

Natural infection of SARS-CoV-2 delta variant in Asiatic lions (*Panthera leo persica*) in India

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Abstract

In May 2021, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was detected in nine Asiatic lions (*Panthera leo persica*) in Arignar Anna Zoological Park, Chennai, Tamil Nadu, India. Sequence and phylogenetic analysis showed that the SARS-CoV-2 viruses belong to a variant of concern (VOC, delta variant, B.1.617.2 lineage) and that these viruses clustered with B.1.617.2 lineage viruses of the same geographical region detected in the same month.

Since the first identification of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019 in the city of Wuhan, Hubei province, China, its associated disease coronavirus disease 2019 (COVID-19) has resulted in 176,531,710 confirmed human cases, with 3,826,181 deaths as of June 17, 2021 (Zhou et al., 2020; World Health Organization, 2021). Because viruses closely related to SARS-CoV-2 have been found in bats and pangolins, they are being debated as possible SARS-CoV-2 origins (Lam et al., 2020; Liu et al., 2020; Zhang et al., 2020). For the reason that SARS-CoV-2 is believed to have a zoonotic origin, finding susceptible animal species, reservoirs, and cross-species transmission events is a subject of global scientific and public interest. Experimental studies showed that SARS-CoV-2 infects and replicates efficiently in domestic cats, ferrets, and fruit bats but poorly in dogs. However, chickens, ducks, and pigs do not seem to support productive SARS-CoV-2 infection (Shi et al., 2020; Kim et al., 2020). Conversely, in natural conditions, SARS-CoV-2 seems to have broader host susceptibility range and as of 31 May, 2021, SARS-CoV-2 infections has now been reported in at least ten animal species, namely, cats, dogs, tigers, lions, snow leopards, pumas, gorillas, pet ferrets, otter and farmed mink from thirty countries resulting in 552 outbreaks worldwide (OIE, 2021). Importantly, in most of these natural infection cases, caretakers or owners of these animal species have been found positive for SARS-CoV-2 infection, pointing the transmission of infection from humans to animals. In rare cases, two way transmissions (transmission of infection from humans to animals, and back to humans) have also been documented in farmed minks in the Netherlands (Oude Munnink et al., 2021).

In this study, we report the first confirmed natural SARS-CoV-2 infections in Asiatic lions (*Panthera leo persica*) caused by a delta variant as per World Health Organization (WHO) nomenclature (PANGO lineage B.1.617.2, Nextstrain clade 21A) in India, and provide a thorough genomic characterization of SARS-CoV-2 obtained from the infected Asiatic lions. These confirmed natural infections in the India occurred in last week of May, 2021, when India was battling an unprecedented surge in COVID-19 cases.

The International Union for Conservation of Nature (IUCN) has classified Asiatic lions as Endangered, and their population is steadily declining owing to poaching and habitat degradation (Bauer et al., 2016). Some Asiatic lions in Arignar Anna Zoological Park, Chennai, Tamil Nadu, India experienced loss of appetite, nasal discharge and occasional coughing symptoms in the last week of May 2021. COVID-19 claimed the lives of two lions, Neela and Pathbanathan, on June 3 and 16, 2021, respectively. Nasal swabs, rectal swabs, and faecal samples were taken from eleven Asiatic lions (6 males and 5 females) during 24 to 29 May, 2021 and sent to the ICAR-National Institute of High Security Animal Diseases in Bhopal, India, for molecular investigations (Table S1). The genome of SARS-CoV-2 was confirmed in nine lions using the VIRALDTECT - II Multiplex Real Time PCR Kit (GENES2ME Pvt. Ltd., India), which enabled identification of SARS-CoV-2 E, N, and RdRp genes. Furthermore, all of the lion samples tested negative for Canine Distemper Virus using the OIE-recommended RT-PCR method (Frisk et al., 1999).

