Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Kim M-C, Cui C, Shin K-R, et al. Duration of culturable SARS-CoV-2 in hospitalized patients with Covid-19. N Engl J Med. DOI: 10.1056/NEJMc2027040

Supplementary Appendix

Table of Contents

Detailed Methods

Study patients and clinical characteristics

A consecutive group of all patients with coronavirus disease 2019 (COVID-19) who were hospitalized in Chung-Ang University Hospital, an 850-bed tertiary hospital with National Designated Isolation Unit Wards[1] in Seoul, South Korea between February and June 2020 were enrolled. The diagnosis of COVID-19 was confirmed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay at admission. In South Korea, patients with COVID-19 have been obliged to be isolated in hospitals with negative pressure rooms or in Community Treatment Centers, regardless of the presence or absence of symptoms and severity of the illness.[2,3] In Chung-Ang University Hospital, the patients were isolated until two consecutive negative or inconclusive real-time RT-PCR results were documented at least 24 hours apart.[2,4] The clinical course, including fever (≥ 37.5℃) and respiratory manifestations such as sore throat, cough, sputum, dyspnea, and oxygen requirement, were monitored. The study protocol was approved by the Chung-Ang University Hospital Institutional Review Board (No. 2031-002-407).

Collecting serial respiratory samples and testing the samples via real-time reverse transcriptase-polymerase chain reaction assay

Viral loads of the patients were monitored by real-time RT-PCR assays from the date of admission and throughout their hospitalization periods. The plan was to collect samples at approximately 2-day intervals, although this was not always possible. Nasopharyngeal and oropharyngeal swab samples obtained from the patients were collected in 2 mL viral transport media (VTM) together. We adopted cycle-threshold value for the N gene of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to measure the viral loads in COVID-19

patients.[5] Viral ribonucleic acid (RNA) was extracted using an eMAGTM system (bioMérieux, Inc.), following the manufacturer's instructions.[6] For real-time RT-PCR testing, we used the Allplex™ 2019-nCoV Assay (Seegene, Inc.) which targets the E, RdRP, and N genes of SARS-CoV-2, and the assays were performed using a CFX96 (Bio-Rad Laboratories, Inc.).[6] The results and cycle-threshold values of the real-time RT-PCR tests were determined according to the manufacturer's instructions.[4,6] The remaining VTM were stored at -80°C until virus culture could be performed. To determine the sensitivity of the real-time RT-PCR assay, SARS-CoV-2 of known viral titer was serially diluted in phosphate buffered saline (PBS) to concentrations ranging from 10^6 to 10^{-1} plaque forming unit (PFU)/mL. RNA extraction and real-time RT-PCR testing were performed in the same manner as that used for the VTM samples. Amplification curves for the dilutions were constructed by plotting relative fluorescence units against the number of cycles of the reaction. The limit of detection was defined as the lowest concentration of PFU/mL that showed positive results above a set threshold level of the relative fluorescence unit.

Viable SARS-CoV-2 isolation via plaque assay

We conducted SARS-CoV-2 cultures using serial nasopharyngeal and oropharyngeal samples until at least two consecutive no growth were documented. The VTM samples were diluted in PBS supplemented with 3% bovine serum albumin (BSA) and 1% penicillin/streptomycin as needed. A plaque assay was performed to isolate the virus from the samples. Briefly, Vero cells seeded in 6-well plates at 9×10^5 cells/well about 24 h earlier were inoculated with diluted samples, incubated for 1 h (37℃, 5% CO_2) with rocking every 15 min, and overlaid with 2 mL of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium containing 0.6% oxoid agar. Plaque formation was determined

after 72 h of incubation. For plaque purification, well-separated plaques were extracted using a pipette and dissolved in 1 mL of PBS with 3% BSA and 1% penicillin/streptomycin. The viruses in the purified plaque were propagated by inoculating Vero cells in a similar manner, but incubation was carried out for 72 h in DMEM containing 2% fetal bovine serum and 1% penicillin/streptomycin. The presence of the targeted virus in the harvested supernatant was confirmed with real-time RT-PCR assay. To determine the sensitivity of the plaque assay using Vero cells for detecting the presence of infectious virions, a SARS-CoV-2 stock of 1×10^8 PFU/mL was serially diluted 10-fold to a stock of 1×10^{-3} PFU/mL, and the dilutions were subjected to a plaque assay.

