

# SARS-CoV-2 ORF8 gene CAA=TAA and AAA=TAA Termination Codon Mutations found mostly in B.1.1.7 Variants was Independent of Popular L84S Point Mutations

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## Abstract

Five VOCs of SARS-CoV-2 mainly caused million deaths worldwide and named as B.1.1.7 (U.K.), B.1.351 (South Africa), P.1 (Brazil), B.1.617.2 (India), and B.1.1.529 (Africa). In HIV mediated pathogenesis, small trans activator proteins (TAT, NEF, REV) modulate transcription of cellular genes. Similarly, preliminary reports indicated that corona virus ORF8 protein acts as histone mimics disrupting chromatic structure with many epigenetic changes and immune modulator functions. ORF8 protein had also some similarities to immunoglobulin domains and inhibited HMC-1 and IFN-beta functions.

During evolution a 382-nucleotide deletion ( $\Delta 382$ ) in the ORF8 region of the corona virus genome leads to weak virus load and weak pathogenicity (accession no. MT374101). We BLAST searched deletion boundary and was selected few ORF8 protein truncated mutants. The C>T base change at 27972nt and another A>T base change at 28095nt created two termination codons (CAA=TAA and AAA=TAA) to produce 26AA and 67AA long ORF8 truncated proteins. Similar Blast-N search with oligonucleotides selected at the mutation boundaries gave many ORF8 mutants with distinct S24L, V32L, P38S, R52I, A65V, Y73C, L84S, K92E and V100L mutations with or without TAA termination mutations.

Major mutations found in B.1.1.7 lineage which had spike 69HV and 145Y mutations and ORF1ab polyprotein 3675KSF deletion. However, one ORF8 mutant (accession no. OW221449) belongs to Omicron BA.2 variant with 24LPP spike deletion and others to Omicron BA.5 variants (accession nos. OP733645 and OP671680) with 24LPP and 69HV deletion in the spike protein. One termination codon mutant (accession no. OP711842) has also 63nt ORF7a/b deletions. Mutation did not change the hairpin structure in the ORF8 gene and ORF8 protein formed dimeric stable globular 3-D structure to interact with many host proteins. Clearly, generation of such abundant B.1.1.7 lineage ORF8 protein truncated mutants may be one of the causes for the extinction of Alpha variant of corona virus in 2021. Roles of ORF8 mutants as host proteins modulator were explained in light of other deletions and mutations in corona virus genome.

**Keywords:** ORF8 protein, SRAS-CoV-2 Mutation, Termination Codon, Truncated ORF8, Host Protein Modulator.

## Introduction

The SARS-CoV-2 is the causative agent of the coronavirus disease 2019 (COVID-19), a large global outbreak with severe public health consequences and about million deaths [1]. The MERS-Cove, responsible for several outbreaks since 2012 also has been well characterized. The novel SARS-CoV-2 shares nearly 96% similarity to the bat coronavirus isolate RaTG13, suggesting these

animals are the likely natural reservoir of the virus [2].

Thus, different animal, birds and whale corona viruses were known since 2003 but human corona viruses were appeared in December, 2019 at Wuhan province of China. Within 2 years few thousand variants were sequenced and divided into Alpha, Beta, Delta, Gamma as well as Omicron variants [3]. Analysis suggested

that spike protein (1273AA) of COVID-19 had gone extensive mutations and deletions than large polyprotein ORF1ab (7096aa). The LPP, HV, VYY, FR, L and Y were major deletions occurred in the spike whereas EPE insertion was also reported in Omicron BA.1 variant.

Among the ORF1ab deletions, KSF deletion in nsp1 domain was found only in omicron BA.4 sub-variants and LSG deletion found in omicron BA.1 sub-variant whereas SGF deletion in nsp6 domain was found in most Omicron and Alpha variants [4]. Dominant point mutations D614G and N501Y were important that increased transmission and pathogenicity whereas ~30 mutations mostly in the RBP domain were reported that were not found in deadly B.1.1.7, B.1.617.2 and AY.103 variants. The E484A, T478K, L452R and K417N/T, were very immune-modular. However, Omicron variants were less pathogenic and usually did not require oxygen support and hospitalization unless co-morbidity. Still pneumonia, cough and cold, chest pain, confusion and headache were different symptoms that affected >630 million people worldwide.

SARS-CoV-2 is a large positive-stranded RNA virus with ~30000 nucleotides genome. It has structural proteins Membrane (M), Envelope (E), Nucleocapsid (N), Spike (S) coded from 3'-1/3 part of the virus independently but RNA-dependent RNA polymerase was coded from nsp12 domain of ORF1ab polyprotein coded from 2/3 of the 5'-parts of the genome and such polyprotein was degraded into sixteen polypeptides (nsp1-nsp16) [5]. The nsp2 protein is RNA topoisomerase whereas Nsp3 and nsp5 are proteases. The nsp6, nsp7, nsp8, nsp9 and nsp10 were small accessory proteins involved in RNA polymerase replication complex [5].

The nsp14 and nsp15 are nucleases and nsp16 is methyltransferase (C and as well as nsp13 is RNA helicase [6, 7]). Nsp11 is a small peptide and function was not known. ORF3a, ORF6, ORF7a/7b, ORF8 and ORF10 small proteins also coded from 3' end of the genome and have roles in regulating cellular genes [8]. Many drugs were discovered against proteases and RNA polymerases but vaccines were only important remedy that halted the corona virus spread.

Attenuated virus vaccine was developed but engineered spike protein DNA vaccine was very successful whereas mRNA vaccine was also reported. Here, we reported that small ORF8 protein synthesis was hampered due to creation of TAA termination codon in mRNA creating 26AA truncated ORF8 protein. This phenomenon was shown to reduce corona virus load and less severe pathogenicity and likely virus was cleared by host immune system.

## Methods

We searched PubMed to get idea on published papers on ORF8 and also searched SARS-CoV-2 NCBI database using BLAST-N and BLAST-X search methods. Multi-alignment of protein was done by

Multiline software [9] and multi-alignment of DNA by CLUSTAL-Omega software (Sievers, F et al., 2011). 1st impression of ORF8 mutants was gained by Blast search of deletion boundary of 120nt sequence and analyzing the sequences with 90-100% similarities.

Blast search of ORF8 full length gene used to get mutant ORF8 proteins with or without termination codon [10]. Then, the other ORF8 mutants were detected by Blast-N search of TAA mutant oligos as well as other oligos selected from point mutation boundaries. Hairpin structure of ORF8 gene 222nt 5'-terminal sequence was done by Oligoaniline 3.1 software (Integrated DNA Technologies). The protein 3-D structure was determined by SWISS-Model software (<https://swissmodel.expasy.org/>) [11].

## Results

The genes and proteins in the corona virus (~30kb) and the sequence of ORF8 protein were shown in figure-1. BLAST-N search first selected three ORF8 gene TAA termination mutants (accession nos. MZ212478, MZ294172 and OK234981) using  $\Delta$ 382 deletion oligo. There was a G>T mutation at 27972nt causing CAA change to 1st TAA termination codon and only 26AA ORF8 protein was produced instead 121 AA. There was a 2nd termination codon created at 68K amino acid codon (AAA=TAA) causing 68AA ORF8 protein.

In accession nos. OP735325, OP735327 and OP735334, there was a C>T mutation at 27964 causing ORF8 S24L mutation. However, there was a creation of another G>T mutation at 28048nt in termination mutants (accession nos. MZ212478, MZ294172 and OK234981) causing R52I AA change. Another Y73C mutation in the ORF8 protein was located nearby 2nd termination codon (figure-2).

Then, we used 1st termination oligo and 2nd termination oligo (Table-1) to select more termination mutants and multi-aligned together (Figure-2A; accession nos. OP711844 and OP711836). The 30kb corona virus genomes alignment was huge (>100 pages) so only the desired portions of the alignment were shown in figures. Figure-2B showed unusual GHVMV deletion (5'-GGTCATGTTATGGTT-3') in the ORF1ab polyprotein in one termination mutant (accession no. OP711844) while all five termination mutants had SGF deletion.

