Proper assignation of reactivation in a COVID-19 recurrence initially interpreted as a reinfection

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Running title: COVID-19 reactivation and nosocomial transmission

Article´s main point: Whole Genome Sequencing revealed that a COVID-19 recurrence, initially considered as a reinfection, corresponded to a reactivation, with major consequences, leading to a more severe second episode with fatal resolution and subsequent nosocomial transmission, with an additional COVID-19-related death.

Abstract

A 77-year-old-male (Case R) who had had a previous diagnosis of mild COVID-19 episode, was hospitalized 35 days later. On Day 23 post-admission, he developed a second COVID-19 episode, now severe, and finally died. Initially, Case R COVID-19 recurrence was interpreted as a reinfection due to the exposure to a SARS-CoV-2 RT-PCR-positive room-mate. However, whole-genome-sequencing indicated that case R recurrence corresponded to a reactivation of the strain involved in his first episode. Case R reactivation had major consequences, leading to a more severe episode, and causing a subsequent transmission to another two hospitalized patients, one of them with fatal outcome.

Keywords: COVID-19, SARS-CoV-2, reactivation, nosocomial transmission, WGS.

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Introduction

Whole genome sequencing (WGS) has been essential to clarify a key aspect in the COVID-19 pandemic, namely, the analysis of recurrences, allowing to identify which are due to reinfections [1, 2]. Genomic research has demonstrated the prolonged persistence of viable SARS-CoV-2 in severely immunosuppressed patients [3, 4], but it has not equally been used to support reactivations, and the scarce reports focus primarily on clinical descriptions [5]. Furthermore, the potential relationship between SARS-CoV-2 reactivation and associated nosocomial outbreaks has not been described to date. In this study we present a SARS-CoV-2 reactivation and its consequences in the nosocomial setting.

Patients and Methods

Clinical data

Baseline characteristics and clinical and laboratory parameters at COVID-19 diagnosis and their outcome were obtained from their electronic medical records. The study was approved (REF: MICRO.HGUGM.2020-042) by the ethical research committee of Gregorio Marañón Hospital.

Diagnostic tests

SARS-CoV-2 RT-PCRs

Viral RNA was extracted and purified from 300 µL of nasopharyngeal exudates with the aid of the KingFisher (Thermo Fisher Scientific, Waltham, Massachusetts) instrument. Next, an RT-PCR was performed, using the TaqPath COVID-19 CE-IVD RT-PCR kit (Thermo Fisher Scientific, USA).

SARS-CoV-2 serology

Determinations of antibodies in sera were performed by specific qualitative detection of anti-SARS-CoV2 IgGs (anti-N), using a chemiluminescent immunoassay of microparticles (CMIA) in the ARCHITECT system (Abbott, Chicago, USA).

Whole genome sequencing

Eleven µL of RNA were used as template for reverse transcription using Invitrogen SuperScript IV reverse transcriptase (ThermoFisher Scientific, Massachusetts, USA) and random hexamers (ThermoFisher Scientific, Massachusetts, USA). Whole genome amplification of the coronavirus was done with an Artic_nCov-2019_V3 panel of primers (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) (artic.network/ncov-2019) and the Q5 Hot Start DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA). Libraries were prepared using the Nextera Flex DNA

Library Preparation Kit (Illumina lnc, California, USA) following manufacturer´s instructions.

Libraries were quantified with the Quantus™ Fluorometer (Promega, Wisconsin, USA), before being pooled at equimolar concentrations (4 nM). Next, they were sequenced in pools of up to 17 libraries on the Miseq system (Illumina Inc, California, USA) and the MiSeq Reagent Micro kit v2 (2x151pb) or in pools of up to 96 libraries with the MiSeq Reagent (2x201 pb).

