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SHORT COMMUNICATION

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SARS-CoV-2 show no infectivity at later stages in a prolonged COVID-19 patient despite positivity in RNA testing

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Abstract

Inpatient coronavirus disease 2019 (COVID-19) cases present enormous costs to patients and health systems in the United States. Many hospitalized patients may continue testing COVID-19 positive even after the resolution of symptoms. Thus, a pressing concern for clinicians is the safety of discharging these asymptomatic patients if they have any remaining infectivity. This case report explores the viral viability in a patient with persistent COVID-19 over the course of a 2-month hospitalization. Positive nasopharyngeal swab samples were collected and isolated in the laboratory and analyzed by quantitative reverse-transcription polymerase chain reactions (qRT-PCR), and serology was tested for neutralizing antibodies throughout the hospitalization period. The patient experienced waning symptoms by hospital day 40 and had no viable virus growth by hospital day 41, suggesting no risk of infectivity, despite positive RT-PCR results which prolonged his hospital stay. Notably, this case showed infectivity for at least 24 days after disease onset, which is longer than the discontinuation of transmission-based precautions recommended by the Center for Disease Control and Prevention. Thus, our findings suggest that the timeline for discontinuing transmission-based precautions may need to be extended for patients with severe and prolonged COVID-19 disease. Additional large-scale

studies are needed to draw definitive conclusions on the appropriate clinical management for these patients.

KEYWORDS
COVID-19, inpatients, patient discharge, real-time polymerase chain reaction, SARS-CoV-2

1 | INTRODUCTION

On March 11, 2020, the World Health Organization declared the coronavirus disease 2019 (COVID-19) a pandemic. COVID-19, caused by order *nidovirales*, family *coronaviridae*, genus *betacoronavirus*, species *severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2), has caused more than 128 million laboratory-confirmed infections worldwide as of March 31, 2021.[1] Inpatient COVID-19 hospitalizations were projected to cost the United States health care system up to $16.9 billion in 2020[2] and impose a large financial burden to individual patients.[3] The median hospital stay for COVID-19 was 10–14 days in the United States.[4] Many hospitalized patients with prolonged viral shedding may test COVID-19 positive even after resolution of symptoms and infectivity, causing an extended hospitalization.[5–8] In addition to measuring viral load, serological tests measuring antibody responses against SARS-CoV-2 are also valuable diagnostic tools. SARS-CoV-2-specific antibodies against the receptor binding domain (RBD), nucleocapsid (N), and spike (S) antigens vary over time, correspond to disease severity, and peak 1 to 2 months after symptom onset.[9] Neutralizing antibodies (Nabs), which function to bind to infectious viruses and minimize virus pathogenesis, have been shown to persist over 3 months, but can also rapidly decline within 2 months.[9,10] Thus, a pressing concern for clinicians is gauging the safety of discharging these asymptomatic patients: whether they have any remaining infectivity and whether they are adequately protected from additional infection.

As of August 2020, the Center for Disease Control and Prevention (CDC) no longer recommends test-based strategies due to prolonged and detectable shedding in patients that no longer have infectivity.[11] The CDC recommends the following guidelines for the discontinuation of transmission-based precautions for persons with severe or critical illness: patients may be discontinued from transmission-based precautions up to 20 days after symptom onset, at least 24 h after the last fever, and improved symptoms.[11] In this case report, we present a patient with critical severity of COVID-19 disease who was still shedding infectious viruses at 24 days after symptom onset during his 2-month long hospitalization.

2 | METHODS

2.1 | Ethics statement

This study was performed under the institutional review board (#2023844) and the Biosafety Level 3 (#20-14), in compliance with the Institutional Biosafety Committee of the University of Missouri-Columbia.

2.2 | Sample collection

The patient's clinical observations were documented at least twice daily and multiple nasopharyngeal swabs and plasma samples were collected and tested to determine viral loads and Nab titers. Periodic national early warning scores (NEWS) were assessed. A score of 7 or higher identifies high-risk patients requiring activation of a medical emergency team.[12] The patient's NEWS scores were between 8 and 12 from Day 33 through Day 45 and then remained below 7 from Day 46 until discharge.

2.3 | COVID-19 diagnosis

COVID-19 was diagnosed using the 2019 novel coronavirus (2019-nCoV) real-time reverse-transcriptase (RT)–PCR diagnostic panel from the International Reagent Resource. A threshold cycle (C_t-value) below 40 is considered COVID-19 positive. Four positive samples were collected throughout the patient's hospital stay, and three samples were successfully recovered for analysis.

