



RESEARCH ARTICLE

Integrated genomic view of SARS-CoV-2 in India [version 1; peer review: 3 approved]

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Abstract

Background: India first detected SARS-CoV-2, causal agent of COVID-19 in late January 2020, imported from Wuhan, China. From March 2020 onwards, the importation of cases from countries in the rest of the world followed by seeding of local transmission triggered further outbreaks in India.

Methods: We used ARTIC protocol-based tiling amplicon sequencing of SARS-CoV-2 (n=104) from different states of India using a combination of MinION and MinIT sequencing from Oxford Nanopore Technology to understand how introduction and local transmission occurred.

Results: The analyses revealed multiple introductions of SARS-CoV-2 genomes, including the A2a cluster from Europe and the USA, A3 cluster from Middle East and A4 cluster (haplotype redefined) from Southeast Asia (Indonesia, Thailand and Malaysia) and Central Asia (Kyrgyzstan). The local transmission and persistence of genomes A4, A2a and A3 was also observed in the studied locations. The most prevalent genomes with patterns of variance (confined in a cluster) remain unclassified, and are here proposed as A4-clade based on its divergence within the A cluster.

Conclusions: The viral haplotypes may link their persistence to geo-climatic conditions and host response. Multipronged strategies

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including molecular surveillance based on real-time viral genomic data is of paramount importance for a timely management of the pandemic.

Keywords

SARS-CoV-2, COVID-19, COVID, viral genomes, MinION, Whole Genome Sequencing



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Introduction

The ongoing pandemic of COVID-19 caused by SARS-CoV-2 following its first appearance in China has pressed the global community to take measures to flatten its transmission (Chan *et al.*, 2020; Zhu *et al.*, 2020). The severe symptoms of infection can include pneumonia, severe acute respiratory syndrome, kidney failure and even death with a coalescence of factors (Young *et al.*, 2020; Zhu *et al.*, 2020). Many COVID-19 cases have been reported to be asymptomatic and may serve as carrier of SARS-CoV-2 (He *et al.*, 2020; Xu *et al.*, 2020). Genome sequences of SARS-CoV-2 suggest its origin and transmission patterns after it enters a new population is proving to be an important step towards formulating strategies for management of this pandemic (Andersen *et al.*, 2020; Chen & Li, 2020).

The first three cases in India were reported in late January and early February, in individuals with a travel history of Wuhan, China. India took drastic steps to contain the further spread of the virus including imposition of travel restrictions to and from the affected countries. There were no new cases of COVID-19 for almost a month. However, while the global focus was on China and other eastern countries like South Korea and Japan; European countries, the Middle East and the USA reported a surge in cases of COVID-19. March 2020 onwards, India also witnessed a surge of imported cases from countries other than China which has been further assisted with local transmission. In March, imposition of nationwide lockdown checked the epidemic curve. Despite these measurements, India is at the verge of a large outbreak as the transmission is rapidly increasing with more than 450,000 reported cases of COVID-19 by the fourth week of June 2020.

We carried out whole genome sequencing of SARS-CoV-2 (n=104) from Pan-India through Surveillance Program of the National Center for Disease Control (NCDC), Delhi. Here, we combine genetic and epidemiological data to understand the genetic diversity, evolution, and epidemiology of SARS-CoV-2 across India. The spectrum of variations would be an important tool towards contact tracing, effective diagnostics and backbone for drug and vaccine development.

Methods

Subject recruitment

The study was conducted jointly by the NCDC and CSIR-Institute of Genomics and Integrative Biology (CSIR-IGIB). Institutional ethical clearance was obtained at both institutes prior to initiation of research; the need for consent from the patients was waived by the committee. A total of 127 laboratory-confirmed cases of COVID-19 from targeted testing and available samples at NCDC which represent different geographic locations or states and travel history from different countries during the early phase of the outbreak (Table 1 and Extended data, Supplementary figure S1 [Kumar *et al.*, 2020b]). were included in the study for genomic analyses. Targeted testing involved suspected cases; having symptoms (fever, cough and breathlessness) with recent travel history to high-risk countries (China, South Asia, Middle East, European countries such as Italy, Spain, UK, France and USA) or positive contacts of COVID-19 cases.

Sample collection and molecular investigations

The nasopharyngeal and oropharyngeal swabs (in viral transport medium) were received at NCDC, Delhi through the Integrated Disease Surveillance Programme were subjected to viral inactivation followed by RNA extraction using QIAamp Viral RNA Mini Kit (Cat. No. 52906, Qiagen). Total RNA content in the elute was quantified using NanoDrop (Thermo Fisher Scientific). The 260/280 ratio ranged between 1.6–2.2 for the majority of the samples. To ensure that sub-optimal RNA samples are also included in the study, we made use of SuperScript IV (Cat. No. 18091050, Thermo Fisher Scientific, Waltham, MA, USA), for superior first strand cDNA synthesis and included them for sequencing.

Molecular diagnosis of COVID-19

A quantitative reverse transcription (RT)-PCR assay was used on purified RNA for detection of SARS-CoV-2 in the samples. Quantitative RT-PCR was carried out using TaqMan assay chemistry on ABI7500 platform. The primer/probe concentrations and reaction conditions for diagnostics were as per the WHO protocols (Corman *et al.*, 2020). Two target genes were used for diagnosis of SARS-CoV-2, envelope (E) gene for screening and RNA dependent RNA polymerase (RdRp gene) for confirmation. The positive samples were analyzed based on the country of origin (traveller), contact with positive case, geographical location (community), gender and age. Samples from each group were selected and further processed for WGS of the SARS-CoV-2.

Whole genome sequencing of SARS-CoV-2

cDNA synthesis: Total RNA from SARS-CoV-2 positive samples were quantified using Nanodrop and 50 ng of the RNA was taken for double-stranded cDNA synthesis. Briefly, first strand cDNA was made using 1.0 µl of random hexamer (50 ng/µl), 1.0 µl of dNTPs (10 nM) and 13.0 µl of total RNA with volume adjusted with nuclease-free water (NFW), followed by incubation at 65°C for 5 mins and cooling on ice. To this, 4.0 µl of 5X SSRT IV Buffer, 1.0 µl of 100 mM DTT, 1.0 µl of ribonuclease inhibitor and 1.0 µl of SSRT IV enzyme (200 U/µl) was added (Cat. No. 18091050, Thermo Fisher Scientific, Waltham, MA, USA) with incubation at 23°C for 10 minutes, 50°C for 10 minutes and 80°C for 10 minutes. 1.0 µl of RNase H was added to this and incubated at 37°C for 20 mins. Next, 20.0 µl of first strand cDNA was heated at 95°C for 3 minutes after addition of 10 pmol of random primers, 10 µM dNTPs and 1X Klenow Buffer, followed by immediate cooling on ice. Soon after, 1.0 µl of Klenow Fragment (Cat. No. M0210S, New England Biolabs) was added with incubation at 37°C for 60 mins, 75°C for 10 mins and 4°C for 10 mins. This was followed by Ampure beads purification (Cat. No. A63881, Beckman Coulter) and quantification using Qubit dsDNA HS assay kit (Cat. No. Q32854, Invitrogen).

Nanopore library preparation and sequencing: A total of 100 ng of double stranded cDNA was taken for next generation sequencing (NGS) using a highly multiplexed PCR amplicon approach for sequencing on the Oxford Nanopore Technologies (ONT) (Oxford, United Kingdom) MinION using V3 primer

Table 1. Frequency and description the variations obtained in the SARS-CoV-2 genome from major identified cluster from 104 sequences.