Subsequent to confirmation of SARS-CoV-2 infection in the lions, whole genome sequencing of SARS-CoV-2 was generated directly from nasal swabs from four lions (Lioness- Jaya, Lion-Shankar, Lioness- Niranjana, and Lion- Pradeep). In a nutshell, tiling PCR spanning over the whole genome of SARS-CoV-2 was carried out with the Artic network primers (<https://artic.network/>). Cleaning and quantification of PCR products were performed and 100ng of each sample was used to create a barcoded sequencing library using the PCR Barcoding Kit (SQK-PBK004). The sequencing was done on an Oxford Nanopore Technologies MinION with a R9.4.1 flowcell, yielding a total of 300MB of data. To assemble the whole genome, guppy v5.0.7 (Oxford Nanopore Technologies) was used for base calling and de-multiplexing, followed by Porechop (<https://github.com/rrwick/Porechop>) for adaptor removal. The readings were mapped to the reference SARS-CoV-2 genome (NC 045512) using minimap2 v2.17 (r941) (Li, 2018), and the variations were called using nanopolish v0.13.2 (Loman et al., 2015). After two rounds of nanopolish, four complete genomes were produced and submitted to NCBI with the GenBank accession numbers MZ363851-MZ363854.

To better understand the temporal dynamics of SARS-CoV-2, complete SARS-CoV-2 genomes with high coverage originating from the Tamil Nadu state of India (n = 310) during 1 January 2021 to 11 June 2021 were downloaded from GISAID on 11 June 2021 (Elbe and Buckland-Merrett, 2017, <https://www.gisaid.org/>). These genomes were clustered at 99.9% identity threshold using UCLUST algorithm to generate a set of representative sequences (Edgar, 2010). PANGO lineages (Rambaut et al., 2020) and Nextstrain clades (<https://clades.nextstrain.org/>) were assigned to these representative sequences. Along with the SARS-CoV-2 genomes in lions of this study and Wuhan-Hu-1 reference sequence (EPI_ISL_402124), these representative sequences were aligned using MAFFT v.7.475 (Katoh and Standley, 2013) and a phylogenetic tree was constructed using the GTR-Gamma model in RAxML v. 8.2.12 (Stamatakis, 2014). In addition, complete SARS-CoV-2 genome having high coverage from the lions (n = 24) across the globe were downloaded from GISAID on 11 June 2021 and a phylogenetic tree was constructed using the GTR-Gamma model in RAxML v. 8.2.12 (Stamatakis, 2014).

Compared to the Wuhan-Hu-1 reference sequence (EPI_ISL_402124), all the four Asiatic lions sequences contained 24 amino acid substitutions and 2 deletions. The tempo-spatial dynamics of substitutions across the multiple proteins encoded by the SARS-CoV-2 detected in lions, as well as their functional roles are provided in Table S2. The spike protein sequence of SARS-CoV-2 in lions had 9 amino acid substitutions and 2 deletions compared with the Wuhan-Hu-1 strain, and are typically matched with a delta variant (PANGO lineage B.1.167.2) of SARS-CoV-2 viz., N-terminal domain (NTD; T19R, G142D, E156del, F157del, R158G), receptor binding motif (RBM; L452R, T478K), Subdomain 2 (SD2, D614G), substitution close to S1/S2 protease cleavage site (P681R), and heptad repeat 1 (HR1, D950N) (Figure 1). Additionally, they carried the K77T substitution in the NTD, which has been reported from 24 countries, including India, with a low frequency of 0.44%, but it occurs 27.42 % of the time (65/237 sequences) in the B.1.167.2 lineage in Tamil Nadu, India (Table S2). SARS-CoV-2 had previously been

documented to cause natural infections in lions from the United States (B, 19A), Spain (B.1.177), and the Czech Republic (B.1.1.7, Alpha), as well as two lineages in tigers from the Czech Republic (B.1.1.7, Alpha) and the United States (B.1) (Figure S1) (McAloose et al., 2020). As a result, our study describes the first confirmed natural SARS-CoV-2 infection in Asiatic lions in India, which was caused by a variant of concern (VOC delta, B.1.167.2 lineage).