FastQ files above the GISAID thresholds were deposited at GISAID EPI_ISL_654287, EPI_ISL_654203, EPI_ISL_654284, EPI_ISL_654176 and EPI_ISL_1173765. An in-house analysis pipeline was applied to analyse the sequencing reads. The pipeline can be accessed at https://github.com/pedroscampoy/covid_multianalysis. Briefly, the pipeline goes through the following steps: 1) removal of human reads with Kraken [https://genomebiology.biomedcentral.com/articles/10.1186/gb-2014-15-3-r46]; 2) pre-processing and quality assessment of fastq files using fastp [https://academic.oup.com/bioinformatics/article/34/17/i884/5093234] v0.20.1 (arguments: --cut tail, --cut-window-size, --cut-mean-quality , -max_len1 ,-max_len2) and fastQC v0.11.9 [Andrews S.; S Bittencourt a, "FastQC: a quality control tool for high throughput sequence data – ScienceOpen," Babraham Inst., p. http://www.bioinformatics.babraham.ac.uk/projects/, 2010.]; 3) mapping with bwa v0.7.17 [H. Li and R. Durbin, "Fast and accurate short read alignment with Burrows-Wheeler transform," Bioinformatics, vol. 25, no. 14, pp. 1754–1760, 2009.] and variant calling using IVAR v1.2.3 [https://genomebiology.biomedcentral.com/articles/10.1186/s13059-018-1618-7] using Wuhan-1 sequence (NC_045512.2) as reference; 4) Recalibration of punctual low

coverage positions using joint variant calling. When necessary, informative non-covered positions were analysed by standard Sanger sequencing with the corresponding flanking primers from the ARTIC set.

Results

Our case (Case R, Figure 1) was a 77-year-old male with hypertension and dyslipidaemia, a diagnosis of cutaneous B-cell lymphoma in remission, a previous stroke, and chronic obstructive pulmonary disease associated with mild interstitial lung disease without exacerbation or need of supplemental oxygen. His first positive SARS-CoV-2 RT-PCR was on July 28, 2020 when he had a mild infection with fever without developing pneumonia or other complications. Hospital admission was not required. SARS-CoV-2 serology was not performed at that time.

On September 1, (35 days after his first positive RT-PCR, Figure 1) he was admitted to the hospital due to an acute obstructive cholangitis secondary to choledocholithiasis that was removed by endoscopy. Chest x-ray on admission showed chronic alterations compatible with idiopathic pulmonary fibrosis. The images were no different from the previous episode. The patient received piperacillin-tazobactam. After the endoscopic procedure, he developed mild acute pancreatitis, hemobilia, and acute kidney injury related to acute tubular necrosis. In addition, he developed catheter-related *Enterococcus faecium* bacteraemia successfully treated with vancomycin. During this time, he obtained two negative SARS-CoV-2 RT-PCR tests (September 1 and 14, Figure 1).

On Day 23 following admission (57 days after his first positive RT-PCR from his previous COVID-19 episode), extensive bilateral lung opacities were identified in a control abdominal computed tomography (CT). After these unexpected radiological findings, SARS-CoV-2 RT-PCRs were performed for two consecutive days, both positive (Ct 19, Ct 21). SARS CoV-2 IgG serology was negative (Figure 1).

Case R developed mild dyspnoea and hypoxemia (oxygen saturation of 92% at room air). He received remdesivir for five days and dexamethasone 20 mg once daily for

four days. After a slight improvement, on Day 29, he developed fever and respiratory worsening. On Day 31, high-flow oxygen therapy and a single 400 mg dose of tocilizumab (IL-6 level: 226pg/mL) were administered. The patient was transferred to the ICU where he received full ventilatory support and continuous changing between prone and supine positions. However, the patient rapidly developed multiorgan failure with hemodynamic instability, mixed metabolic and respiratory acidosis, and renal impairment requiring continuous renal replacement therapy. Body CT scan revealed non-specific colitis and worsening of the bilateral pulmonary opacities with pleural effusion. A colonoscopy ruled out ischemic colitis. Despite all therapeutic interventions, the patient developed refractory multi-system organ failure and finally died on Day 34. Retrospectively, we recovered three sera specimens (from days 23, the day the nasopharyngeal RT-PCR result was positive, 27, and 30) and all were positive for SARS-CoV-2 by RT-PCR (Ct value in all three was 37). Clinical outcomes are shown in Figure 1.

