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BRIEF REPORT

Long-Term Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infectiousness Among Three Immunocompromised Patients: From Prolonged Viral Shedding to SARS-CoV-2 Superinfection

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Background. Guidelines for stopping coronavirus disease 2019 patient isolation are mainly symptom-based, with isolation for 10 to 20 days depending on their condition.

Methods. In this study, we describe 3 deeply immunocompromised patients, each with different clinical evolutions. We observed (1) the patients' epidemiological, clinical, and serological data, (2) infectiousness using viral culture, and (3) viral mutations accumulated over time.

Results. Asymptomatic carriage, symptom resolution, or superinfection with a second severe acute respiratory syndrome coronavirus 2 strain were observed, all leading to prolonged infectious viral shedding for several months.

Conclusions. Understanding underlying mechanisms and frequency of prolonged infectiousness is crucial to adapt current guidelines and strengthen the use of systematic polymerase chain reaction testing before stopping isolation in immunocompromised populations.

Keywords. COVID-19; immunocompromised patients; isolation; SARS-CoV-2; viral shedding.

The coronavirus disease 2019 (COVID-19) pandemic has severely disrupted healthcare systems and socioeconomic activities. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused large outbreaks in, bars, workplaces, households, and healthcare institutions. In the latter, patients' management and isolation is critical.

Several important questions, which are important to determine prevention policies, remain unanswered regarding the duration of infectiousness and, consequently, the duration of isolation in healthcare institutions. The Centers for Disease Control and Prevention recommends a 10-day isolation period for afebrile COVID-19 patients with mild and/or moderate clinical presentation and improvement of other symptoms for at least 24 hours. This period is extended for up to 20 days for patients with severe infection and/or severe immunosuppression. A negative SARS-CoV-2 reverse-transcription polymerase chain reaction (RT-PCR) control is not mandatory but is encouraged in immunocompromised patients before stopping isolation [1]. For hospitalized patients in France, the isolation period should be at least 14 days after symptom onset and 48 hours after their resolution. This period is extended up to 24 days for severe infections or immunocompromised patients [2]. Several studies described prolonged positive RT-PCR for more than 15 days postsymptom onset for less than 5% of hospitalized patients, but without viral culture testing [3]. However, 3 cases of long-term infectious shedding with high viral loads were recently reported in the literature with viral shedding for 35, 70, and 119 days [4–6].

In this study, we describe 3 deeply immunocompromised patients, each presenting a different clinical evolution. All 3 had prolonged viral shedding with high viral load for several months. We explored (1) their epidemiological, clinical, and serological data, (2) infectiousness using viral culture, and (3) viral mutations accumulated over time.

METHODS

Samples and Patients

Respiratory samples and sera were collected from the patients as a part of their routine clinical care. The research was approved by the local ethics committee, number CER-2020-6.

Severe Acute Respiratory Syndrome Coronavirus 2 Polymerase Chain Reaction

The respiratory samples were tested using either the Cobas SARS-CoV-2 (Roche, Rotkreuz, Switzerland) [7] or the NeumoDX (QIAGEN, Hilden, Germany) using the IP2 Institute Pasteur and the World Health Organization E gene primers [8]. E gene cycle threshold (Ct) value was used as a proxy for viral load.

Antibody Testing

Anti-SARS-CoV-2 nucleocapsid (N) and spike (S) immunoglobulin G were detected using a chemiluminescent microparticle

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immunoassay (Architect; Abbott, Chicago, USA) and an enzyme-linked immunosorbent assayassay (EuroImmun, Lubeck, Germany), respectively.

Viral Culture

Vero E6 cells (American Type Culture Collection [ATCC], reference no. R CRL-1586) were cultured in Dulbecco's modified Eagle's medium ([DMEM] Gibco) with 10% of heat-inactivated fetal bovine serum ([FBS] Gibco) at 37°C and 5% of CO_2. In brief, 200 µL of respiratory samples in viral transport media mixed with 800 µL DMEM were filtered and inoculated in 12-well plates containing 1.10^5 cells for 1 hour before adding of 500 µL DMEM with 4% of FBS. After a 6-day incubation, cytopathogenic effect assessment and RT-PCR quantification in cell supernatant were performed to assess the production of new virions. A previously cultured SARS-CoV-2 strain was systematically added as a positive control to ensure cell sensitivity.