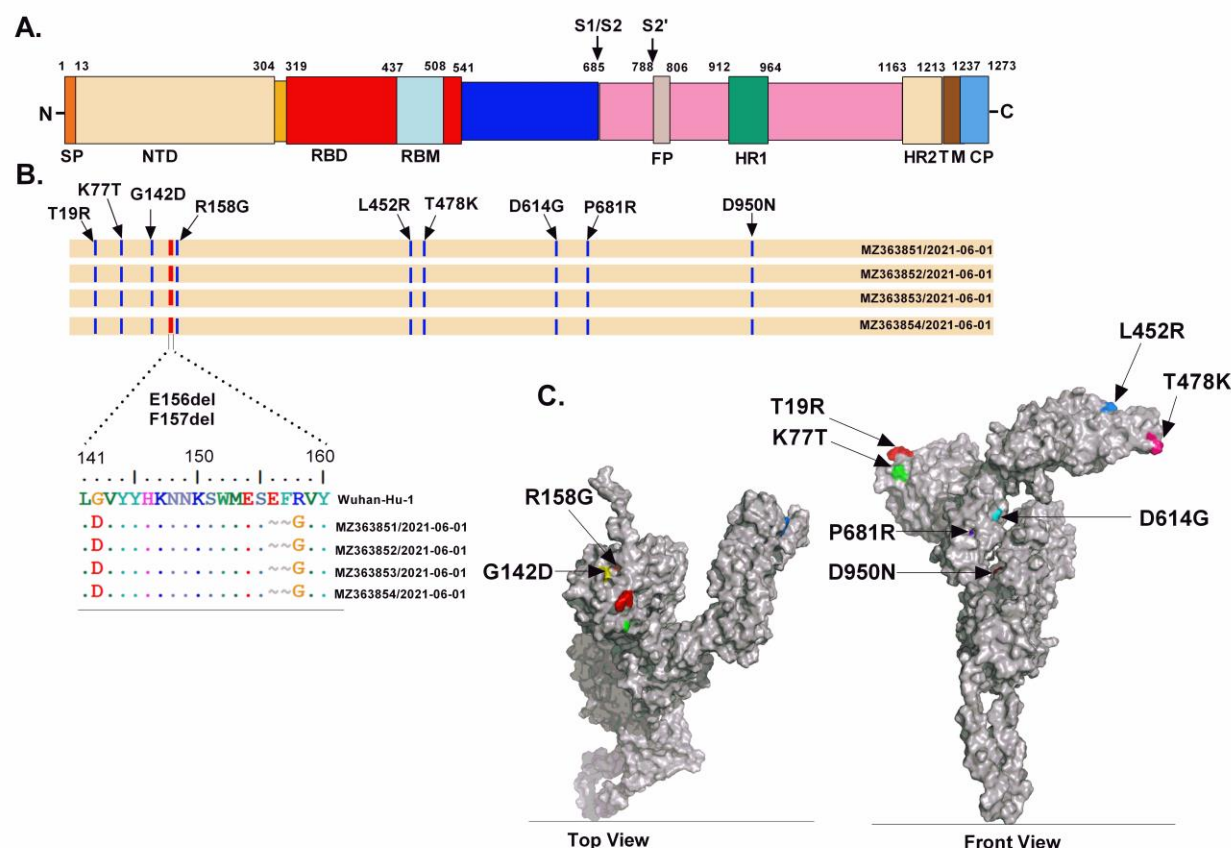


Figure 1. (A) shows schematic representation of functional domains of the spike (S) protein for SARS-CoV2 Wuhan-Hu-1; (B) A comparison of amino acid changes detected in the S protein of SARS-CoV-2 in Asiatic lions with respect to Wuhan-Hu-1 reference sequence (EPI_ISL_402124); (C) Mapping of amino acid substitutions noted in Asiatic lion SARS-CoV-2 (GenBank accession no. MZ363851) on to the structural model of S protein, build using I-TASSER (Iterative Threading ASSEmbly Refinement) on a template (PDB: 6acc) (Yang et al., 2015).