Whole genome sequencing analysis (WGS)

Prior to having the WGS data, several findings, i.e., chronology of SARS-CoV-2 infections, dates of symptom onset, positive SARS-CoV-2 RT-PCRs, and room coincidences, led clinicians to assume that Case R recurrence was a reinfection due to the exposure to a patient with whom he had shared the hospital room (Case A) and who had been admitted 11 days before due to an intestinal obstruction, had a bilateral pneumonia and subsequent positive SARS-CoV-2 RT-PCR. However, WGS data (obtained in a larger study analysing a wide nosocomial outbreak in the Gastroenterology ward, under evaluation) indicated that fully different strains were identified in Case A and Case R (Figure 2a). In addition, Case R was part of Cluster which also included Cases S and T, infected by an identical strain (0 SNPs, Figure 2a). Cases S and T had shared a room, but

Case R at the time of his positive-RT-PCR was in a different one. However, tracking back his previous movements revealed that Case R had shared room with case S seven days before, confirming a link between them; SARS-CoV-2 infection in Case S had a fatal outcome.

WGS data ruled out our initial hypothesis of reinfection after nosocomial exposure and led us to consider, alternatively, Case R as a reactivation, causing a subsequent nosocomial transmission. The sequences of the positive specimens collected from Case R first and second episodes (July and September, 2020) belonged to the same lineage (B.1.177) and showed nearly identical sequences; they shared 16 SNPs and differed in two (Figure 2a and 2c, Supplementary Table). The marked diversity of circulating SARS-CoV-2 in the second COVID-19 wave (Figure 2b), the differences between the strains circulating in July and September and the high similarity between the Case R´s sequences and those from the two related nosocomial cases, altogether, strongly supports that Case R recurrence most likely represented a reactivation causing subsequently a nosocomial transmission.

177 **Discussion**

178 This study shows the importance of WGS-based analysis to correctly understand
179 COVID-19 recurrences and, additionally, the true links within nosocomial transmission
180 events. This technique provided key data to describe a COVID-19 reactivation, which
181 was subsequently responsible for another two nosocomial cases.

182 The similarities between the strains infecting Case R in the July and September
183 episodes may be explained by either a persistently active infection or a reactivation after
184 a clinical resolution.

185 The persistently active infection hypothesis was less likely out because the patient
186 fully recovered from mild clinical symptoms experienced during his first episode.
187 Furthermore, X-rays at admission did not show abnormal SARS-CoV-2-related findings
188 and two sequential negative PCRs just before being diagnosed again in September (at
189 admission and 14 days later) were obtained. Finally, during the 23 days of hospital stay
190 before reactivation, the patient had close contact with four roommates, none of which had
191 a COVID-19 diagnosis.

192 All the previous findings make more likely the alternative explanation, namely
193 reactivation, for the high sequence similarities between the specimens collected during
194 the two episodes experienced by Case R. The subtle differences (two different SNPs and
195 16 identical SNPs) found for this case are similar to those described in a reactivation
196 reported elsewhere [6]. The reactivation hypothesis means that SARS-CoV-2 should have
197 have stayed undetected (or unsampled) in some kind of reservoir between the two
198 sequential episodes. The presence of SARS-CoV-2 in extra-pulmonary tissues (eyes,
199 gastrointestinal tract, liver, and brain) has been reported [7], due to the ubiquity of the
200 ACE2 receptors. However, reservoirs for SARS-CoV-2 after the resolution of a COVID-
201 19 episode have not been defined yet and the presence of SARS-CoV-2 in non-respiratory

tissues from asymptomatic cases [8] suggests that further studies are needed to identify other viral reservoirs [9].