POS	Gene	Gene	clade	Aminoacid change	Amino Acid changes	effect	Global Frequency					Variant count in this study
							A	T	G	C	N	
11083	NSP6	orf1ab	A3	Leu3606Phe	QHD43415.1:p.3606L>F; QHD43415.1:p.3606-	LOW; MODERATE; MODIFIER	0	1137	7544	0	41	68
13730	NSP12/RdRP	orf1ab	-	Ala4489Val	QHD43415.1:p.4489A>V	MODERATE	0	99	0	8622	1	65
6312	NSP3	orf1ab	-	Thr2016Lys	QHD43415.1:p.2016T>K; QHD43415.1:p.2016T>I; QHD43415.1:p.2016T>R	MODERATE	93	3	0	8625	1	62
28311	N-capsid	N	-	Pro13Leu	QHD43423.2:p.13P>L; QHD43423.2:p.13P>R; QHD43423.2:p.13-	MODERATE; MODIFIER	0	160	1	8560	1	53
23929	S-Protein	S	-	Tyr789Tyr	QHD43416.1:p.789Y; QHD43416.1:p.789-	LOW; MODIFIER	0	88	0	8627	7	46
14408	NSP12/RdRP	orf1ab	A2a	Pro4715Leu	QHD43415.1:p.4715P>L; QHD43415.1:p.4715-	MODERATE; MODIFIER	0	5325	0	3381	16	26
23403	S-Protein	S	A2	Asp614Gly	QHD43416.1:p.614-; QHD43416.1:p.614D>G	MODIFIER; MODERATE	3346	0	5356	0	20	26
241	5'UTR	5'UTR	A2	0	QHD43415.1	MODIFIER; DISTANCE=25	0	5194	0	3149	379	24
3037	NSP3	orf1ab	A2	Phe924Phe	QHD43415.1:p.924F; QHD43415.1:p.924-	LOW; MODIFIER	1	5348	0	3352	21	23
6310	NSP3	orf1ab	-	Ser2015Arg	QHD43415.1:p.2015S>R; QHD43415.1:p.2015-; QHD43415.1:p.2015S	MODERATE; MODIFIER; LOW	28	17	0	8672	5	22
25563	ORF3a	ORF3a	A2a2	Gln57His	QHD43417.1:p.57Q>H; QHD43417.1:p.57-	MODERATE; MODIFIER	0	2300	6415	1	6	9
1397	NSP2	orf1ab	A3	Val378Ile	QHD43415.1:p.378V>I	MODERATE	137	0	8584	0	1	7
18877	NSP14/Exonuclease	orf1ab	-	Leu6205Leu	QHD43415.1:p.6205L	LOW	0	208	0	8512	2	7
884	NSP2	orf1ab	-	Arg207Cys	QHD43415.1:p.207R>C	MODERATE	0	39	0	8682	1	6
8653	NSP4	orf1ab	-	Met2796Ile	QHD43415.1:p.2796M>I	MODERATE	0	37	8680	0	5	6
26735	M-Protein	M	-	Tyr71Tyr	QHD43419.1:p.71Y; QHD43419.1:p.71-	LOW; MODIFIER	0	41	0	8677	4	6
28688	N-capsid	N	A3	Leu139Leu	QHD43423.2:p.139L; QHD43423.2:p.139-	LOW; MODIFIER	0	8586	0	130	6	6
29742	3'UTR	3'UTR	A3	0	QHI42199.1	MODIFIER; DISTANCE=68	61	120	8268	0	273	6
12685	NSP8	orf1ab	-	Gln4140His	QHD43415.1:p.4140Q>H; QHD43415.1:p.4140-	MODERATE; MODIFIER	0	0	8720	0	2	5
16993	NSP13/Helicase	orf1ab	-	Tyr5577His	QHD43415.1:p.5577Y>H	MODERATE	0	8715	0	6	1	5
22444	S-Protein	S	-	Asp294Asp	QHD43416.1:p.294D	LOW	0	13	0	8438	271	5
25461	ORF3a	ORF3a	-	Ala23Ala	QHD43417.1:p.23A	LOW	0	8714	0	6	2	5
28854	N-capsid	N	-	Ser194Leu	QHD43423.2:p.194S>L; QHD43423.2:p.194-	MODERATE; MODIFIER	0	64	0	8654	4	5
1706	NSP2	#N/A	-	Ser481fs	#N/A	#N/A	0	8721	0	0	1	4
1707	NSP2	orf1ab	-	Ser481Phe	QHD43415.1:p.481S>F; QHD43415.1:p.481-	MODERATE; MODIFIER	0	3	0	8716	3	4
658	NSP1	orf1ab	-	Ala131Ala	QHD43415.1:p.131A; QHD43415.1:p.131-	LOW; MODIFIER	0	8706	0	14	2	3
1820	NSP2	orf1ab	-	Gly519Ser	QHD43415.1:p.519G>S; QHD43415.1:p.519-	MODERATE; MODIFIER	13	0	8708	0	1	3
7621	ORF1a/b	orf1ab	-	Cys2452Cys	QHD43415.1:p.2452C	LOW	0	8720	0	0	2	3
8782	NSP4	orf1ab	-	Ser2839Ser	QHD43415.1:p.2839S; QHD43415.1:p.2839-	LOW; MODIFIER	0	1370	0	7344	8	3
14805	NSP12/RdRP	orf1ab	A1a1	Tyr4847Tyr	QHD43415.1:p.4847Y; QHD43415.1:p.4847-	LOW; MODIFIER	0	892	0	7813	17	3

POS	Gene	Gene	Gene	Gene	clade	Amino acid change	Amino Acid changes	effect	Global Frequency					Variant count in this study
									A	T	G	C	N	
18486	NSP14/Exonuclease	orf1ab	-	Leu6074Leu	-	QHD43415.1:p.6074L		LOW	0	3	0	8717	2	3
19524	NSP14/Exonuclease	orf1ab	-	Leu6420Leu	-	QHD43415.1:p.6420L; QHD43415.1:p.6420-		LOW; MODIFIER	0	33	0	8501	188	3
21792	S-Protein	S	-	Lys77Met	-	QHD43416.1:p.77K>M		MODERATE	8717	0	0	0	5	3
27191	M-Protein	M	-	Ter223Ter	-	QHD43419.1:p.223-; QHD43419.1:p.223*		MODIFIER; LOW	8717	0	2	0	3	3
28881	N-capsid	N	A2a1	Arg203Lys	-	QHD43423.2:p.203R>K; QHD43423.2:p.203R>M; QHD43423.2:p.203-		MODERATE; MODIFIER	1357	1	7347	0	17	3
28882	N-capsid	N	A2a1	Arg203Arg	-	QHD43423.2:p.203R; QHD43423.2:p.203R>S; QHD43423.2:p.203-		LOW; MODERATE; MODIFIER	1353	0	7353	0	16	3
28883	N-capsid	N	A2a1	Gly204Arg	-	QHD43423.2:p.204-; QHD43423.2:p.204G>R		MODIFIER; MODERATE	0	0	7353	1353	16	3
1059	NSP2	orf1ab	A2a2a	-	-	QHD43415.1:p.265T>I; QHD43415.1:p.265-		MODERATE; MODIFIER	0	1900	0	6816	6	2
1281	NSP2	orf1ab	-	Ala339Val	-	QHD43415.1:p.339A>V; QHD43415.1:p.339-		MODERATE; MODIFIER	0	3	0	8717	2	2
1947	NSP2	#N/A	-	-	-	#N/A		#N/A	0	8720	0	0	2	2
2480	NSP2	orf1ab	-	Ile739Val	-	QHD43415.1:p.739-; QHD43415.1:p.739I>V		MODIFIER; MODERATE	8419	0	292	0	11	2
2558	NSP2	orf1ab	A1a1b	Pro765Ser	-	QHD43415.1:p.765P>S; QHD43415.1:p.765-		MODERATE; MODIFIER	0	321	0	8390	11	2
7600	ORF1a/b	orf1ab	-	Cys2445Cys	-	QHD43415.1:p.2445C; QHD43415.1:p.2445-		LOW; MODIFIER	0	0	0	8721	1	2
13920	NSP12/RdRP	#N/A	-	Lys4552Lys	-	#N/A		#N/A	0	0	8721	0	1	2
16355	NSP13/Helicase	orf1ab	-	Lys5364Arg	-	QHD43415.1:p.5364K>R		MODERATE	8721	0	0	0	1	2
18395	NSP14/Exonuclease	orf1ab	-	-	-	QHD43415.1:p.6044A>V		MODERATE	0	2	0	8719	1	2
18803	NSP14/Exonuclease	orf1ab	-	Ser6180Ile	-	QHD43415.1:p.6180S>I		MODERATE	0	0	8721	0	1	2
19684	EndoRNAse	orf1ab	-	Val6474Leu	-	QHD43415.1:p.6474V>L		MODERATE	0	43	8663	0	16	2
21137	O-ribose methyltransferase	orf1ab	-	Lys6958Arg	-	QHD43415.1:p.6958-; QHD43415.1:p.6958K>R		MODIFIER; MODERATE	8705	0	14	0	3	2
22289	S-Protein	S	-	Ala243Ser	-	QHD43416.1:p.243A>S		MODERATE	0	2	8716	0	4	2
26144	ORF3a	ORF3a	A1a	Gly251Val	-	QHD43417.1:p.251G>V; QHD43417.1:p.251-		MODERATE; MODIFIER	0	820	7890	0	12	2
28144	ORF8	ORF8	B	Leu84Ser	-	QHD43422.1:p.84L>S; QHD43422.1:p.84-		MODERATE; MODIFIER	0	7346	0	1353	23	2
28144	ORF8	ORF8	B	Leu84Ser	-	QHD43422.1:p.84L>S; QHD43422.1:p.84-		MODERATE; MODIFIER	0	7346	0	1353	23	2
29555	orf	Intergenic	-	-	-	0 QH142199.1		MODIFIER,DISTANCE=3	0	1	0	8710	11	2
29555	orf	Intergenic	-	-	-	0 QH142199.1		MODIFIER,DISTANCE=3	0	1	0	8710	11	2
29729	3'UTR	3'UTR	-	-	-	QH142199.1		MODIFIER,DISTANCE=55	0	8457	1	0	264	2
84	5'UTR	5'UTR	-	-	-	-		MODIFIER	1	3	0	8137	581	1
167	5'UTR	5'UTR	-	-	-	QHD43415.1		MODIFIER,DISTANCE=99	0	1	8337	1	383	1
203	5'UTR	5'UTR	-	-	-	QHD43415.1		MODIFIER,DISTANCE=63	0	3	1	8355	363	1
337	NSP1	orf1ab	-	-	-	QHD43415.1:p.24R		LOW	0	2	0	8686	34	1
507	NSP1	orf1ab	-	-	-	QHD43415.1:p.81-86FHGHVMM>H		MODERATE	8711	0	0	0	11	1
509	NSP1	orf1ab	-	-	-	QHD43415.1:p.82-85GHVM>V		MODERATE	0	0	8710	0	12	1
561	NSP1	#N/A	-	-	-	#N/A		#N/A	0	0	8716	0	6	1

