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Original Article

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ARTICLE INFO

Keywords:

SARS-CoV-2

Severe coronavirus disease

Virus shedding

ABSTRACT

Background: Approximately 5% of patients with coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 develop severe COVID-19. Severe COVID-19 requires respiratory management with mechanical ventilation and an extended period of treatment. Prolonged infectious virus shedding is a concern in severe COVID-19 cases, but few reports have examined the duration of infectious virus shedding. Therefore, we investigated the duration of infectious virus shedding in patients transferred to Hiroshima University Hospital with severe COVID-19 requiring mechanical ventilation.

Methods: Nasopharyngeal swab specimens were collected and analyzed using both viral culture and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) tests between December 2020 and February 2021.

Results: Of the 23 patients tested, the proportions of those with positive test results at first specimen collection (the median number of days to first specimen collection after symptom onset was 10) on RT-qPCR and viral culture tests were 95.7% and 30.4%, respectively. All six patients with positive viral culture test results who were followed-up tested negative 24 days after symptom onset but remained positive on RT-qPCR. Viral loads based on PCR testing did not decrease over time, but those determined via culture tests decreased over time. The longest negative conversion time was observed in a dialysis patient on immunosuppressive drugs.

Conclusions: This study indicated that patients with severe COVID-19 remain culture positive for ≥ 10 days after symptom onset. Additionally, immunosuppressed patients with severe COVID-19 could consider isolation for ≥ 20 days.

1. Introduction

Cases of coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continue to increase worldwide. Approximately 5% of patients with COVID-19 develop severe COVID-19 and require mechanical ventilation [1]. Numerous reports have shown that viral culture test results become negative 10 days after symptom onset [2,3]. It has also been reported that specimens collected more than 8 days after symptom onset were culture-negative,

even when a high viral load was detected on quantitative polymerase chain reaction (PCR) testing [4,5]. In mild cases, from day 10 onwards, isolation is recommended for 24 h after the disappearance of symptoms such as fever. In contrast, severe cases are believed to exhibit prolonged infectious virus shedding, and isolation for about 20 days is recommended by the Centers for Disease Control and Prevention (CDC) and the European Centre for Disease Prevention and Control [6,7]. Nevertheless, an insufficient number of reports have examined the duration of infectious virus shedding in severe cases, and determining when to end

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<https://doi.org/10.1016/j.jiac.2021.09.006>

Received 22 April 2021; Received in revised form 26 August 2021; Accepted 7 September 2021

Available online 10 September 2021

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isolation remains a major concern for medical institutions treating patients with severe COVID-19. A positive virus culture test result has been reported from a specimen collected 20 days after symptom onset [8]. A positive viral culture test result has even been reported on around day 70 after symptom onset in a patient with T-cell deficiency [9].

We assessed the duration of virus shedding in patients with severe COVID-19 by performing viral culture and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) testing of patients with severe COVID-19 who required mechanical ventilation and were admitted to Hiroshima University Hospital for treatment.

2. Methods

2.1. Study participants and procedure

The study included 23 patients with severe COVID-19 who required mechanical ventilation and were admitted to Hiroshima University Hospital for treatment between December 2, 2020 and February 5, 2021. Hiroshima University Hospital accepts hospital transfers of patients with severe COVID-19 who require intubation management in Hiroshima Prefecture and provides treatment in its intensive care unit.

Nasopharyngeal swab specimens were collected with a swab and 1 mL of physiological saline. The first specimen was collected within one to two days after transfer to Hiroshima University Hospital. When the first viral culture test result was positive and circumstances allowed for follow-up, the duration of infectious virus shedding was investigated by collecting specimens every three to four days until negative viral culture conversion was observed. Patient information (date of symptom onset, medical history, medications, body mass index [BMI]) was extracted from the hospital's electronic medical records. Medical history of patients was only included in this study when there was evidence of a link between having a medical history and the occurrence of severe COVID-19 [10].