2.4 | Tissue culture infectious dose ($TCID_{50}$)

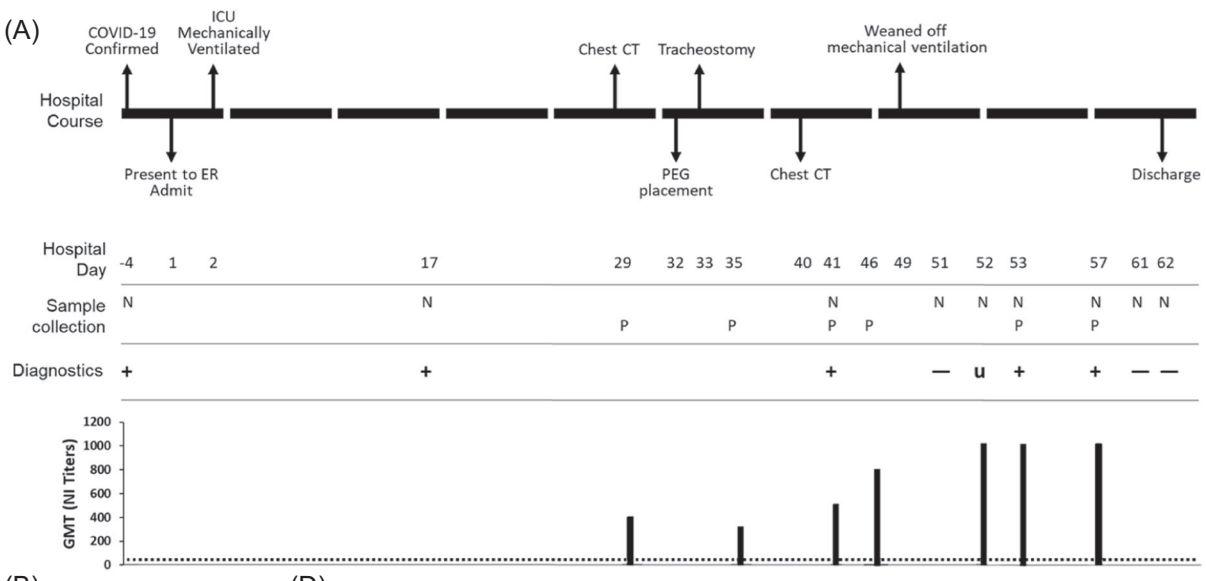
To test the viability of live virus in each of the viral samples at different time points of the patient's hospitalization, the viral samples were serially diluted from $1:10^1$ to, at most, $1:10^{12}$ in opti-minimal essential medium reduced-serum medium. Two hundred microliters of diluted virus were placed in four wells of Vero E6 cells that were seeded in 96-well plates for each dilution for 1 day and incubated at 37°C in 5% CO_2 for 3 days. Cytopathic effects were recorded. TCID50 represents the viral loads causing a cytopathic effect in 50% of the wells as calculated by the Reed–Muench method.[13]

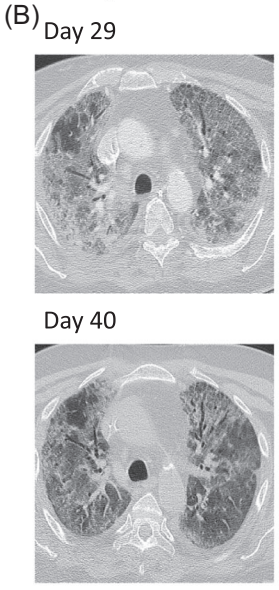
Additional methods are available in the Supporting Information Material.