POS	Gene	Gene	clade	Aminoacid change	Amino Acid changes	effect	Global Frequency					Variant count in this study
							A	T	G	C	N	
683	NSP1	orf1ab	-	-	QHD43415.1:p.140-143LKSF>L; QHD43415.1:p.140L	MODERATE; LOW	0	3	0	8716	3	1
851	NSP2	orf1ab	-	-	QHD43415.1:p.196Y>H	MODERATE	0	8721	0	0	1	1
1281	NSP2	orf1ab	-	Ala339Val	QHD43415.1:p.339A>V; QHD43415.1:p.339-	MODERATE; MODIFIER	0	3	0	8717	2	1
1601	NSP2	orf1ab	-	-	QHD43415.1:p.446L>I	MODERATE	1	0	0	8719	2	1
1601	NSP2	orf1ab	-	-	QHD43415.1:p.446L>I	MODERATE	1	0	0	8719	2	1
1887	NSP2	orf1ab	-	-	QHD43415.1:p.541A>V; QHD43415.1:p.541-	MODERATE; MODIFIER	0	2	0	8717	3	1
1912	NSP2	orf1ab	-	-	QHD43415.1:p.549S	LOW	0	17	0	8703	2	1
1912	NSP2	orf1ab	-	-	QHD43415.1:p.549S	LOW	0	17	0	8703	2	1
2040	NSP2	orf1ab	-	-	QHD43415.1:p.592T>I	MODERATE	0	1	0	8719	2	1
2485	NSP2	orf1ab	-	-	QHD43415.1:p.740I	LOW	0	2	0	8719	1	1
2558	NSP2	orf1ab	A1a1b	Pro765Ser	QHD43415.1:p.765P>S; QHD43415.1:p.765-	MODERATE; MODIFIER	0	321	0	8390	11	1
3145	NSP3	orf1ab	-	-	QHD43415.1:p.960L>F	MODERATE	0	9	8710	0	3	1
3176	NSP3	orf1ab	-	-	QHD43415.1:p.971P>S	MODERATE	0	4	0	8712	6	1
3429	NSP3	orf1ab	-	-	QHD43415.1:p.1055T>I	MODERATE	0	1	0	8720	1	1
3604	NSP3	orf1ab	-	-	QHD43415.1:p.1113H	LOW	0	0	0	8721	1	1
4144	NSP3	#N/A	-	-	#N/A	#N/A	0	0	8718	0	4	1
4680	NSP3	#N/A	-	-	#N/A	#N/A	0	0	0	8721	1	1
4859	NSP3	#N/A	-	-	#N/A	#N/A	0	0	8720	0	2	1
5008	NSP3	orf1ab	-	-	QHD43415.1:p.1581T	LOW	0	0	8720	0	2	1
5151	NSP3	orf1ab	-	-	QHD43415.1:p.1629V>A	MODERATE	0	8720	0	0	2	1
5657	NSP3	orf1ab	-	-	QHD43415.1:p.1798V>I; QHD43415.1:p.1798V>L	MODERATE	1	0	8720	0	1	1
5730	NSP3	orf1ab	-	-	QHD43415.1:p.1822T>I	MODERATE	0	10	0	8711	1	1
6395	NSP3	orf1ab	-	-	QHD43415.1:p.2044L	LOW	0	3	0	8709	10	1
7071	NSP3	#N/A	-	-	#N/A	#N/A	1	0	8718	0	3	1
7350	ORF1a/b	#N/A	-	-	#N/A	#N/A	0	0	0	8720	2	1
7734	ORF1a/b	#N/A	-	-	#N/A	#N/A	0	8717	0	0	5	1
8595	NSP4	orf1ab	-	-	QHD43415.1:p.2777T>I	MODERATE	0	0	0	8721	1	1
9477	NSP4	orf1ab	-	-	QHD43415.1:p.3071F>Y	MODERATE	150	8567	0	0	5	1
9634	NSP4	orf1ab	-	-	QHD43415.1:p.3123L>F	MODERATE	8710	4	0	0	8	1
10039	NSP4	orf1ab	-	-	QHD43415.1:p.3258T; QHD43415.1:p.3258-	LOW; MODIFIER	0	1	0	8717	4	1
10449	Mpro	orf1ab	-	-	QHD43415.1:p.3395P>L; QHD43415.1:p.3395-	MODERATE; MODIFIER	0	3	0	8717	2	1
11074	NSP6	orf1ab	-	-	QHD43415.1:p.3604F>X; QHD43415.1:p.3603F; QHD43415.1:p.3603F>FX;	HIGH; LOW; MODERATE; MODIFIER	0	22	0	86	3	1
11109	NSP6	orf1ab	-	-	QHD43415.1:p.3615A>V	MODERATE	0	20	0	8699	3	1
12929	NSP9	orf1ab	-	-	QHD43415.1:p.4222-	MODIFIER	0	0	8720	0	2	1

POS	Gene	Gene	clade	Aminoacid change	Amino Acid changes	effect	Global Frequency					Variant count in this study
							A	T	G	C	N	
13038	NSP10	#N/A	-	-	#N/A	#N/A	0	0	0	8720	2	1
13862	NSP12/RdRP	orf1ab	-	-	QHD43415.1.p.4533T>I; QHD43415.1.p.4533-	MODERATE; MODIFIER	0	13	0	8707	2	1
14220	NSP12/RdRP	orf1ab	-	-	QHD43415.1.p.4652D	LOW	0	1	0	8720	1	1
15290	NSP12/RdRP	#N/A	-	-	#N/A	#N/A	0	0	0	8721	0	1
15344	NSP12/RdRP	#N/A	-	-	#N/A	#N/A	0	0	0	8721	1	1
15435	NSP12/RdRP	orf1ab	-	-	QHD43415.1.p.5057-	MODIFIER	8720	0	0	0	2	1
15669	NSP12/RdRP	#N/A	-	-	#N/A	#N/A	0	8720	0	1	1	1
16221	NSP12/RdRP	orf1ab	-	-	QHD43415.1.p.5319P	LOW	0	0	0	8721	0	1
16377	NSP13/Helicase	orf1ab	-	-	QHD43415.1.p.5371P	LOW	0	5	8716	0	1	1
17122	NSP13/Helicase	orf1ab	-	-	QHD43415.1.p.5620A>T; QHD43415.1.p.5620A>S	MODERATE	1	0	8719	0	2	1
17122	NSP13/Helicase	orf1ab	-	-	QHD43415.1.p.5620A>T; QHD43415.1.p.5620A>S	MODERATE	1	0	8719	0	2	1
17403	NSP13/Helicase	orf1ab	-	-	QHD43415.1.p.5713A	LOW	0	2	0	8718	2	1
17415	NSP13/Helicase	orf1ab	-	-	QHD43415.1.p.5717A	LOW	0	8721	0	0	1	1
17415	NSP13/Helicase	orf1ab	-	-	QHD43415.1.p.5717A	LOW	0	8721	0	0	1	1
17656	NSP13/Helicase	orf1ab	-	-	QHD43415.1.p.5798M>V	MODERATE	8700	0	3	0	19	1
17747	NSP13/Helicase	orf1ab	B1a1	-	QHD43415.1.p.5828P>L; QHD43415.1.p.5828-	MODERATE; MODIFIER	0	856	0	7842	24	1
17858	NSP13/Helicase	orf1ab	B1a	-	QHD43415.1.p.5865Y>C	MODERATE	7845	0	876	0	1	1
18060	NSP14/Exonuclease	orf1ab	B1	-	QHD43415.1.p.5932L; QHD43415.1.p.5932-	LOW; MODIFIER	0	886	0	7832	4	1
18078	NSP14/Exonuclease	orf1ab	-	-	QHD43415.1.p.5938K	LOW	1	0	8720	0	1	1
18312	NSP14/Exonuclease	orf1ab	-	-	QHD43415.1.p.6016V	LOW	1	2	0	8718	1	1
18312	NSP14/Exonuclease	orf1ab	-	-	QHD43415.1.p.6016V	LOW	1	2	0	8718	1	1
18573	NSP14/Exonuclease	orf1ab	-	-	QHD43415.1.p.6103S	LOW	0	8720	0	1	1	1
19644	EndoRNase	#N/A	-	-	#N/A	#N/A	0	8697	0	0	25	1
19861	EndoRNase	orf1ab	-	-	QHD43415.1.p.6533A>T	MODERATE	1	0	8681	0	40	1
19862	EndoRNase	orf1ab	-	-	QHD43415.1.p.6533A>V; QHD43415.1.p.6533-	MODERATE; MODIFIER	0	5	0	8676	41	1
19875	EndoRNase	#N/A	-	-	#N/A	#N/A	0	0	0	8682	40	1
20255	EndoRNase	orf1ab	-	-	QHD43415.1.p.6664-; QHD43415.1.p.6664D>G	MODIFIER; MODERATE	8720	0	0	0	2	1
20429	EndoRNase	orf1ab	-	-	QHD43415.1.p.6722P>L	MODERATE	0	1	0	8720	1	1
20580	EndoRNase	orf1ab	-	-	QHD43415.1.p.6772V; QHD43415.1.p.6772-	LOW; MODIFIER	1	3	8715	0	3	1
20749	O-ribose methyltransferase	#N/A	-	-	#N/A	#N/A	0	0	8721	0	1	1
21707	S-Protein	S	-	-	QHD43416.1.p.49H>Y	MODERATE	0	29	0	8687	6	1
21989	S-Protein	S	-	-	QHD43416.1.p.143-144VY>D; QHD43416.1.p.143V>F	MODERATE	0	1	8714	0	7	1
21990	S-Protein	S	-	-	QHD43416.1.p.143-144VY>V	MODERATE	0	8712	0	0	10	1
22430	S-Protein	#N/A	-	-	#N/A	#N/A	0	0	8518	0	204	1