2.2. Cell and viral culture

SARS-CoV-2 was cultured in Vero cells expressing TMRPSS2 (VeroE6/TMRPSS2 cells, procured from JCRB cell bank, Japan). Cultures were observed for seven days and checked for cytopathic effects (CPEs). Infectivity titer was measured based on 50% tissue culture infectious dose (TCID₅₀), while TCID₅₀/mL was calculated using a method described in a previous report [11]. When no CPEs were observed within seven days, the culture was considered negative.

2.3. RT-qPCR

RT-qPCR tests were performed using a method described in a previous report [11]. SARS-CoV-2 nucleic acids were extracted using a Viral Total Nucleic Acid Purification Kit (Promega Corporation, USA), while RT-qPCR was performed using a One Step PrimeScript™ III RT-qPCR mix (Takara Bio Inc., Japan). N genes were targeted using the forward primer (2.4 μM), 5'-AAA TTT TGG GGA CCA GGA AC-3', reverse primer (3.2 μM), 5'-TGG CAG CTG TGT AGG TCA AC-3', and probe (0.4 μM) 5'-FAM-ATG TCG CGC ATT GGC ATG GA-BHQ-3'. RT-qPCR was run according to the manufacturer's protocol. A known quantity of SARS-CoV-2 RNA was used as a positive control, and copies/mL were calculated from cycle threshold (Ct) values.

2.4. Ethics

This research was approved by the Ethical Committee for Epidemiology of Hiroshima University (approval number: E-2157). The requirement for written informed consent was waived because only the patients' clinical specimens were used in this study, and the patients had the right to opt-out from the use of their surplus patient material and medical data for research.

2.5. Statistical analysis

Fisher's exact test and the Mann Whitney *U* test were used to compare patient characteristics. Statistical significance was set at *p* < 0.05. Statistical analysis was performed using JMP software version 15 (SAS Institute Inc., USA).

3. Results

3.1. Patient characteristics

Thirty-three nasopharyngeal swab specimens were collected from 23 patients. Characteristics of patients included in the study are shown in Table 1. Patients were categorized into culture-positive (seven patients, 30.4%) and culture-negative (16 patients, 69.6%) groups, depending on initial viral culture test results. The median number of days prior to the first specimen collection was 10 days after symptom onset (culture-positive group: nine days, culture-negative group: 10.5 days; *p* = 0.21). The median patient age was 64.0 years (culture-positive group: 67.0 years, culture-negative group: 62.5 years; *p* = 0.66), and 82.6% of the patients were men (culture-positive group: 66.7%, culture-negative group: 87.5%; *p* = 0.35). The median BMI was 28.4 kg/m² (culture-positive group: 26.4 kg/m², culture-negative group: 30.8 kg/m²; *p* = 0.23), the most frequent comorbidity was hypertension (culture-positive group: 57.1%, culture-negative group: 50.0%; *p* = 0.56), and 30.4% of patients had diabetes mellitus (culture-positive group: 14.3%, culture-negative group: 37.5%; *p* = 0.28). Table 2 shows the viral culture results, PCR test results, and treatment according to the time since symptom onset. All specimens were collected before withdrawing mechanical ventilation. The median viral load of the first specimen was 4.0

Table 1
Patient characteristics (N = 23).

Patient No.	Age (years)	Sex	BMI (kg/m ²)	Medical history
Culture-positive group (N = 7)				
P3	61	Male	20.3	Rheumatoid arthritis (receiving prednisolone and abatacept), end-stage renal failure (undergoing dialysis), autoimmune leukopenia
P7	48	Male	28.4	Fatty liver
P8	52	Male	32.4	Diabetes mellitus (HbA1C: 11.2%)
P11	70	Male	26.4	Hypertension
P12	67	Male	25.7	Hypertension
P14	73	Female	24.5	Hypertension
P22	68	Female	27.9	Rheumatoid arthritis (receiving Janus kinase inhibitor and prednisolone), hypertension
Culture-negative group (N = 16)				
P1	53	Male	23.8	Diabetes mellitus (HbA1C: 7.6%), interstitial pneumonitis
P2	42	Male	34.7	Not significant
P4	50	Male	40.7	Diabetes mellitus (HbA1C: 9.8%)
P5	66	Male	31.0	Diabetes mellitus (HbA1C: 8.7%)
P6	72	Male	29.7	Diabetes mellitus (HbA1C: 11.9%), hypertension
P9	68	Male	30.9	Diabetes mellitus (HbA1C: 7.2%), hypertension
P10	65	Male	26.1	Chronic hepatitis C
P13	57	Male	23.5	Hypertension
P15	81	Male	24.6	Hypertension
P16	77	Female	33.2	Diabetes mellitus (HbA1C: 7.4%)
P17	68	Male	31.7	Hypertension
P18	64	Male	22.3	Emphysema, hypertension
P19	59	Male	23.7	Hypertension
P20	54	Male	30.7	Hypertension
P21	29	Male	36.4	Crohn's disease
P23	61	Female	32.6	Rheumatoid arthritis (receiving methotrexate)

