omicron	-	✓	-	-
37.	ACVI/COVID/17	Omicron	✓	✓	-	-
38.	ACVI/COVID/18	Omicron	-	-	-	-
39.	ACVI/COVID/19	Omicron	-	✓	✓	-
40.	ACVI/COVID/20	Omicron	-	✓	-	-
41.	ACVI/COVID/21	Omicron	-	✓	-	-
42.	ACVI/COVID/22	Omicron	-	✓	-	-
43.	ACVI/COVID/23	Omicron	-	-	-	-
44.	ACVI/COVID/24	Omicron	-	-	-	✓
45.	ACVI/COVID/25	Omicron	-	✓	-	-
46.	ACVI/COVID/26	Omicron	-	✓	-	-
47.	ACVI/COVID/27	Omicron	-	✓	-	-
48.	ACVI/COVID/28	Omicron	-	✓	-	-
49.	ACVI/COVID/29	Omicron	-	✓	-	-
50.	ACVI/COVID/30	Omicron	✓	✓	✓	✓
51.	ACVI/COVID/39	Omicron	-	-	-	-
52.	ACVI/COVID/40	Omicron	-	✓	-	-
53.	ACVI/COVID/41	Omicron	-	-	-	-
54.	ACVI/COVID/53	Omicron	-	-	-	-
55.	ACVI/COVID/68	Omicron	-	-	-	-
56.	ACVI/COVID/69	Omicron	-	-	-	-
	Total: 33		**9 (27.77%)**	**21 (63.63%)**	**8 (24.24%)**	**11 (33.33%)**
57.	ACVI/COVID/37	Delta	-	✓	-	-
58.	ACVI/COVID/38	Delta	-	✓	-	-
59.	ACVI/COVID/54	Delta	-	✓	-	-
60.	ACVI/COVID/55	Delta	-	✓	-	-
61.	ACVI/COVID/56	Delta	-	✓	-	-
62.	ACVI/COVID/57	Delta	-	✓	-	-
63.	ACVI/COVID/58	Delta	-	✓	-	-
64.	ACVI/COVID/59	Delta	-	-	-	-
65.	ACVI/COVID/60	Delta	-	✓	-	-
66.	ACVI/COVID/61	Delta	-	✓	-	-
67.	ACVI/COVID/62	Delta	-	✓	-	-
68.	ACVI/COVID/65	Delta	-	✓	-	-
69.	ACVI/COVID/66	Delta	-	✓	-	-
70.	ACVI/COVID/67	Delta	-	✓	-	-
71.	ACVI/COVID/70	Delta	-	✓	-	-

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72.	ACVI/COVID/71	Delta	-	✓	-	-
73.	ACVI/COVID/72	Delta	-	✓	-	-
74.	ACVI/COVID/73	Delta	-	✓	-	-
75.	ACVI/COVID/74	Delta	-	✓	-	-
76.	ACVI/COVID/75	Delta	-	✓	-	-
	Total: 20		**0 (0%)**	**19 (95.0%)**	**0 (0%)**	**0 (0%)**

Discussion

The footprints of the early form of Coronavirus, which was supposedly 2000 years old, have been seen in our genome (Farhud et al., 2021; Farhud and Mojahed, 2022). This suggests how our system has evolved successfully, contributing to fitness in spite of these viral epidemics (Enard and Petrov, 2020). The Coronavirus during its evolution from commonly circulating strains in the form of Human coronavirus 229E, Human coronavirus NL63, Human coronavirus OC43, Human coronavirus HKU1 to the virulent strains in the form of SARS-CoV-1, MERS and SARS-CoV-2 has led to the inclusion of many mutations in each of the structural and non-structural proteins (unpublished data, 2023). The SARS-CoV-2 being a RNA virus adds to the fact that there are many possibilities from evolution, ecology and epidemiology points of view that change/modify the course of the virus's transmission, mutation rate, and infective nature (Pybus and Rambaut, 2009). Some of these variations remain, while some are lost due to the bottleneck effect (Clarke et al., 1993). It has been reported in a study done by Ghafari and co-workers that with time, there was a drop in the substitution rate of the virus by nearly 50% (Ghafari et al., 2022; Markov et al., 2023). D614G was the first substitution seen in the spike protein of the SARS-CoV-2 virus (Volz et al., 2021).

Along similar lines, we focused on the consistence of viral structural proteins in various cases of SARS-CoV-2 infection, i.e., Wuhan, Delta, and Omicron variants. Of the four genes taken for the study, it was observed that the appearance of the genes was sometimes different in all the samples. Interestingly, it was observed that it is the Envelope (E) protein that exhibited maximum stability. During the entry into the host cell, it is the structural proteins that interact with various receptors and proteins present on the surface of the cell. Then, the genome takes entry to start the process of replication and multiplication (Santos-Mendoza, 2023). Thus, the structural proteins are of utmost importance and the Envelope protein is one part of the important structural components and its presence in almost 84.21% of the cases justifies this as new drug target.

The present study sensitizes many virological and host-virus interactions after observing the persistence and depletion of four structural proteins in infection caused by three different SARS-CoV-2 strains viz; Wuhan, Delta and Omicron strains mainly responsible for the three consecutive waves of COVID-19 Pandemic. The persistence of E protein in the majority of the clinical samples in all three pandemic strains highlights its conserved nature, as also reported by other studies (Rahman et al., 2021), and also establishes that immunological neutralization of this proteins by the host system could not be successful. Our observations further comprehend the presence of E protein in 91.3 % of samples of the Wuhan strain, 63.6 % in the Omicron strain, and 95% of samples of the Delta strain. The epidemiological data (Shahbaz et al., 2023) suggest that of the three strains, Wuhan and Delta caused the most severities and mortalities whereas Omicron caused only mild morbidity. The strains responsible for severities due to COVID-19 could be due to the presence of E protein in them and this could be the preferred drug target to reduce the severities of infection. For the selection of a particular pathogenic protein, to interrupt its replication within the host cell, the E protein has emerged as the best choice as a drug target and our observations could attract the development of a drug target against this viral protein. There are in-silico studies and reviews on the possible role of E protein as a drug target (Mandala et al., 2020; Chernyshev, 2020; Park et al., 2021; Das et al., 2021; Xin et al., 2021), however, we report for the first time the evidence of wet lab studies on this protein and relate its persistence with the corresponding severities caused by different strains.

Conclusion

Intracellular synthesis of viral proteins causes cell damage and deprives the infected organs of their essentially required peptides. The foreignness of the viral proteins for their antigenic molecular weight sensitizes adaptive immunity. However, lighter proteins escape the immunogenic response and may prove more pathogenic. We report the role of the E protein (75 amino acids) as the cause of the severity of COVID-19 and recommend

the need for the development of appropriate drug molecules against the E protein.

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Conflict of Interest

The authors declare no conflict of interest.

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