The spike (E156del, F157del, and R158G) and NS3 (V88I) deletions and substitutions noted in all four lion sequences were not found in human virus sequences from the same geographic location. This could be due to insufficient and small-scale sequencing of SARS-CoV-2 in this geographic region, causing the prevalence of these deletions and substitutions to be underestimated. Although NS3 (V88I) substitution frequency is very low in India as well as world, the B.1.36.8 lineage in Punjab, a northern state of India carried this substitution in July 2020 (Table S2). It is possible that NS3 (V88I) was inserted at random during virus replication. Furthermore, these deletions and substitutions in spike and NS3 were not seen in previously reported lion sequences (from the United States, Spain, and the Czech Republic), ruling out the

possible host-adapted mutations. However, experimental investigations are needed to see if deletions and substitutions in spike protein, namely E156del, F157del, R158G, and K77T, are escape mutants or related with increased transmissibility and/or pathogenicity.

Blasting of all the four lions viral genome sequences against the available sequences in the GISAID closely matched with a SARS-CoV-2 sequence (GISAID accession no. EPI_ISL_2463770) found in a patient belonging to the same geographical region in May 2021. Furthermore, phylogenetic analysis of viral genome sequences from the lions, as well as all the sequences available from Tamil Nadu state of India, clustered them into the human B.1.617.2 lineage (VOC Delta, GISAID clade - G/478K.V1, Nextstrain clade - 20A/S:478K) (Figure S2). Importantly, viral genome sequences from all the lions closely matched a representative SARS-CoV-2 sequence (GISAID accession no. EPI_ISL_2463770, which represents 152 viral genome pools of the same geographical region), corroborating with the lions' sample collection month (Figure 2). In the absence of any recent new animal introductions to the zoo, no public access to the zoo during COVID-19 imposed strict lockdown and consistent use of personal protective equipment (PPE) by the zoo keepers, the source of SARS-CoV-2 infection in the Asiatic lions cannot be ascertained and can only be presumed to be via unpremeditated contaminated fomites. Seven of nine infected lions belong to the lion safari of the zoo and hence share a common habitat including shelter, food space and water source; the other two lions are display animals who share a common moat. In the presence of shared habitat offering several opportunities of close physical contact, identification of infection with genetically identical SARS-CoV-2 in these lions in short period of time, clearly indicates the likelihood of lion-to-lion transmission, which might be of grave concern.

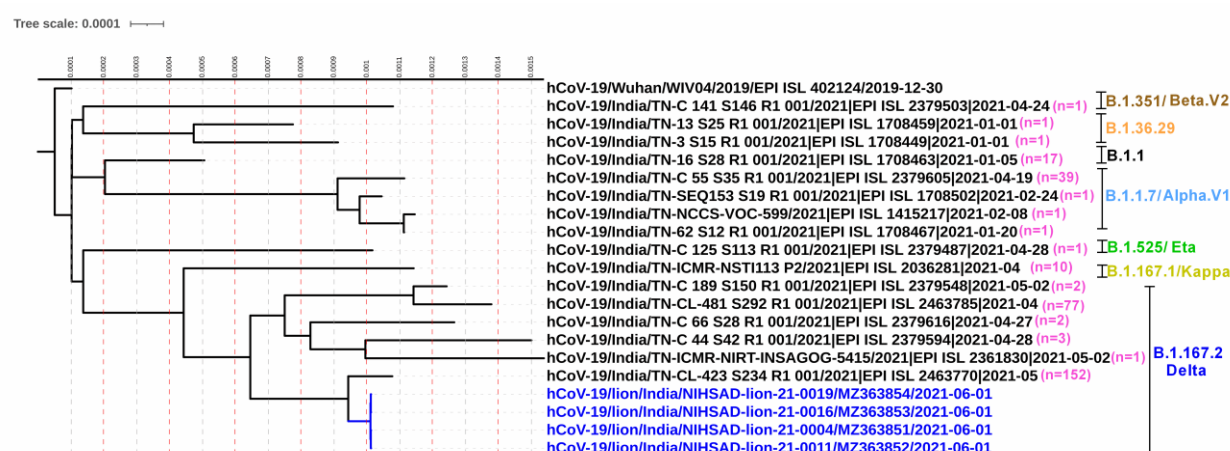


Figure 2. Complete genome phylogenetic analysis of Asiatic lions SARS-COV-2 sequences with Wuhan-Hu-1 reference sequence (EPI_ISL_402124) and representative sequences of different clusters (generated at 99.9% identity threshold) from the available SARS-COV-2 sequences from Tamil Nadu state of India. The Maximum Likelihood (ML) tree was rooted to Wuhan-Hu-1. The number in parentheses indicates the number of SARS-CoV-2 genome sequences clustered at 99.9% identity threshold.

