Notes from Field

An Immunocompetent Patient with High Neutralizing Antibody Titers Who Shed COVID-19 Virus for 169 days — China, 2020

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Few reports are available about prolonged shedding of coronavirus disease (COVID-19) virus, also known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), by immunocompetent patients. We report a case of an infected, immunocompetent person with 169 days of COVID-19 virus shedding during which time he had two brief periods when he tested negative for COVID-19 followed by again testing positive. We describe relevant clinical, immunological, and genomic features. We found that continuous and prolonged viral replication and infectivity existed in an immunocompetent COVID-19 patient, despite having high neutralizing antibody titers.

INTRODUCTION

Previous case reports have described immunocompromised patients shedding COVID-19 virus RNA for 105 days and 153 days, and to our knowledge, the longest reported duration of COVID-19 virus shedding in an immunocompetent COVID-19 patient was 61 days (*1*–*3*). In this report, we describe an immunocompetent COVID-19 case in Beijing with 169 days of viral shedding.

CASE REPORT

A 64-year-old man with coronary atherosclerotic heart disease, hypertension, and type-2 diabetes, with a 40-year history of smoking and alcohol consumption, was confirmed as a COVID-19 case on February 12, 2020. He was identified in a family cluster of COVID-19 virus infection (Figure 1A), in which 4 of 7 family members were confirmed to be COVID-19 cases and whose COVID-19 virus viral sequences were highly homologous. Overall, 3 family members recovered and were discharged by March, but the subject of this case report, who had moderately severe COVID-19, was isolated at the hospital due to persistent COVID-19 virus positivity until August of 2020, with an exception of two 2-week periods when he tested negative (*4*) (Figure 1A, Supplementary Table S1 available in http://weekly.chinacdc.cn/).

The patient had chills, fever (38.6 °C), sore throat, and loss of appetite from February 1 to his admission on February 12. Clinical examination revealed decreased white blood cell (WBC) and lymphocyte counts (Supplementary Table S2 available in http://weekly.chinacdc.cn/). Chest computed tomography (CT) showed patchy ground-glass opacities in the upper and the lower lobes under the pleura of both lungs and in the middle lobe of the right lung (Supplementary Figure S2 available in http://weekly.chinacdc.cn/). Throughout his hospitalization, no abnormalities were observed in his liver or kidney function or in routine blood examinations (Supplementary Table S3 available in http://weekly.chinacdc.cn/). Supportive evidence that he was immunocompetent was that all absolute cluster of differentiation 4 (CD4) counts were above 350/μL, the CD4/cluster of differentiation 8 (CD8) ratio was above 1, and he was HIV negative; however his CD8 cell counts and natural killer (NK) cell counts were low at times (Supplementary Table S3).

The patient's neutralizing antibody titer was 1∶2,048 measured 13 days after illness onset; the titer peaked at 1∶8,192 on day 24 and subsequently declined, staying at 1∶384 for over 9 months (Figure 1B; Supplementary Table S4 available in http://weekly.chinacdc.cn/). The highest viral loads, as assessed by PCR cycle thresholds (Ct) in sputum (Ct=17.4) and nasopharyngeal specimens (Ct=23.4), occurred 4 to 5 months (days 114 and 132) after illness onset (Figure 1C, Supplementary Tables S5–S6 available in http://weekly.chinacdc.cn/). Overall, 3 sputum samples (days 107, 112, and 131) were positive for sub-genomic RNA (sgRNA), with respective Ct values of 32.25, 38.15, and 38.30 (Figure 1C). Detailed methods are in Supplementary Materials (available in http://weekly.chinacdc.cn/).