BMI, body mass index; HbA1C, glycated hemoglobin; P, patient.

Table 2
Timing of SARS-CoV-2 testing and treatment according to days since symptom onset (N = 23).

Patient No.	Days since symptom onset		Mechanical ventilation	Antiviral drugs	Corticosteroid therapy
	First specimen collection (copies/mL)	Negative culture result (copies/mL)			
Culture-positive group (N = 7)					
P3	12 (2.1×10^9)	24 (4.8×10^6)	11–44	Favipiravir: 3–10	8–18
P7	9 (2.7×10^8)	16 (3.0×10^4)	9–16	Favipiravir: 3–8	4–11; High-dose pulse therapy: 9
P8	10 (1.9×10^5)	N.D.	9–11	None	6–11
P11	9 (4.1×10^7)	13 (4.5×10^7)	7–34	None	4–13
P12	10 (5.3×10^6)	13 (1.8×10^6)	10–14	None	9–15
P14	8 (9.7×10^8)	15 (8.0×10^4)	6–17	Favipiravir: 4–5	4–17; High-dose pulse therapy: 4–6, 12–14
P22	4 (2.7×10^7)	11 (4.7×10^5)	4–29	Remdesivir: 3	3–10; High-dose pulse therapy: 3
Culture-negative group (N = 16)					
P1	14 (6.4×10^3)		10–15	Favipiravir: 1–6	3–16; High-dose pulse therapy: 5–7
P2	11 (7.3×10^6)		8–13	None	5–13
P4	11 (7.0×10^4)		11–18	None	8–11
P5	9 (4.6×10^4)		9–13	Favipiravir: 5–7; Remdesivir: 7–8	7–9; High-dose pulse therapy: 9
P6	11 (4.9×10^6)		11–17	Favipiravir: 7–9; Remdesivir: 9–10;	7–10; High-dose pulse therapy: 10
P9	9 (3.7×10^7)		9–12	Favipiravir: 0–3; Remdesivir: 3–7	3–7
P10	8 (2.7×10^5)		9–25	None	2–8; High-dose pulse therapy: 5–7
P13	12 (3.9×10^6)		9–24	None	7–15; High-dose pulse therapy: 7–8
P15	12 (3.2×10^3)		3–15	None	3–12
P16	9 (6.5×10^3)		2–9	None	2–8
P17	10 (2.7×10^6)		5–28	None	9–15
P18	12 (1.6×10^6)		7–25	None	7–13
P19	6 (–)		1–11	None	1–10
P20	10 (1.3×10^3)		4–20	None	4–10
P21	13 (4.8×10^4)		11–20	None	8–13; High-dose pulse therapy: 9–11
P23	8 (1.1×10^5)		8–13	Favipiravir: 3–6; Remdesivir: 6–7	7–13

High-dose pulse therapy: methylprednisolone 1000 mg/day.

The timing of the first specimen collection, the first specimen collection with a negative culture result, mechanical ventilation, antiviral drug administration, and corticosteroid therapy are shown according to the number of days since symptom onset.

Copies/mL were detected using polymerase chain reaction testing.