In figure-2C, we demonstrated that all five TAA mutants had HV and Y deletions in the spike protein indicating Alpha (B.1.1.7) variant type. Surely such variant also contained N501Y and D614G dominant mutations in the spike protein required for enhanced transmission and disease severity (data not shown). We further aligned the spike proteins to confirmed the above finding through genetic alignment that indeed 69HV and 145Y deletions occurred (figure-2D).

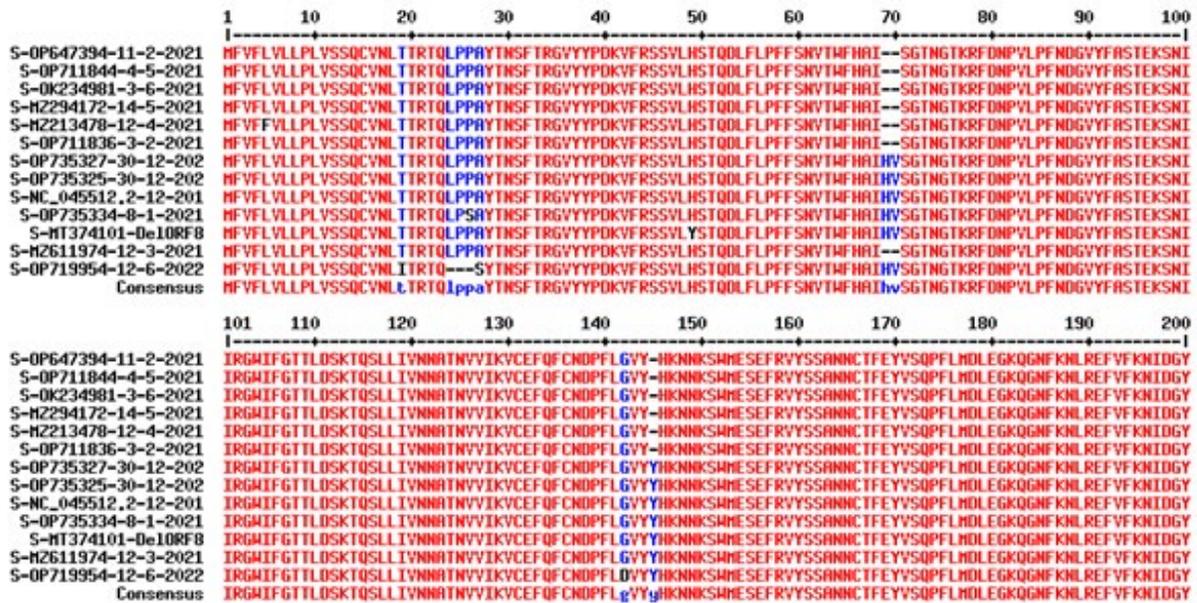


Acc. no. Date of isolation	ORF1ab region
MZ213478-12-4-2021	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-537
MZ294172-14-5-2021	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-538
OK234981-3-6-2021	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-468
OP711836-2nd	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-486
OP711844-1st	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-471
MZ611974-12-3-2021	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-501
OA982176-10-9-2020	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-540
MW593419-20-5-2020	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-498
B-NC_045512.2-12-2019	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-540
OP735325-30-12-2020	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-502
OP735325-8-1-2021	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-502
OP735327-30-12-2020	acggttcgggatgctcgaactgcacccatggtcatggtatgggttagctggtagcagaact-503
	*****
MZ213478-12-4-2021	tagtttg--SGF-----aagctaaaaagactggtggtatgtagcagctgtaggttact-11328
MZ294172-14-5-2021	tagtttg-----aagctaaaaagactggtggtatgtagcagctgtaggttact-11329
OK234981-3-6-2021	tagtttg-----aagctaaaaagactggtggtatgtagcagctgtaggttact-11259
OP711836-2nd	tagtttg-----aagctaaaaagactggtggtatgtagcagctgtaggttact-11277
OP711844-1st	tagtttg-----aagctaaaaagactggtggtatgtagcagctgtaggttact-11262
MZ611974-12-3-2021	tagtttgctcgggttttaagctaaaaagactggtggtatgtagcagctgtaggttact-11301
OA982176-10-9-2020	tagtttgctcgggttttaagctaaaaagactggtggtatgtagcagctgtaggttact-11340
MW593419-20-5-2020	tagtttgctcgggttttaagctaaaaagactggtggtatgtagcagctgtaggttact-11298
B-NC_045512.2-12-2019	tagtttgctcgggttttaagctaaaaagactggtggtatgtagcagctgtaggttact-11340
OP735325-30-12-2020	tagtttgctcgggttttaagctaaaaagactggtggtatgtagcagctgtaggttact-11302
OP735325-8-1-2021	tagtttgctcgggttttaagctaaaaagactggtggtatgtagcagctgtaggttact-11302
OP735327-30-12-2020	tagtttgctcgggttttaagctaaaaagactggtggtatgtagcagctgtaggttact-11303
	*****

**Figure 2B:** Deletion in the ORF1ab protein in few ORF8 TAA termination codon mutants.

Acc. No. Date of isolation	Spike protein region
MZ213478-12-4-2021	cttggtccttaccctttcctttccaatggttacttgggtccatgctatc--HV--tctgggac-21762
MZ294172-14-5-2021	cttggtccttaccctttcctttccaatggttacttgggtccatgctatc-----tctgggac-21763
OK234981-3-6-2021	cttggtccttaccctttcctttccaatggttacttgggtccatgctatc-----tctgggac-21693
OP711836-2nd TAA	cttggtccttaccctttcctttccaatggttacttgggtccatgctatc-----tctgggac-21711
OP711844-1st TAA	cttggtccttaccctttcctttccaatggttacttgggtccatgctatc-----tctgggac-21696
MZ611974-12-3-2021	cttggtccttaccctttcctttccaatggttacttgggtccatgctatc-----tctgggac-21735
OA982176-10-9-2020	cttggtccttaccctttcctttccaatggttacttgggtccatgctatcacatgctctctgggac-21780
MW593419-20-5-2020	cttggtccttaccctttcctttccaatggttacttgggtccatgctatcacatgctctctgggac-21738
B-NC_045512.2-12-2019	cttggtccttaccctttcctttccaatggttacttgggtccatgctatcacatgctctctgggac-21780
OP735325-30-12-2020	cttggtccttaccctttcctttccaatggttacttgggtccatgctatcacatgctctctgggac-21742
OP735325-8-1-2021	cttggtccttaccctttcctttccaatggttacttgggtccatgctatcacatgctctctgggac-21742
OP735327-30-12-2020	cttggtccttaccctttcctttccaatggttacttgggtccatgctatcacatgctctctgggac-21743
	*****
MZ213478-12-4-2021	tcaattttgtaaatgatccatttttgggtggtt-Y-taccacaaaaacaacaaaagttggat-21999
MZ294172-14-5-2021	tcaattttgtaaatgatccatttttgggtggtt---taccacaaaaacaacaaaagttggat-22000
OK234981-3-6-2021	tcaattttgtaaatgatccatttttgggtggtt---taccacaaaaacaacaaaagttggat-21930
OP711836-2nd	tcaattttgtaaatgatccatttttgggtggtt---taccacaaaaacaacaaaagttggat-21948
OP711844-1st	tcaattttgtaaatgatccatttttgggtggtt---taccacaaaaacaacaaaagttggat-21933
MZ611974-12-3-2021	tcaattttgtaaatgatccatttttgggtggttattaccacaaaaacaacaaaagttggat-21975
OA982176-10-9-2020	tcaattttgtaaatgatccatttttgggtggttattaccacaaaaacaacaaaagttggat-22020
MW593419-20-5-2020	tcaattttgtaaatgatccatttttgggtggttattaccacaaaaacaacaaaagttggat-21978
B-NC_045512.2-12-2019	tcaattttgtaaatgatccatttttgggtggttattaccacaaaaacaacaaaagttggat-22020
OP735325-30-12-2020	tcaattttgtaaatgatccatttttgggtggttattaccacaaaaacaacaaaagttggat-21982
OP735325-8-1-2021	tcaattttgtaaatgatccatttttgggtggttattaccacaaaaacaacaaaagttggat-21982
OP735327-30-12-2020	tcaattttgtaaatgatccatttttgggtggttattaccacaaaaacaacaaaagttggat-21983
	*****

**Figure 2C:** Multi-alignment of SARS-CoV-2 sequences as in (figure-2A) to show the deletion in the spike protein of ORF8 termination codon mutants. Deletion of 69HV and 145Y indicated that ORF8 mutants were B.1.1.7 variant.



