An immunocompetent 169-day prolonged SARS-CoV-2 shedding patient with high neutralizing antibody

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Article

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Abstract

Information with prolonged SARS-CoV-2 shedding among immunocompetent patients is limited. We describe a twice repositive 169-day prolonged SARS-CoV-2 shedding in an immunocompetent patient and explore potential factors from clinical, immunological and genomic perspectives. We found that continuous viral replication and infectivity could exist in an immunocompetent COVID-19 patient with high neutralizing antibody.

Introduction

Previous identification of immunocompromised cases with 105- and 153-day persistent RNA shedding suggest immunocompromised patients are more likely to have prolonged virus shedding. Here, we described a twice repositive immunocompetent COVID-19 case in Beijing with 169-day viral shedding.

Results

Using data available in the patient’s medical record, the 64-year-old male patient had underlying diseases of coronary atherosclerotic heart disease, hypertension and type 2 diabetes with a history of daily smoking and alcohol consumption for 40 years. He was identified from a family cluster of SARS-CoV-2 infection on Feb 12 (Fig. 1A), in which 4 out of 7 were confirmed as COVID-19 cases whose viral sequences were highly homologous. While his 3 family members recovered and were discharged by March, the patient who had non-severe pneumonia (i.e., moderate severity) was quarantined at the hospital due to persistent positivity until August with the exception of two 2-week periods when he tested negative.

After identification as a COVID-19 case, he was immediately admitted to the hospital for quarantine. On February 25, he was discharged following two consecutive negative PCR tests (> 24hr apart) but re-admitted due to a repositive result from routine follow-up testing on March 13th. Because he was still positive on day-105, he was transferred May 16 to Ditan Hospital which has more experience treating COVID-19 cases. He was discharged July 14 after two consecutive negative tests but was readmitted when found repositive again July 28. He was discharged permanently the next week (August 5; Fig. 1A). He was treated with antivirals, interferon, and traditional Chinese medicine during the whole course of illness.

The patient reported chills, fever (38.6°C), sore throat and loss of appetite from February 1 to first admission on Feb 12. Clinical examination revealed decreased WBC and lymphocytes (Table S1). CT images showed patchy ground-glass opacity in the upper lobe and the lower lobe under the pleura of both lungs and the middle lobe of the right lung (Figure S1). Throughout his hospitalization, no obvious abnormality or changes were observed in his liver or kidney functions or in routine blood examinations (Table S2). All CD4 absolute counts were above 350/µl, CD4/CD8 was above 1, and he was HIV negative,
indicating he was immunocompetent, although CD8 cell count and Natural Killer (NK) cells were relatively low at some timepoints (Table S2).

The patient’s neutralizing antibody was 1:2048 13 days from disease onset, peaked at 1:8192 on day 24 and declined in the follow-up period but remained at 1:384 for over 9 months (day 299) (Fig. 1B). The highest viral loads as assessed by cycle threshold (Ct) in both sputum (Ct 17.4) and nasopharyngeal specimens (Ct 23.4) occurred 4 to 5 months after disease onset (Tables S5-S6, Fig. 1C). Positive sgRNA was detected in three sputum samples collected on days 107, 112 and 131, with Ct values of 32.25, 38.15 and 38.30, respectively (Fig. 1C).

Phylogenetic analysis showed all viruses belonged to lineage B 5 and shared a nucleic identity of 99.95–99.98% with reference strain (NC 045512), excluding possibility of reinfection (Fig. 2A). Five mutations were observed when the case was diagnosed, and this cluster shared an identical sequence among his two family members (the viral load of the other family members was too low to be sequenced). However, mutations accumulated across the SARS-CoV-2 genome (GGG28881-28883AAC, etc.) and reached to 14 sites on day 151. The detected mutations turned the lineage from B to B.1.1 (Fig. 2B and C).

**Discussion**

To our knowledge, we reported the longest period of viral shedding (169 days) and intrahost variants (151 days). The intrahost mutation rate was comparable to that of other interhost variants 6,7. However, unlike previous studies which were of shorter infection periods, the mutations identified in this case appeared across the whole genome rather than select hotspots, such as S and ORF8 1,3.