If the reservoir hypothesis were correct, we would expect reactivations to be mainly associated with immunosuppression, which would trigger the replication of the latent strain. Few studies have proposed reactivation as the explanation for COVID-19 recurrence [5, 10], some involving immunosuppression. However, only two were supported with viral genome analyses [11] [6]. Several factors suggest the presence of immunosuppression in Case R. Firstly, he had stayed hospitalized 23 days suffering of severe conditions before his first positive RT-PCR. Acute care settings is a risk factor of malnutrition. Before the diagnosis of COVID-19, Case R had lymphopenia for 12 days; this may impair immunity, a factor associated to increased morbidity and mortality [12, 13]. Secondly, the patient suffered of severe gastrointestinal conditions (acute cholangitis, post-ERCP acute pancreatitis, and gastrointestinal bleeding requiring blood transfusion) that could have worsened his immune system. Finally, he presented two infections (cholangitis and a catheter-related infection) and acute kidney injury that might have further worsened his already weakened immune system. SARS-CoV-2 IgG determination was negative at the time of the second episode diagnosis, which might be consistent with immunosuppression; although we should also consider that the detection of specific responses months after acute infection sometimes may be not optimal.

A relevant retrospective finding in Case R is the positive SARS-CoV-2 RT-PCR in three serum specimens taken the same day he had his first diagnostic SARS-CoV-2 RT-PCR, and four and six days later. SARS-CoV-2 may be detected in plasma samples from patients with respiratory disease and this may have value to predict the severity of the disease [14]. However, SARS-CoV-2 RNAemia has not been found close to diagnosis, even in cases with pneumonia [15]. Therefore, the presence of SARS-CoV-2

227 in plasma just at the initial diagnosis of the second episode experienced by Case R, would
228 suggest that we are not facing a new infection but a likely longer-term disease, which may
229 support the reactivation scenario.

230 In summary, we report genomic viral analysis allowed to identify a reactivation
231 case with major consequences, leading to a more severe second episode with fatal
232 resolution and subsequent nosocomial transmission of the same strain with an additional
233 COVID-19-related death.

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Conflict of interests

The authors do not have commercial or other associations that might pose a conflict of interest.

Data availability

The data that support the findings of this study (FastQ files) are openly available in GISAID at https://www.gisaid.org/ . Reference numbers EPI_ISL_654287, EPI_ISL_654203, EPI_ISL_654284, EPI_ISL_654176 and EPI_ISL_1173765.

Figures

Figure 1. Clinical timeline for Case R. ERCP: endoscopic retrograde cholangiopancreatography; RT-PCR: Reverse-transcription polymerase chain reaction; S: serum sample; NP nasopharyngeal sample; (+) Positive result; (-) Negative result; RBC: red blood cells transfusion. CT: computerized axial tomography scan. MO failure: multiorgan failure; HFNC: high-flow nasal cannulas; O. intubation: orotracheal intubation

Figure 2. a) Network of relationships obtained from whole genome sequencing analysis for the outbreak strains. Each dot corresponds to a single nucleotide polymorphism. When two or more cases share identical genome (zero single nucleotide polymorphisms between them) they are included in the same box. mv: median vector: not sampled recent common ancestor for the two branches. REF: Wuhan-1 reference strain. **b)** Phylogenetic tree including 183 representative sequences from SARS-CoV-2 circulating in July 2020 (case R´s first episode) and September 2020 (case R´s second episode). The two sequences from case R are indicated and also those from the two other cases involved in the nosocomial outbreak. c)Distribution along the SARS-CoV-2 chromosome of the single nucleotide polymorphisms identified in the two sequential episodes of Case R. Each vertical bar corresponds to a single nucleotide polymorphism.