Determination of the duration of SARS-CoV-2 shedding

We compared the time relative to the onset of illness between the viral culture and the real-time RT-PCR assay. The time from symptom onset to viral clearance in viral culture and real-time RT-PCR assay was estimated. We treated the time to viral clearance as interval-censored data and fit a Kaplan–Meier distribution for the data using nonparametric maximum-likelihood estimations, along with 1,000 bootstrap replicates.[7,8] In order to estimate the time to viral clearance in the culture and real-time RT-PCR tests, we identified the shortest and longest possible time to viral clearance in each test in the patients.[9,10] The day of viral clearance in the culture was defined as the first day of two consecutive no growth results, and the day of viral clearance in the real-time RT-PCR assay was defined as the first day of two consecutive negative real-time RT-PCR results. If a positive culture was not documented in a patient, he or she was left censored (censoring when viral clearance in culture had occurred before the time of admission).[9] If real-time RT-PCR clearance had not yet been documented during the hospitalization period, we treated the data as being right censored (censoring when real-time RT-PCR clearance might occur after the time of discharge).[9] We compared the

estimated time from the date of symptom onset to the date of viral clearance between the culture and real-time RT-PCR tests in the 50th percentile of the patients. Data manipulation was conducted using R software (version 4.0.3; R foundation for Statistical Computing).

Detailed Results

Study patients and serial respiratory samples

A total of 21 patients with COVID-19 were consecutively enrolled during the study period. The clinical characteristics, disease severity, treatments, and outcomes of the 21 patients are described in Table S1. A total of 165 samples were tested for real-time RT-PCR assay throughout the hospitalization period. Sampling intervals varied from 1 to 5 days: the median sampling interval was 2 days (interquartile range, 2-3 days). Among them, we performed viral culture in 89 samples. From the 21 patients with COVID-19, viable SARS-CoV-2 was cultured in 29 samples. The timing of real-time RT-PCR and culture tests, whether viable SARS-CoV-2 was isolated or not in the samples, kinetics of the viral loads, and detailed information regarding clinical characteristics over time, including fever and respiratory manifestations, in each patient are specified in Table S2.

Duration of viable virus shedding for SARS-CoV-2

The presence or absence of viable virus according to the time from symptom onset and the cycle-threshold values in the serial respiratory samples are shown in Figure 1. SARS-CoV-2 was cultured so far as 12 days from symptom onset (in Patient 6). Viable virus was grown until 3 days after the resolution of fever (≥ 37.5℃) (in Patient 14). Viral culture was positive only in samples with cycle-threshold values ≤ 28.4 for the N gene (Table S2 and Table S3). We estimated the time to viral clearance in the culture and real-time RT-PCR tests in the 21 patients. The shortest and longest possible time to viral clearance in viral culture and real-time RT-PCR assay in each patient are summarized in Table S4. The nonparametric maximum-likelihood estimates for the distribution function of patients with SARS-CoV-2 shedding in viral culture and real-time RT-PCR assay are depicted in Figure S1. From the

date of symptom onset, the time to viral clearance in culture was 7 days (95% confidence interval, 5-10 days), compared to 34 days (the lower 95% confidence interval was 24 days, but the upper 95% confidence interval was not computable) in real-time RT-PCR assay in 50% of the patients.

Culture positivity rate as a function of the time from symptom onset and cycle-threshold values

We further dissected culture positivity rates according to the time from symptom onset and cycle-threshold values (Table S3). Culture positivity rates constantly decreased as the time from symptom onset increased: the culture positivity rate was 67% within 4 days, and it subsequently decreased to 40% in 5-8 days, 23% in 9-12 days, and 0% after 13 days of symptoms onset. A gradual decline in culture positivity rates was observed with increasing cycle-threshold values: a viable virus was identified in 100% of the cultures when the cycle-threshold value was ≤ 22, 78% when the cycle-threshold value was > 22 and ≤ 24, 67% when the cycle-threshold value was > 24 and ≤ 26, 46% when the cycle-threshold value was > 26 and ≤ 28, 14% when the cycle-threshold value was > 28 and ≤ 30, and 0% when the cycle-threshold value was > 30.