Although previous studies suggest repositive COVID-19 patients have lower or no infectiousness 8,9, our evidence suggests infectiousness may last until 151 days after symptom onset. Although lack of lab facilities prevented virus isolation and culture was not performed, the observed accumulated mutations as well as positive sgRNA 3–4 months after infection suggests on-going viral replication in the course of infection and therefore potential for transmission 1. More attention to the infectiousness of repositive patients is therefore warranted.

The patient did not have severe clinical symptoms which documents that prolonged viral shedding can occur in moderately ill cases 10,11. Whether the administration of traditional Chinese medicine or interferon for COVID-19 treatment influences the occurrence of repositivity is unknown. Because such frequent nucleic acid testing is only done in people with COVID-like symptoms, we may be underdetecting the frequency of these long-term virus shedding cases 4.

To our knowledge, this is the first case of long-term shedding in an immunocompetent case, which previously has only been reported in immunocompromised cases 1,2. We are aware of only this one case so the evidence might have limited generalizability, but occurred despite his neutralizing antibody titer being much higher than that of other patients evaluated at our facility 3, suggesting immune responses
other than the humoral immune response may have an important role in viral clearance. This may explain some vaccine failures if vaccine-induced antibody alone is insufficient to eliminate viral infections in some people. For persistent viral infections, perhaps cellular immune responses and innate immune function play a vital role in eventual clearance. The low CD8 T-cell and NK cell counts may have prolonged the time to eradication or may have been the result of an exhausted immune response, but remains unclear. The dynamic interaction of killer T-cells and SARS-CoV-2 infection as well as the individual’s immunological function need to be evaluated to understand the risk factors for prolonged infectious SARS-CoV-2 shedding. This may also include evaluating the role of the genetic background of the patient or virus-host interactions (potential viral reverse-transcription and integration) in contributing to prolonged viral shedding. 

**Methods**

Before enrollment, a written informed consent was obtained from this patient.

**Clinical presentations**

All available data on white blood cell (WBC), lymphocyte, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL) from liver function tests; albumin (ALB), blood urea nitrogen (BUN) and creatinine level (Cr) from kidney function tests, and computerized tomography (CT) images were collected from the past clinical charts and tests results.

**Laboratory examinations**

**Neutralizing Antibody**

A total of 10 blood samples were collected. Antibodies were determined with a modified cytopathogenic neutralization assay (NA) based on live SARS-CoV-2. The dynamics of neutralizing antibody levels at different time points were analyzed. We conducted neutralization assay (NA) to evaluate antibody level according to the Reed-Muench method on day 5. The presence of neutralizing antibody was determined by a modified cytopathogenic assay. Serum samples were inactivated at 56°C for 30 minutes and serially diluted with cell culture medium in 2-fold steps. The diluted serums were mixed with a virus suspension of 100 median tissue culture infective dose in 96-well plates at a ratio of 1:1, followed by 2 hours’ incubation at 36.5°C in a 5% carbon dioxide (CO2) incubator; 1–2 × 104 Vero cells were then added to the serum-virus mixture, and the plates were incubated for 5 days at 36.5°C in a 5% CO2 incubator. Cytopathic effect of each well was recorded under microscopes, and the neutralizing titer was calculated by the dilution number of 50% protective condition. A titer of ≥ 1:4 indicated seropositivity.

**Viral Load**
Viral loads were obtained from 33 nasopharyngeal swabs and 20 sputum samples collected at different time points during the patient's hospitalization. All available Cycle threshold (Ct) values for determining viral load were abstracted from 7500 software v2.3 from two hospitals.

In addition, subgenomic RNA (sgRNA) was monitored to evaluate the transcription of SARS-CoV-2. 15 nasopharyngeal swabs and four sputum samples collected from day 100 to day 157 were analyzed.

Phylogenetic analysis

Longitudinal nasopharyngeal swabs and sputum samples were collected after diagnosis, and then sequenced by Next Generation Sequencing (NGS). A total of 14 viral genome sequences were obtained from serial samples, including nine genomes from sputum samples and three genomes from nasopharyngeal swabs collected from the study case, and two from nasopharyngeal swabs collected from his two family members.