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Figure1

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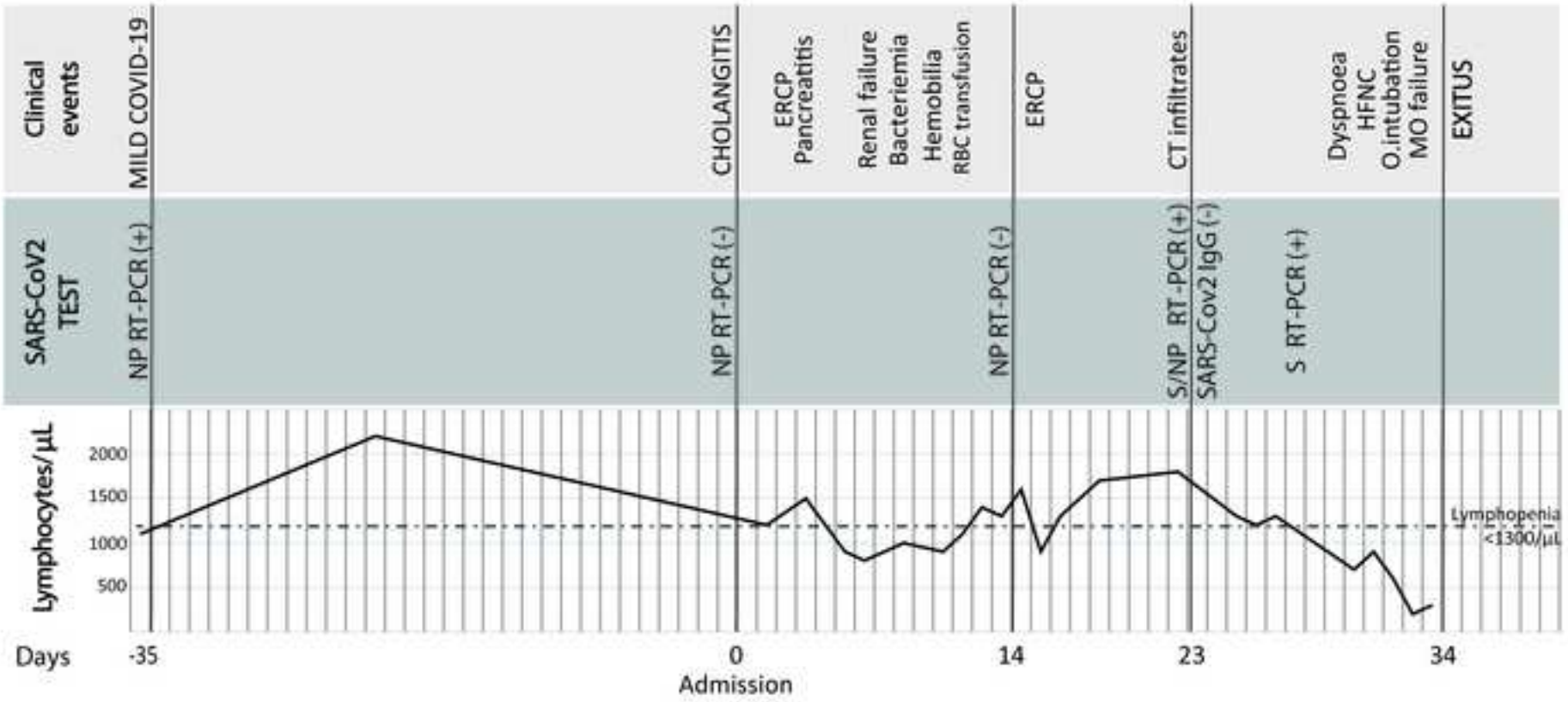

