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First case of within-host co-infection of different SARS-CoV-2 variants in Ecuador

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Abstract

Background: COVID-19 infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can cause mild symptoms to severe illness and death. Co-infections of SARS-CoV-2 with other respiratory viruses have been described. However, two SARS-CoV-2 lineage co-infection have been rarely reported.

Methodology: A genotyping analysis and two different types of whole genome sequencing were performed (Illumina MiniSeq and ONT MinION). When examining the phylogenetic analysis in NextClade and Pangolin webservers, and considering the genotyping findings, conflicting results were obtained.

Results: The raw data of the sequencing was analyzed, and nucleotide variants were identified between different reads of the virus genome. B.1 and P.1 lineages were identified within the same sample.

Conclusions: We concluded that this is a co-infection case with two SARS-CoV-2 lineages, the first one reported in Ecuador.

Keywords: COVID19, co-infection, genome sequencing, NGS, SARS-CoV-2
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Introduction

Co-infection cases with different variants of SARS-CoV-2 have been identified in countries like Brazil, Portugal, and Belgium [1–3]. Considering that recombination is a well-known feature of coronaviruses and it happens when a cell is infected with multiple distinct viral lineages, the study of co-infections is important to understand the evolution of SARS-CoV-2 [4].

In this work, we report the first Ecuadorian case of SARS-CoV-2 co-infection with different variants in a 45-year-old Ecuadorian female. She had an Oxford/AstraZeneca complete two-dose COVID-19 vaccine and presented mild symptoms.

Materials and methods

Human subject: Case report and bioethics committee approval

The study protocol was approved by the Institutional Review Board of the Universidad San Francisco de Quito P2020–022IN (CEISH No. 1234) and by the Ecuadorian Ministry of Public Health MSP-CGDES-2020-0121-O. The patient provided

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written informed consent for sample analysis and publication. A family consisting of three people was diagnosed positive by RT-qPCR for COVID-19 on July 22, 2021. Husband, a 48-year-old man with healthy conditions and vaccinated with Oxford/AstraZeneca (ChAdOx1 nCoV-19) developed mild symptoms. The 45-year-old woman, also healthy and vaccinated with two doses of Oxford/AstraZeneca developed mild symptoms, starting with heaviness in the forehead and around the eyes, headache and followed by smell and taste loss. While her 9-year-old daughter was not vaccinated, she presented rhinorrhea and cutaneous manifestations. Only in the case of the woman sample the virus genome was sequenced due to a Ct value less than 25 and its higher concentration of viral genetic material.

Case detention and genotyping analysis

The molecular analysis of the sample Ecu2002 was performed on the molecular laboratory INTERLAB. The sample was collected from oropharyngeal swabs, preserved in viral transport media, and stored at -20°C until further analysis. Briefly, the RNA extraction was performed on a KingFisher Flex Purification System with MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit. Two RT-qPCR reactions were performed by triplicate to detect the following mutations: 69-70del, E484K, K417T, K417N and L452R. In the first RT-qPCR, the TaqPath COVID-19 CE-IVD Kit was used to identify the presence of SARS-CoV-2 and signal the presence of S gene 69-70del mutation. In the second RT-qPCR, the Applied Bio-system TaqMan SNP genotyping methodology was used to account the signal of E484K, K417T, K417N and L452R mutations. Both RT-qPCR reactions were performed on a QuantStudio 5 Real-Time PCR system (Applied Biosystems) and data was analyzed with COVID-19 Interpretive Software 2.5 (Thermo Fisher Scientific) for the first RT-qPCR and Design and Analysis Software 2.5.1 (Thermo Fisher Scientific) for the second RT-qPCR.

Whole genome sequencing of SARS-CoV-2 sample

We performed three different sequencing procedures of the same sample in two sequencing platforms. On one hand, the Omics Sciences Laboratory at Universidad de Especialidades Espiritu Santo (UEES) in Guayaquil, Ecuador, generated two different sequencing through Illumina MiniSeq platform. On the other hand, to corroborate the data obtained in the previous sequencing, the Instituto de Microbiología USFQ in Quito, Ecuador, generated one sequencing through Oxford Nanopore MinION device. For the three different consensus sequences obtained, the online tool NextClade (v.1.6.0) [5] was used to assign the sequences to clades, identify mutations, and to determine sequence quality control. The three genomes were also uploaded to the web application Pangolin COVID-19 Lineage Assigner (v.3.1.11) [6] for lineage classification. Finally, Tablet alignment viewer (v.1.21.02.08) [7] and AliView [8] were used to visualize the sequences using the sorted .bam files.