Phylogenetic analyses showed that all viruses

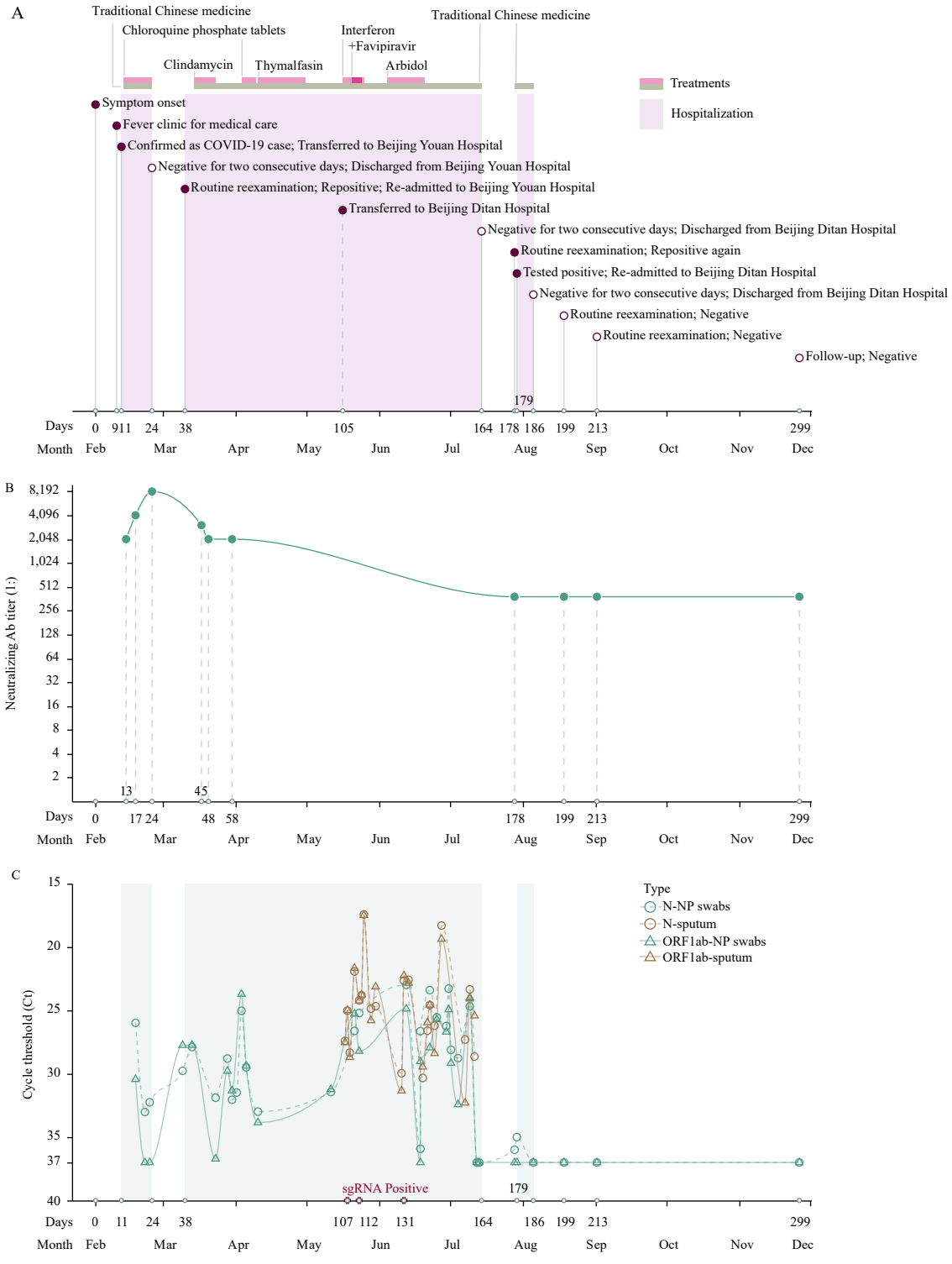

FIGURE 1. The timeline of the course of treatment and conducting assays for neutralizing antibody titers and PCR cycle threshold values of the COVID-19 patient from illness onset to 299 days after illness onset. (A) Time course of diagnosis and treatments of the patient. (B) Geometric mean titer (GMT) of neutralizing antibodies; 10 blood samples (days 13, 17, 24, 45, 48, 58, 178, 199, 213, and 299) were tested in triplicates. (C) Cycle threshold (Ct) values from detecting N and ORF1ab genes of COVID-19 virus from 33 nasopharyngeal (NP) swabs and 20 sputum samples.
Note: PCR was considered negative when the Ct value was ≥37. Days with positive sgRNA assessed in three sputum samples are noted with red circles. N denotes N gene; NP denotes for nasopharyngeal samples, sputum denotes for sputum samples. ORF1ab denotes for open-reading-frame 1ab gene.