Viral Whole-Genome Sequencing

Full genome viral sequencing was conducted from primary clinical samples. Reverse transcription was performed with SuperScript IV with random hexamers after MagnaPure extraction. Tiling PCR amplification was performed according to the Artic protocol (nCoV-2019 sequencing protocol v2) with 2 pools of primers (ARTIC nCoV-2019 V3 panel). Libraries were prepared with NEBNext Companion Module for Oxford Nanopore Technologies, Ligation Sequencing (SQK-LSK 109) and sequenced using MinION R9.4.1 flow cells. All sequences obtained have been deposited on GISAID (EPI_ISL_833191 to EPI_ISL_833200).

Viral Genomes Analysis

Reads were filtered using the Nanofilt and Nanostat python scripts [9]. They were mapped on the reference genome Wuhan Hu-1 (GenBank identification number NC_045512.2) using minimap2. Alignment coverage, depth, and general quality were assessed using in-house R scripts. Variant calling was performed with bcftools suite, and the proportions of each variant in each sample were retrieved with an in-house R script. Finally, for patient 3, mutations located on the same amplicon tiles were recovered using R to assess their linkage.

RESULTS

Patient 1

A 66-year-old African male was admitted on June 4, 2020 for loss of autonomy and confusion. He was diagnosed with human immunodeficiency virus-1 infection (plasma viral load at 275 000 copies/mL and CD4 cell count at 0/mm^3, CD19 cell count diminished at 60/mm^3) and progressive multifocal leukoencephalopathy with positive cerebrospinal fluid (CSF) PCR for JC virus and compatible brain imagery. All other viral PCR in CSF, including SARS-CoV-2, were negative (Table 1). He was diagnosed with SARS-CoV-2 infection with positive nasal PCR (Ct value 22) and typical computed tomography (CT) chest findings. Despite efficient multi-antiretroviral therapy, the patient had persistent CSF positive PCR for JC, no CD4 cell count increase, and progressive neurological deterioration responding only to painful stimulation after 3 months (Glasgow coma scale = 8).

Throughout his hospital stay, the patient did not experience any dyspnea nor respiratory symptoms and showed increasing COVID-19 lesions. Nasopharyngeal (NP) PCR were constantly positive (Ct from 15 to 25) until day 111. Viral cultures were positive between days 43 and 95 (Figure 1). The first negative SARS-CoV-2 RT-PCR was obtained at day 124, and subsequent SARS-CoV-2 serology remained negative.

Sequences, obtained at 4 time points up to day 75, did not show any mutations except for a transient appearance of a C23718T mutation (Figure 1). The viral strain differed from the Wuhan reference strain by 14 mutations.

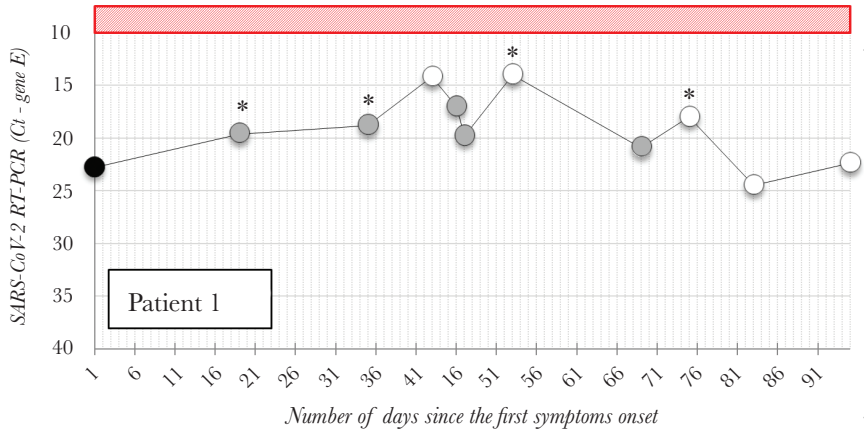
Patient 2

On April 15, patient 2, a heart-transplanted 71-year-old European male patient receiving an immunosuppressive treatment (prednisone, mycophenolic acid, belatacept), was hospitalized for asthenia, dry cough, myalgia, and low-grade fever for 1 week. He also presents with diabetes mellitus and chronic kidney disease (glomerular filtration rate, 35 mL/minute). He had neither dyspnea nor oxygen requirements throughout his hospital stay. The NP swabs tested positive for SARS-CoV-2 at admission and day 14 with minimal COVID-19 involvement on CT scan (<10%). At day 39, he was discharged after clinical improvement, despite persistent positive PCR at day 32 (21 Ct) (Table 1).