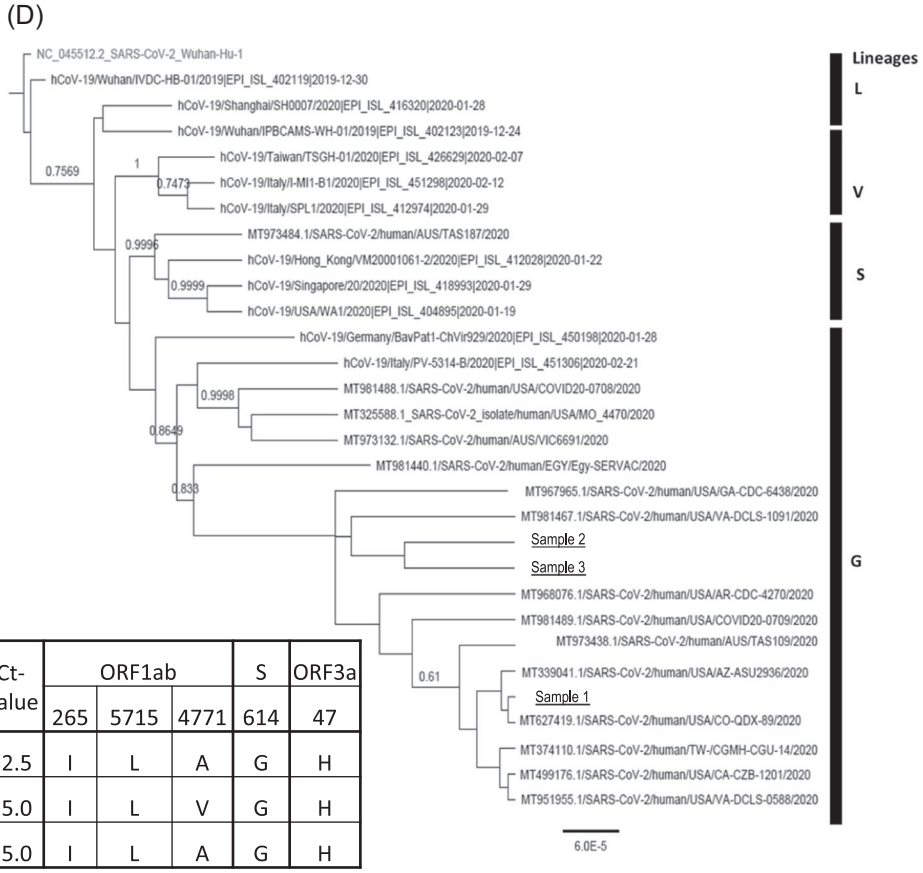
3 | RESULTS

3.1 | COVID-19 disease course

In March 2020, a 65-year-old Caucasian male presenting to urgent care with fever, weakness, fatigue, rhinorrhea, and cough was diagnosed SARS-CoV-2 positive 3 days after disease onset. The patient had recently returned from Europe and had comorbidities of







(C)

Sample	GISAID Clade	Ct-Value	ORF1ab			S	ORF3a
			265	5715	4771	614	47
1 (Day 17)	GH	32.5	I	L	A	G	H
2 (Day 41)	GH	35.0	I	L	V	G	H
3 (Day 57)	GH	25.0	I	L	A	G	H

FIGURE 1 Time course of clinical, virological, and immunological responses in a COVID-19 patient with a prolonged clinical course. (A) Clinical outcomes, SARS-CoV-2 tests, and neutralizing antibody responses demonstrate the course of clinical improvement and are associated with the elevation of neutralizing antibodies. The dashed line denotes the titer of 1:40 which was used to define seroconversion. (B) CT chest demonstrates diffuse bilateral ground glass opacities (white arrow) and patchy areas of subpleural consolidation (black arrow) (left image, Day 29), and follow-up CT chest (right, Day 40) shows interval improvement in the airspace opacities. (C) Summary of amino acid mutations in the viruses from clinical samples 1, 2, and 3. (D) Phylogenetic tree of the viruses with complete genomes. The phylogenetic tree was rooted with the SARS-CoV-2 isolate Wuhan-Hu-1 (Genbank accession No: NC_045512.2) (gray). Bayesian posterior probabilities are indicated in the nodes. Scale bar shows the average number of substitutions per nucleotide site. The sequences from three clinical samples are underlined. COVID-19, coronavirus disease 2019; CT, computerized tomography; ER, emergency room; GMT, geometric mean titer; ICU, intensive care unit; NI Titers, neutralizing titers; N, nasopharyngeal swab; PEG, percutaneous endoscopic gastrostomy; P, plasma; +, SARS-CoV-2 positive by RT-PCR; RT-PCR, reverse-transcription polymerase chain reaction; -, SARS-CoV-2 negative by RT-PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; u, SARS-CoV-2 inconclusive by RT-PCR

TABLE 1 Comparison of neutralizing antibodies using microneutralization assays with live SARS-CoV-2 virus, neutralization analyses with pseudovirus expressing spike proteins, and ELISA assays