POS	Gene	Gene	clade	Aminoacid change	Amino Acid changes	effect	Global Frequency					Variant count in this study
							A	T	G	C	N	
22458	S-Protein	S	-	-	QHD43416.1:p.299T>I	MODERATE	0	1	0	8469	252	1
23042	S-Protein	S	-	-	QHD43416.1:p.494-	MODIFIER	0	8686	0	1	35	1
23108	S-Protein	#N/A	-	-	#N/A	#N/A	0	0	8678	1	43	1
23660	S-Protein	#N/A	-	-	#N/A	#N/A	0	0	8721	0	1	1
23677	S-Protein	S	-	-	QHD43416.1:p.705V	LOW	0	8721	0	0	1	1
24166	S-Protein	#N/A	-	-	#N/A	#N/A	8719	0	0	0	3	1
24622	S-Protein	S	-	-	QHD43416.1:p.1020-	MODIFIER	0	8719	0	0	3	1
24694	S-Protein	S	B1a1a	-	QHD43416.1:p.1044G	LOW	8634	87	0	0	1	1
24904	S-Protein	S	-	-	QHD43416.1:p.1114-; QHD43416.1:p.1114I	MODIFIER; LOW	0	5	0	8715	2	1
25318	S-Protein	S	-	-	QHD43416.1:p.1252S	LOW	0	0	0	8719	3	1
25318	S-Protein	S	-	-	QHD43416.1:p.1252S	LOW	0	0	0	8719	3	1
25350	S-Protein	S	A2a10	-	QHD43416.1:p.1263P>L; QHD43416.1:p.1263-	MODERATE; MODIFIER	0	51	0	8661	10	1
25642	ORF3a	ORF3a	-	-	QHD43417.1:p.84L	LOW	0	1	0	8720	1	1
25793	ORF3a	ORF3a	-	-	QHD43417.1:p.134R>H; QHD43417.1:p.134R>L	MODERATE	0	3	8706	0	13	1
25826	ORF3a	#N/A	-	-	#N/A	#N/A	8706	1	0	0	15	1
25919	ORF3a	ORF3a	-	-	QHD43417.1:p.176T>I	MODERATE	0	1	0	8711	10	1
25979	ORF3a	ORF3a	-	-	QHD43417.1:p.196G>V; QHD43417.1:p.196-	MODERATE; MODIFIER	0	149	8570	0	3	1
27191	M-Protein	M	-	Ter223Ter	QHD43419.1:p.223-; QHD43419.1:p.223*	MODIFIER; LOW	8717	0	2	0	3	1
27195	orf	#N/A	-	-	#N/A	#N/A	8720	0	0	0	2	1
27208	ORF6	ORF6	-	-	QHD43420.1:p.3H>Y; QHD43420.1:p.3-	MODERATE; MODIFIER	0	3	0	8716	3	1
27364	ORF6	ORF6	-	-	QHD43420.1:p.55E>*; QHD43420.1:p.55E>Q	HIGH; MODERATE	0	1	8720	0	1	1
27384	ORF6	ORF6	-	-	QHD43420.1:p.61D; QHD43420.1:p.61-	LOW; MODIFIER	0	8682	0	38	2	1
27874	ORF7b	Intergenic	-	-	QHD43422.1	MODIFIER;DISTANCE=20	0	0	0	8705	17	1
28115	ORF8	ORF8	-	-	QHD43422.1:p.74I	LOW	0	1	0	8703	18	1
28115	ORF8	ORF8	-	-	QHD43422.1:p.74I	LOW	0	1	0	8703	18	1
28253	ORF8	ORF8	-	-	QHD43422.1:p.120F>L; QHD43422.1:p.120F; QHD43422.1:p.120-; QHD43422.1:p.121I>X	MODERATE; LOW; MODIFIER; HIGH	3	5	2	8703	9	1
28657	N-capsid	N	-	-	QHD43423.2:p.128D; QHD43423.2:p.128-	LOW; MODIFIER	0	151	0	8569	2	1
28863	N-capsid	N	-	-	QHD43423.2:p.197S>L; QHD43423.2:p.197-	MODERATE; MODIFIER	0	149	0	8570	3	1
29574	ORF10	#N/A	-	-	#N/A	#N/A	0	8711	0	0	11	1
29614	ORF10	ORF10	-	-	QHI42199.1:p.19C	LOW	0	8	0	8703	11	1
29614	ORF10	ORF10	-	-	QHI42199.1:p.19C	LOW	0	8	0	8703	11	1
29807	3'UTR	#N/A	-	-	#N/A	#N/A	1	80	0	1	68	1

pools (ARTIC Protocol, see <https://artic.network/resources/ncov/ncov-amplicon-v3.pdf>). Amplification was done using Takara LA Taq® DNA Polymerase Hot-Start Version (Cat. No. RR042B, Takara) along with 2.5 mM dNTPs, 10X Buffer II LA Takara, 10 µM primer pool, with the final volume adjusted to 25 µl using NFW. Primers were made into two pools, pool A and pool B, with 5 µl of each primer from the 100 µM primer stock. The stock (100 µM) was diluted with NFW in order to obtain a working stock of each pool at 10 µM. PCR was performed with initial denaturation at 98°C for 30 secs followed by denaturation at 98°C for 15 secs, 65°C for 5 mins, for a total of 35 cycles, with hold at 4°C. Post PCR pooling (pool A and pool B) of PCR amplicons was followed by purification using Ampure beads. Following purification, 1.0 µl of the library was run on a DNA1000 Agilent bioanalyzer (Cat. No. 5067-1504, Agilent) to check for a size of ~400 bp. Next, 125 ng of each sample was taken forward for End prep with NEBNext Ultra II End Repair/dA tailing module (Cat. No. E7546, New England Biolabs). The reaction mix was incubated on a thermal cycler at 20°C for 5 mins followed by 65°C for 5 mins. Following this, 1.5 µl of End prep DNA was taken forward for native barcode ligation using native barcodes (EXP-NBD104 and EXP-NBD114, ONT) and Blunt/TA Ligase master mix (Cat. No. M0367, New England Biolabs). The mix was incubated at room temperature for 15 mins followed by purification using Ampure beads. The purified product was used for adaptor ligation using Adapter Mix II and Quick T4 DNA ligase (Cat. No. M2200L, New England Biolabs). After adaptor ligation, it was purified using a combination of short fragment buffer and Ampure beads resulting in a sequencing ready library. Library quantification was conducted using the Qubit dsDNA HS assay kit (Cat. No. Q32854, Invitrogen) and 70 ng of the library was used for sequencing. Barcoding, adaptor ligation, and sequencing were performed on samples with Ct values between 16–31. A ‘no template control’ was created at the cDNA synthesis step and amplicon generation step to detect cross-contamination between samples. Controls were barcoded and sequenced with both the high- and low-titer sample groups. The sequencing flowcell was primed and used for sequencing using MinION Mk1B.