Illumina MiniSeq sequencing protocol. The library preparation for SARS-CoV-2 whole-genome sequencing on the Illumina platform was performed using two different protocols.

For the first one, the library was prepared from ~50 ng purified total RNA using the CleanPlex® SARS-CoV-2 Research and Surveillance Panel (Paragon Genomics, Hayward, CA, USA) with a two-pooled multiplex PCR approach to cover the entire genome. All the products were purified by CleanMag magnetic beads (Paragon Genomics, Hayward, CA, USA) following the manufacturer's protocol.

For the second one, the Illumina COVIDSeq test (Illumina, San Diego, CA, USA) was used according to the manufacturer's recommendations. The cDNA was synthesized from RNA using random hexamer and amplified by two primers pools producing 98 amplicons across the SARS-CoV-2 genome [9] and 11 additional targets from human mRNA as an internal control. The PCR product was processed for tagmentation reaction with bead-linked transposomes and adapter ligation using IDT for Illumina PCR index set 1. Further, enrichment and cleanup steps were performed according to the manufacturer's instructions and eluted in a 50 µL resuspension buffer.

In both procedures, the library was quantified using the Qubit dsDNA High Sensitivity assay kit on a Qubit 4.0 instrument (Life Technologies). Each sample was normalized to 4 nM, pooled together, and denatured with 5 µL of 0.2 N sodium hydroxide. The 1.2 pM library was spiked with 5% PhiX control (PhiX Control v3) and sequenced on an Illumina MiniSeq platform (Illumina, San Diego, CA, USA), using a MiniSeq system mid-output kit (300 cycles).

MinION sequencing protocol. The RNA was extracted in a type II biosafety chamber with HEPA filters in the Instituto de Microbiología USFQ. The Quick-RNA™ Viral Kit (Zymo, USA) was used to extract the total RNA of the samples, following the manufacturer instructions. The RNA retrotranscription to cDNA was performed using the ARTIC protocol [9]. Target enrichment was done following the Artic Network protocol with the SARS-CoV-2 primer scheme (V3). The product of this reaction was purified using the AMPure XP magnetic beads (Beckman Coulter, USA), following manufacturer instructions. The purified product was quantified using a Qubit (Thermo Fisher Scientific) with a Qubit RNA Assay Kit (Thermo Scientific, Invitrogen, USA). The preparation of the cDNA library was performed by using the Native Barcoding Expansion 96 kit (EXP-NBD196) (Oxford Nanopore Technologies), then, the library was loaded into the MinION flow cell (FAQ 61532).

The sequencing run was programmed with the MinKNOW software for 35 hours, with real-time base calling activated, and with the final output format in FASTQ. The RAMPART software (v1.0.5) from ARTIC Network (https://github.com/artic-network/rampart) was used to monitor the sequencing in real-time. Once the sequencing finished, Porechop (v 0.2.4) (https://github.com/rrwick/Porechop) was used to carry out demultiplexing and adapter removal. Then, the Medaka pipeline from the ARTIC Network bioinformatics pipeline was employed for variant calling [9]. To generate consensus genomes, the reads were mapped against the reference genome Wuhan-Hu-1 (GenBank accession number MN908947).

Co-infection analysis

To analyze the nucleotide frequency at different positions of the SARS-CoV-2 genome, a single bam file was generated using the raw data from the Illumina sequencing.

Due to the different taxonomic assignments of the consensus sequences, we generated four new consensus sequences using iVar consensus [10] with different consensus frequency thresholds. In this step, only the data generated in Illumina were considered and a value of 0.2 and 0.8 of consensus frequency thresholds were selected.

Results

Ecu2002 sample was positive for SARS-CoV-2 and negative for S gene 69-70del mutation (Table 1), K417T and L452R mutations (Table 2). Regarding K417T and E484K mutations, allele 2 values were higher in relation to allele 1 with a manual call of positive for those mutations.