belonged to lineage B (*5*) and were 99.95% to 99.98% homologous with reference strain NC 045512 — evidence that is incompatible with reinfection (Supplementary Figure S1A available in http://weekly.chinacdc.cn/) (*6*). Five single nucleotide variants (SNVs) were observed when the case report subject was diagnosed, and sequences among two other family members were identical (the viral load of the third family member was too low to be sequenced). Mutations accumulated across the COVID-19 virus genome, increasing to 14 mutation sites on Day 151. In total, 3 amino acid substitutions in the S protein were observed in serial samples, including H655Y, Y200C, and D614G substitutions. The mutations effectively changed the lineage from B to B.1.1 (Supplementary Figure S1B–C).

Previous case reports have described immunocompromised patients shedding COVID-19 virus RNA for 105 days and 153 days; the longest, previously-reported duration of COVID-19 virus shedding in an immunocompetent COVID-19 patient was 61 days (*1*–*3*). To our knowledge, the patient we describe has the longest duration of viral shedding (169 days) with intra-host variants (151 days). The intra-host mutation rate was comparable to that seen with inter-host variants (*7*–*8*). However, unlike previous studies, which describe shorter infection periods, the mutations identified in this case appeared across the entire genome rather than in select hotspots, such as S and ORF8 genes (*1*–*2*).

Although previous studies suggested that COVID-19 patients positive for COVID-19 virus RNA following a period of being negative have little or no infectiousness (*9*–*10*), our evidence suggests infectiousness may last up to 151 days after symptom onset. Although lack of laboratory facilities precluded virus isolation and culture, the observed accumulated mutations and positive sgRNA 3–4 months after infection suggests continuous, on-going viral replication and therefore potential for transmission (*1*). We believe that more attention to the infectiousness of patients testing positive after a period of testing negative is warranted.

The patient did not have severe clinical symptoms, indicating that prolonged viral shedding can occur in moderately ill cases (*11*–*12*). Because frequent nucleic acid testing is normally only done in people with COVID-19-like symptoms, we may be under-detecting occurrence of long-term virus shedding (*4*).

We anticipate additional follow-up of this individual. Although a single case report may have limited generalizability, our observation that he shed virus despite his neutralizing antibody titers being much higher than titers we have seen with other patients at our facility (*13*) suggests that immune responses other than humoral responses may have important roles in virus clearance. Perhaps cellular immune responses and innate immune functions are important for eventual clearance of persistent infections. The low CD8 T-cell and NK cell counts may have prolonged the time required for virus elimination or may indicate an exhausted immune response; however, it is not clear which of these two possibilities is at play. Dynamic interactions of killer T-cells, COVID-19 virus infection, and the individual's immunological function need to be evaluated holistically to understand risk factors for prolonged and infectious COVID-19 virus shedding. It may also be important to evaluate the role of the genetic background of the patient or of virus-host interactions and their contributions to prolonged viral shedding (*14*).

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Ethics: This study has obtained consent from participant and is approved by the Medical Ethical Committee of Beijing YouAn Hospital, Capital Medical University (approval number [2020]036).

Conflicts of Interest: No potential conflicts of interest was reported by the authors.

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SUPPLEMENTARY MATERIALS

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 obtained from clinical charts and tests results.

Laboratory Examinations

Neutralizing Antibody

Ten blood samples were collected. Antibodies were assessed with a modified cytopathogenic neutralization assay (NA) using live COVID-19 virus. 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. Serum samples were inactivated at 56 °C for 30 minutes and serially diluted with cell culture medium in 2-fold steps. Diluted sera 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 of incubation at 36.5 °C in a 5% carbon dioxide (CO_2) incubator; 1–2 × 10^4 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% CO_2 incubator. Cytopathic effects in each well were recorded by microscope; neutralizing titers were calculated by the dilution number yielding a 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 the 2 hospitals (Thermo Fisher Scientific, Beijing, China).