Illumina library preparation and sequencing

A common pool of cDNA was used for making both Illumina and Nanopore sequencing libraries and subsequent sequencing. cDNA (100 ng) was used to construct the Illumina library using the Nextera XT protocol, as per manufacturer’s instructions (15031942 v05, Illumina Inc). Briefly, tagmentation of cDNA was done which tagged and fragmented the cDNA by addition of amplicon tagment mix (ATM) and tagment DNA buffer, as per manufacturer’s protocol, Illumina Inc with incubation at 55°C for 5 mins with heated lid option. Tagmentation was stopped by addition of neutralization tagment buffer. This was followed by the addition of unique index adapters (i7 and i5 adapters) to the samples. Index adapters are then used for PCR amplification at 72°C for 3 mins, 95°C for 30 secs and 12-cycles of 95°C for 10 secs, 55°C for 30 secs, 72°C for 30 secs; and 72°C for 5 mins. The PCR product was purified using AgencourtAMPure XP beads. The quantity of the sequencing ready library was measured using Qubit dsDNA HS assay

kit (Cat. No. Q32854, Invitrogen) and quality by Agilent DNA HS kit (Cat. No. 5067-4626, Agilent). Illumina’s MiSeq platform was used for sequencing.

Analysis pipeline for nanopore sequencing data

The raw fast5 files were base-called and demultiplexed using the Guppy basecaller (version 3.5.2). The fastq files were normalized by read length, thereby eliminating possible chimeric reads. Pre-alignment quality control was carried out to assess the read quality using Nanopack tools (version 1.29.0) (De Coster *et al.*, 2018). Minimapp2 (version 2.17) has been used to align the raw reads with the reference (MN908947.3) (Li, 2018). Nanopolish were used for accurate variant calling from the aligned output (Loman, 2015) with options, minimum flanking sequence - 10, ploidy - 1 and minimum candidate frequency - 0.15. The possible heterozygous variants are filtered out as a separate group after the variants have been called. Post-alignment QC was then performed with Nanopack tools as well as the seaborn (version 0.10.1) package in python to create the distribution of amplicon quality and CT-value vs coverage and depth. Finally, a consensus fasta was created, wherein genomic regions with low coverage and low quality were masked using BCFtools (version 1.9).

Miseq data analysis

The raw reads from the miseq were quality-checked by FASTQC (version 0.11.8). Trimalore was used to trim the reads containing bad quality and the minimum length of 40 base pairs was kept as a threshold for the reads. HISAT2 is used to map the reads to the human genome (GRCh37) to remove potential the human rRNA reads for the contamination with default parameters [Kim *et al.*, 2019]. The unmapped reads from the human are converted from bam to fastq using bam2fastq (version 2.29) to align to the SARS-CoV-2. HISAT2 was used to align unmapped reads with the SARS-CoV-2 reference genome (MN908947.3 build). Using both samtools (version 1.9) and BCFtools from the SARS-CoV-2 aligned bam files the consensus fasta was generated. The variants in the samples were called using BCFtools and VarScan.

Phylogeny and network analysis

The fasta sequences were aligned using MAFFT (version 7.455) considering the MN90894.3 version as the reference sequence. Phylogenetic trees were constructed using the Neighbour joining algorithm as statistical method and maximum composite likelihood as model in MEGA X software. FIGTREE (version 1.4.4) was used for the graphical visualisation of phylogenetic analysis. Pheatmap (version 1.0.12) and ComplexHeatmap (version 1.10.2) packages from R 3.6.2 were used to plot the heatmaps. Haplotype network analysis was conducted using PopART (version 1.7) [Leigh & Bryant, 2015].

Protein based annotation

In order to categorize the specific amino acid change and the proteins containing the variants, they were annotated with SnpEff (version 4.5) [Cingolani *et al.*, 2012]. The annotation was performed according to the known reference genome of SARS-CoV-2 (i.e. NC_045512) in the NCBI database

[Wang *et al.*, 2020]. SARS-CoV-2 polypeptide ORF1ab encodes 16 non-structural proteins (nsp) as a result of proteolytic processing. Hence, for better mapping of the variants present in ORF1ab, we annotated the variants according to the respective nsp residue number.

Further, conservation analysis of the full-length sequences of proteins harbouring these mutations was done on the basis of the six other coronaviruses. The multiple sequence alignment of seven protein sequences was performed with **Clustal Omega** [Madeira *et al.*, 2019]. The conservation score of ORF3a and ORF8 were calculated with low confidence due to introduced gaps at these positions during alignment. The amino acid type was defined as hydrophobic (G, A, V, L, I, M, P, F, W), polar (S, T, N, Q) or charged (H, K, R, D, E). With this definition, the type of change of residues was calculated.

Three-dimensional protein models

To map the high frequency mutations on proteins, we took protein structure models of SARS-CoV-2 from the Swiss Model repository (<https://swissmodel.expasy.org/repository/species/2697049>) [Waterhouse *et al.*, 2018] and models were generated through comparative modeling and by using Robetta prediction server [Kim *et al.*, 2004]. In total three structural models were obtained from swiss model repository and the nsp12 structure was obtained from RCSB [PDB ID 6M71] [Gao *et al.*, 2020]. The details of missing residues or structural domains of each protein is described below. The Spike protein exists as a homotrimer consisting of 1273 residues in each chain with a total of 3819 amino acids. While electron microscopy structures are available for different conformations of Spike protein, in particular S1 region, many residues within the S1 and S2 stalks are missing [Walls *et al.*, 2020; Wrapp *et al.*, 2020]. Therefore, to map the mutations onto the structure we obtained the S1 stalk model of the spike protein (residues 15-1137). Similarly, nsp3 also known as PL-PRO (papain-like proteinase) is a large multi-domain transmembrane protein. For mapping the nsp3 mutations, we considered the model for the nucleic acid binding domain (residue 1089-1203), which is conserved in betacoronaviruses [Angelini *et al.*, 2013]. The Nucleocapsid protein comprise of N-terminal and C-terminal domains connected by linker region. However, structural information for the linker region is unknown.

Results

Demographic details and travel history of the subjects

The majority of the SARS-CoV-2-positive samples were obtained from New Delhi, covering the national capital region of Delhi, India and a few clusters identified by the surveillance team (covering the states of Delhi, Tamil Nadu, Maharashtra, Uttar Pradesh, Andhra Pradesh, West Bengal, Bihar, Orissa, Rajasthan, Haryana, Punjab, Assam and Union territory of Ladakh). The mean (standard deviation) age of the total 127 subjects was 41.4±17.5 years with age range 0.5–76 years and median of 39 years. The male-to-female gender ratio of in the age group <39 years was 35:28, while the remaining 46 subjects >40 years had the ratio of 58:6. Exposure to COVID-19 was suggestive of travel history of subjects to Europe, West Asia and East Asia. A minority of subjects were from foreign countries: Indonesia

(n=14), Thailand (n=2) and Kyrgyzstan (n=2). The identified localities of the subjects will further help in molecular surveillance of SARS-CoV-2 in respective geographical regions.

Profile of SARS-CoV-2 genome sequences

The average amplicon coverage for the V3 ARTIC primers used in the study was more than 1000X coverage across the majority of the samples (*Extended data*, Supplementary figure S2 [Kumar *et al.*, 2020b]). We also looked into whether lower Ct values are a good indicator of genome coverage using a minimal set of virus mapping reads. We plotted genome coverage and average sequencing depth across Ct value of both the genes (E and RdRp). It was observed that higher Ct values (27 onwards) have increased possibility of lower genome coverage (*Extended data*, Supplementary figure S2 [Kumar *et al.*, 2020b]), although some lower Ct value samples also had incomplete genome coverage. We sequenced a subset of samples on orthogonal platforms and sequencing methods (shotgun and amplicon) using ONT and Illumina platform. Significantly, we observed that the genetic variants were common between both the platforms.

NGS analysis and construction of phylogeny network for SARS-CoV-2 sequences

A total of 104 samples passed the quality threshold for mapping full genome coverage threshold for SARS-CoV-2 genome <0.05 N content with median coverage ~1500x (see *Underlying data* for each accession number [Kumar *et al.*, 2020a]). A total of 23 samples that did not qualify the threshold criteria were excluded from strain identification. The phylogenetic analysis of 104 high quality sequences reveal all the strains to be grouped into two major clades, a sub-clade and other clades (**Figure 1** and *Extended data*, Supplementary figure S3 [Kumar *et al.*, 2020b]). From variants perspectives, we observed 163 variants representing singletons (107 variants), rare: 2-5% (45 variants), and common variants: >5% (11 variants). The common variants observed were 241 (Leader sequence), 3037 (NSP3), 6310 (NSP3), 6312 (NSP3), 11083 (NSP6), 13730 (NSP12/RdRp), 14408 (NSP12/RdRp), 23403 (S-Protein), 23929 (S-Protein), 25563 (ORF3a), 28311 (N-capsid). The following cluster-based segregation of SARS-CoV2 sequences was observed.