$\times 10^5$ copies/mL (culture-positive group: 2.7×10^7 copies/mL; culture-negative group: 1.1×10^5 copies/mL; $P = 0.067$). The median duration of corticosteroid therapy until the first specimen collection was 6 days (culture-positive group: 5 days; culture-negative group: 6 days; $p = 0.14$). Nine of the 23 patients (39.1%) received high-dose (methylprednisolone 1000 mg/day) pulse therapy (culture-positive group: 3/7 [42.9%]; culture-negative group: 6/16 [37.5%]; $p > 0.099$). Further, 9 of the 23 patients (39.1%) received antiviral drugs (culture-positive group: 4/7 [57.1%]; culture-negative group: 5/16 [31.3%]; $p = 0.36$). No significant difference between the two groups was found in any characteristics.

3.2. Test results by specimen

At first specimen collection, 95.7% of the 23 patients tested positive via RT-qPCR results, and 30.4% of the patients tested positive via viral culture. Of the 33 specimens tested, the proportion of those with positive viral culture test results was 33.3%. The longest time to produce a positive viral culture test result was 21 days after symptom onset, and the infectivity titer ranged from 1.1×10^2 to 3.6×10^6 TCID₅₀/mL. Of the 33 specimens considered, the proportion of those with a positive RT-qPCR test result was 97.0% and detected viral loads ranged from 1.3×10^3 to 2.1×10^9 copies/mL (Fig. 1). RT-qPCR test results showed no change in viral load over time, but viral culture test results showed a decrease in infectivity titer over time.

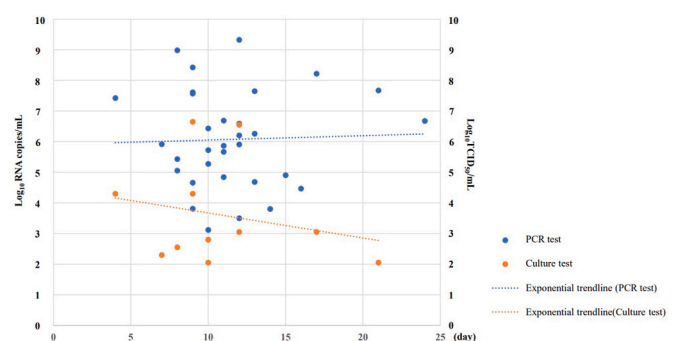


Fig. 1. SARS-CoV-2 viral load results and exponential trendline based on quantitative polymerase chain reaction testing and culture (N = 33). PCR, polymerase chain reaction.

Of the seven patients with an initial positive viral culture test result, specimens were collected and viral culture and RT-qPCR testing were performed in six patients until negative viral culture conversion occurred (Fig. 2). Viral culture testing revealed that the infectivity titer decreased over time and negative viral culture conversion occurred in all six patients (median negative conversion time: 14 days). PCR testing revealed viral loads of at least 10^4 copies/mL, even in specimens with a negative viral culture test result. One of the seven patients, whose

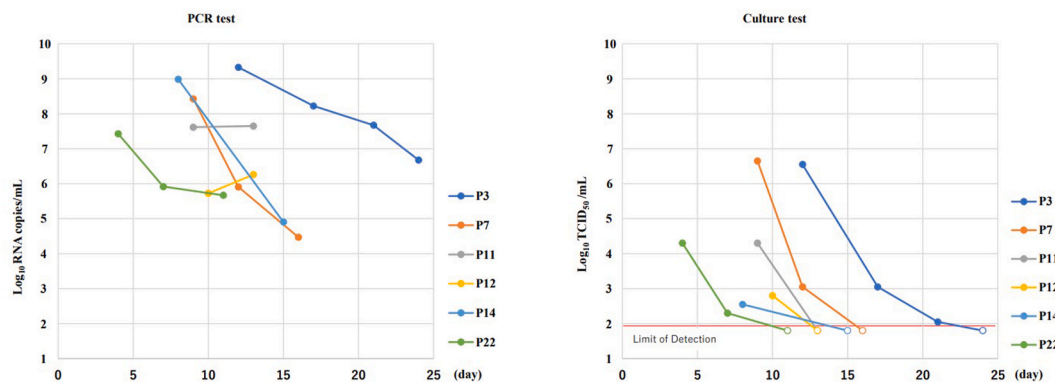


Fig. 2. Time-course of SARS-CoV-2 polymerase chain reaction and culture results in patients with one or more culture-positive results PCR, polymerase chain reaction.

negative viral culture conversion time was 24 days, had been receiving prednisolone and abatacept for rheumatoid arthritis, undergoing dialysis, and had underlying autoimmune leukopenia.