The phylogenetic analysis of the consensus sequence generated in the first Illumina MiniSeq sequencing found that the sample belonged to clade 20B but lineage B.1, while the second consensus sequence from the second Illumina sequencing classified the sequence to clade 20J and lineage P.1. Due to this contradiction, the third sequencing was performed with the MinION and it revealed that the sample Ecu2002 had a variant be-longing to clade 20A and lineage B.1.540. The nucleotide and amino acid mutations of the Illumina sequencing analyses are described in Table 3.

Due to the three different phylogenetic analysis obtained from the same sample, we manually analyzed the raw data in Tablet and identified various single nucleotide variants between the different mapped reads (Figure A1). Using the merged.bam file from the two Illumina sequencing we identified the different nucleotide frequencies at different positions of the SARS-CoV-2 genome (Fig. 1).

The consensus sequences generated with different frequency thresholds in iVar produced similar phylogenetic classifications to those obtained previously (Table 4). This information suggests a coinfection between two different variants of SARS-CoV-2.

Discussion

Evidence of SARS-CoV-2 recombination was reported previously [11,12]. In this regard, the study of co-infections is

TABLE 1. TaqPath COVID-19 CE-IVD for SARS-CoV-2 and S gene 69-70del mutation

Sample name	ORF1ab Ct	N gene Ct	S gene Ct[a]	MS2 Ct	Interpretative result
Ecu2002	16.507	15.926	18.705	27.377	Positive SARS-CoV-2
Extraction control	—	—	—	26.861	SARS-CoV-2 Not Detected
Negative control	—	—	—	27.993	—
Positive control	24.748	25.886	25.381		—

[a]A positive amplification equals absence of S gene 69-70del mutation.

TABLE 2. TaqMan SNP genotyping for SARS-CoV-2 mutations

Sample name	Sample type	SNP assay	Confidence	Allele 1	Allele 2	Call	Manual call
Ecu2002	Nasopharyngeal	K417T	0.93	0.207	2.094	Positive allele 2	K417T positive
Ecu2002	Nasopharyngeal	K417N	1	0.448	0.034	Positive allele 1	K417N negative
Ecu2002	Nasopharyngeal	E484K	0.92	0.278	2.650	Positive allele 2	E484K positive
Ecu2002	Nasopharyngeal	L452R	1	0.345	0.021	Positive allele 1	L452R positive
NC K417T	Water	K417T	1	0.189	0.011	No amplification	No amplification
NC K417N	Water	K417N	1	0.005	0.001	No amplification	No amplification
NC E484T	Water	E484K	1	0.005	0.007	No amplification	No amplification
NC L452R	Water	L452R	1	0.001	0.006	No amplification	No amplification

TABLE 3. Illumina MiniSeq sequencing: Mutations found in SARS-CoV-2 coinfected genomes from Ecuador compared to Wuhan-Hu-1 (GenBank accession number MN908947)

Sample ID	Gisaid ID	Locality	QC	N's (missing data)	Clade	Lineage	Gen	Mutation	Amino acid replacement
Ecu2002 (First seq)			Good	556	20J	P.1	N	C28851G	P80R
								G28881A	R203K G204R
								G28882A	
								G28883C	
								G28975A	M234I
								11288-11296	S3675-
							ORF1a		G3676-
									F3677-
							ORF1b	C14408T	P314L
								G17259T	E1264D
							ORF3a	C25517T	P42L
								G25563T	Q57H
								T25676C	L95S
							ORF8	C27925T	T11I
								G28167A	E92K
							ORF9b	C28512G	Q77E
								C21621A	T20N
								C21638T	P26S
								A22320G	D253G
								A22812C	K417T
							S	G23012A	E484K
								A23063T	N501Y
								A23403G	D614G
								G25088T	V1176F
Ecu2002 (Second seq)		Good		241	20B	B.1	N	C28851G	P80R
								G28881A	R203K G204R
								G28882A	
								G28883C	
								G28975A	M234I
							ORF1a	11288-11296	S3675-
									G3676-
									F3677-
								A5648C	K1795Q
								C7011T	A2249V
							ORF1b	C c14408T	P314L
								G17259T	E1264D
							ORF3a	G25563T	Q57H
							ORF8	C27925T	T11I
								G28167A	E92K
							ORF9b	C28512G	Q77E
								C21846T	T95I
								A22320G	D253G
								A22812C	K417T
							S	G23012A	E484K
								A23063T	N501Y
								A23403G	D614G
								C24642T	T1027I

important to understand the evolution of SARS-CoV-2. We reported a case of SARS-CoV-2 co-infection in a woman in Ecuador.