**Figure 2D:** Multi-alignment of Spike proteins of ORF8 mutants as discussed above. The LPP deletion but not HV deletion indicated accession no. OP719954 belong to an Omicron BA.2 variant. The 69HV and 145Y deletions indicated B.1.1.7 lineage.

Figure-3 demonstrated the presence of R52I (AGA=ATA) mutation in ORF8 termination codon mutants while in figure-4 similar Y73C mutation (TAC=TGC) located after 2<sup>nd</sup> TAA termination codon was presented (accession nos. MZ213478, MZ294172 and OK234981). In figure-5, we also showed the S24L amino acid change in ORF8 gene without creating TAA termination codons. But in other search we found S24L mutants with creating ORF8 1<sup>st</sup> termination codon (see, figure-7B). Such findings were done by BLAST-X search of 60nt sequence with distinct mutation selected through multi-alignment.

Next, we demonstrated the abundant ORF8 protein L84S mutants as well as one 119DF two amino acids deletion mutant without generating TAA termination codons (figure-6A). No 1<sup>st</sup> (not shown here) and 2<sup>nd</sup> (boxed) TAA termination codons were found in ORF8 gene L84S mutants. These sequences were selected through BLAST-N search using L84S oligo (Table-1). Further, we confirmed the variant type of such mutants demonstrating ORF1ab and spike proteins deletions (figure-6B). The data indicated the presence of LPP and HV deletions in the spike as well as 31ERS deletion in the nucleocapsid (N) protein of corona virus confirming Omicron BA.5 variants (accession nos. OP733645, OP671680).

While accession no. OW221449 had only 24LPP deletion in the spike plus 31ERS deletion in the N protein being an Omicron BA.2 variant. Note that no 141KSF ORF1ab protein deletion was found which was a character of Omicron BA.4 variant. Neither EPE insertion in the spike was found which was a confirmation of Omicron BA.1 variant. Surprisingly, three Omicron variants ORF8 L84S mutants had 26nt 3'UTR deletion and characterization of such deletion never been discussed before [12]. The other accession numbers had no 157FR deletion in the spike and such variant were earlier variants like Beta and P.1 with D614G mutation.

However, Δ382 deletion mutant used to select first TAA mutants had D614 indicating primitive (2020) variant and such big deletion in the ORF8 gene was limited!

Next, we selected more S24L ORF8 mutations with 1<sup>st</sup> termination codon oligo plus S24L oligo by BLAST-N search (figure-7). In figure-7A we showed the alignment of many accession numbers with 100% homology and 100% cover with the oligo and many 1<sup>st</sup> TAA mutants of the ORF8 gene selected (figure-7B) whereas no mutation in the 2<sup>nd</sup> termination area was found. Given to the fact, we yet to get a 2<sup>nd</sup> termination mutant only without 1<sup>st</sup> TAA mutation.

Because, presence of 1<sup>st</sup> TAA created only 26AA ORF8 protein and need for the 2<sup>nd</sup> TAA not required then. It may happen that there is a sub-genomic initiation site up front of 2<sup>nd</sup> termination site creating more smaller spliced ORF8 mRNA and proteins [13].

In figure-7C we confirmed that among the many S24L ORF8 mutants accession numbers ON113700 and OU200773 were B.1.1.7 lineage with spike HV and 1Y deletions as well as SGF ORF1ab protein deletion. But no Delta variant and Omicron variant were detected in this search and thus other accession numbers might be primitive corona virus isolates. As we continued our search for TAA termination ORF8 mutants, we used 2<sup>nd</sup> TAA termination codon Y73C oligo. It appeared again that although we used 2<sup>nd</sup> termination oligo, we selected both 1<sup>st</sup> and 2<sup>nd</sup> TAA termination codons (figure-8). All B.1.1.7 variants were selected with SGF deletion in ORF1ab protein as well as 69HV+145Y spike deletions (data not shown).

Only OP711842 variant has 63nt deletion in ORF7a/7b region. Our attempts to get mutant with only ORF8 2<sup>nd</sup> termination codon was failed. We do not understand what is the necessary of 2<sup>nd</sup> termination codon as active 1<sup>st</sup> termination codon means no ORF8

protein generates. We found that search with R52I mutation oligo could not generated with a termination codon mutant. Further, we used full length ORF8 gene (normal vs TAA mutated plus point mutation vs TAA repaired plus point mutation) to BLAST-X search and identified few mutant ORF8 alleles which demonstrated in Figure-9A and figure-9B. In this search, we got V32L, P38S, R52I, A65V, Y73C, K92E and V100L mutations in the ORF8 gene.

Then, we aligned the ORF8 mutant protein as shown in Figure-10A and a phylogenetic analysis shown in figure-10B. Similarly, we addressed the RNA structure in the ORF8 gene region and found that no basic changes in hairpin structure was occurred between normal and TAA mutated RNAs (figure-11). We further addressed the overall 3-D protein structures of mutated ORF8 protein but with limitation, a compact folding was found as compared to

animal corona virus ORF8 protein available (PDB ID:7F8I). Ramachandran plot also confirmed the alpha-helical globular structure of the ORF8 protein. Such compact folded structure of ORF8 protein was necessary to interact with host proteins.

In summary, our search generated many ORF8 TAA 1<sup>st</sup> termination codon plus TAA 2<sup>nd</sup> termination codon mutants (accession numbers, MZ213478, MZ394172, OK234981, OP711836, OP711844, OP683545, OP711842, OP711837, OX020484, OP585720 and OW999874). Similarly, we demonstrated few only 1st TAA termination codon mutants (accession numbers, MZ497707, ON113700, OU200773, MW98643, OP735332 and ON084987). This confirmed that such ORF8 mutation created no ORF8 protein and likely reduced the severity of corona virus mediated pathogenesis.



**Figure 3:** Detection of R52I (AGA=ATA) mutation in ORF8 termination codon mutants (accession nos. MZ213478, MZ294172 and OK234981).

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    Acc.no.Date of isolation                                ORF8 2nd TAA region Y73C
M2213478-12-4-2021    atgaggctgggtcttaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28119
M2294172-14-5-2021    atgaggctgggtcttaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28120
OK234981-3-6-2021     atgaggctgggtcttaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28050
M2611974-12-3-2021    atgaggctgggtcttaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28094
OA982176-10-9-2020    atgaggctgggtcttaaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28140
MWS593419-20-5-2020   atgaggctgggtcttaaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28098
B-NC_045512.2-12-2019 atgaggctgggtcttaaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28140
OP735325-30-12-2020  atgaggctgggtcttaaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28102
OP735324-8-1-2021    atgaggctgggtcttaaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28102
OP735327-30-12-2020  atgaggctgggtcttaaatcaccattcagtgcatcgatcgcgtaattatacagtttccct-28103
*****

M2213478-12-4-2021  atgaggctgggtcttaatcaccattcagtgcatcgatcgcgtaattatacagtttccct 28119
blastX-ORF8
ORF8 protein [Severe acute respiratory syndrome coronavirus 2]
Sequence ID: QVU06321.1Length: 121.
Score          Expect      Method          Identities      Positives
39.3 bits(90)  0.030      Composition-based stats.  18/19(95%)     18/19(94%)
Query 3      EAGS*SPIQCIDIGNYTVS 59
              EAGS SPIQCIDIGNYTVS
Sbjct 64     EAGSKSPIQCIDIGNYTVS 82

B-NC_045512.2-12-2019 atgaggctgggtcttaatcaccattcagtgcatcgatcgcgtaattatacagtttccct 28140
blastX-Wuhan
ORF8 protein, partial [Severe acute respiratory syndrome coronavirus 2]Sequence ID: UHR93329.1Length: 90.
Score          Expect      Method          Identities      Positives
43.9 bits(102) 3e-04      Composition-based stats.  19/19(100%)    19/19(100%)
Query 3      EAGSKSPIQYIDIGNYTVS 59
              EAGSKSPIQYIDIGNYTVS
Sbjct 64     EAGSKSPIQYIDIGNYTVS 82

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**Figure 4:** Demonstration of ORF8 gene Y73C mutation (TAC=TGC) located after 2nd TAA termination codon. (A) Multi-alignment of ORF8 mutants and (B) BlastX search to get mutant AA.