In conclusion, we reported the first confirmed natural SARS-CoV-2 infections in Asiatic lions (*Panthera leo persica*) in India, which were caused by a delta variant (B.1.617.2 lineage). This finding justifies increased surveillance for this VOC in other wild species, as well as strict biosecurity measures to check sick/asymptomatic handlers/keepers/visitors from entering the area.

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Figure S1. ML tree showing the phylogenetic relationship among the Asiatic lions SARS-COV-2 sequences of this study with other available lions and tigers sequences in the GISAID.

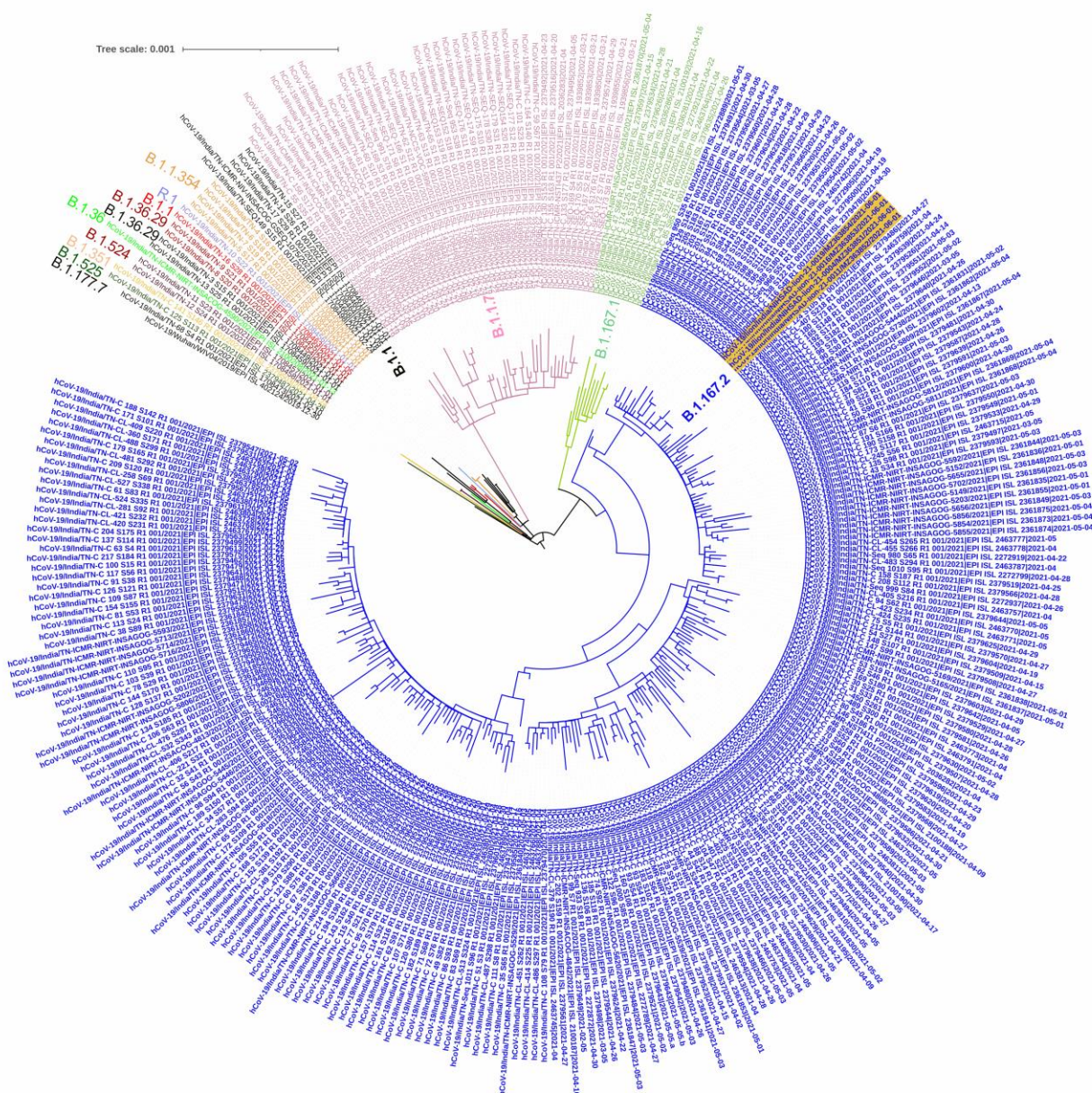


Figure S2. The phylogenetic analysis of Asiatic lions SARS-COV-2 sequences with all the available SARS-COV-2 sequences from Tamil Nadu state of India. The PANGO lineages were differently colored.

Table S1. Details of samples collected from 11 Asiatic lions in Arignar Anna Zoological Park, Chennai, Tamil Nadu, India

S. No	Identity of Lions	Age (years)	Type of sample	RT-qPCR Results (C _t Value)			Results
				E gene	RdRp	N	
1.	Lioness- Jeya	3	Nasal Swab	20.16	19.65	19.97	Positive
			Rectal Swab	19.23	18.27	19.75	Positive
2.	Lion- Padmnabhan	12	Nasal Swab	18.90	18.10	18.50	Positive
			Rectal Swab	27.41	26.75	27.79	Positive
