COVID-19 re-infection by a phylogenetically distinct SARS-CoV-2 variant, first confirmed event in South America.

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Abstract

The permanence of rt-PCR positivity after a long time in Covid-19 patients has prompted the question of whether SARS-CoV-2 could cause a persistent infection or patients can become re-infected by this virus. Both possibilities could have critical implications for the management and control of Covid-19. Here we present the first confirmed case of SARS-CoV-2 reinfection in Ecuador and South America.

Materials and methods: Our diagnostic laboratory detected a potential re-infection in one patient who was SARS-COV2 rt-PCR positive twice (in May and July 2020).

The first laboratory-confirmed infection presented with mild symptoms and full recovery, reaffirmed by a negative RT-PCR test result obtained two weeks after symptom onset. More severe COVID-19-like symptoms presented again four weeks after the first event, and a third RT-PCR test was performed which resulted positive.

The total RNA extraction (from the samples collected on both occasions) was sequenced in an Oxford Nanopore MinION using a tilling PCR protocol developed by the ARTIC-Network, and the reads were analyzed using the artic-medaka consensus generation tool. Anti SARS-CoV-2 IgM and IgG antibodies were investigated.
Results: different SARS-CoV-2 variants were identified in each infection event. For the first infection, the genome was assigned to the B1.p9 GISAID clade while the variant associated with the second episode was assigned to the A.1.1 GISAID clade. High levels of both SARS-CoV-2 specific IgM and IgG were observed during the second event.

Discussion: a patient with two COVID-19 events presented two different SARS-CoV-2 variants on each event, confirming reinfection. This phenomenon is still considered rare.

Keywords: SARS-CoV-2, COVID-19, reinfection, whole-genome sequencing.
Introduction

The current pandemic caused by the newly discovered SARS-CoV-2 has brought many questions to clinical, basic, and applied researchers which are still unknown. One critical observation was the long persistence of symptoms and rt-PCR positivity in many Covid19- patients. This observation may be a result of viral persistence or re-infection. Persistent infections with Sarbecoviruses like MERS-CoV have been observed in bats (Banerjee et al., 2020). Also, reinfections with human betacoronaviruses, causing cold-like syndromes, have been reported previously (Galanti and Shaman, 2020). The distinction between these 2 phenomena is crucial because both require different therapeutical and public health control approaches.

To resolve this controversy, we have been actively looking for patients showing signs of recurrent disease and recurrent rt-PCR positivity. The confirmation of reinfection events requires the demonstration that different viral genetic variants are present in each of the different disease episodes. Similar cases investigated with this technology have been reported in Hong Kong, Belgium, Netherlands, and the United States of America (Kai-Wang To et al., 2020). Here we report the first confirmed case of SARS-CoV-2 reinfection in South America caused by two different viral genetic variants.

Materials and Methods

Sample collection

Two oropharyngeal swabs were collected at two distinct time points corresponding to the period when the patient presented COVID-19-like symptoms. The samples were collected in 1.5 ml Eppendorf tubes with 1X DNA/RNA Shield (Zymo, USA), to preserve the contained genetic material and ensure viral inactivation. Sample
positivity to COVID-19 was determined using the RT-PCR method, with the Veri-Q PCR 316 kit (Mico Biomed, South Korea), that target ORF3a and N genes. Both samples were stored in RNAsheild (Zymo, USA) at -80°C after PCR analysis. The results were officially reported to the Ecuadorian Ministry of Public Health (MSP) and the National Institute of Public Health and Research (INSPI) following local protocols.

**RNA extraction**

RNA was extracted in a type II biosafety chamber with HEPA filters, at the Virology Laboratory of the IMUSFQ (Microbiology Institute of Universidad San Francisco de Quito). Quick-RNA™ Viral Kit (Zymo, USA) was used to extract the total RNA from each sample, following manufacturer instructions. Retrotranscription of RNA to cDNA was carried out using the ARTIC protocol (Quick, 2020).