Analytical sensitivity of real-time reverse transcriptase-polymerase chain reaction assay

Amplification curves of the real-time RT-PCR assay for the N gene of SARS-CoV-2 of known viral titers that were subjected to 10-fold serial dilutions from 10^6 to 10^{-1} PFU/mL are shown in Figure S2. Cycle-threshold values corresponding to the serially diluted SARS-CoV-2 titers are described in Table S5. There was excellent linearity between viral loads and cycle-threshold values, with a coefficient (R^2) of 0.9994. The limit of detection was of single (10^0)

PFU/mL.

Analytical sensitivity of plaque assay

Serially diluted SARS-CoV-2 stocks from 10^8 to 10^{-3} PFU/mL were subjected to a plaque assay to assess the assay's sensitivity in detecting the presence of infectious virions (Figure S3). If the plaque assay was reproducible and sensitive, the dilution with a concentration of 10^0 PFU/mL should theoretically form a single plaque. In duplicate experiments, viable virus was detected in the dilutions until the concentration of stock reached 10^0 PFU/mL.

Supplementary Figures and Tables

Figure S1. Estimates of the time to viral clearance. The nonparametric maximum-likelihood estimation of the proportion of patients with SARS-CoV-2 shedding in 21 patients hospitalized with COVID-19 in viral culture (Panel A) and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay (Panel B) are shown. The orange rectangles represent proportions of patients with the corresponding intervals censored for viral clearance. The gray lines represent the 95% confidence interval (CI) of the fitted distributions. The 95% CI of the time to viral clearance (the red point) for the 50th percentile of the patients is indicated by the red horizontal line. From the date of symptom onset, culture clearance occurred by 7 days (95% CI, 5-10 days) and real-time RT-PCR clearance occurred by 34 days (the lower limit of the 95% CI was 24 days, but the upper limit of the 95% CI was not computable) in 50% of the patients.