In addition, sub-genomic RNA (sgRNA) was monitored to evaluate transcription of COVID-19 virus. Fifteen 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 9 genomes from sputum samples and 3 genomes from nasopharyngeal swabs collected from the study case, and 2 from nasopharyngeal swabs collected from his 2 family members.

Viral Genomic RNA and Subgenomic RNA Detection

A series of nasopharyngeal swabs and/or sputum samples were collected after diagnosis. Viral RNA was extracted by automated nucleic acid purification (KingFishe Flex Purification System, Thermo, Waltham, MA, USA) and diluted in 90 μL RNase-free H_2O. 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 (https://www.protocols.io/view/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 × 150 paired-end sequencing kits (Illumina). Negative control

samples were processed and sequenced in parallel for each sequencing run as contamination control.

COVID-19 Virus Genome Analysis

Quality control and adaptor trimming was done CLC Genomics Workbench (v10.0, Qiagen, Germany). The clean reads were mapped to the reference COVID-19 virus 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 N-J model with bootstrap of 1000, including the patient COVID-19 virus 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 iTOl (https://itol.embl.de/). The viral genomes reported in this study have been deposited in GISAID (https://www.gisaid.org).

SUPPLEMENTARY TABLE S1. The timeline of the course of patient treatment from illness onset to 299 days after onset.

Date	Days from illness onset	Event
2/1/20	0	Symptom onset
2/10/20	9	Fever clinic for medical care
2/12/20	11	Confirmed as COVID19 case; transfer to Youan Hospital
2/26/20	24	Negative for two consecutive days; discharged from Youan hospital
3/13/20	38	Routine reexamination; positive again; re-admitted to Youan Hospital
5/16/20	105	Transfer to Ditan hospital
7/14/20	164	Negative for two consecutive days; discharged from Ditan hospital
7/28/20	178	Routine reexamination; positive again
7/29/20	179	Last day tested positive; re-admitted to Ditan hospital
8/5/20	186	Negative for two consecutive days; discharged from Ditan hospital
8/18/20	199	Routine reexamination; negative
9/1/20	213	Routine reexamination; negative
11/26/20	299	Follow-up; negative

SUPPLEMENTARY TABLE S2. Clinical laboratory information of the patient from past tests.

Date	Days from illness onset	WBC (10^9/L)	N (10^9/L)	L (10^9/L)	L (%)	NLR	HB (g/L)	ALT (U/L)	AST (U/L)	TBIL (μmol/L)	DBIL (μmol/L)	ALB (g/L)	BUN (mmol/L)	Cr (μmol/L)
2/13	12	1.96*	1.32*	0.36*	18.3*	3.7	121	51*	145*	25.8*	7.3*			
2/18	17	3.3*	1.96*	1.02	30.9	1.9	129	70*	123*	11.5	3.6			
2/25	24	3.68*	2.33	1.08	29.3	2.2	143	19	40	12.8	2.5			
3/12	40	3.92*	2.4	1.05	26.8	2.3	156	24	23	22.7*	4.8			
3/13	41	3.91*	2.62	0.88*	22.5	3.0	135	11	22	16.9	5.3			
3/16	44	4.8	2.77	1.42	29.6	2.0	154	34	22	22.6*	4.6			
3/17	45	3.32*	2.19	0.81*	24.4	2.7	143							
3/20	49		2.19					36	25	20.6*	4.9			
3/30	58	2.24*	1.07*	0.76*	33.9	1.4	136	16	30	17.5	2.1			
5/17	106	3.26*	1.75*	1.14	35	1.5	148	16.1	21.2	31.4*	11.0	49	3.1	49.1*
5/21	109	3.33*	1.95*	0.91*	27.3	2.1*	147	15.6	21.9	21.2*	8.9*	45	3.8	51.8*
5/25	113	3.81*	2.19	1.12	29.4	2.0	154	23.1	28.8	21.7*	8.8*	49	4.1	51.9*
5/27	116												4.6	49.2*
5/30	119												4.5	51.2*
6/4	124	3.76*	2.16	1.27	33.8	1.7	161*	47.1	45.6*	21.3*	7.2*	47	4.5	48.7*
6/16	136	3.25*	2.18	0.84*	25.8	2.6*	146	47.5	49.7*	16.5	7.2*	42	4.5	45.1*
6/28	147							21.7	31.6	25.3*	10.9*	44	4.1	49.3*
7/28	178	3.86*	2.82	0.68*	17.6*	4.2*	152	30.8	30.2	22.1*	8.7*	46	3.7	46.4*
8/3	184	3.53*	2.07	1.05	29.7	2.0	161*						5.3	55.5
Reference*		4–10	2–8	1–5	20–40	1.5–2.0	120–160	9–50	15–40	0–18.8	0–6.8	40–55	3.1–8	57–97