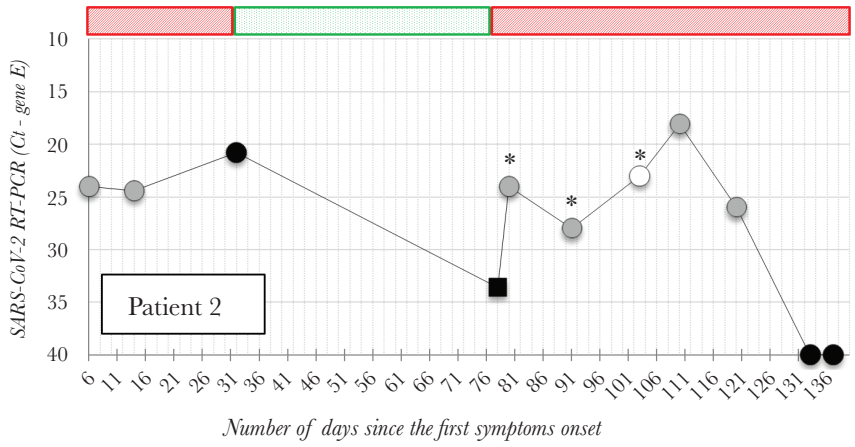
On June 23, 76 days after initial symptoms' onset, the patient presented with dry cough, dyspnea, and oxygen requirement. He was admitted to the intensive care unit for cardiac decompensation due to underlying respiratory infection. The CT scan showed worsened COVID-19 compatible lesions (40%). He had lymphopenia with CD4 <200/mm^3 and CD19 <20/mm^3. Bronchoalveolar (BAL) and NP samples collected at readmission (day 78) were positive for SARS-CoV-2 (Ct at 33 and 24, respectively). During his stay, multiple SARS-CoV-2 PCR and viral cultures were performed (Figure 1). Viral culture was positive on an NP sample collected at day 103. The last NP SARS-CoV-2 RT-PCR-positive sample was collected on day 120. The patient had negative serology throughout his illness. No viral sequence could be obtained from the first episode to assess the possibility of a new infection during the second episode. Sequences from day 80, 91, and 103 show minimal evolution during this 2-month period (Figure 1).

Patient 3

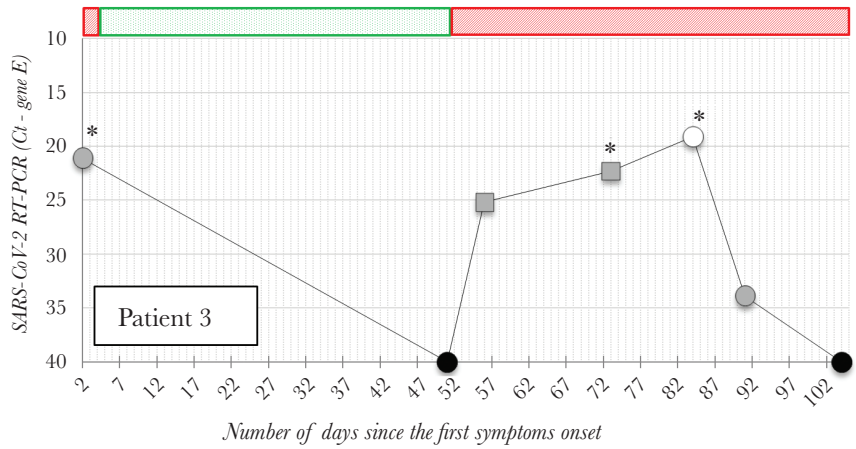
A 35-year-old Tunisian patient with rheumatoid arthritis and under treatment with rituximab (B lymphopenia with zero $CD19^+$ cells/mm^3) presented on April 28, 2020 with fever, cough,



Position	Region	Mutation	Days from 1st S.O. 19	35	53	75
241	non-coding	C-T	P	P	P	P
2416	ORF1ab	C-T (syn.)	P	P	P	P
3037	ORFlab	C-T (syn.)	P	P	P	P
7834	ORFlab	C-T (syn.)	P	P	P	P
14322	ORFlab	C-T (syn.)	94%	P	P	P
14408	ORFlab	C-T	P	P	P	P
18880	ORF1ab	G-T	P	P	P	P
23403	S	A-G	P	P	P	P
23718	S	C-T	-	-	62%	-
25207	S	C-T (syn.)	91%	P	P	P
25563	ORF3a	G-T	P	P	P	P
26038	ORF3a	T-C	P	P	P	P
26600	M	C-T (syn.)	P	P	P	P
27970	ORF8	C-A	P	P	P	P