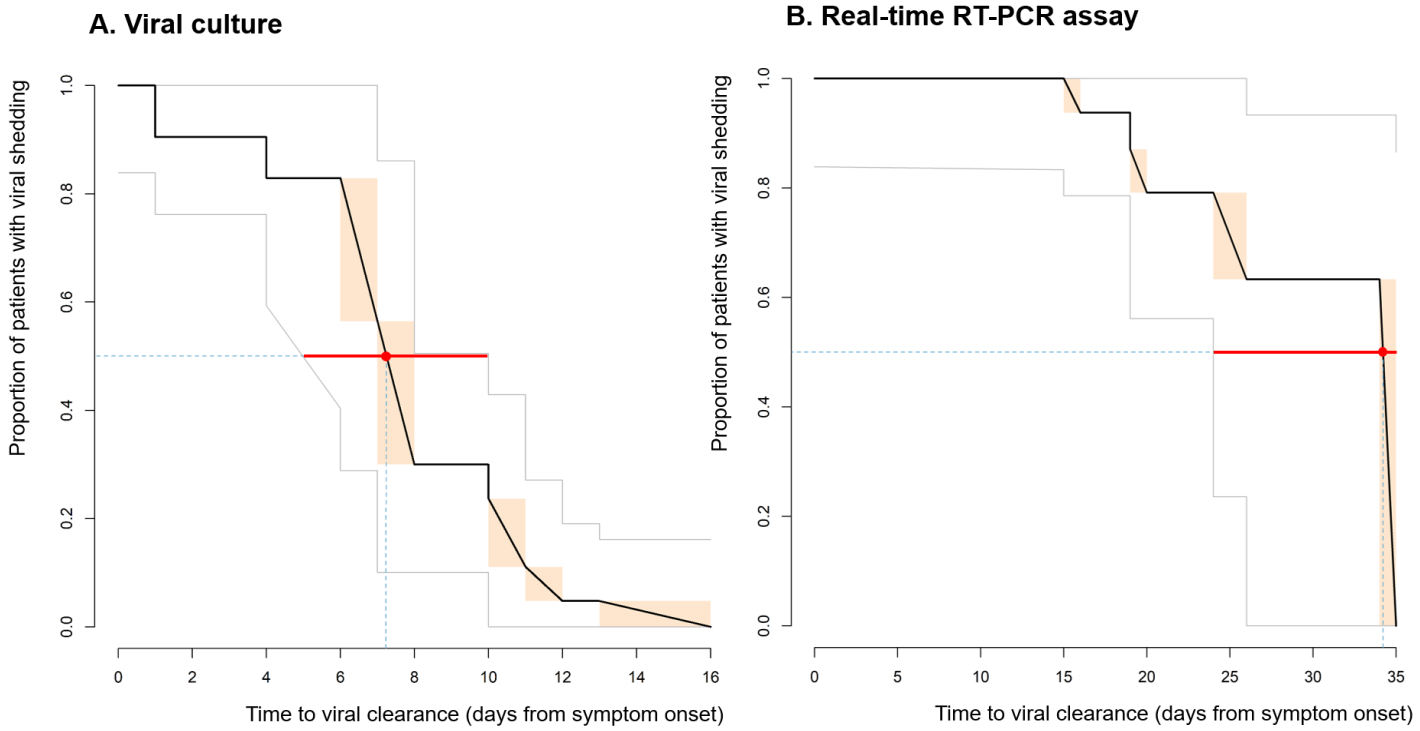

Figure S2. Amplification curves for real-time reverse transcriptase-polymerase chain reaction assay for the N gene of SARS-CoV-2. Amplification curves are generated by plotting relative fluorescence units (RFU) against cycle numbers as the reaction progresses. SARS-CoV-2 RNA diluted serially from 10^6 to 10^{-1} PFU/mL was tested to determine the cycle-threshold value. The cycle-threshold value for each concentration is determined by the cycle at a RFU threshold value (the horizontal line).