Viral genomic RNA and subgenomic RNA detection

A serial of nasopharyngeal swabs and/or sputum samples were collected after diagnosis. The viral RNA was extracted by automated nucleic acid purification (KingFisher Flex Purification System, Thermo, Waltham, MA, USA) and diluted in 90 µL RNase-free H2O. The viral genomic RNA (gRNA) was amplified by real-time RT-PCR assay using 5 µL input RNA and commercial kits (Bojie, Shanghai, China). The subgenomic RNA (sgRNA) was detected by real-time RT-PCR assay using 5 µL of RNA, TaqMan Fast Virus 1-Step Master Mix (Thermo), and sgRNA specific primer/probe sets. The results were determined according to the manufacturer's instruction. Negative and positive controls were applied to ensure the quality of the tests.

Next generation sequencing

Viral RNA was extracted from patient nasopharyngeal swabs and sputum samples using automated nucleic acid purification (KingFisher Flex Purification System). First strand cDNA synthesis was performed with the SuperScript IV First Strand Synthesis System (Invitrogen, MA, USA), using 8 µL input RNA and random hexamers. Then tiled-PCR amplicons were generated by 25–32 PCR cycles using ARTIC nCoV-2019 sequencing protocol v3. The primers for Pool 1 and 2 were synthesized by Sangon (Shanghai, China). NGS libraries were prepared by Nextera XT Library Prep Kit (Illumina, San Diego, CA, USA) and sequenced on MiniSeq using 2 x 150 paired-end sequencing kits (Illumina). Negative control samples were processed and sequenced in parallel for each sequencing run as contamination control.

SARS-CoV-2 genome analysis

Quality control and adaptor trimming was done CLC Genomics Workbench (v10.0, Qiagen, Germany). The clean reads were mapped to the reference SARS-CoV-2 genome (GenBank: MN908947.3) using Bowtie2 and SAMtools. Variant calling was performed using Genome Analysis Toolkit (GATK, version 4.0.10). Single nucleotide polymorphic variants were filtered for quality (QUAL) > 200 and quality by depth (QD) >
20 and indels were filtered for QUAL > 500 and QD > 20 using the filter tool in bcftools, v1.9. Phylogenetic analysis was conducted using MAFFT v7. A maximum likelihood tree was inferred by NJ model with bootstrap of 1000, including the patient SARS-CoV-2 genomes, the reference genome sequence (GenBank: MN908947.3) and 494 representative genomes randomly selected from NCBI virus dataset by regions of interest. The final figure was made using iTOI (https://itol.embl.de/). The viral genomes reported in this study have been deposited in the GISAID (https://www.gisaid.org).

**Data Availability**

The data that support this study are available on request from the corresponding author [LW and QW] upon reasonable request.

**Declarations**

**Acknowledgement**

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Ethics Declarations

Competing interests

The authors declare no competing interests.

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Figures
Figure 1

The time course of treatments, neutralizing antibody and cycle threshold of the patient. (A) Time course of diagnosis and treatments of the patient. (B) Neutralizing antibody of the patient in Geometric Mean Titer (GMT). A total of 10 blood samples (Day 13, 17, 24, 45, 48, 58, 178, 199, 213 and 299) was collected and tested. The experiment was performed in triplicates concurrently. (C) Cycle threshold (Ct) values of detecting N and ORF1ab genes from SARS-CoV-2 from 33 nasopharyngeal (NP) swabs and 20 sputum
samples of the patient. The test was considered negative when the Ct value was \( \geq 37 \). The days of positive sgRNA assessed in three sputum samples are noted on the timeline by red circles.

**Figure 2**

The genomic features of SARS-CoV-2 in serial samples. (A) Phylogenetic tree of SARS-CoV-2 in serial samples from the study case (red stars) and single specimens from two of his family members (blues and green squares). The tree was constructed by the N-J method with bootstrap values determined by 1000 replicates. (B) The number of mutations in serial samples derived from study case. (C) The distribution of mutations across the full SARS-CoV-2 genome. Missense mutations and synonymous mutations were indicated in orange and green, respectively. Sputum samples and throat swab samples were shown in dark blue and light blue, respectively.

**Supplementary Files**
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- FigureS1.pdf
- Supplements0401.docx