The genotyping and phylogenetic classification of the consensus sequences product of the different sequencing generated conflicting results. Therefore, when analyzing the raw sequences on Tablet and the nucleotide frequencies, several nucleotide variations were detected between different sequencing reads (Fig. 1 and Figure A1). Performing three different sequencing that generated reads with the same nucleotide variations allows us to rule out any type of sample contamination. Furthermore, by generating different consensus sequences with different minimum frequency threshold values, we were able to generate more evidence about the presence of at least two SARS-CoV-2 variants within the same host.

The results of the whole viral genome sequencing from Ecu2002 sample identified two variants: B.1 lineage with shared mutations with P1 lineage. Lineage B.1 has been re-ported mainly in the United States of America (48.0%) followed by United Kingdom (9.0%) [13]. While the P.1 lineage has been circulating in the population since the end of March 2021 and has been widely distributed throughout Ecuador [13]. These results show that the patient presents a coinfection with two variants, a variant of concern (P.1) and a variant close to the original SARS-CoV-2 lineage (B.1).

It has been reported that a coinfection with different viruses at the same time can have a negative impact on the development of the disease. However, when different lineages of the same virus co-infect a person, the symptomatology is unknown. In this study, the woman patient developed heaviness in the

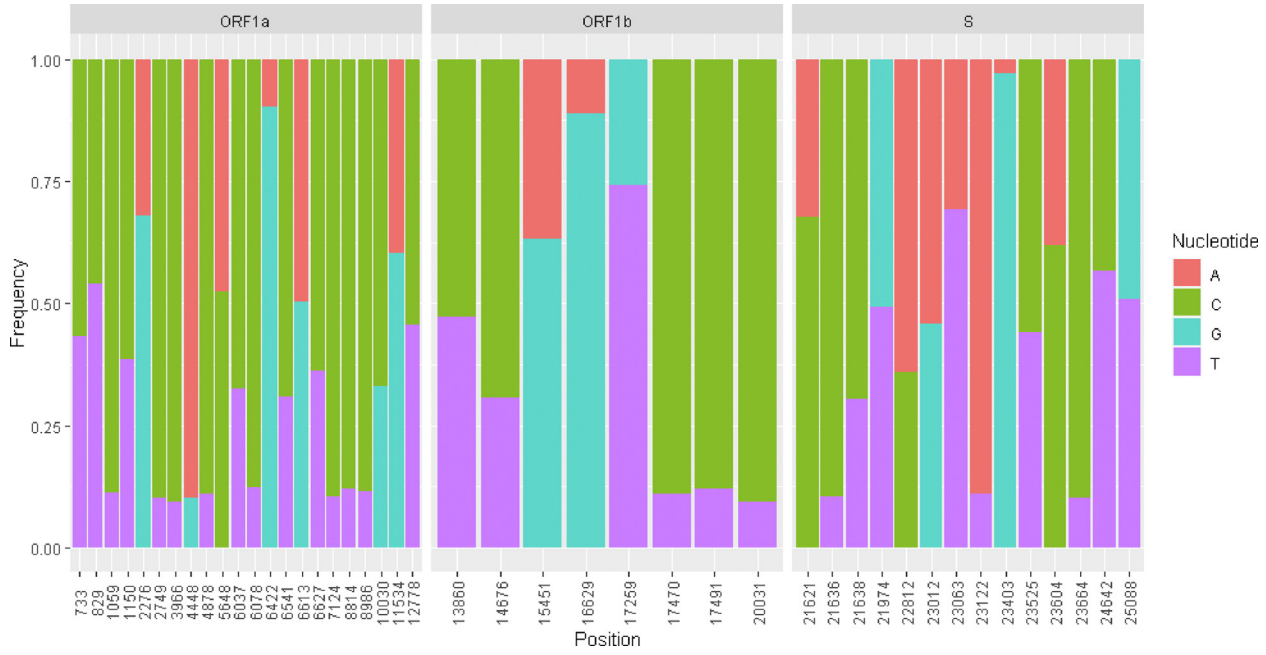


FIG. 1. Nucleotide frequencies of the the sample Ecu2002 generated from the Illumina sequencing. Distinct frequencies of SNVs suggesting the existence of at least two haplotypes.