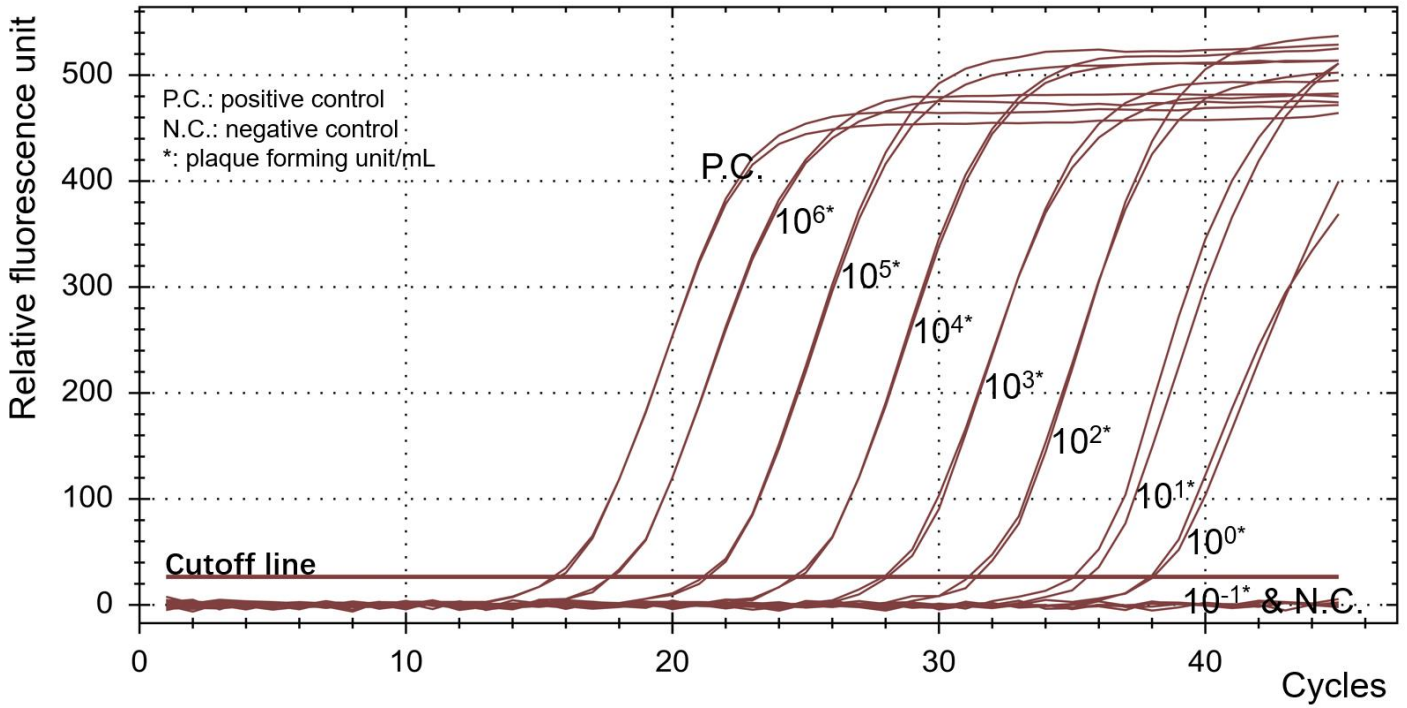

Figure S3. Plaque assays for detecting the presence of viable SARS-CoV-2 in serially diluted stocks. SARS-CoV-2 stock of 1×10^8 PFU/mL was serially diluted 10-fold to 1×10^{-3} PFU/mL. Plaque assay for the dilutions was performed to detect infectious virions. If the plaque assay was reproducible and sensitive, the dilution with a concentration of 10^0 PFU/mL should theoretically form a single plaque. The viable virus was detected in dilutions with a concentration of 10^0 PFU/mL or more.

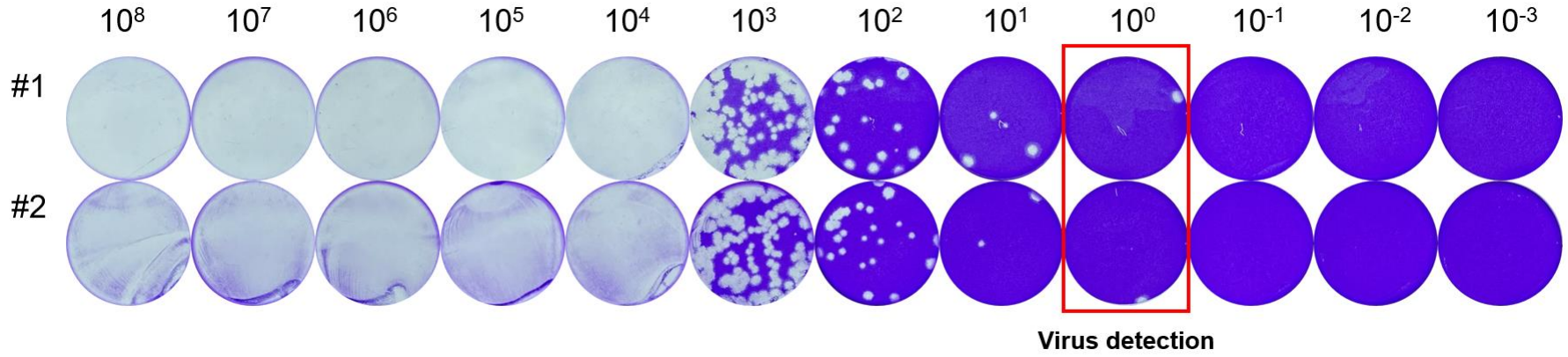

Table S1. Clinical characteristics, disease severity, treatments, and outcomes in 21 patients with COVID-19