**Whole-genome sequencing**

Target enrichment for further whole-genome sequencing was performed through the primer scheme approach developed by the ARTIC Network for nCoV-2019, using the V3 primer sets (Quick, 2020). The product of this reaction was purified by using AMPure XP magnetic beads (Beckman Coulter, USA), following manufacturer instructions; and it was quantified using QuBit (Thermo Fisher Scientific) with a Qubit RNA Assay Kit (Thermo Scientific, Invitrogen, USA). After normalization, cDNA library preparation was carried out by using the Rapid Barcoding kit (SQK-RBK004) (Oxford Nanopore Technologies), then, the library was loaded into the MinION flow cell (FLO-MIN 106).

To monitor sequence in real-time, the RAMPART software (v1.0.5) from the ARTIC Network ([https://github.com/artic-network/rampart](https://github.com/artic-network/rampart)) was used. After sequencing, Nanoplot (De Coster et al., 2018) and Porechop (version 0.2.4) ([https://github.com/rrwick/Porechop](https://github.com/rrwick/Porechop)) were used to determine sequence quality.
scores and to carried out demultiplexing and adapter removal, respectively. Then, the ARTIC Network bioinformatics pipeline was employed for variant calling (Quick, 2020). To generate consensus genomes, the reads were mapped against the reference strain Wuhan-Hu-1 (GenBank accession number MN908947). Tablet alignment viewer (version 1.19.09.03) (https://ics.hutton.ac.uk/tablet) was used to visualize the mapped sequence. The online tool NextClade (v0.4.0) (Hadfield et al., 2018) was used to assign the sequences to clades. Finally, the two genomes were uploaded to the CoV-GLUE online resource (Singer et al., 2018), for lineage classification and mutations determination.

**Immunological test**

A COVID-19 Qualitative antibody IgG/IgM Rapid Test (SAFECARE BIO-TECH, China) was performed by “AMC Laboratorio Clínico Pasteur”, a private laboratory in Quito during the first infection event. During the second event, an ELISA antibody test was performed by the medical clinic SIME (Sistemas Médicos Universidad San Francisco de Quito), using NovaLisa® SARS-CoV-2 IgG and NovaLisa® SARS-CoV-2 IgM (NovaTec Immundiagnostica GmbH, Germany).

**Ethical 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 Health MSP-CGDES-2020-0121-O. The patient provided written informed consent for sample analysis and publication.
Results

Patient clinical evolution

The patient is a 46-year-old man who, on May 12th, 2020, presented with 3 days of intense headache and drowsiness. His wife and daughter reported the same symptoms. He underwent an RT-PCR SARS-CoV-2 test which was performed seven days after the onset of symptoms on May 20, 2020, in the Institute of Microbiology of the USFQ (IM-USFQ). The result came back positive (gene ORF3a, Cq=36.85).

Subsequently, his symptoms improved, and a second RT-PCR test was performed on June 3, 2020, at the IM-USFQ (21 days after symptom onset) and it was negative. Over one month later, by the end of July, the patient reports having close contact with a relative that was diagnosed afterward with COVID-19. Two days after this contact, on July 20th, the patient presented once again with symptoms suggestive of COVID-19. This time, the symptoms were more severe and included odynophagia, nasal congestion, fever of 38.5°C, strong back pain, productive cough, and dyspnea. For this reason, he was advised to take another RT-PCR test which was also performed at IM-USFQ on July 22, 2020, with a positive result. Despite his moderate symptoms and dyspnea, the patient did not require hospitalization and his clinical status improved. Finally, a fourth RT-PCR test was performed on August 4, 2020, which came back negative and the patient has been since asymptomatic.