Cluster 1: The sequences (n=26) in this cluster belonged to the G-clade [variant 23403 (Spike protein D614G) as per GISAID nomenclature (Global Initiative on Sharing All Influenza Data)] Based on Nextstrain classification these 26 sequence belonged to A2a clade [(denoted by positions C241T; C3037T, A23403G (S: D614G); C14408T (ORF1b/RdRp: P314L)] (**Figure 1A**). The additional frequent variants in this cluster observed were 25563 (ORF3a, n=9), 18877 (NSP14/Exonuclease, n=7), 26735 (M-protein, n=6), 22444 (S-protein, n=5), and 28854 (N-capsid, n=5). One novel variant 1947 T>C (NSP2) was observed in two strains in this cluster.

Cluster 2: In our study large numbers of strains (n=65) belong to this unclassified cluster (as per GISAID and Nextstrain). The strains in this cluster had the predominant variants, G11083T variant (NSP6) (n=65), C13730T in RdRp (n=65), C28311T (N-capsid); n=65, C6312A (NSP3 variant); n=64 (one sequence

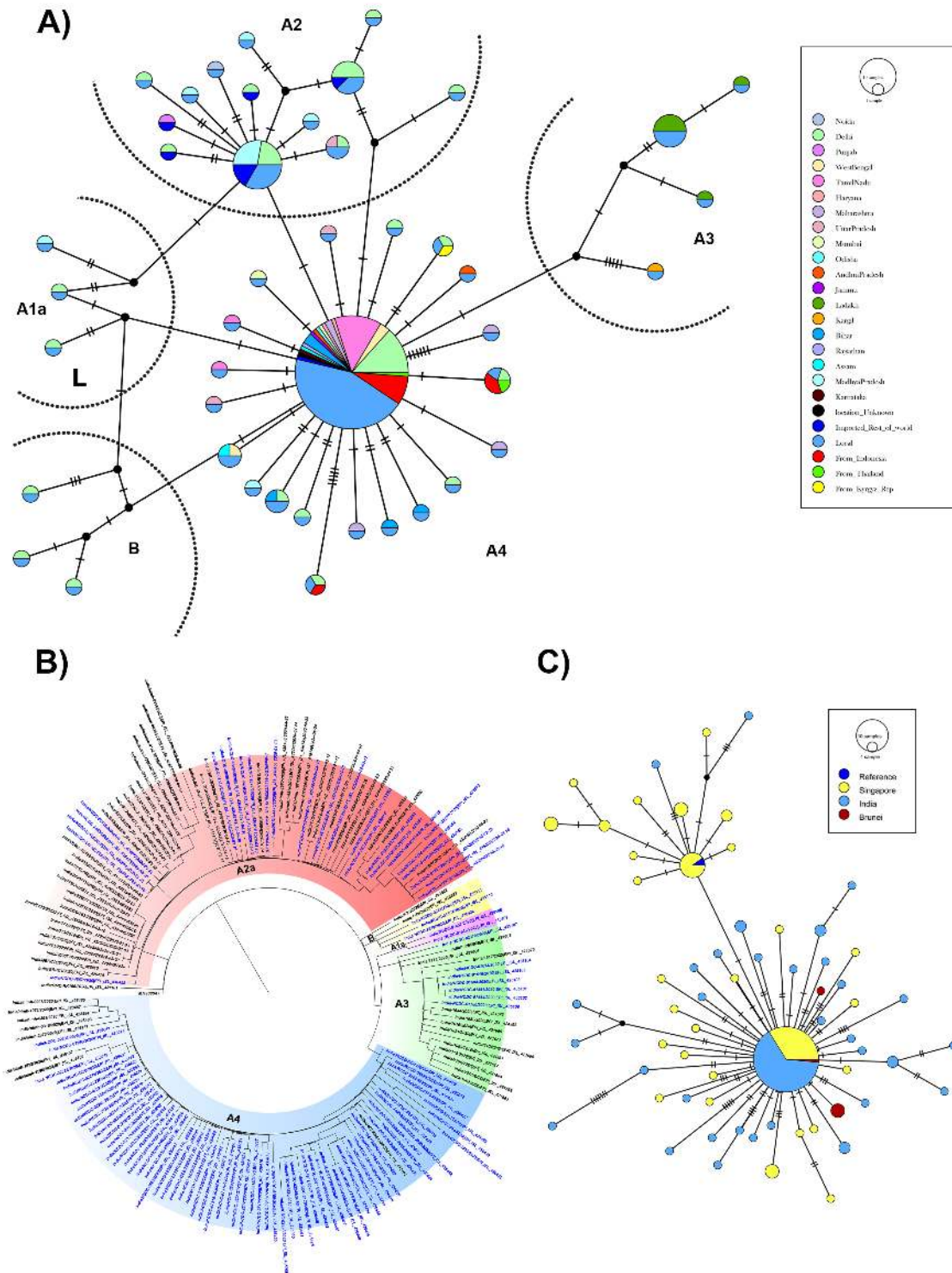


Figure 1. Haplotype network and phylogenetic analysis of SARS-CoV-2 sequences. (A) The network analysis (integer neighbourhood joining network at reticulation tolerance value of 0.5, popART) of SARS-CoV-2 sequences from this study showing distinct clades with their geographical locations. A4 clade described in this study for the first time has widespread geographical affiliations. A3 being more confined to Ladakh and mostly these clusters represent introduction of the infection through travel history. Mutations are marked with hatch lines connecting the nodes. **(B)** Phylogenetic tree (generated using MEGA) of SARS-CoV-2 genomes from sequences submitted from across India depicting major clade-based distribution of SARS-CoV-2 in India. **(C)** Network analysis (Median spanning network) of A4 clade sequences submitted from Indian and neighbouring East Asian countries.

being called N), C23929T (S-protein), n=50 (other being low depth/N bases). The variant C6310A (NSP3); n=22 being observed as another frequent alteration (Figure 1B). We also observed few novel variants, G12685T (NSP8); n=5 and TC1706T (NSP2); n=4, T7621C (ORF1a/b); n=3, A21792T (S protein); n=3, G13920A (NSP12/RdRp); n=2, A16355G (NSP13/Helicase); n=2 and G18803T (NSP14/Exonuclease); n=2 in this cluster. The majority of the key cluster variants 11083, 13730, 28311, 6312, 23929 are also shared in sequences submitted from Singapore and Brunei; additionally, similar clade sequences were observed in India submitted by National Institute of Mental Health and Neuro-Sciences (NIMHANS) and Gujarat Biotechnology Research Centre (GBRC) cohort (see *Underlying data* for details of all accession numbers [Kumar *et al.*, 2020a]). Based on the geographical location of the subjects of this cluster, a considerable number of Indonesians (n=7) and two each from Thailand and Kyrgyzstan were part of this cluster from our study site. This probably suggests introduction of this particularly from East Asian countries into India.

Cluster 3: This subclass of strains (n=7) harbouring a common variant G11083T (NSP6), G1397A (NSP2) and T28668C (N-capsid) are described for the A3 clade (Nextstrain) in additions to G29742T (Figure 1A). Other mutated positions, i.e. C884T (NSP2), G8653T (NSP4) were observed in 5 samples, whereas T16993C (NSP13), n=4; T25461C (ORF3a), n=4 and A27191G (M-protein), n=2 are putative novel sites.

The phylogeny analysis of these clusters segregated with the other Indian SARS-CoV-2 genome sequences as recently reported (GISAID) (Figure 1B).

Other SARS-CoV-2 genomes: Two SARS-CoV-2 belonging to the A1a clade had a SNP profile of 11083(NSP6)/14805 (NsP12/RdRp)/2480 (NSP2)/2558 (NSP2)/26144 (ORF3a). In addition, we observed three B clade sequences having position 8782 C>T (NSP4) and 28144 T>C (ORF8; S clade GISAID) mutated and with one sequence with an additional C18060T B1 variant. One genome from Maharashtra had no variants and probably represented the first genome sequenced from Wuhan, China.

Redefining cluster 2 with neighbourhood re-joining

With over represented variants in cluster 2 for variants 11083/13730/28311/6312/23929, we defined this cluster with A4 clade. This has similarity with sequences submitted from Singapore, Brunei and other Indian sequences submitted. The haplotype network analysis suggests that these sequences are having a common origin from East Asia/South-East Asia (Figure 1C and Figure 2). This A4 clade has multiple variants in important region of viral genome, RdRp (A97V), N-capsid (P12L), NSP3 (T2016K), NSP6 (L37F) and NSP3 (S1197R) variants. In our cohort of samples, the majority of subjects were from Tamil Nadu, Delhi and Indonesia and others were from various other states (Figure 2).