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    Acc.no.Date of isolation                                2nd termination codon ORF8 protein region
M2213478-12-4-2021    agtcatgtacttaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27999
M2294172-14-5-2021    agtcatgtacttaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt28000
OK234981-3-6-2021     agtcatgtacttaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27990
M2611974-12-3-2021    agtcatgtactcaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27974
OA982176-10-9-2020    agtcatgtactcaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt28020
MWS593419-20-5-2020   agtcatgtactcaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27978
B-NC_045512.2-12-2019 agtcatgtactcaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt28020
OP735325-30-12-2020  agtcatgtactcaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27982
OP735324-8-1-2021    agtcatgtactcaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27982
OP735327-30-12-2020  agtcatgtactcaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27982
*****
M2213478-12-4-2021    agtcatgtacttaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27999
blastX-ORF8
ORF8 protein, partial [Severe acute respiratory syndrome coronavirus 2]Sequence ID: UAM18414.1Length: 55.
Score          Expect      Method          Identities      Positives
42.0 bits(97)  0.001      Compositional matrix adjust.  18/19(95%)     18/19(94%)
Query 3      SCT*HQPYWDDPCPIHFY 59
              SCT HQPYWDDPCPIHFY
Sbjct 24     SCTQHQPYWDDPCPIHFY 42
OP735327-30-12-2020  agtcatgtactcaaacatcaaccatagttagttgatgaccggtgtcctattcacttctatt27982
blastX-ORF8
ORF8 protein, partial [Severe acute respiratory syndrome coronavirus 2]Sequence ID: UDH89230.1Length: 84.
Score          Expect      Method          Identities      Positives
45.4 bits(106) 8e-05      Compositional matrix adjust.  19/19(100%)    19/19(100%)
Query 3      LCTQHQPYWDDPCPIHFY 59
              LCTQHQPYWDDPCPIHFY
Sbjct 24     LCTQHQPYWDDPCPIHFY 42

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**Figure 5:** Demonstration of S24L amino acid change in ORF8 mutant without creating TAA termination codons. But in other search we found S24L mutants with creating ORF8 1st termination codon (see, figure-7B).

Acc. no. Date of virus isolation			
OW221449-8-3-2022	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28122	ORF8 protein region
OP732645-4-10-2022	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28114	
OP671680-5-10-2022	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28077	
OP581726-3-2-2021	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28077	
OP471800-26-3-2020	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28086	
ONS32661-15-2-2022	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28092	
ON653598-12-9-2020	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28129	
OW863380-6-2-2022	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28129	
NC_045512.2-12-2019	atgaggctgggttcaaaatcaccattcagttacatcgatatcggtaattatacagtttcct	28140	
	***** 2nd termination zone *****		
OW221449-8-3-2022	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28182	
OP732645-4-10-2022	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28174	
OP671680-5-10-2022	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28137	
OP581726-3-2-2021	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28137	
OP471800-26-3-2020	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28146	
ONS32661-15-2-2022	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28152	
ON653598-12-9-2020	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28189	
OW863380-6-2-2022	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28189	
NC_045512.2-12-2019	gttcaccttttacaattaaatgcccaggaaacctaaattgggtagctctgtagtcgctgtgt	28200	
	***** L84S *****		
OW221449-8-3-2022	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28242	
OP732645-4-10-2022	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28234	
OP671680-5-10-2022	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28197	
OP581726-3-2-2021	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28191	
OP471800-26-3-2020	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28206	
ONS32661-15-2-2022	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28212	
ON653598-12-9-2020	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28249	
OW863380-6-2-2022	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28249	
NC_045512.2-12-2019	cgttctatgaagacttttttagagtatcatgacgttcggtgtgttttagatttcactctaaa	28260	
	***** ORF8 termination codon *****		

**Figure 6A:** Demonstration of abundant ORF8 protein L84S mutants as well as one 119DF deletion mutant without generating TAA termination codons. No 1st termination codon (not shown here) and 2nd termination codon (boxed) were found in ORF8 gene L84S mutants.

OW221449-8-3-2022	tagtttg-----aaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11331	
OP732645-4-10-2022	tagtttg---8GF---aaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11329	
OP671680-5-10-2022	tagtttg-----aaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11292	
OP581726-3-2-2021	tagtttgctgggtttaaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11286	
OP471800-26-3-2020	tagtttgctgggtttaaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11286	
ONS32661-15-2-2022	tagtttgctgggtttaaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11292	
ON653598-12-9-2020	tagtttgctgggtttaaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11329	
OW863380-6-2-2022	tagtttgctgggtttaaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11329	
NC_045512.2-12-2019	tagtttgctgggtttaaagctaaagactgtgttatgtatgcatcagctgtagttgtact	11340	
	*****		
OW221449-8-3-2022	tcagtggtttaaacttataaccagaactcaat-----catabacactaaatctttcac	21642	
OP732645-4-10-2022	tcagtggtttaaacttataaccagaactcaat---LFP---catabacactaaatctttcac	21640	
OP671680-5-10-2022	tcagtggtttaaacttataaccagaactcaat-----catabacactaaatctttcac	21603	
OP581726-3-2-2021	tcagtggtttaaacttataaccagaactcaatbaccctcgcatacactaaatctttcac	21606	
OP471800-26-3-2020	tcagtggtttaaacttataaccagaactcaatbaccctcgcatacactaaatctttcac	21606	
ONS32661-15-2-2022	tcagtggtttaaacttataaccagaactcaatbaccctcgcatacactaaatctttcac	21612	
ON653598-12-9-2020	tcagtggtttaaacttataaccagaactcaatbaccctcgcatacactaaatctttcac	21649	
OW863380-6-2-2022	tcagtggtttaaacttataaccagaactcaatbaccctcgcatacactaaatctttcac	21649	
NC_045512.2-12-2019	tcagtggtttaaacttataaccagaactcaatbaccctcgcatacactaaatctttcac	21660	
	*****		
OW221449-8-3-2022	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21762	
OP732645-4-10-2022	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21754	
OP671680-5-10-2022	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21717	
OP581726-3-2-2021	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21726	
OP471800-26-3-2020	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21726	
ONS32661-15-2-2022	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21732	
ON653598-12-9-2020	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21769	
OW863380-6-2-2022	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21769	
NC_045512.2-12-2019	cttgttctaaccttcttttccaatggttactgtggttccatgctatacatgctctctgggac	21780	
	*****		
OW221449-8-3-2022	ggttgggtggaccctcagattcaactggcagtaaacagaaatg---ERS---gtggggcgcg	28353	
OP732645-4-10-2022	ggttgggtggaccctcagattcaactggcagtaaacagaaatg-----gtggggcgcg	28345	
OP671680-5-10-2022	ggttgggtggaccctcagattcaactggcagtaaacagaaatg-----gtggggcgcg	28308	
OP581726-3-2-2021	ggttgggtggaccctcagattcaactggcagtaaacagaaatgggagaacgcagtgggggcgcg	28311	
OP471800-26-3-2020	ggttgggtggaccctcagattcaactggcagtaaacagaaatgggagaacgcagtgggggcgcg	28326	
ONS32661-15-2-2022	ggttgggtggaccctcagattcaactggcagtaaacagaaatgggagaacgcagtgggggcgcg	28332	
ON653598-12-9-2020	ggttgggtggaccctcagattcaactggcagtaaacagaaatgggagaacgcagtgggggcgcg	28369	
OW863380-6-2-2022	ggttgggtggaccctcagattcaactggcagtaaacagaaatgggagaacgcagtgggggcgcg	28369	
NC_045512.2-12-2019	ggttgggtggaccctcagattcaactggcagtaaacagaaatgggagaacgcagtgggggcgcg	28380	
	*****		
OW221449-8-3-2022	gggaggacttgaagagcaccacatcttttcacc-----3' UTR-----t	29707	
OP732645-4-10-2022	gggaggacttgaagagcaccacatcttttcacc-----t	29699	
OP671680-5-10-2022	gggaggacttgaagagcaccacatcttttcacc-----t	29662	
OP581726-3-2-2021	gggaggacttgaagagcaccacatcttttcaccgagggccacacggagtagcagtcagagtg	29691	
OP471800-26-3-2020	gggaggacttgaagagcaccacatcttttcaccgagggccacacggagtagcagtcagagtg	29706	
ONS32661-15-2-2022	gggaggacttgaagagcaccacatcttttcaccgagggccacacggagtagcagtcagagtg	29712	
ON653598-12-9-2020	gggaggacttgaagagcaccacatcttttcaccgagggccacacggagtagcagtcagagtg	29749	
OW863380-6-2-2022	gggaggacttgaagagcaccacatcttttcaccgagggccacacggagtagcagtcagagtg	29749	
NC_045512.2-12-2019	gggaggacttgaagagcaccacatcttttcaccgagggccacacggagtagcagtcagagtg	29760	
	*****		