Immunological tests

Antibody tests were performed during the first and second courses of disease. A rapid qualitative (positive/negative) antibody test (Covid19 IgG/IgM) was performed on May 16, 2020, results were positive for Covid19 IgM and negative for IgG. At the time of the second infection, an ELISA quantitative antibody test was performed on August 18, 2020. It produced the following results: Ac. Anti SARS-CoV2 IgG: 34.1

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NTU and Ac. Anti SARS-CoV2 IgM: 54.2 NTU. These results are interpreted as positive for both IgM and IgG.

**Whole Genome Sequencing**

The sequences obtained from the first and second infections have been uploaded to the global repository of SARS-CoV-2 genomes hosted by GISAID under the accession numbers EPI_ISL_525430 (for the sample from the first infection) and EPI_ISL_516650 (for the sample from the second infection).

Phylogenetic analysis identifies the variant from the first infection event as belonging to the 20A clade according to NextClade, and to the B1.p9 lineage in GISAID. The second infection variant belongs to the 19B clade according to NextClade, and the A.1.1 lineage in GISAID (Figure 1).

**Figure 1.** Phylogenetic assignment of two samples recovered from the same patient at different times. Sequences were aligned to a representation of the global SARS-CoV-2 genetic diversity using a banded Smith-Waterman algorithm with an affine gap-penalty. The genomes from the first and second infections are highlighted in red. The tree was obtained from NextClade v0.4.0.
When compared to the Wuhan-Hu-1 reference genome, the sequence from the first event has eight SNPs: C2113T, C3037T, C7765T, C14408T, C17690T, C18877T, A23403G, G25563T; these involve changes in four amino acids: P323L (in nsp12), S485L (in nsp13), D614G (in S) and Q57H (in ORF 3a). On the other hand, the genome from the second infection episode has ten SNPs: C1457T, C8782T, T9445C, T17531C, C17747T, A17858G, C18060T, G18756T, A24694T, T28144C; which determine five amino acid replacements: R218C (in nsp2), I432T, P504L, Y541C (in nsp13) and L84S (in ORF8) (Figure 2). No shared mutations were observed between the two sequences, further suggesting that both variants resulted from distinct evolutionary trajectories.

**Figure 2.** Genome annotation and amino acid changes were identified for each of the whole genome sequences obtained from the first and second infection events, compared to the Wuhan-Hu-1 (GenBank accession number MN908947) reference genome. In red: amino acid changes of the first genome sequence, In blue: amino acid changes of the second genome sequence.

**Discussion**

We describe the first case of SARS-Cov-2 reinfection in Ecuador and Latin-America which adds to the reinfection evidence presented by others in Hong Kong, Netherlands, and the USA. Even though reinfections with the human common cold-coronavirus have been reported previously, the symptoms of reinfected patients were milder than the primary infections (Callow et al., 1990). It was surprising, then,
that this patient showed a more severe disease in the second infection. In other reports subsequent infection was asymptomatic (Parry, 2020). However, it is possible that the patient described here was suffering from an additional condition during the second infection.

Reinfection events are rare, but they have been described in other RNA viruses (Miller, 1990). This appears to be the case for SARS-CoV-2, illustrated by the large number of cases that have recovered from the disease worldwide compared to a small number of reported reinfections.

The protective immune response to SARS-CoV-2 infection is also not fully understood, however, protection against severe disease has been previously demonstrated in animal models and inferred in humans infected with this virus (Ladhani et al., 2020). Interestingly, the antibody test performed during the first infection event showed the presence of specific anti-SARS-CoV-2 IgM, and no IgG was detected. The sample for this test was taken four days after the onset of symptoms, therefore it is not possible to determine if this infection was able to produce a subsequent IgG response. The second event showed high anti-SARS-CoV-2 IgM and IgG. The increase in IgG could be associated with a previous humoral response compared to the negative qualitative IgG test in the first infection. It has been proposed the first wave of an immune response, characterized by early short-lived plasma cells; and then a second wave, of a smaller number of longer-lived plasma (Alter and Seder, 2020). It is important to remark that it is not possible to determine whether a protective immune response was developed using conventional antibodies test.
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