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 web servers, 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.

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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 (CEISIH No. 1234) and by the Ecuadorian Ministry of Public Health MSP-CGDES-2020-0121-O. The patient provided
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 detection 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 (UES) in Guayaquil, Ecuador, generated one sequencing through Oxford Nanopore MinION platform. 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 ampliers across the SARS-CoV-2 genome 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 belonging 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.

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 | MS2 Ct | Interpretative result            |
|----------------------|-----------|-----------|-----------|--------|----------------------------------|
| Ecu2002              | 16.507    | 15.926    | 18.705    | 27.377 | Positive SARS-CoV-2              |
| Extraction control   | —         | —         | —         | —      | SARS-CoV-2 Not Detected          |
| Negative control     | —         | —         | —         | —      |                                  |
| Positive control     | 24.748    | 25.886    | 25.381    | 24.748 |                                  |

*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     | 0.92       | 0.448    | 0.034    | Positive allele 1 | K417N negative |
| Ecu2002              | Nasopharyngeal | E484K     | 0.345      | 0.278    | 2.650    | Positive allele 2 | E484K positive |
| NC K417T             | Water       | K417T     | 0.189      | 0.005    | 0.001    | No amplification | No amplification |
| NC K417N             | Water       | K417N     | 0.005      | 0.005    | 0.001    | No amplification | No amplification |
| NC E484T             | Water       | E484K     | 0.005      | 0.005    | 0.001    | No amplification | No amplification |
| NC L452R             | Water       | L452R     | 0.005      | 0.005    | 0.001    | No amplification | No amplification |

Discussion

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

### 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.I N C28851G G28881A G28882A G28883C G28975A 11288-11296 ORF1a | G2C203 K204R C343 G367-367- F367- P314L E1264D C42 G57H L95S T1 I1 ORF1b C14408T G17259T C25517T G25543T T3676C ORF2a C27927T G28167A C28512G C21638T A22320G A22812C G23012A A23063T A23403G G25088T ORF3b C27925T G28167A C28512G C28883C G28975A 11288-11296 ORF4a A5448C C7011T ORF4b C14408T G17259T G25543T ORF5 T27927T G28167A C28512G C21846T A22200G A22812C G23012A A23063T A23403G C24642T |
| Ecu2002 (Second seq) Good 241 20B B.I N C28851G G28881A G28882A G28883C G28975A 11288-11296 ORF1a | M234I G367-367- F367- K1795Q A2249V ORF1b C14408T G17259T G25543T ORF2a C27927T G28167A C28512G C21846T A22200G A22812C G23012A A23063T A23403G C24642T |

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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%7C9782847a49e3d4a5225108da5de21d61%7C9274ee3f94254109a27f9fb15c10675d%7C0%7C63792552299017372%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJXVCI6Mn0%3D%7C3000%7C%7C&sdata=RcP%2FBfJlo6%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.

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