**Figure 6B:** Demonstration of differential ORF1ab, S, N and 3'-UTR deletions in ORF8 L84S mutants. The accession nos. OW221449, OP732645, OP671680 were Omicron variant with ERS N-protein deletion. The OW221449 is omicron BA.2 with no 69HV deletion whereas both OP732645 and OP671680 are omicron BA.5 as no 141KSF deletion for BA.4 variant and no EPE insertion for BA.1 variant. However, we did not find B.1.1.7 variant here.

Sequences producing significant alignments		Download	Select columns	Show	100				
Description		Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/1650757/2021_ORF1ab_poly	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19778	ON113700.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/19349/2020_ORF1ab_polyse	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19607	ON084987.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/NCoV_19_USA_ID_IVREF_7	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19619	OU966688.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/MN-CDC-18X264153753205	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19664	MZ717946.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/WY119508/2021_ORF1ab_e	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19609	MZ497707.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/NVNSPHL-LV_308458/202	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19782	MZ473687.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 genome assembly_chromosome_1	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19654	OU200773.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 genome assembly_chromosome_1	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19654	OU201727.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/IL-CDC-1C0070592/2021_O	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19546	MZ412275.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 genome assembly_chromosome_1	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19684	OU055692.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 genome assembly_complete genome: monoarctic	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19684	OU056078.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/CO-CDC-MMR016689131/20	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19782	MZ298065.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 genome assembly_chromosome_1	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19684	OU0334614.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 genome assembly_chromosome_1	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19684	OU0334612.1
<input checked="" type="checkbox"/>	Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/human/USA/TX-CDC-ASC210037499/20	Severe acute res...	58.0	58.0	100%	2e-05	100.00%	19658	MZ282053.1

Figure 7A: Selection of more S24L ORF8 mutation with 1st termination codon oligo by BLAST-N search.

Acc. no. Date of isolation	S24L	1st termination codon generated in most	
MZ497707-24-4-2021	agt	tatgtact	28008
ON113700-30-3-2021	agt	tatgtact	27963
OU200773-3-5-2021	agt	tatgtact	27972
MW731336-11-2-2021	agt	yatgtact	28017
MW986843=8-3-2021	agt	tatgtact	27990
OP735332-8-1-2021	agt	tatgtact	27982
ON084987-7-10-2020	agt	tatgtact	27984
NC_045512.2-12-2019	agt	ccatgtact	28020
MZ497707-24-4-2021	ctaaa	tggtatattagagtaggagct	28068
ON113700-30-3-2021	ctaaa	tggtatattagagtaggagct	28023
OU200773-3-5-2021	ctaaa	tggtatattagagtaggagct	28032
MW731336-11-2-2021	ctaaa	tggtatattagagtaggagct	28077
MW986843=8-3-2021	ctaaa	tggtatattagagtaggagct	28050
OP735332-8-1-2021	ctaaa	tggtatattagagtaggagct	28042
ON084987-7-10-2020	ctaaa	tggtatattagagtaggagct	28044
NC_045512.2-12-2019	ctaaa	tggtatattagagtaggagct	28080
MZ497707-24-4-2021	atgaggct	gggttct	28128
ON113700-30-3-2021	atgaggct	gggttct	28083
OU200773-3-5-2021	atgaggct	gggttct	28092
MW731336-11-2-2021	atgaggct	gggttct	28137
MW986843=8-3-2021	atgaggct	gggttct	28110
OP735332-8-1-2021	atgaggct	gggttct	28102
ON084987-7-10-2020	atgaggct	gggttct	28104
NC_045512.2-12-2019	atgaggct	gggttct	28140
MZ497707-24-4-2021	cgttctat	gaa	28248
ON113700-30-3-2021	cgttctat	gaa	28203
OU200773-3-5-2021	cgttctat	gaa	28212
MW731336-11-2-2021	cgttctat	gaa	28257
MW986843=8-3-2021	cgttctat	gaa	28230
OP735332-8-1-2021	cgttctat	gaa	28222
ON084987-7-10-2020	cgttctat	gaa	28224
NC_045512.2-12-2019	cgttctat	gaa	28260

ORF8 termination codon

Figure 7B: Selection of more S24L ORF8 mutant with 1st TAA termination codon oligo but no 2nd termination codon was generated here.

Acc. no. Date of isolation	ORF1ab protein region
MZ497707-24-4-2021	tagtttgctcgggttttaagctaaaagactggttatgtagcatcagctgtagtggtact 11328
ON113700-30-3-2021	tagtttg---SGF---aagctaaaagactggttatgtagcatcagctgtagtggtact 11292
OU200773-3-5-2021	tagtttg-----aagctaaaagactggttatgtagcatcagctgtagtggtact 11301
MW731336-11-2-2021	tagtttgctcgggttttaagctaaaagactggttatgtagcatcagctgtagtggtact 11337
MW986843-8-3-2021	tagtttgctcgggttttaagctaaaagactggttatgtagcatcagctgtagtggtact 11310
OP735332-8-1-2021	tagtttgctcgggttttaagctaaaagactggttatgtagcatcagctgtagtggtact 11302
ON084987-7-10-2020	tagtttgctcgggttttaagctaaaagactggttatgtagcatcagctgtagtggtact 11304
NC_045512.2-12-2019	tagtttgctcgggttttaagctaaaagactggttatgtagcatcagctgtagtggtact 11340
//	*****
	Spike protein region
MZ497707-24-4-2021	cttggtcttacctttctttttccaatggtacttggtccatgctatacatgtctctgggac 21768
ON113700-30-3-2021	cttggtcttacctttctttttccaatggtacttggtccatgctatc-HV---tctgggac 21726
OU200773-3-5-2021	cttggtcttacctttctttttccaatggtacttggtccatgctatc-----tctgggac 21735
MW731336-11-2-2021	cttggtcttacctttctttttccaatggtacttggtccatgctatacatgtctctgggac 21777
MW986843-8-3-2021	cttggtcttacctttctttttccaatggtacttggtccatgctatacatgtctctgggac 21750
OP735332-8-1-2021	cttggtcttacctttctttttccaatggtacttggtccatgctatacatgtctctaggac 21742
ON084987-7-10-2020	cttggtcttacctttctttttccaatggtacttggtccatgctatacatgtctctgggac 21744
NC_045512.2-12-2019	cttggtcttacctttctttttccaatggtacttggtccatgctatacatgtctctgggac 21780
	*****
MZ497707-24-4-2021	tcaattttgtaatgatccatttttgggtggttattaccacaaaaacaacaaaagtggat 22008
ON113700-30-3-2021	tcaattttgtaatgatccatttttgggtggtt-Y-taccacaaaaacaacaaaagtggat 21963
OU200773-3-5-2021	tcaattttgtaatgatccatttttgggtggtt--taccacaaaaacaacaaaagtggat 21972
MW731336-11-2-2021	tcaattttgtaatgatccatttttgggtggttattaccacaaaaacaacaaaagtggat 22017
MW986843-8-3-2021	tcaattttgtaatgatccatttttgggtggttattaccacaaaaacaacaaaagtggat 21990
OP735332-8-1-2021	tcaattttgtaatgatccatttttgggtggttattaccacaaaaacaacaaaagtggat 21982
ON084987-7-10-2020	tcaattttgtaatgatccatttttgggtggttattaccacaaaaacaacaaaagtggat 21984
NC_045512.2-12-2019	tcaattttgtaatgatccatttttgggtggttattaccacaaaaacaacaaaagtggat 22020
	*****

**Figure 7C:** Deletions in ORF1ab and spike proteins of S24L mutants to demonstrate few ORF8 S24L mutants are Alpha variant (accession nos. ON113700 and OU200773) but not Delta or Omicron variants.