Variables	
Age, median years (IQR)	62 (40-69)
Male sex	16 (76)
Underlying comorbidity	
Previously healthy	7 (33)
Hypertension	10 (48)
Diabetes	3 (14)
Chronic lung disease	2 (10)
Immunocompromised condition	1[a] (5)
Clinical manifestation	
Pneumonia[b]	15 (71)
Upper respiratory infection	5 (24)
Asymptomatic	1 (5)
Severity of illness at admission	
NEWS2 score, median (IQR)	1 (0-4)
SOFA score, median (IQR)	0 (0-2)
APACHE II score, median (IQR)	5 (3-8)
Laboratory findings at admission	
White blood cell, median /mm^3 (IQR)	4,400 (3,700-5,500)
Hemoglobin, median g/dL (IQR)	14.7 (13.1-15.6)
Platelet × 10^3, median /mm^3 (IQR)	178 (145-262)
Serum creatinine, median mg/dL (IQR)	0.84 (0.77-0.95)
Total bilirubin, median mg/dL (IQR)	0.6 (0.5-0.6)
C-reactive protein, median mg/dL (IQR)	1.0 (0.2-2.4)
Oxygen therapy	8 (38)

Mechanical ventilation	1 (5)
Antiviral treatment	
Conservative care	7 (33)
Hydroxychloroquine	9 (43)
Duration of hydroxychloroquine use, median days (IQR)	10 (7-10)
Lopinavir/ritonavir	9 (43)
Duration of lopinavir/ritonavir use, median days (IQR)	8 (6-10)
Remdesivir	0
Adjuvant corticosteroid use	0
Convalescent plasma therapy	0
Acute respiratory distress syndrome	1 (5)
Shock	1 (5)
In-hospital mortality	0

NOTE. Data are presented as the number of patients with percentage (%) unless otherwise indicated.

[a] The patient was treated with weekly methotrexate due to rheumatoid arthritis

[b] Pneumonia is defined as the involvement of lung parenchyma on high-resolution computed tomography. All patients underwent high-resolution computed tomography at admission.

Abbreviations: APACHE II = Acute Physiology and Chronic Health Evaluation II; COVID-19 = coronavirus disease 2019; IQR = interquartile range; NEWS2 = National Early Warning Score 2; SOFA = Sequential Organ Failure Assessment

Table S2. Timing of real-time reverse transcriptase-polymerase chain reaction assay and viral culture, presence or absence of viable SARS-CoV-2, kinetics of the viral loads, and clinical course in hospitalized patients with COVID-19

Patient	Age/ sex	Disease	Day 1[a]	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19
1	65/ male	Pneumonia												29.6 Adm				32.3			32.4
2[b]	55/ male	Pneumonia								30.2 Adm					34.7			28.4		39.7	
3	43/ female	Pneumonia				17.2 Adm				28.6			29.7		37.7		37.0			39.3	Dis
4[c]	82/ female	Pneumonia with ARDS									31.7 Adm		32.0			33.5			39.1		
5	35/ male	URI					30.0 Adm				24.2		26.4		31.3			37.7		30.5	
6	91/ male	Pneumonia										22.4 Adm		27.4				29.9	36.6		30.8
7	85/ female	Pneumonia								26.1 Adm				33.1		26.0		33.7			34.5
8	63/ female	Pneumonia						24.6 Adm		28.6			32.1		33.3		28.3			40.0	
9	46/ male	Asymptomatic[d]	36.9 Adm		39.8				38.4	Dis											
10	69/ female	Pneumonia								36.6 Adm		39.7		37.6	Dis						
11	68/ male	Pneumonia								28.2 Adm			30.3			33.2	40.0			35.9	40.0

Patient	Age/ sex	Disease	Day 1[a]	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19
12	30/ male	URI			22.3 Adm	19.6		28.2		31.8		29.2	30.5		36.5			36.1 Dis			
13	37/ male	URI			22.0 Adm	23.6		27.0			27.9	30.2	23.8		36.0			40.0 Dis			
14	24/ male	URI		30.8 Adm		22.3		18.9		17.4	20.9		31.0			34.9	40.0	Dis			
15	62/ male	URI					22.9 Adm		26.0		32.0			32.5		29.1			33.7		38.7
16	70/ male	Pneumonia		19.2 Adm		23.8			25.9		37.9		33.0		37.2			40.0		40.0	Dis
17	62/ male	Pneumonia			20.1 Adm	26.6			22.4		26.9		28.9		35.5			40.0	Dis		
18	32/ male	Pneumonia			23.8 Adm	27.0			24.6		37.2		30.0		36.0			40.0		37.6	Dis
19	66/ male	Pneumonia	35.2 Adm	34.9	34.7		35.2		36.4		35.8			40.0		35.6		40.0			37.9 Dis
20	60/ male	Pneumonia		18.2 Adm	20.5	20.3		25.4		28.4		26.9			36.2		37.9		39.1		
21	59/ male	Pneumonia									25.5 Adm	29.2		31.9		35.5		39.5			40.0