Abbreviations: WBC=white blood cell; N=neutrophil; L=lymphocytes; HB=hemoglobin; ALT=alanine aminotransferase; AST=aspartate aminotransferase; TBIL=total bilirubin; DBIL=direct bilirubin; ALB=albumin; BUN=blood urea nitrogen; Cr=creatinine.
* Laboratory values that are outside of the normal reference values for general population at Ditan Hospital.

SUPPLEMENTARY TABLE S3. Immune cells information in the peripheral blood from the patient.

Phenotype	Results of different date surveillance				Normal reference
Date (days after onset)	5/18 (107)	5/25 (114)	6/19 (139)	7/1 (151)	
CD3+ T cells	698*	801*	1,008*	767*	1,027–2,086 cells/μL
CD8+ absolute counts	297*	277*	227*	294*	320–1,250 cells/μL
CD8+/CD45+	28.1%	25.7%	15.8%	25.7%	15%–34%
CD4+ absolute counts	375*	487*	791	454*	706–1,125 cells/μL
CD4+/CD45+	35.4%	45.2%	54.8%*	39.7	30%–54%
CD4+/CD8+ ratio	1.3	1.8	3.5*	1.6	1–2
CD16+CD56+ absolute counts	113	140	48	NA	90–590 cells/μL
CD16+CD56+/CD45+	11	13	4*	NA	5%–27%
CD19+ absolute counts	111	87*	165	NA	90–660 cells/μL
CD19+/CD45+	11	8	13	NA	6%–25%

Note: For acquired immunodeficiency HIV patients, CD4 >350 cells/μL indicates immunocompetence.
Abbreviation: NA=not applicable.
* Values outside of the reference range in general population.

SUPPLEMENTARY TABLE S4. Neutralizing antibody titer in Geometric Mean Titers (GMT) from 10 blood samples from the patient.