Acc. no. Date of isolation	ORF8 gene region of SARS-CoV-2
NC_045512.2-12-2019	agtcattgactcaacatcaaccatattgtagttgatgaccggtgctcattcacttctatt 28020
OP683545-27-6-2021	agtcattgacttaacatcaaccatattgtagttgatgaccggtgctcattcacttctatt 27963
OP711842-13-4-2021	agtcattgacttaacatcaaccatattgtagttgatgaccggtgctcattcacttctatt 27884
OP711837-3-2-2021	agtcattgacttaacatcaaccatattgtagttgatgaccggtgctcattcacttctatt 27948
OX020484-24-4-2021	agtcattgacttaacatcaaccatattgtagttgatgaccggtgctcattcacttctatt 28002
OP585720-1-1-2021	agtcattgacttaacatcaaccatattgtagttgatgaccggtgctcattcacttctatt 27948
OW999874-26-2-2021	agtcattgacttaacatcaaccatattgtagttgatgaccggtgctcattcacttctatt 28002
	*****
	Termination codon
NC_045512.2-12-2019	ctaaatggtatattagagtaggagctagaaaaatcagcacctttaattgaaatgtgctggtg 28080
OP683545-27-6-2021	ctaaatggtatattagagtaggagctataaaaaatcagcacctttaattgaaatgtgctggtg 28023
OP711842-13-4-2021	ctaaatggtatattagagtaggagctataaaaaatcagcacctttaattgaaatgtgctggtg 27944
OP711837-3-2-2021	ctaaatggtatattagagtaggagctataaaaaatcagcacctttaattgaaatgtgctggtg 28008
OX020484-24-4-2021	ctaaatggtatattagagtaggagctataaaaaatcagcacctttaattgaaatgtgctggtg 28062
OP585720-1-1-2021	ctaaatggtatattagagtaggagctataaaaaatcagcacctttaattgaaatgtgctggtg 28008
OW999874-26-2-2021	ctaaatggtatattagagtaggagctataaaaaatcagcacctttaattgaaatgtgctggtg 28062
	*****
	Point mutation and no termination codon
NC_045512.2-12-2019	atgaggctggttctaaatcaccattcagtcacatcgatcggtaattatacagtttct 28140
OP683545-27-6-2021	atgaggctggttctaaatcaccattcagtcacatcgatcggtaattatacagtttct 28083
OP711842-13-4-2021	atgaggctggttctaaatcaccattcagtcacatcgatcggtaattatacagtttct 28004
OP711837-3-2-2021	atgaggctggttctaaatcaccattcagtcacatcgatcggtaattatacagtttct 28068
OX020484-24-4-2021	atgaggctggttctaaatcaccattcagtcacatcgatcggtaattatacagtttct 28122
OP585720-1-1-2021	atgaggctggttctaaatcaccattcagtcacatcgatcggtaattatacagtttct 28068
OW999874-26-2-2021	atgaggctggttctaaatcaccattcagtcacatcgatcggtaattatacagtttct 28122
	*****
	Termination codon      point mutation

**Figure 8:** Selection of ORF8 mutants using 2nd TAA termination codon oligo with Y73C mutation. All B.1.1.7 variants were selected with 3675SGF deletion in ORF1ab protein as well as 69HV+145Y spike deletions (data not shown). Only OP711842 variant has 63nt deletion in ORF7a/7b region. We found that R52I mutation could not generated with a termination codon.

(A) E-NC\_045512.2-12-2019 (Wuhan corona virus ORF8 gene)  
 atgaaattcttctgtttcttaggaatcaccacaactgtagctgcatttcaccaagaatgtagtttacagtcagtcactcaacatcaaccatagtagttgatgacc  
 gtgtcttattacccttcttattcctaaatggtatattagagtaggagctagaaaatcagcacccttaattgaattgctgctggatgaggtgggtctcaatcacccttc  
 agtcacatcgatctggtaattatcacagttctctgtttaccctttacaatttaattgcccagggaacctaaattgggtagctctgtagtgctgctgtctctatgaagac  
 ttttagagatcatgacgctcgtgctgttttagatttcataa

ORF8 protein [SARS-CoV-2, Accession no. NC\_045512.2] Sequence ID: [YP\\_009724396.1](#) Length: 121.  
 Score Expect Method Identities Positives  
 251 bits(642) 3e-84 Compositional matrix adjust. 121/121(100%) 121/121(100%)  
 Query 1 MKFLVFLGIITTVAAAFHQECSLQ9CTQHQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL 100  
 MKFLVFLGIITTVAAAFHQECSLQ9CTQHQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL  
 Sbjct 1 MKFLVFLGIITTVAAAFHQECSLQ9CTQHQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL 60

Query 101 CVDEAGS\*SPIQYIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI 361  
 CVDEAGS\*SPIQYIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI  
 Sbjct 61 CVDEAGS\*SPIQYIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI 121

(B) OR234991-3-6-2021 (ORF8 gene with two TAA termination codons)  
 atgaaattcttctgtttcttaggaatcaccacaactgtagctgcatttcaccaagaatgtagtttacagtcagtcactcaacatcaaccatagtagttgatgacc  
 gtgtcttattacccttcttattcctaaatggtatattagagtaggagctagaaaatcagcacccttaattgaattgctgctggatgaggtgggtctcaatcacccttc  
 agtcacatcgatctggtaattatcacagttctctgtttaccctttacaatttaattgcccagggaacctaaattgggtagctctgtagtgctgctgtctctatgaagac  
 ttttagagatcatgacgctcgtgctgttttagatttcataa

ORF8 protein [SARS-CoV-2, Accession no. OL739852] Sequence ID: [UFX85754.1](#) Length: 121.  
 Score Expect Method Identities Positives  
 243 bits(619) 1e-80 Compositional matrix adjust. 119/121(98%) 119/121(98%)  
 Query 1 MKFLVFLGIITTVAAAFHQECSLQ9CT\*HQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL 100  
 MKFLVFLGIITTVAAAFHQECSLQ9CT HQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL  
 Sbjct 1 MKFLVFLGIITTVAAAFHQECSLQ9CTQHQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL 60

Query 101 CVDEAGS\*SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI 361  
 CVDEAGS SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI  
 Sbjct 61 CVDEAGS\*SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI 121

ORF8 protein [SARS-CoV-2, Accession no. MZ013607] Sequence ID: [QUE21848\\_1](#) Length: 121.  
 Score Expect Method Identities Positives  
 239 bits(610) 3e-79 Compositional matrix adjust. 117/121(97%) 118/121(97%)  
 Query 1 MKFLVFLGIITTVAAAFHQECSLQ9CT\*HQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL 100  
 MKFLVFLGIITTVAAAFHQECSLQ9CT HQPYYVVDPCPIHFYSEWYIRVGA KSAPLIEL  
 Sbjct 1 MKFLVFLGIITTVAAAFHQECSLQ9CTQHQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL 60

Query 101 CVDEAGS\*SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI 361  
 CVDEAGS SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI  
 Sbjct 61 CVDEAGS\*SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI 121

ORF8 protein [SARS-CoV-2, Accession no. MZ341638] Sequence ID: [QWE64732\\_1](#) Length: 121.  
 Score Expect Method Identities Positives  
 239 bits(610) 3e-79 Compositional matrix adjust. 117/121(97%) 118/121(97%)  
 Query 1 MKFLVFLGIITTVAAAFHQECSLQ9CT\*HQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL 100  
 MKFLVFLGIITTVAAAFHQECSLQ9CT HQPYYVVDPCPIHFYSEWYIRVGA KSAPLIEL  
 Sbjct 1 MKFLVFLGIITTVAAAFHQECSLQ9CTQHQPYYVVDPCPIHFYSEWYIRVGARKSAPLIEL 60

Query 101 CVDEAGS\*SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI 361  
 CVDEAGS SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI  
 Sbjct 61 CVDEAGS\*SPIQCIDIGNYTVSCLPFTINQEPKLGSLVVRCSFYEDFLEYHDRVVLDPI 121

**Figure 9A:** Demonstration of new ORF8 mutation by BLAST-X search of full length ORF8 gene. (A) Wuhan corona virus ORF8 gene analysis and (B) two TAA termination ORF8 gene analysis.