Figure2

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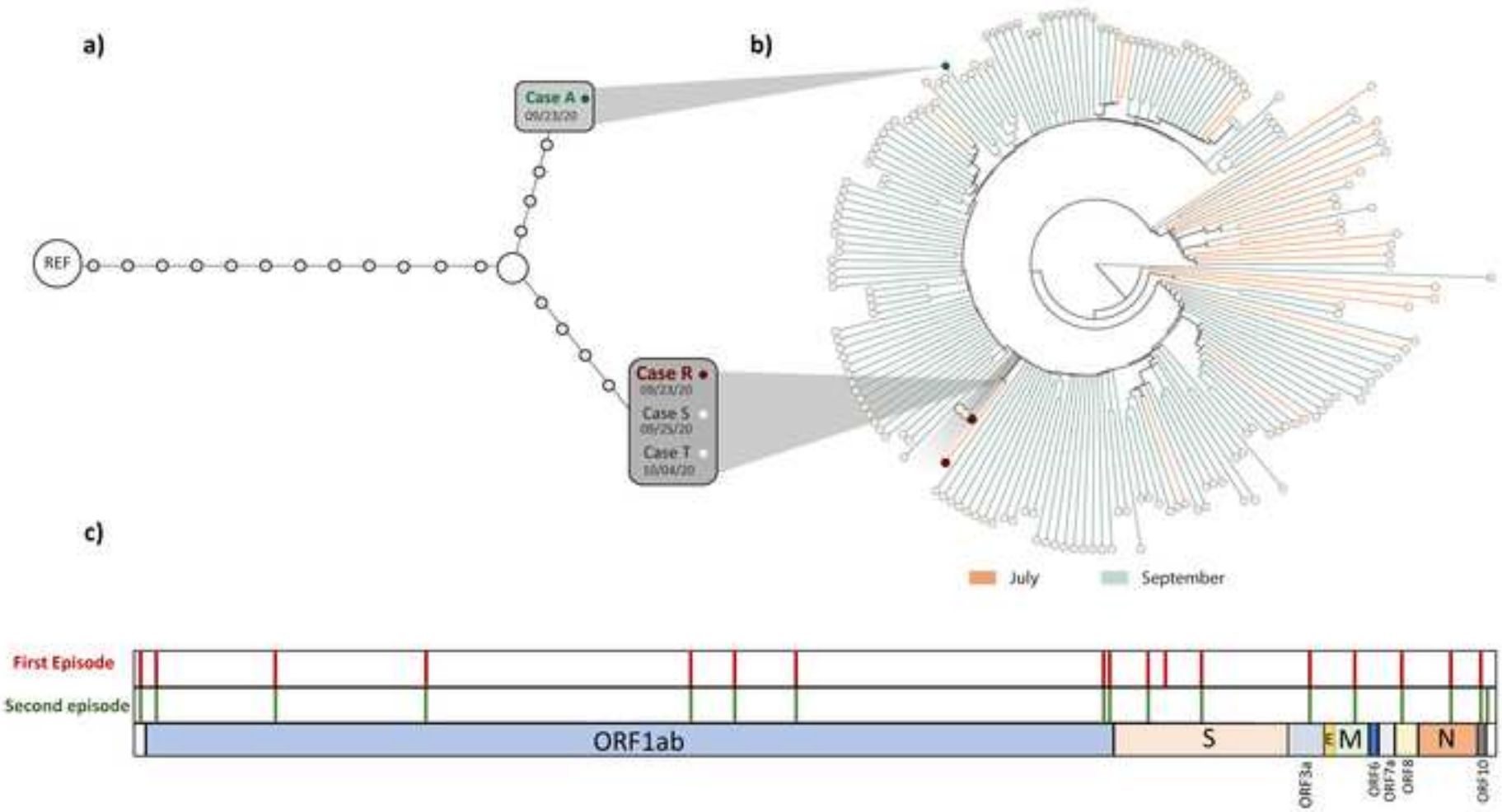

Supplemental Material

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SNP analysis of the sequential episodes from Case R

*Sanger sequencing

	Episode 1	Episode 2
Position	28/07/2020	23/09/20202
T|22669|C	1.0	0
G|29692|T	0.0*	1.0
C|241|T	1.0	1.0
C|3037|T	1.0	1.0
C|14408|T	1.0	1.0
A|21222|T	1.0	1.0
G|21255|C	1.0	1.0
C|27944|T	1.0	1.0
G|29645|T	1.0	1.0
C|6286|T	1.0	1.0
C|25889|T	1.0	1.0
C|22227|T	1.0	1.0
T|445|C	1.0	1.0
C|12119|T	1.0	1.0
C|13115|T	1.0	1.0
A|23403|G	1.0	1.0
C|26801|G	1.0	1.0
C|28932|T	1.0	1.0