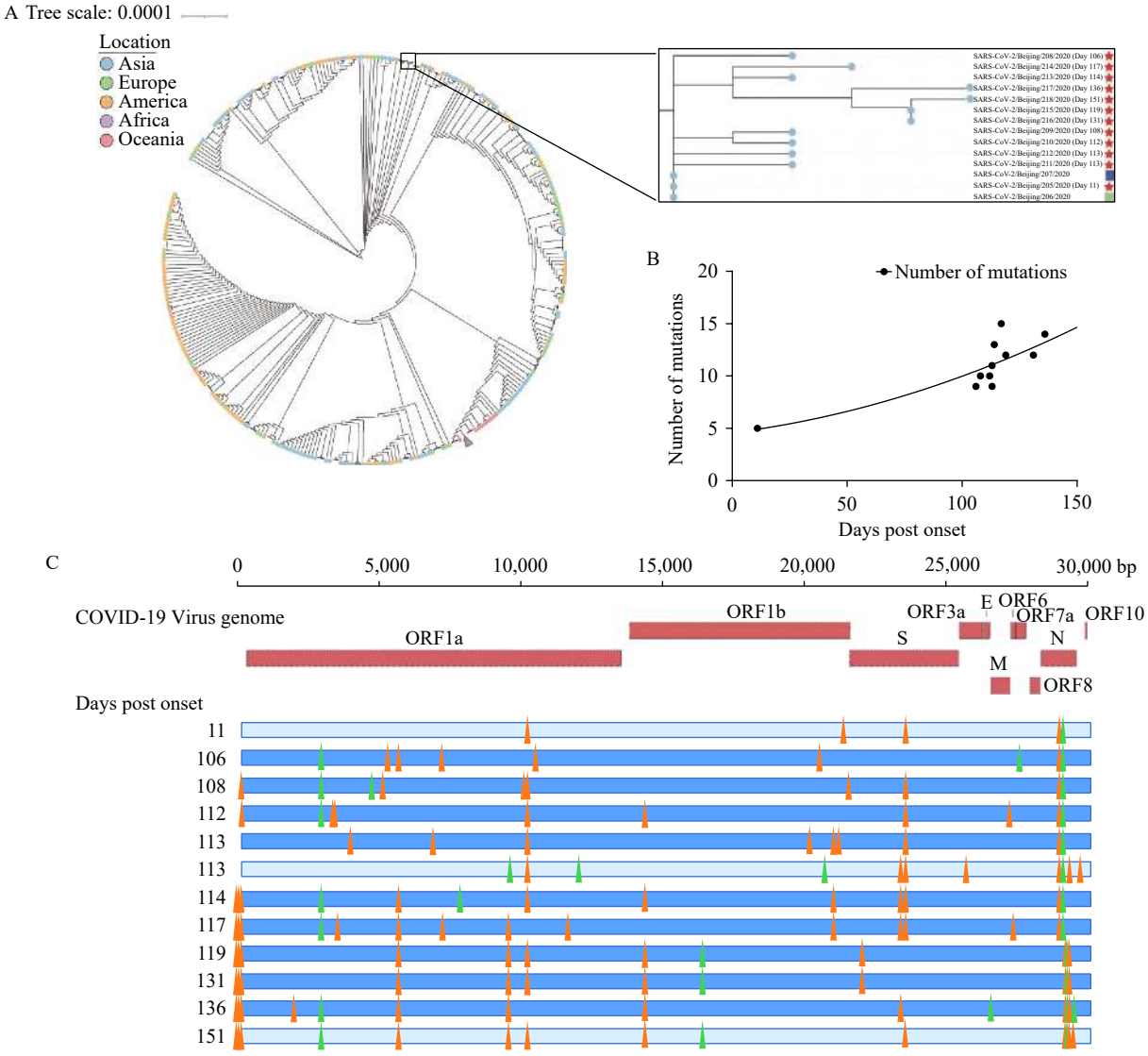
Date	Days from illness onset	Neutralizing Ab titer (1:)
2/14/20	13	2048
2/18/20	17	4,096
2/25/20	24	8,192
3/17/20	45	3,072
3/20/20	48	2,048
3/30/20	58	2,048
7/28/20	178	384
8/18/20	199	384
9/1/20	213	384
11/26/20	299	384

SUPPLEMENTARY TABLE S6. Cycle Threshold (Ct) values from 20 sputum samples of the patient.

Date	Date from illness onset	N gene	ORF 1ab gene
5/17/20	106	27.4	27.53
5/18/20	107	25.02	24.99
5/19/20	108	28.33	28.7
5/21/20	110	21.93	21.66
5/23/20	112	24.21	24.23
5/24/20	113	23.81	23.77
5/25/20	114	17.43	17.49
5/28/20	117	24.86	25.8
5/30/20	119	24.67	23.14
6/10/20	130	29.96	31.34
6/11/20	131	22.65	22.25
6/13/20	133	22.58	22.84
6/19/20	139	30.33	29.45
6/21/20	141	26.6	25.96
6/22/20	142	24.58	24.57
6/24/20	144	26.21	28.39
6/27/20	147	18.3	19.37
7/7/20	157	27.3	32.3
7/9/20	159	23.34	23.99
7/11/20	161	28.64	25.42