Continued (patient 1-11)

Patient	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28	Day 29	Day 30	Day 31	Day 32	Day 33	Day 34	Day 35	Day 36	Day 37	Day 38
1		34.9		37.3			39.7	Dis											
2	31.9			34.3			40.0				40.0	Dis							
3																			
4		39.5		40.0		39.1			40.0				Dis						
5	40.0			40.0	Dis														
6			35.6		36.6		35.8			38.6		40.0		37.5	Dis				
7		35.0		37.7			Dis												
8		39.4 Dis																	
9																			
10																			
11	40.0	Dis																	

Continued (patient 12-21)

Patient	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26	Day 27	Day 28	Day 29	Day 30	Day 31	Day 32	Day 33	Day 34	Day 35	Day 36	Day 37	Day 38
12																			
13																			
14																			
15		36.3		35.2			36.2		40.0		34.7	40.0		35.7		40.0		40.0	Dis
16																			
17																			
18																			
19																			
20	39.2	Dis																	
21		40.0		38.0	Dis														

NOTE. Data are the cycle-threshold values for the N gene of SARS-CoV-2 in real-time reverse transcriptase-polymerase chain reaction assay. Viral loads for the patients were monitored from the date of admission and throughout the hospitalization periods.

- Pink-colored box indicates positive culture of SARS-CoV-2.
- Blue-colored box indicates negative culture of SARS-CoV-2.
- White-colored box indicates that the viral culture was not conducted.
- Red-colored line indicates the period of fever (≥ 37.5℃).
- Yellow-colored line indicates the period of respiratory manifestations such as sore throat, cough, sputum, dyspnea, and oxygen requirement.

[a] Day 1 indicates the date of symptom onset in patients with SARS-CoV-2 infection.

[b] The patient had a medical history of rheumatoid arthritis and was treated with weekly methotrexate.

[c] This patient received mechanical ventilation from day 10 to day 14.

[d] The date of diagnosis was assigned as day 1 in the asymptomatic patient.

Abbreviations: Adm = admission; ARDS = acute respiratory distress syndrome; COVID-19 = coronavirus disease 2019; Dis = discharge; URI = upper respiratory infection; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

Table S3. Culture positivity rates of viable SARS-CoV-2 according to time from symptom onset and cycle-threshold value

Variables	Number of tested real-time RT-PCR assay	Number of positive viral culture / number of tested viral culture	Culture positivity rates (%)
Time from symptom onset			
≤ 4 days	21	14/21	67
5-8 days	23	8/20	40
9-12 days	34	7/31	23
13-16 days	34	0/13	0
17-20 days	22	0/4	0
> 20 days	31	0/0	NA
Cycle-threshold value for the N gene of SARS-CoV-2			
≤ 22	11	11/11	100
> 22 and ≤ 24	9	7/9	78
> 24 and ≤ 26	6	4/6	67
> 26 and ≤ 28	11	5/11	46
> 28 and ≤ 30	16	2/14	14
> 30 and ≤ 32	14	0/13	0
> 32 and ≤ 34	12	0/7	0
> 34	86	0/18	0
Total	165	29/89	33

Abbreviations: RT-PCR = reverse transcriptase-polymerase chain reaction; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

Table S4. The shortest and longest possible time to viral clearance in viral culture and real-time reverse transcriptase-polymerase chain reaction assay

Patient	C_L	C_R	P_L	P_R
1	1	12	27	NA
2	1	8	24	26
3	5	8	19	NA
4	1	9	26	NA
5	10	11	19	20
6	13	16	34	NA
7	9	12	24	NA
8	7	8	22	NA
9	1	1	8	NA
10	1	8	13	NA
11	1	8	19	19
12	7	8	17	NA
13	7	9	14	NA
14	10	11	15	NA
15	6	7	34	35
16	5	7	14	16
17	5	7	14	NA
18	4	4	19	NA
19	1	1	20	NA
20	11	13	21	NA
21	10	10	24	NA