TABLE 4. Phylogenetic analysis with different frequency thresholds values generated in iVar

Sequencing	Frequency threshold	Lineage	Clade
Illumina first seq	0.8	P.1	20J
Illumina first seq	0.2	P.1	20A
Illumina second seq	0.2	P.1	20B
Illumina second seq	0.8	B.1	20A

forehead and around the eyes, headache and smell and taste loss.

In the context of the SARS-CoV-2 pandemic, it is important to notice that an active genomic surveillance and analyzing bigger data sets is necessary to understand the relationship between a co-infection and the disease progression.

In conclusion, this report shows the first reported co-infection with different SARS-CoV-2 lineages in Ecuador.

Author contributions

Conceptualization, JCFC, GT, VB, and PC; methodology, MC, EM, MBPV, SM, JJG, MBW, BG, GML.; software MC, EM, MBPV, SM, JJG, MBW, BG, GML; validation, PC, GT, PRS, VB, DA.; formal analysis, JCFC, MC; writing original draft preparation, all the authors. All authors have read and agreed to the published version of the manuscript.

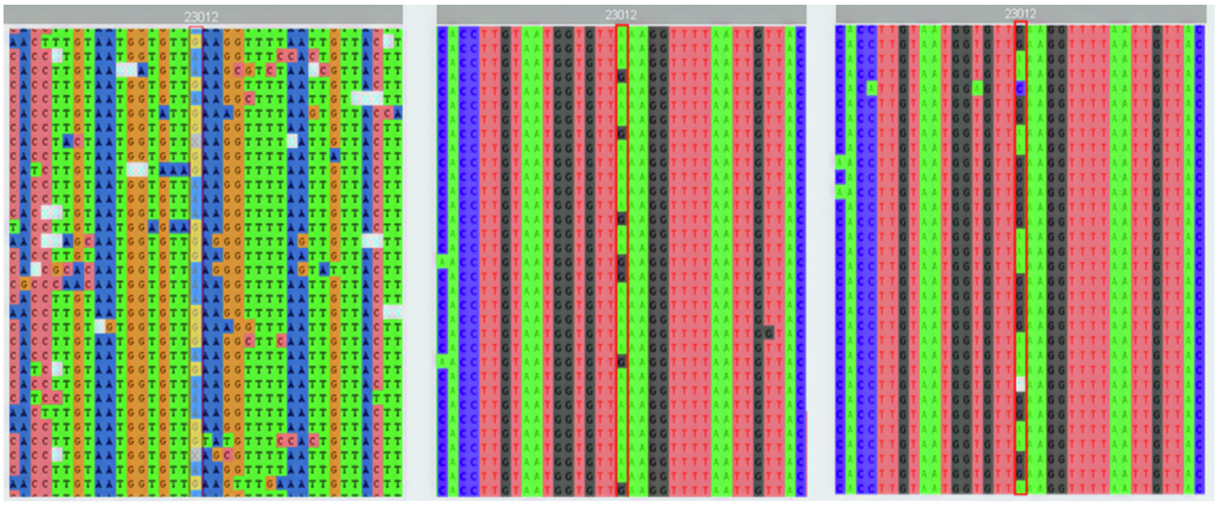
Data availability statement

Consensus sequences have been uploaded to GISAID.org https://nam11.safelinks.protection.outlook.com/?url=http%3A%2F%2Fgisaid.org%2F&data=05%7C01%7CV.Balraj%40elsevier.com%7C982847a49e3d4a52253108da5de21d61%7C9274ee3f94254109a27f9fb15c10675d%7C0%7C0%7C637925522990417372%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C3000%7C%7C%7C&sdata=RcP%2FBfJIo6%2BpnuDFn39OUsK9qS8DZ%2Be2kp3aN2ofpck%3D&reserved=0 under the accession numbers EPI_ISL_13508358 to EPI_ISL_13508362.

Transparency declaration

The authors declare no conflict of interest. This research was funded by USFQ and UEES.

Appendix A.



(a) (b) (c)

FIGURE A1. Tablet visualization alignment: a) Oxford Nanopore MinION sequencing: Nucleotide changes between different mapped reads in the position 23.012 of sample Ecu2002; b-c) Illumina MiniSeq sequencing: Nucleotide changes between different mapped reads in the position 23.012 of sample Ecu2002 first sequence and Ecu2002 second sequence respectively.