Day	NI-live GMT (± SD)	NI-pseduo (mean tters)	ELISA (mean titers)											
			RBD			S (full length)			S1			S2		
			IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
29	403 (±1.49)	8600	8100	300	900	8100	300	900	8100	100	300	24,300	300	900
35	320 (±1.00)	10,148	8100	300	900	8100	300	900	8100	100	300	24,300	300	600
41	508 (±1.49)	7287	8100	300	900	16,200	300	900	8100	100	300	8100	300	300
46	806 (±1.49)	–	–	–	–	–	–	–	–	–	–	–	–	–
52	1016 (±1.49)	10,746	8100	100	900	16,200	300	900	8100	100	900	8100	100	300
53	1016 (±1.49)	–	–	–	–	–	–	–	–	–	–	–	–	–
56	1016 (±1.49)	–	–	–	–	–	–	–	–	–	–	–	–	–
57	–	8773	16,200	100	300	8100	300	900	8100	100	900	8100	100	200
Negative control	<1:40	<1:40	50	50	50	50	50	50	50	50	50	50	50	50

Note: NI-live assays were performed in triplicate, and geometric mean values were calculated. NI-pseudo assays were performed in triplicate, and titers were calculated using regression analyses to correspond to 50% inhibition. ELISA assays were performed in duplicate, and the mean values were calculated.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; GMT, geometric mean titer; IgG, immunoglobulin G; NI-live, neutralization inhibition assays using live virus; NI-Pseudo, neutralization inhibition assays using pseudovirus; –, not available; RBD, receptor binding domain; *SD*, standard deviation.

hypertension, hyperlipidemia, and prediabetes. He did not have any pre-existing conditions causing him to be immunocompromised. Four days later (7 days after disease onset), the patient was admitted to University of Missouri Health Care Hospital (Day 1) with increased shortness of breath (Figure 1A). X-ray revealed bilateral, patchy ground glass opacities consistent with viral pneumonia. Hydroxychloroquine and broad-spectrum antibiotics were initiated during the first week of admission.

On Day 2 (D2), the patient developed acute hypoxic respiratory failure and was transferred to the intensive care unit (ICU), requiring endotracheal intubation, mechanical ventilation, and intermittent vasopressors. His hospital course was complicated by secondary bacterial pneumonia, eosinophilic bronchiolitis, and oral candidiasis. A computerized tomography (CT) scan performed on D29 showed extensive diffuse bilateral ground glass opacities and subpleural consolidation consistent with COVID-19-associated respiratory failure (Figure 1B). A percutaneous endoscopic gastrostomy and tracheostomy were placed on D32 and D33, respectively. The patient had another fever on D36–38 due to Klebsiella pneumonia which was treated with broad spectrum antibiotics. A follow-up CT chest scan on D40 showed interval improvement in the airspace opacities (Figure 1B).

3.2 | Nasopharyngeal swab test results

Viral RNA was undetectable on D51 and inconclusive on D52. An inconclusive PCR result indicated that one of the controls from the PCR test was unsuccessful. Thus, an additional nasopharyngeal swab performed the following day (D53) and was positive again. A follow-up nasopharyngeal swab was collected and was still positive on D57 with markedly increased viral RNA load (Figure 1C). On D61 and D62, two nasopharyngeal swabs revealed undetectable viral RNA. The patient was discharged on D63 in stable condition.

3.3 | Genome sequencing and elevated viral load

Due to the positive then negative then positive COVID-19 test results of this patient, the viruses were recovered and sequenced using one-step RT-PCR amplification with an integrated microfluidic system and next-generation sequencing[14] (Supporting Information Methods) to check for reinfection. Viruses were recovered from the clinical samples collected on D17 (Sample 1, threshold cycle [C_t] = 32.5), which was 24 days postdisease onset. The sequences recovered for Sample 1 contained 29,904 nucleotides (GenBank accession No: MW004168), Sample 2 (D41, C_t = 35.0) contained fragments totaling 13,499 nucleotides, and Sample 3 (D57, C_t = 25.0) contained fragments totaling 15,556 nucleotides. The complete genomic sequence of Sample 1 was 99.980% identical to SARS-CoV-2 isolate Wuhan-Hu-1 (Genbank accession No: NC_045512.2). The pairwise nucleotide identities of the three clinical samples were 99.970% (Samples 1 vs. 2), 99.974% (Samples 1 vs. 3), and 99.973% (Samples 2 vs. 3) in the approximately 10 kb overlapping region. Growth assays for these three samples were performed to test for infectious virus, and only Sample 1at D17 (24 days after symptom onset) was viable.