NOTE. C_L and C_R represent the shortest and longest possible time (days) to viral clearance from symptom onset in viral culture. P_L and P_R represent the shortest and longest possible time (days) to viral clearance from symptom onset in real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The date of viral clearance in viral culture and real-time RT-PCR assay was defined as the first day of two consecutive negative results of each test. When viral clearance in real-time RT-PCR assay was not observed due to discharge, the latest limit is indicated as "NA".

Abbreviations: NA = not available; RT-PCR = reverse transcriptase-polymerase chain reaction

Table S5. Cycle-threshold values corresponding to known viral titers of SARS-CoV-2 with serial dilution in real-time reverse transcriptase-polymerase chain reaction assay

Virus titer (log_{10} PFU/mL)	Cycle-threshold value[a] for the N gene
-1	Not determined
0[b]	38.5
1	35.3
2	31.3
3	28.0
4	24.6
5	21.2
6	17.7

[a] The Allplex™ 2019-nCoV Assay (Seegene, Inc.) was used for the real-time reverse transcriptase-polymerase chain reaction assay.

[b] Limit of detection of the real-time reverse transcriptase-polymerase chain reaction assay was 10^0 PFU/mL.

Abbreviations: PFU = plaque forming unit; SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

References

1. The Facility Guidelines on National Designated Isolation Unit Wards. 17 January 2020. (https://www.cdc.go.kr/board/board.es?mid=a20507020000&bid=0019&act=view&list_no=365783&tag=&nPage=1.)

2. Response guidelines to prevent the spread of COVID-19. 8-1 ed: Korea Disease Control and Prevention Agency; 20 May, 2020. (In Korean) (http://ncov.mohw.go.kr/board/doFileDownload.do?file_name=%E2%98%85%EC%BD%94%EB%A1%9C%EB%82%98%EB%B0%94%EC%9D%B4%EB%9F%AC%EC%8A%A4%EA%B0%90%EC%97%BC%EC%A6%9D-19%20%EB%8C%80%EC%9D%91%20%EC%A7%80%EC%B9%A8%20(%EC%A7%80%EC%9E%90%EC%B2%B4%EC%9A%A9)%E3%80%8D(8-1%ED%8C%90)_1.pdf&file_path=/upload/ncov/file/202006/1590974972535_20200601102937.pdf&seq=4306).

3. Kang E, Lee SY, Jung H, Kim MS, Cho B, Kim YS. Operating Protocols of a Community Treatment Center for Isolation of Patients with Coronavirus Disease, South Korea. Emerg Infect Dis 2020;26.

4. Hong KH, Lee SW, Kim TS, et al. Guidelines for Laboratory Diagnosis of Coronavirus Disease 2019 (COVID-19) in Korea. Ann Lab Med 2020;40:351-60.

5. He X, Lau EHY, Wu P, et al. Temporal dynamics in viral shedding and transmissibility of COVID-19. Nat Med 2020;26:672-5.

6. Allplex 2019-nCoV assay: instructions for use. Seoul, South Korea: Seegene, 2020. (Cat. no. RP10250X/RP10252W.)

7. Reich NG, Lessler J, Cummings DA, Brookmeyer R. Estimating incubation period distributions with coarse data. Stat Med 2009;28:2769-84.

8. Dugue AE, Pulido M, Chabaud S, Belin L, Gal J. How to Deal with Interval-Censored Data Practically while Assessing the Progression-Free Survival: A Step-by-Step Guide Using SAS and R Software. Clin Cancer Res 2016;22:5629-35.

9. Lessler J, Reich NG, Cummings DA, et al. Outbreak of 2009 pandemic influenza A (H1N1) at a New York City school. N Engl J Med 2009;361:2628-36.

10. Assiri A, McGeer A, Perl TM, et al. Hospital outbreak of Middle East respiratory syndrome coronavirus. N Engl J Med 2013;369:407-16.