OK234981-3-6-2021 repaired two termination codons (full length orf8 gene)

(A) `atgaaattctctgtttcttaggaatcatcacaactgtagctgcatttcaccaagaatgtagtttacagtcattgactcaacatcaacccatgtagttgatgacc  
gtgtccatctcactctcttcttaaatggtatattagagtaggagctctaaaatcagcacccttaattgtaattgtgcgtggatgagcctggtctcaatcaccatc  
agtgcacgataatcggaatatacagttctctgtttaccttttacaattaattgccaggaaacctaaattgggtagctctgtgagtcggtgtctcgtttcatgaagac  
tttttagagatcatgacgcttctgtgttttagatttcactaa`

ORF8 protein [SARS-CoV-2, Accession no. MW644355] Sequence ID: [QRX60686.1](#) Length: 121.

Score	Expect	Method	Identities	Positives
250 bits(639)	1e-83	Compositional matrix adjust.	121/121(100%)	121/121(100%)
Query 1	MKFLVFLGIITTVAAFHQECSLQSC	TQHQPYYVDDPCPIHFYSKWYIRVGAIKSAPLIEL	180	
Sbjct 1	MKFLVFLGIITTVAAFHQECSLQSC	TQHQPYYVDDPCPIHFYSKWYIRVGAIKSAPLIEL	60	

(B) Query 181 CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 361  
CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 361  
Sbjct 61 CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 121

ORF8 protein [SARS-CoV-2, Accession no. MW781335] Sequence ID: [QTD14049.1](#) Length: 121.

Score	Expect	Method	Identities	Positives
245 bits(625)	1e-81	Compositional matrix adjust.	119/121(98%)	119/121(98%)
Query 1	MKFLVFLGIITTVAAFHQECSLQSC	TQHQPYYVDDPCPIHFYSKWYIRVGAIKSAPLIEL	180	
Sbjct 1	MKFLVFLGIITTVAAFHQECSLQSC	TQHQPYYVDDPCPIHFYSKWYIRVGAIKSAPLIEL	60	

(C) Query 181 CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 361  
CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 361  
Sbjct 61 CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 121  
**A65V Y73C**

ORF8 protein [SARS-CoV-2, Accession no. MZ242532] Sequence ID: [QVM81399.1](#) Length: 121

Score	Expect	Method	Identities	Positives
245 bits(625)	2e-81	Compositional matrix adjust.	118/121(98%)	120/121(99%)
Query 1	MKFLVFLGIITTVAAFHQECSLQSC	TQHQPYYVDDPCPIHFYSKWYIRVGAIKSAPLIEL	180	
Sbjct 1	MKFLVFLGIITTVAAFHQECSLQSC	TQHQPYYVDDPCPIHFYSKWYIRVGAIKSAPLIEL	60	

(D) Query 181 CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 361  
CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 361  
Sbjct 61 CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 121  
**V32L Y73C V100L**

ORF8 protein [SARS-CoV-2, Accession no. MZ473538] Sequence ID: [QXE76672.1](#) Length: 121.

Score	Expect	Method	Identities	Positives
244 bits(624)	2e-81	Compositional matrix adjust.	119/121(98%)	119/121(98%)
Query 1	MKFLVFLGIITTVAAFHQECSLQSC	TQHQPYYVDDPCPIHFYSKWYIRVGAIKSAPLIEL	180	
Sbjct 1	MKFLVFLGIITTVAAFHQECSLQSC	TQHQPYYVDDPCPIHFYSKWYIRVGAIKSAPLIEL	60	

(E) Query 181 CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 361  
CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 361  
Sbjct 61 CVDEAGSKSPIQCIDIGNYTVSCLPFTINCQEPKLGSLVVRCSFYEDFLEYHDRVVLDFT 121  
**P38S Y73C**

Figure 9B: Selection more ORF8 mutants by BLAST-X search using mutant ORF gene but repairing two TAA mutations. Here, MW644355 mutant may be B.1.1.7 lineage.

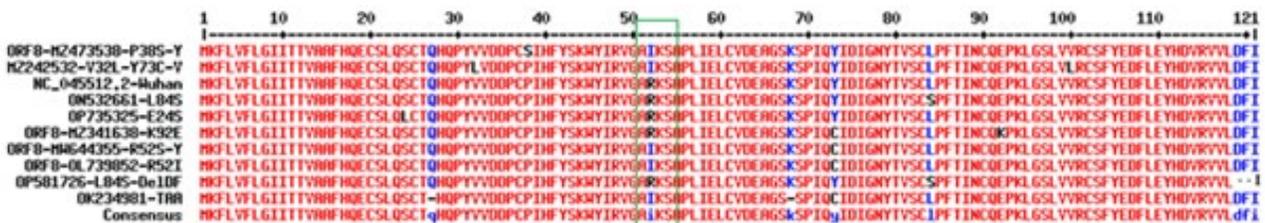
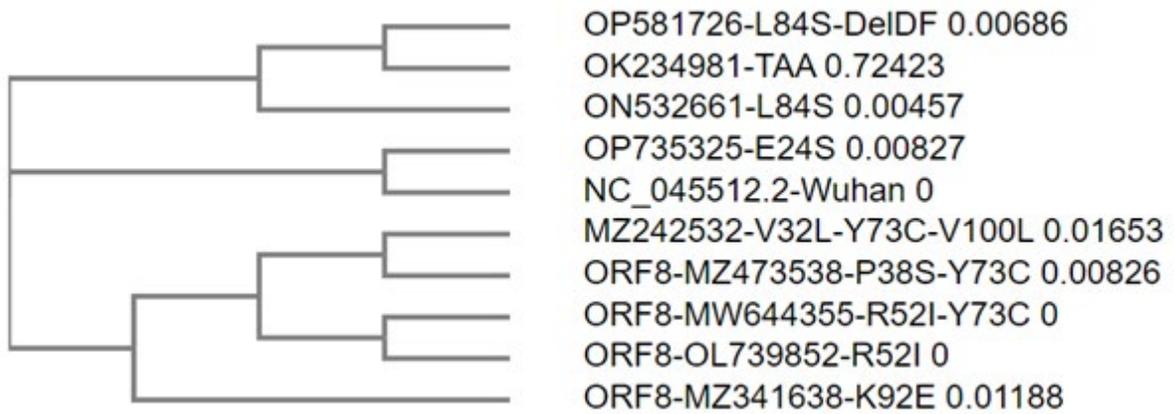
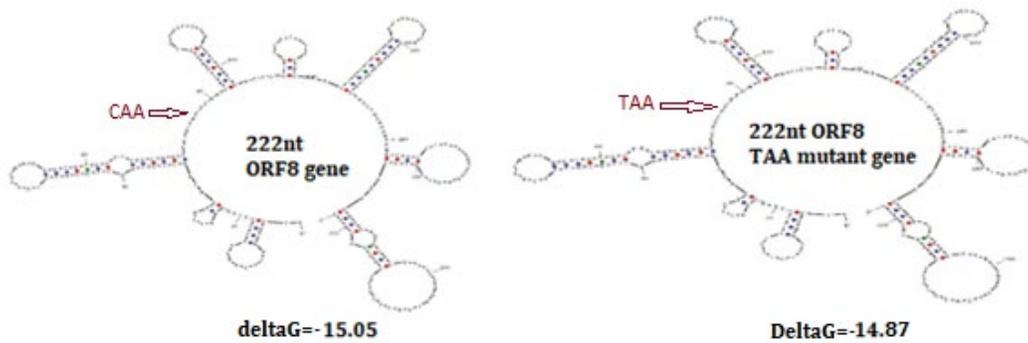