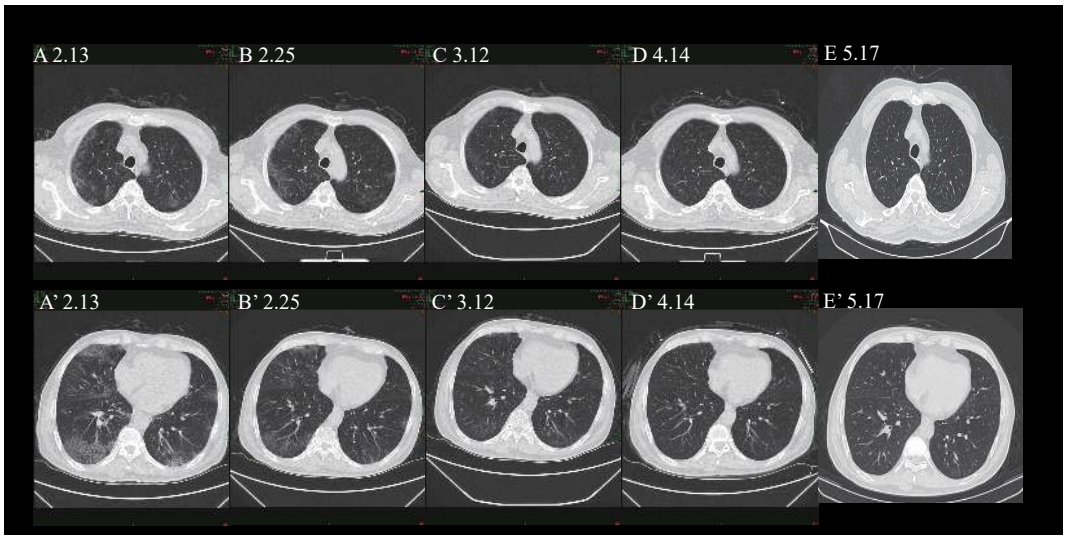
SUPPLEMENTARY TABLE S5. Cycle Threshold (Ct) values from 33 nasopharyngeal swab samples from the patient.

Date	Days from illness onset	N gene	ORF 1ab gene
2/18/20	17	25.98	30.44
2/22/20	21	33.02	>37
2/24/20	23	32.25	>37
3/12/20	37	29.77	27.75
3/13/20	41	27.89	27.74
3/23/20	51	31.89	36.71
3/28/20	56	28.8	29.79
3/30/20	58	32.05	31.33
4/1/20	60	31.48	>37
4/3/20	62	25.03	23.71
4/5/20	64	29.49	29.38
4/10/20	69	32.99	33.85
5/11/20	100	31.44	31.23
5/21/20	110	26.62	25.28
5/23/20	112	25.21	28.21
6/12/20	132	22.99	24.88
6/18/20	138	35.92	>37
6/18/20	138	26.64	29.03
6/22/20	142	23.4	27.94
6/25/20	145	25.54	25.71
6/29/20	149	26.23	26.7
6/30/20	150	23.28	24.94
7/1/20	151	28.11	29.16
7/4/20	154	28.77	32.43
7/9/20	159	24.68	24.08
7/12/20	162	>37	>37
7/13/20	163	>37	>37
7/28/20	178	36	>37
7/29/20	179	35	>37
8/5/20	186	>37	>37
8/18/20	199	>37	>37
9/1/20	213	>37	>37
11/26/20	299	>37	>37



SUPPLEMENTARY FIGURE S1. Genomic features of COVID-19 virus in serial samples of the patient from Day 11 to Day 151 after illness onset. (A) Phylogenetic tree of COVID-19 virus in serial samples from the study case (red stars) and single specimens 2 two of his family members (blues and green squares). The tree was constructed by the N-J method with bootstrap values determined with 1,000 replicates. (B) The number of mutations in serial samples derived from study case. (C) The distribution of mutations across the full COVID-19 virus genome.

Note: Missense mutations and synonymous mutations were indicated in orange and green, respectively. Sputum samples and throat swab samples are shown in dark blue and light blue, respectively.



SUPPLEMENTARY FIGURE S2. Computerized tomography scans change of the patient from February 13, 2020 to May 17, 2020 in 2 hospitals in Beijing. (A) and (A'), CT scans of the patient's upper and lower lung on Feb 13. (B) and (B'), CT scans of upper and lower lung on Feb 25. (C) and (C'), CT scans of upper and lower lungs on March 12, the second hospital admission. (D) and (D') CT scan of upper and lower lungs on April 14. (E) and (E') CT scans of upper and lower lung on May 15.
Note: No signs of pneumonia were observed.