3.	Lioness- Kavitha	18	Nasal Swab	23.79	22.87	23.89	Positive
			Rectal Swab	32.19	31.44	31.90	Positive
			Faecal sample	27.56	27.20	27.72	Positive
4.	Lion- Shankar	18	Nasal Swab	19.26	18.66	18.82	Positive
			Rectal Swab	30.22	29.41	29.45	Positive
5.	Lioness- Neela	9	Nasal Swab	22.71	23.22	22.57	Positive
			Rectal Swab	25.09	24.74	24.46	Positive
			Faecal sample	-	-	-	Negative
6.	Lioness- Niranjana	2	Nasal Swab	19.68	19.04	19.17	Positive
			Rectal Swab	38.61	38.14	33.69	Negative
			Faecal sample	-	-	-	Negative
7.	Lion- Pradeep	2	Nasal Swab	17.59	16.85	17.35	Positive
			Rectal Swab	29.05	28.60	29.02	Positive
			Faecal sample	-	-	-	Negative
8.	Lion- Vishnu	4	Faecal sample	24.34	23.80	24.72	Positive
			Nasal swab	26.60	25.43	26.74	Positive
9.	Lioness- Jeya@Bhuvana	12	Faecal sample	28.11	27.39	28.49	Positive
			Nasal swab	27.06	26.11	27.36	Positive
10.	Lion- Veera	10	Faecal sample	-	-	-	Negative
11.	Lion- Shiva	12	Nasal swab	-	-	-	Negative
			Throat swab	-	-	-	Negative
			Trachea sample	-	-	-	Negative
			Lymph node sample	-	-	-	Negative
			Lungs sample	-	-	-	Negative

Table S2. Amino acid substitutions identified in different proteins encoded by the SARS-CoV-2 of lions in comparison to Wuhan-Hu-1 reference sequence (GISAID accession no. EPI_ISL_402124), along with their functional roles