Position	Region	Mutation	Days from 1st S.O. 80	91	103
241	non-coding	C-T	P	P	94%
1059	ORF1a	C-T	56%	55%	77%
3037	ORF1a	C-T (syn.)	82%	87%	94%
14408	ORF1b	C-T	58%	53%	72%
23403	S	A-G	92%	92%	89%
25563	ORF3a	G-T	48%	60%	75%



Position	Region	Mutation	Days from 1st S.O. 2	73	84
241	non-coding	C-T	95%	93%	94%
1059	ORF1a	C-T	74%	86%	86%
3037	ORF1a	C-T (syn.)	90%	P	P
5147	ORF1a	C-T	68%	-	-
14408	ORF1b	C-T	71%	78%	85%
23403	S	A-G	92%	92%	93%
25563	ORF3a	G-T	80%	69%	88%
9409	ORF1a	A-G (syn.)	-	-	66%
9438	ORF1a	C-T	-	-	65%
15593	ORF1b	A-G	-	-	78%
21585	S	T-G	-	-	72%
25688	ORF3a	C-T	-	-	84%
26333	E	C-T	-	-	67%
26527	M	C-T	-	-	71%

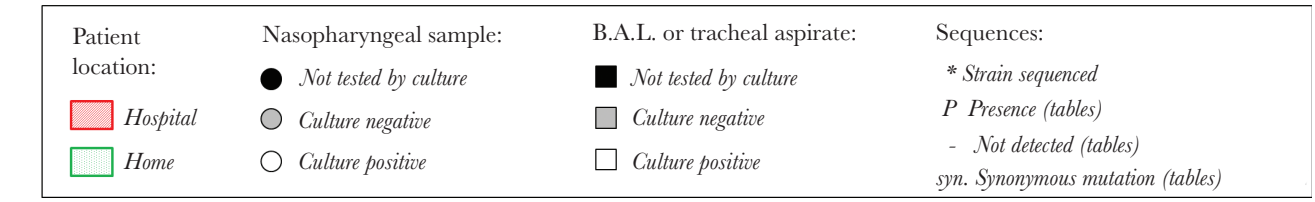


Figure 1. Virological follow up of the 3 patients. For each patient, the viral load in respiratory samples are indicated by the observed cycle threshold (Ct) values. The nature of sample is indicated by the point shape, and viral culture status is indicated by the color. The viral strains successfully sequenced are indicated by an asterisk, and the sequence differences with the reference strain are indicated in the table along each graphic. B.A.L., bronchoalveolar; RT-PCR, reverse-transcription polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

and mild dyspnea. He had a positive NP sample for SARS-CoV-2 (21 Ct) with COVID-19 CT scan lesions (25% lung involvement) (Table 1). He did not require oxygen therapy and was discharged at day 3. On day 49, he was readmitted for persistent cough, exertional dyspnea, and intermittent fever. The CT scan presented different topography of COVID-19 lesions. Severe acute respiratory syndrome coronavirus 2 PCR was negative on an NP swab collected on day 51 but positive on a BAL performed on day 56 was (25 Ct). Taking note of imaging findings, negative bacterial investigations, and the lack of antibiotics response, diagnosis of post-COVID-organized pneumonia was established. A 1-week corticosteroid treatment was initiated on day 66 with significant clinical improvements. On day 73, the patient had recurrence of fever, cough, and increased inflammatory markers (C-reactive protein at 125 mg/L) that gradually improved within 4 weeks. An NP swab collected on day 84 was positive on RT-PCR (19 Ct) and viral culture. Severe acute respiratory syndrome coronavirus 2 PCR was negative at day 104. The patient's serology remained negative up to day 121.

Severe acute respiratory syndrome coronavirus 2 whole genome was obtained from (1) an NP swab at day 2, (2) a lower respiratory tract sample at day 73, and (23) an NP swab after symptom's relapse at day 84. Sequences from days 2 and 73 were similar, aside from a C5147T mutation detected in at 68% frequency at day 2 (Figure 1). On day 84, we detected the appearance of 7 mutations at frequencies close to 70%. Two pairs of close mutations were present in the same PCR tiles. The mutations in these pairs were strongly linked, ie, approximately 99% of tiles amplicons contained either none or both mutations (Supplementary Table 1), suggesting a coinfection with a second viral strain presenting 7 additional mutations, linked with a symptom relapse at day 73.