References

[1] Francisco R da S, Benites LF, Lamarca AP, de Almeida LGP, Hansen AW, Gularte JS, et al. Pervasive transmission of E484K and emergence of VUI-NP13L with evidence of SARS-CoV-2 co-infection events by two different lineages in Rio Grande do Sul, Brazil. Virus Res 2021 Apr 15;296:198345.

[2] Pedro N, Silva CN, Magalhães AC, Cavadas B, Rocha AM, Moreira AC, et al. Dynamics of a dual sars-cov-2 lineage co-infection on a prolonged viral shedding COVID-19 case: insights into clinical severity and disease duration [Internet]. [cited 2021 Sep 8] Microorganisms 2021 Feb 1;9(2): 1–10. Available from: https://pubmed.ncbi.nlm.nih.gov/33540596/.

[3] Vankeerberghen A, Holderbeke A, Boel A, Vaerenbergh K Van, Beenhouwer H De, Cattoir L. Case report: a 90-year-old lady infected with two CoVID-19 VoCs: 20I/501Y.V1 and 20H/501Y.V2. In: ECCMID ABSTRACT 04978. European Congress of Clinical Microbiology & Infectious Diseases; 2021.

[4] Jackson B, Rambaut A, Pybus O, Robertson D, Connor T, Loman N, et al. Recombinant SARS-CoV-2 genomes involving lineage B.1.1.7 in the UK - SARS-CoV-2 coronavirus/SARS-CoV-2 Molecular Evolution [Internet] Virological 2021 [cited 2021 Sep 13]. Available from: https://virological.org/t/recombinant-sars-cov-2-genomes-involving-lineage-b-1-1-7-in-the-uk/658.

[5] Hadfield J, Megill C, Bell SM, Huddleston J, Potter B, Callender C, et al. NextStrain: real-time tracking of pathogen evolution. Bioinformatics 2018 Dec 1;34(23):4121–3.

[6] OToole A, Scher E, Underwood A, Jackson B, Hill V, McCrone JT, et al. Assignment of epidemiological lineages in an emerging pandemic using the Pangolin tool. Virus Evol 2021 Jul 5.

[7] Milne I, Stephen G, Bayer M, Cock PJA, Pritchard L, Cardle L, et al. Using tablet for visual exploration of second-generation sequencing data [Internet]. [cited 2021 Jun 14] Brief Bioinform 2013 Mar;14(2): 193–202. Available from: https://pubmed.ncbi.nlm.nih.gov/22445902/.

[8] Larsson A. AliView: a fast and lightweight alignment viewer and editor for large datasets [Internet] Bioinformatics 2014;30(22):3276–8. https://doi.org/10.1093/bioinformatics/btu531. Available from:.

[9] Quick J, Barnes K. nCoV-2019 sequencing protocol [Internet]. ARTIC Coronavirus Method Devolopment Community; 2020 Jan [cited 2021 Sep 12]. Available from: https://www.protocols.io/view/ncov-2019-sequencing-protocol-bbmuik6w.

[10] Grubaugh ND, Gangavarapu K, Quick J, Matteson NL, De Jesus JG, Main BJ, et al. An amplicon-based sequencing framework for accurately measuring intrahost virus diversity using PrimalSeq and iVar [Internet]. [cited 2021 Dec 25] Genome Biol 2019 Jan 8;20(1):1–19. Available from: https://genomebiology.biomedcentral.com/articles/10.1186/s13059-018-1618-7.

[11] Yi H. 2019 novel coronavirus is undergoing active recombination [Internet]. [cited 2021 Oct 14] Clin Infect Dis 2020 Jul 28;71(15):884. Available from: https://academic.oup.com/cid/article/71/15/884/5781085.

[12] Gribble J, Stevens LJ, Agostini ML, Anderson-Daniels J, Chappell JD, Lu X, et al. The coronavirus proofreading exoribonuclease mediates extensive viral recombination [Internet]. [cited 2021 Oct 14] PLOS Pathog 2021 Jan 19;17(1):e1009226. Available from: https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1009226.

[13] Alaa Abdel Latif, Julia L. Mullen, Manar Alkuzweny, Ginger Tsueng, Marco Cano, Emily Haag, et al., And the center for viral systems biology. outbreak.info, (Available at: outbreak.info 2). Accessed 14 October 2021