Amino acid substitutions	Times reported (% of sequences with this gene showing this mutation)	Reported from no. of countries	Date of collection of first strain with this mutation	First strain to show the mutation	Functional roles of amino acid substitutions
M_I82T	51777 (2.75%)	99	Apr-20	hCoV-19/Indonesia/GO-NIHRD-PME20208/2020	-
N_D377Y	72190 (3.83%)	106	Feb-20	hCoV-19/USA/OH-ODH-SC1040172/2020	-
N_D63G	36273 (1.93%)	62	Apr-20	hCoV-19/Indonesia/GO-NIHRD-PME20208/2020	-Antigenic Drift -Viral Oligomerization Interfaces
N_R203M	40703 (2.16%)	73	Mar-20	hCoV-19/Spain/NC-IBV-001370/2020	-
NS3_S26L	42135 (2.24%)	83	Mar-20	hCoV-19/USA/NY-NYCPHL-000016/2020	-
NS3_V88I	71 times (0.00%)	14	Mar-20	hCoV-19/Austria/CeMM0388/2020 hCoV-19/India/PB-ICMR-148040/2020 EPI_ISL_1165106 (B.1.36.8 , Punjab, JUL-2020)	Viral Oligomerization Interfaces
NS7a_T120I	40866 (2.17%)	77	Feb-20	hCoV-19/Scotland/CVR01/2020	-
NS7a_V82A	39371 (2.09%)	66	Apr-20	hCoV-19/Indonesia/GO-NIHRD-PME20208/2020	Antigenic Drift
NSP12_P323L	1802848 (95.76%)	182	Oct-20	hCoV-19/Italy/MAR-UnivPM30_45476/2020	-
NSP15_K259R	4692 (0.25%)	54	Jul-20	hCoV-19/USA/CA-IGI-0320/2020	-

NSP2_P129L	13166 (0.70%)	91	Mar-20	hCoV-19/Japan/PG-1597/2020	-
NSP3_P822L	14468 (0.77%)	77	Feb-20	hCoV-19/USA/CA-CDPH018/2020	-Host Cell Protein/RNA Interaction -Viral Oligomerization Interfaces
NSP4_D217N	2516 (0.13%)	55	Mar-20	hCoV-19/England/BIRM-61F00/2020	-
NSP4_F375S	1136 (0.06%)	33	Jun-20	hCoV-19/USA/WA-UW-10769/2020	-
NSP6_H11Q	3639 (0.19%)	48	Apr-20	hCoV-19/Italy/LOM-Pavia-41147/2020	-
Spike_D614G	1839357 (97.70%)	185	Oct-20	hCoV-19/Italy/MAR-UnivPM30_45476/2020	-Antigenic Drift and Virulence and Host Change -Ligand Binding -Viral Oligomerization Interfaces Viral Oligomerization Interfaces
Spike_D950N	36277 (1.93%)	70	Mar-20	hCoV-19/Iran/K1r-108/2020	-
Spike_E156G	32810 (1.74%)	62	Mar-20	hCoV-19/Panama/328688/2020	-
Spike_F157del	33063 (1.76%)	63	Jul-20	hCoV-19/USA/TX-HMH-MCoV-40913/2020	-
Spike_G142D	25756 (1.37%)	61	Mar-20	hCoV-19/England/BRIS-124CD4/2020	-
Spike_K77T	777 (0.04%)	24	Dec-20	hCoV-19/Switzerland/ZH-ETHZ-431373/2020	-
Spike_L452R	101257 (5.38%)	108	Mar-20	hCoV-19/Denmark/ALAB-HH65/2020	-Host Change and Others and Antigenic Drift -Antibody Recognition Sites -Increased rate of membrane fusion, internalization and thus better transmissibility
Spike_P681R	45877 (2.44%)	91	Apr-20	hCoV-19/Indonesia/GO-NIHRD-PME20208/2020	-Antibody Recognition Sites
Spike_R158del	33071 (1.76%)	63	Jul-20	hCoV-19/USA/TX-HMH-MCoV-40913/2020	-Antibody Recognition Sites

Spike_T19R	320114(1.07%)	64	Apr-20	hCoV-19/Indonesia/GO-NIHRD-PME20208/2020	-Removes a potential N-glycosylation site which may also affect antigenic and other properties of this strain
Spike_T478K	56250 (2.99%)	79	April 2020	hCoV-19/Indonesia/GO-NIHRD-PME20208/2020	-Host Change and Others and Antigenic Drift. -Host Surface Receptor Binding -Antibody Recognition Sites -Viral Oligomerization Interfaces