Protein-wise analysis of SARS-CoV-2 variants

To provide quantitative insights into the mutant proteins, we characterized amino acid substitutions across the 104 viral

genomes. Of the 53 point mutations identified, 29 were missense that resulted in amino acid substitutions. *Extended data*, Supplementary figure S4 [Kumar *et al.*, 2020b] plots the occurrence of these mutations as a function of each viral protein. The frequency of amino acid variations was highest in nsp6 (L37F), present in 68 genomes, followed by nsp12 (A97V) in 65, nsp3 (T1198K) in 62 and nucleocapsid (P13L) in 53 genomes. Interestingly, the D614G mutation in spike protein, which is considered as a prevalent global mutation [Bhattacharyya *et al.*, 2020; Korber *et al.*, 2020], was present in only 26 of the 104 sequenced genomes.

The analysis of occurrence of each mutation with the type of amino acid change have shown that ~45% of these are synonymous changes (*Extended data*, Supplementary figure S5 [Kumar *et al.*, 2020b]). Within frequently occurring mutations, P13L, L37F, A97V also showed no major residue alterations. However, T1198K in nsp3 involve acquisition of a charged group along with the key S protein mutation (D614G) also involves loss of the charged group. These mutations that lead to positively charged groups may cause more severe structural and functional effects.

We also compared SARS-CoV-2 mutation sites with other six coronavirus sequences (*Extended data*, Supplementary figure S5b [Kumar *et al.*, 2020b]). Most of the mutations were present in variable locations. Out of 29 mutations, 10 are present on highly conserved residue locations. Interestingly, a higher frequency of mutations are at positions that evolve faster/are variable across the coronaviruses, except for A97L and L37F, which are present on conserved locations.

The structural analysis of different viral proteins, nucleocapsid, nsp3, nsp12, and spike protein was conducted and analysis of nucleocapsid protein [Kang *et al.*, 2020] showed its variants were present in the linker region (Figure 3A). The observed mutations in nsp12 (a highly conserved protein) are overlaying onto the interface (P323L) and NiRAN (A97L) region. The latter is critical as it contains a Zn⁺ binding site; however, little is known about the exact functional output. In contrast, the P323L mutation is present on protein interaction junctions where a hydrophobic cleft is known to bind to inhibitors (Figure 3B).

The amino acid change from proline to leucine may result in significant backbone changes, due to the absence of unique proline-induced distortions in the protein backbone. Next, we mapped mutations within Nsp3 protein (Figure 3C). In particular, the mutations were present on the NAB domain of nsp3, which is a nucleic acid binding domain and also interacts with nsp12 [Jian *et al.*, 2018]. This mutation may impact RNA synthesis machinery; however, little is known about its exact mechanism of action. Lastly, the D614G mutation in spike protein is an interesting substitution and has been reported with increased tally (Figure 3D) [Bhattacharyya *et al.*, 2020; Korber *et al.*, 2020]. Structurally, this mutation is located in the S1 subunit that also contains the RBD domain. Although present outside the functional region, the proximity of D614G around S1 cleavage site implicates an important change in the local environment.

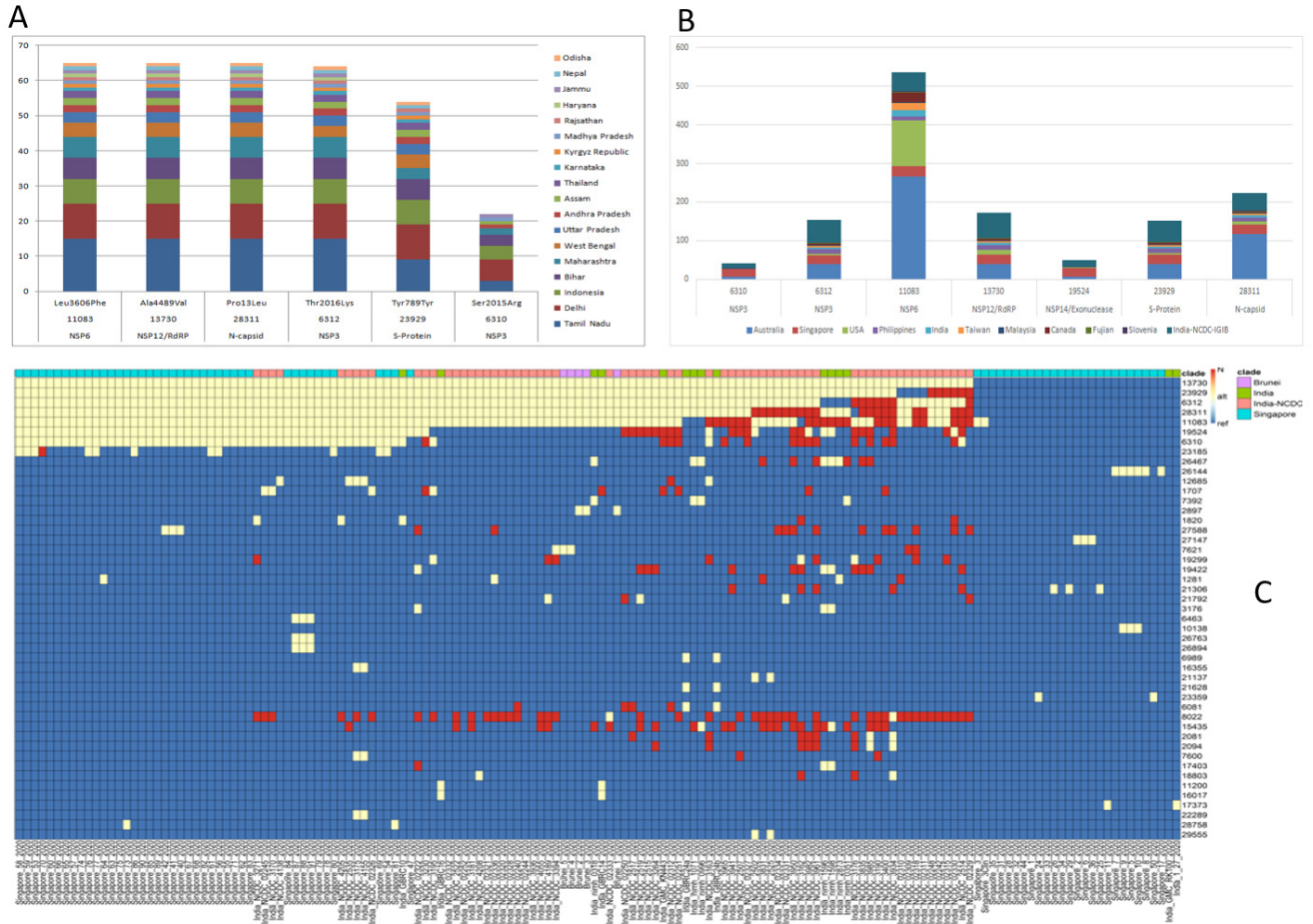


Figure 2. Distribution of A4 clade within India and globally. (A) Distribution of A4 clade variants across different geographical regions from the cohort. (B) Distribution of A4 clade variants across different geographical regions across the globe. (C) Comparison of A4 cluster sequences across the South-East Asian region showing sharing of variants and haplotype across South-East Asian region.

Discussion

This is the first comprehensive genomic picture of the SARS-CoV-2 prevalent in the Indian population during the early phase of outbreaks. The understanding is important keeping in view the vast geographical expanse and population density of India. There were three major waves of viral entry in India associated with multiple outbreaks (*Extended data*, Supplementary figure S6 [Kumar *et al.*, 2020b]). The first wave includes importation of SARS-CoV-2 (A2a cluster) through travelers from Europe (Italy, UK, France, etc.) and the USA. Second wave of SARS-CoV-2 (A3 cluster) was linked with the Middle East (Iran and Iraq). The third wave comprises combined viral (haplotype redefined as A4) entries from Southeast Asia (Indonesia, Thailand and Malaysia) and Central Asia (Kyrgyzstan). The study, taken together with those of other reported genomes (Potdar *et al.*, 2020), revealed that the A4 cluster (previously unclassified) is the most prevalent in the available genome sequences from India. The observed distinct A4 genome lineage of SARS-CoV2 in the Indian Subcontinent, which is present in East Asian Countries like Singapore and Indonesia, may allow further research and investigation to understand the evolution of SARS-CoV-2 genomes in Southeast Asian countries. Many

novel mutations identified may be specific to Indian conditions, but more genomic data is needed to strengthen the assumption to rule out sampling bias and other factors (Lu *et al.*, 2020). However, a more detailed analysis of these genomes might provide information whether these variations need to be considered during design of diagnostic primers as the need for testing shoots up. It may allow for creation of cost-effective panels to trace the movement of lineage specific strains across geographical regions more rapidly and effectively. Lots of efforts are ongoing to identify suitable vaccine candidates through docking studies. These observations are important to consider the variants that map to the Indian genomes during such prioritization studies, since these strains would now form a major fraction of the genomes that are likely to become more prevalent in India after lockdown. Mapping of these variant genomes in conjunction with the clinical history in terms of recovery, hospitalization and co-morbidity might allow identification of variants that should be actionable and would also have relevance for prognosis. It is imperative that robust genomic data based on large sample size, including rural populations with even distribution can bring out the real scenario once correlated with epidemiological data eventually helping in drafting of further management policies.