DISCUSSION

This work reports 3 severely immunocompromised COVID-19 patients shedding infectious viruses up to 4 months postsymptom onset, illustrating different situations leading to long-term infectiousness. This highlights the need for caution and virological controls in deeply immunocompromised populations.The first patient presented a single continuous infection with high viral load and regular positive culture during 123 days. Long-term replication, lack of respiratory symptoms, and absence of mutation selection can be explained by the patient's complete immunosuppression. The second patient presented a positive RT-PCR for 121 days and a positive viral culture on day 103 (27 days after his readmission). Unfortunately, we could not sequence the viral genome during the first hospitalization to rule out a potential reinfection. The last patient presented a quickly resolved COVID-19 episode followed by a post-COVID organized pneumonia with still active viral replication in the lower respiratory tract. At day 84, 32 days after his second hospitalization, he presented a probable superinfection with symptom relapse, high viral loads, positive viral culture, and 7 new mutations (also see the phylogenetic reconstruction in Supplementary Figure 1). Those mutations seem unlikely to have arisen during the 11-day period from the previous sequence, especially because SARS-CoV-2 presents an evolution speed estimated to be 1.10^{-3} mutations per nucleotide per year (ie, 2 to 3 mutations per month) and as highlighted by the extremely low number of mutations selected in our 2 other immunocompromised patients over large periods of time. Those 7 new mutations were never present at a 100% frequency. Moreover, we observed a strong linkage for 2 pairs of these mutations. This, along with the symptom relapse, reinforces the hypothesis of a superinfection with a probable cohabitation of 2 viral strains. The patient's homeless condition also allowed multiple re-exposure until day 52; however, the symptom relapse on day 73 suggests a nosocomial infection. Sequence data from the other patients and from healthcare workers of the ward could not be used to explore the source of infection. Isolation precautions were maintained and observed during the entire hospitalization.

Two cases of prolonged viral shedding for more than 100 days were previously described in 2 patients presenting B-cell immunodeficiency [4, 10]. It is interesting to note that all of our patients also presented deep CD19 depletion. Convalescent plasma to reduce viral shedding in such populations could be evaluated. The T-cell immunity, which could not be explored in our patients, may also play a role in prolonged viral shedding. Several observational studies identified patients with positive SARS-CoV-2 RT-PCR 100 days after their initial detection [11, 12]. However, differentiation between reinfections and prolonged viral shedding was not established. Moreover, these studies did not follow patients' infectiousness using viral culture, as we did here. Although several case reports published in different countries showed SARS-CoV-2 reinfection [13–15], a superinfection with a second SARS-CoV-2 strain has not been described to date.

CONCLUSIONS

In conclusion, immunodeficiency plays a major role in prolonged viral shedding that can be observed in immunocompromised patients without any respiratory symptom (patient 1), late symptom relapse (patient 2), or with SARS-CoV-2 superinfection (patient 3). Further studies are needed to better understand the frequency and dynamics of long-term viral infectiousness. Menwhile, guidelines should recommend virological assessment of infectiousness, using viral culture and/or Ct value measure (low CT value), before stopping isolation for immunocompromised patients.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to

Table 1. Main Characteristics of the 3 Patients Presenting Prolonged Infectious Viral Shedding

Patient's Characteristics	Patient 1	Patient 2	Patient 3
Age (years)	66	71	35
Sex	Male	Male	Male
Immunocompromised condition	HIV	Cardiac transplantation	Rheumatoid arthritis
Comorbities	None	Obstructive sleep apnea, gout disease, osteoarthritis, chronic kidney disease, arterial hypertension, diabetes	None
Characteristics at Initial Admission			
Symptoms	Confusion	Asthenia, dry cough, myalgia, low-grade fever	Fever, cough,mild dyspnea
White blood cells (/mm^3)	2570	4070	4560
Neutrophils (/mm^3)	1600	2400	2580
Lymphocytes (/mm^3)	490	840	1150
Eosinophils (/mm^3)	30	10	260
Hemoglobin (g/dL)	11.2	10.9	13.5
Platelets (/mm^3)	290 000	209 000	250 000
CRP (mg/L)	48	26	25
Creatinine (µmol/L)	64	207	60
SGOT (UI/L)	59	38	18
SGPT (UI/L)	21	34	28
Bilirubin (mg/L)	8	9	5
LDH (U/L)	453	234	199
CD4 (/mm^3)	10	110	1150
CD8 (/mm^3)	270	650	810
CD19 (/mm^3)	60	20	0
Coinfections			
Respiratory	*Mycobacterium gordonae*	None	None
CSF	JC virus	None	None
Blood	CMV, EBV		
Other	Oral candidiasis	None	None

Abbreviations: CMV, cytomegalovirus; CRP, C-reactive protein; CSF, cerebrospinal fluid; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; LDH, lactate dehydrate; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase.

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Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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