4. Discussion

In patients with severe COVID-19 requiring mechanical ventilation, a large difference in the percentage of positive results between viral culture and PCR testing was revealed. Viral culture testing revealed that viral load decreased over time, while decreases were not observed via PCR testing. Previous reports have also shown that PCR test results remain positive for an extended period, up to 40 days or more in some cases [12,13]. The CDC does not recommend ending isolation based on PCR testing [6]. Although the PCR viral load tended to be higher in culture-positive specimens than in culture-negative specimens, we found many culture-negative specimens with viral loads of $\geq 10^5$ copies/mL. In the culture-positive group, the viral load did not decrease significantly before becoming culture-negative. The number of infectious viruses decreases with time, but inactivated viruses may remain in the body or nasal cavity and can be detected by PCR. Therefore, there may be a difference in the viral load obtained using PCR testing and culture. The results of this study also showed that it is difficult to evaluate whether a patient is infectious based on PCR testing, even if the viral load is known.

Improvement of symptoms is also a criterion for lifting isolation in mild cases. It is difficult to confirm the improvement of symptoms while patients are on mechanical ventilation. In severe cases, long-term mechanical ventilation is often required. In this study, all culture-negative specimens were those taken before withdrawing mechanical ventilation.

In terms of infectivity titers, we confirmed that both the viral load and percentage of positive results decreased over time. One patient required 24 days for negative viral culture conversion to occur. In this patient, factors associated with immunosuppression, including treatment with prednisolone and abatacept, and dialysis for renal failure likely influenced the results. The likelihood that these factors caused severe COVID-19 has already been identified. Previous studies revealed that a high proportion of patients receiving prednisolone for rheumatism developed severe COVID-19 [14], and the mortality rate of COVID-19 is reportedly higher in patients who are on dialysis than in their counterparts [15]. Nevertheless, the possibility that these factors may lead to viral culture test positivity for prolonged periods has not been fully examined. Though this affected only one patient in this study, the finding suggests that these factors may prolong the negative viral culture conversion time.

For all the other patients in this study, there was no difference in age, BMI, or medical history between the culture-positive and culture-negative groups, and negative viral culture conversion occurred between days 6 and 16 after symptom onset. Furthermore, in this study,

approximately one third (30.4%) of all patients with severe COVID-19 were believed to have been shedding the infectious virus 10 days after symptom onset.

This study has a few limitations. This was a single-center study of a group with a small sample size, and only seven patients had positive viral culture test results. Patients were transferred to our hospital when they required mechanical ventilation and the timing of the first specimen collection was different for each patient. A few specimens were collected within seven days after symptom onset, and we could not investigate the percentage of positive viral culture test results obtained prior to initiating therapy. In addition, we did not collect specimens every day; hence, the time to culture negativity might have been overestimated. SARS-CoV-2 infectivity was assessed with a viral culture test that used Vero cells expressing TMPRSS2. Vero cells are well suited for SARS-CoV-2 culture [16], and Vero cells expressing TMPRSS2 are even more susceptible to SARS-CoV-2 infection [17]. However, additional knowledge is required to investigate what factors inhibit viral culture and the detection limits of viral culture.

This study showed that 30% of the critically-ill patients remained culture positive for ≥ 10 days after symptom onset. Further, one immunosuppressed patient required 24 days of testing before negative viral culture conversion occurred, suggesting that a longer period of isolation is required for patients with impaired cellular immunity. Further research investigating patients with COVID-19 who exhibit prolonged infectious virus shedding is required.

Author contribution

This study was designed and supervised by NS, TS, and HO. T. Nomura, HK, KO, and NS collected samples and clinical data, which were analyzed by T. Nomura, MK, and TN. All authors contributed to interpreting the data. The final manuscript was reviewed by all authors.

Funding

The authors received no funding for this study.

Declaration of competing interest

We declare no competing interests.

Acknowledgments

None.

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