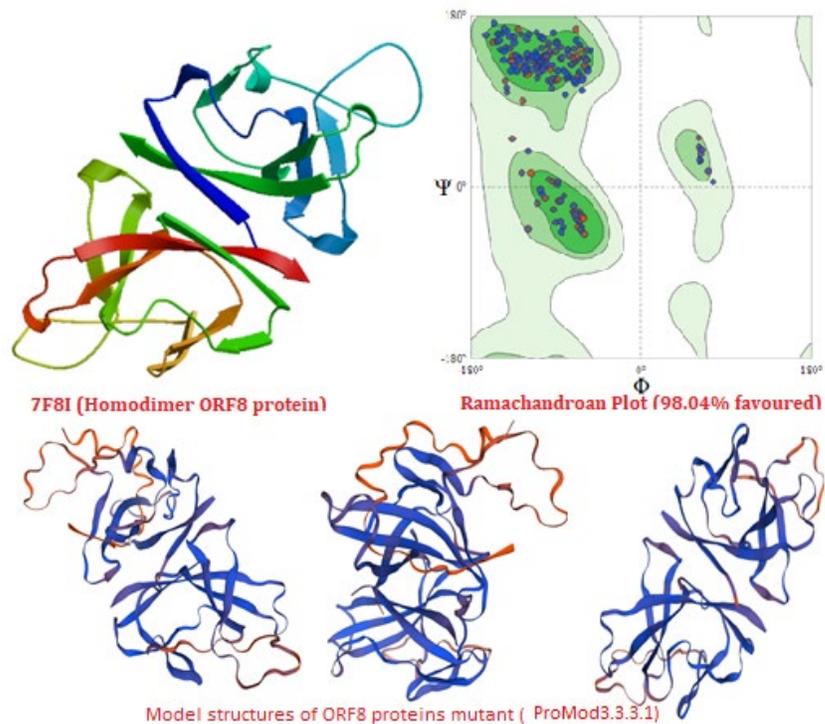
Figure 10A: Multi-alignment of mutated different ORF8 proteins of COVID-19. However, “ARKS” domain (green boxed) similarities with histones was abolished in R52I mutants lowering the interaction with host chromatin (Kee, J et al., 2022). This could be stabilize the corona virus load increasing virus severity.



**Figure 10B:** A phylogenetic tree of corona virus ORF protein mutants.



**Figure 11:** Demonstration of identical hairpin structure of 222nt ORF8 gene of normal and termination mutant of SARS-CoV-2.



**Figure 12:** Model structures of Mutated ORF8 proteins (below) and a model of bat ORF8 protein (7F8I) with 98% similarity (upper) and Ramachandran plot to show stable structure of ORF8 dimer (SWISS-Model)

## Discussion

Over time, the coronavirus has undergone mutations and deletions and different variants reported in different parts of the world with different time since December, 2019. These variants and mutants are Alpha (B.1.1.7), B.1.177, B.1.258, Beta (B.1.351), Gamma (P.1/B.1.1.28.1), Delta (B.1.617.2), Kappa (B.1.617.1), Epsilon (B.1.427/B.1.429), Zeta (P.2); Eta (B.1.525), B.1.1.20, Iota (B.1.526) and B.1.1.298 (Mink Variant). These mutations are of major health concerns, as they reportedly exacerbate the infectious rate of the virus [4]. We found here major ORF8 truncated mutants were B.1.1.7 lineage. Interestingly, RNA recombination generated few omicron lineages with ORF8 protein TAA termination codon mutants.

Initially we selected few TAA termination mutants (figure-2) but BLAST-N search with mutant oligos selected many of such mutants. Whereas, BLAST-X search with mutated full length ORF8 gene contributed more such mutants to characterize. In truth hundred of TAA termination codon mutants detected in the NCBI Database but many of them were partially sequenced and we avoided such sequences in our multi-alignment data (see, figure-6A; figure-7B). RNA sequencing was done extensively and Delta, BA.2.75, BA.4.6, BA.5.2.1, BF.7 and BE.1.1 were predominantly circulating now. We have attempted to characterize the 121 AA long ORF8 protein in the database and such protein was not expressed in many corona virus variants [14].

The ORF8 L84S mutant was more stable and more soluble than wild type and in higher temperature it existed as aggregated conformation. Moreover, ORF8 precipitated at acidic pH and this precipitation was reversed when the solution pH was shifted to neutral [15]. However, we did not find TAA termination codon mutant in L84S mutants but L24S mutants. Preliminary study indicated few dozen proteins like PVR, IRF3, ATF6, Belin 1, FK506-binding protein 10, EDEM, vitronectin, OPJ94, Sec62163, VIP36, TRFT3 and PLAT etc interacted with ORF8 protein regulating protein folding, apoptosis and interferon production. Such process likely favours COVID-19 survival in host cells inhibiting immune control mechanisms [16]. Genetic analysis pointed a severe deletion in ORF8 ( $\Delta$ 382) caused less severe corona infections likely due to low viral load and increased immune clearance [17-20]. However, in cell culture study with such deletion mutant contradicted the finding of lower viral load with no change of cellular transcriptional profile [21].

A recent study suggested that ORF8 mediates immune evasion by downregulating MHC-I molecules like HLA-A2 [22,23] and such finding clearly established the IgG domains similarity of ORF8 to modulate host immune functions and chromatin structure [24]. Dominant mutation of small regulatory protein ORF7a limits viral suppression of the interferon response [25] and we detected one such 62nt deletion abolishing the production of both ORF7a and ORF7b proteins (see, accession no. OP711842).

The ORF8 protein also activates the IL-17 and NLRP3 signalling

pathways, upregulates proinflammatory factors and inhibits the production of IFN types I and II. The study also investigated the ability of compounds isolated from *C. volubilis* leaves to interact with the initiation and termination codons of ORF8 mRNAs isolated from the whole genome of SARS-CoV-2 using computational tools.

The ORF8 mRNA sequences of the translation initiation sites and translation termination sites encoding ORF8 amino acids were retrieved from the full genome of SARS-CoV-2. Molecular docking studies revealed strong molecular interactions of the isolated compounds with the ORF8 mRNA. The immunomodulatory potentials of the isolated compounds were investigated on neutrophil phagocytic respiratory bursts using luminol-amplified chemiluminescence technique [26].

There are also reports that ORF8 modulates vesicular traffic through the unfolded protein response and, therefore, ER stress by stimulating the ATF6 and IRE1 pathways through the upregulation of the GRP78 and GRP94 chaperones [27]. Another small corona virus protein ORF9b interacted with Tom70 mitochondrial chaperone as revealed by cryo-electron microscopy [28]. Similarly, study indicated ORF3a also has trans activator function interacting MHC molecules [29].

Computational analysis of targeted ORF8 RNA device could inhibit the replication of SARS-CoV-2 [30]. Thus, ORF8 is a hotspot for genetic variation in coronaviruses [31]. The clinical effect of deletions in this region appears to be a milder infection with less systemic release of proinflammatory cytokines [32]. An RNA-RNA interaction was reported between SARS-CoV-2 spike and ORF8 genes suggesting ORF8 gene silencing may be required for immune-escape and virus spread [33-34].

## Conclusion

Our preliminary study confirmed the presence of many ORF8 truncated mutants in B.1.1.7 lineages of SARS-CoV-2. Although contradictory, we found ORF8 protein L24S mutation in TAA termination codon mutants but no association of L84S mutation was detected. It appeared that such changes neither changed the RNA tertiary structure nor globular interface of ORF8 protein. However, R52I mutation may destroy the histone mimics of ORF8 protein as "ARKS" AA sequence appeared conserved in cellular histones as well as in ORF8 protein of corona virus. None the less, our study indicated a vivid self-destruction process of corona virus due to point mutations eliminating functions of ORF8 protein.

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## Competent Interest

The author declares no competing interest

## Ethical Issues

The data presented here was computer generated and no animal or human was used.

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