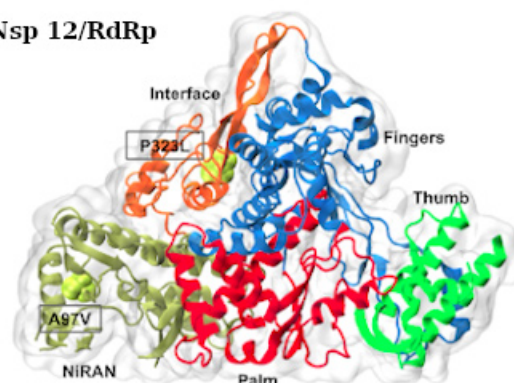
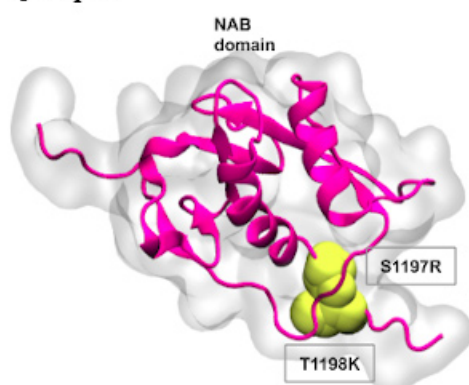
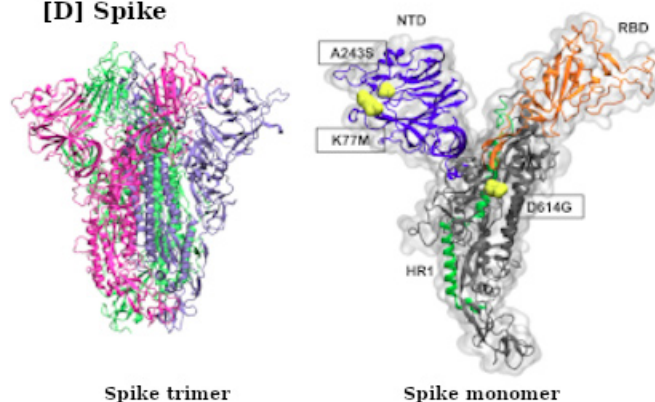
[A] Nucleocapsid**[B] Nsp 12/RdRp****[C] Nsp 3****[D] Spike**

Figure 3. Mapping of high frequency mutations on proteins displaying top-most mutations in SARS-CoV2. (A) Nucleocapsid, the N- and C-terminal domains are coloured in red and purple, respectively, where the missing linker region is shown as dotted line. (B) NSP12/RdRp, showing the five of its domains, NIRAN (olive), Palm (red), Thumb (green), Fingers (blue) and Interface (orange). (C) Nucleic acid-binding domain (NAB) of Nsp3 protein shown in pink color. (D) The trimeric spike protein showing 3 chain like structure. The right panel shows a single chain where the NTD, RBD and HR1 domains are coloured in orange, purple and green, respectively, whereas the rest of the structure is coloured grey. The mutations in all the proteins are marked within black boxes shown in yellow spheres.

Data availability**Underlying data**

All sequence data have been deposited with [GISAID](https://gisaid.org/) [Shu & McCauley, 2017].

Figshare: Acknowledgement table GISAID and Accession. <https://doi.org/10.6084/m9.figshare.12624815.v4> [Kumar *et al.*, 2020a].

This project contains all GISAID accession numbers generated and analysed in this study.

Extended data

Figshare: Supplementary figures S1-S6. <https://doi.org/10.6084/m9.figshare.12631880.v1> [Kumar *et al.*, 2020b].

This file contains the following extended data:

- Supplementary Figure S1: A schematic diagram showing numbers of samples with their geographical affiliations with respect to states of India.
- Supplementary Figure S2: Sequencing data quality parameters and orthogonal platform validation.

- Supplementary figure S3: A heat map representation of SARS-CoV-2 variants per sample and their respective segregation in respective clusters.
- Supplementary Figure S4: Protein annotation of the amino acid substitutions.
- Supplementary Figure S5: Amino acid properties marked as a function of mutations.
- Supplementary Figure S6: Scheme showing importation of prevalent SARS-CoV-2 genomes (3 major waves) in India.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

Author information

Pramod Kumar, Rajesh Pandey, Pooja Sharma and Mahesh S. Dhar are joint first authors. Mohammed Faruq, Anurag Agrawal and Partha Rakshit are joint senior authors.

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Reviewer Report 28 September 2020

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Sushil Kumar Sharma

Division of Virology, Defence Research and Development Establishment, Gwalior, Madhya Pradesh, India

This is an extensive study revealing the comprehensive genomic picture of the SARS-CoV-2 prevalence in the Indian population during the early phase of outbreaks explaining the three major waves of viral entry in India associated with multiple outbreaks *i.e.* first wave (A2a cluster from Europe and USA), second wave (A3 cluster from Middle East); and the third wave (A4 cluster from Southeast Asia) of importation of SARS-CoV-2 from various countries. The study has been designed and executed meticulously with logical conclusions and publication-worthy merit.

1. **Results: Demographic details and travel history of the objects:** “The majority of the SAS-CoV-2...” may please be replaced with “The majority of the SARS-CoV-2...”
2. The Supplementary Figure S6: “Scheme showing importation of prevalent SARS-CoV-2 genomes (3 major waves) in India” may be included in the main text.
3. D614G mutation has been reported in several studies. For example in one of the studies it has been claimed by Mr Babu V Bassa that the frequency of G614 variant in India is at least 90% as of June, 2020 (Bassa and Uppu, 2020¹) as may be seen at the biorxiv.org site (<https://www.biorxiv.org/content/10.1101/2020.06.04.128751v1>). This data from different regions of India needs to be discussed for better comprehension.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molekular biology,

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 21 September 2020

<https://doi.org/10.21956/wellcomeopenres.17692.r40420>

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Suresh Singh Yadav 

Department of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel

The study entitled 'Integrated genomic view of SARS-CoV-2 in India' explores the comprehensive genomic analysis of the SARS-CoV-2 early outbreaks in India. The study is highly relevant to the current epidemic and may add important information for further research and strain evolution of SARS-CoV-2, especially in India. Along with other clades previously known (named A2a, A2 and A3...), the authors reported that A4 (previously unclassified) is the most prevalent in the available genome sequences from India. The authors discussed that several novel mutations identified may be exclusive to the Indian population/conditions. It can also be helpful to trace the movement of lineage-specific strains across geographical regions more efficiently. The study is well designed, executed and concluded, and has significant merit to be published. The authors may address the following minor comments before indexing:

1. The authors should mention what was the positive and negative control for the qPCR.
2. Abstract - Methods: Sentence may be refreshed into two small sentences.
3. Methods: subheading 'Sample collection and molecular investigations' line seven - may be

as 'Absorbance ratio (260/280 nm) was ranged between 1.6–2.2 for most of the samples indicating its good quality

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular biology, cell signalling, epigenetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 24 August 2020

<https://doi.org/10.21956/wellcomeopenres.17692.r39810>

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✓ **Vijay Nema** 

Division of Molecular Biology, ICMR-National AIDS Research Institute, Pune, Maharashtra, India

This manuscript is well designed and executed to explore importation of SARS-CoV-2 genome with respect to major clade depicting A2a, A3 and A4 wave to India however, SARS-COV-2 Cluster B as well as A1a though minor is not mentioned for its entry in India and the Indian subcontinent.

The following amino acid substitutions are reported predominantly in Orf1ab region of the SARS-CoV-2 genome and its occurrence is in the following order L37F > A97V > T1198K > D614G > P323L > S1197R, however only D614G from spike region seems to be showing more infectivity and

transmission efficiency in Europe while rest of the amino acid substitutions need to be explored for their infectivity and transmission efficiency. This will provide significant association between different mutation patterns and transmission profile with respect to India and the Indian subcontinent.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Biology, Metagenomics, Tuberculosis drug discovery

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