The viruses from all three samples belong to the GH lineage of SARS-CoV-2 (Figure 1D) with D614G in the S protein, P4715L in nonstructural protein (nsp) 12, T265I at nsp2, and Q57H at

open reading frame 3a (ORF3a), consistent with the prevalent D614G/Q57H/T265I subclade in the United States.[15] A limited number of polymorphisms were identified among these three viruses (Figure 1C). Of note, A4771V at ORF1ab was identified only in Sample 2 but not in Samples 1 and 3.

3.4 | Analysis of neutralizing antibodies

Plasma samples were collected between D29 and D57 for neutralization/inhibition (NI) and enzyme-linked immunosorbent assay (ELISA) determination (Figure 1A, Table 1). Live SARS-CoV-2-based NI assays showed highly positive and constant Nab titers, ranging from 1:403 to 1:320, until D41. On D52, when the patient retested indeterminate then positive, the Nab geometric mean titer increased to 1:1016, remaining elevated until discharge. Pseudotyped NI results also indicated raised Nab titers on D52.

ELISA results showed elevated RBD, S, S1, and S2-specific IgM, IgG, and IgA titers for the first 45 days (Table 1). While other antibodies remained stable or began declining following the first negative test, RBD-specific IgG and S1-specific IgA titers continued rising throughout the hospitalization (Table 1). Elevated RBD-specific IgG and S1-specific IgA titers correlated with the patient retesting positive on D52.

4 | DISCUSSION

In current CDC interim guidance on duration of isolation and precautions for adults with COVID-19, persons with severe or critical illness are recommended to be removed from transmission-based precautions 20 days after initial disease onset, at least 24 h after their last fever without fever-reducing medications, and improved symptoms.[11] Viral shedding in COVID-19 patients can last up to 3 months after disease onset,[14] but viral shedding is not necessarily correlated with infectious virus.[16] Thus, PCR results are not a sufficient measure for infectivity. In this case report, we show that it is possible for a not immunocompromised patient with severe and persistent COVID-19 symptoms to continue shedding infectious virus beyond 20 days after symptom onset with cell-viability assays, even after the patient had been afebrile for multiple weeks. This patient presented with viable virus growth 24 days after symptom onset, suggesting that patients with severe and persistent COVID-19 may have a longer viral infectivity than originally recognized.

The patient also presented with fluctuating viral loads and increased antibody titers during his severe acute infection and extended persistent infection. A strength of this study is the use of three different serological assays to monitor antibody development. The boosted Nab titers and viral RNA from nasopharyngeal swabs on D52 after a RNA-negative result, along with high sequence identities among three viruses and a limited number of polymorphisms (Figure 1D) suggest thepatient likely experienced a recurrent or persistent infection during his prolonged hospitalization. It is possible that the negative test may have been a false-negative test or a second infection, which may have been caused by reactivated viruses in this patient, even while the patient's symptoms improved. Intriguingly, only RBD-specific IgG and S1-based IgA titers clearly increased via ELISA during the recurrent infection period, consistent with Nab titers using live virus (Table 1). Although the pseudotyped NI assay detected Nabs, potential discrepancies from the live virus-based NI assay, as in this study, indicate that caution is needed when interpreting pseudotyped NI data. Overall, our serological and genomic tests were used to determine that this patient demonstrated viable virus growth at least 24 days after symptom onset and experienced elevated neutralizing antibodies during the later stages of his disease.

The primary limitation of this study is that the available data encompasses a single patient. Confounding factors include the multiple pharmaceutical treatments in addition to fluid maintenance and pain control (Table S1) that the patient received. Bacterial pneumonia was treated with broad spectrum antibiotics from D3-42. Antifungals were given to treat oral candidiasis D45-56, and methylprednisolone (steroid) was administered from D45 to D56 for eosinophilic bronchiolitis. In summary, the patient was on steroid medication and had just completed antifungal therapy when they retested positive for SARS-CoV-2 between D54 and D56.

In summary, this report follows the clinical and serological timeline of a patient with severe and persistent COVID-19. This patient demonstrated viable virus growth at least 24 days after symptom onset. These findings suggest that separate discontinuation of transmission-based precaution guidelines for patients with persistent symptoms and extended hospital stays may be necessary.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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