

SARS-CoV-2 Virus Culture and Subgenomic RNA for Respiratory Specimens from Patients with Mild Coronavirus Disease

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We investigated 68 respiratory specimens from 35 coronavirus disease patients in Hong Kong, of whom 32 had mild disease. We found that severe acute respiratory syndrome coronavirus 2 and subgenomic RNA were rarely detectable beyond 8 days after onset of illness. However, virus RNA was detectable for many weeks by reverse transcription PCR.

Severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) is causing a global pandemic and affecting global health and the world economy. Virus RNA might be detectable by reverse transcription PCR (RT-PCR) many weeks after clinical recovery (1,2), which affects the duration of isolation of patients. Similar findings were seen with SARS during 2003 (3). A large proportion of transmission occurs before and soon after onset of illness (4). However, the duration of contagiousness after the onset of clinical symptoms remains poorly understood. This duration is relevant to determining policy for discharge of patients from containment in hospitals.

Viral RNA detection by RT-PCR does not prove the presence of infectious virus; culture isolation of virus is a better indication of contagiousness. Recent studies on experimentally infected hamsters showed efficient transmission of SARS-CoV-2 to contact hamsters on day 1 after challenge when virus culture results were positive in nasal washes, but not at day 6

when nasal washes were culture negative, although viral load determined by RT-PCR was still high ($>6.0 \log_{10}$ RNA copies/mL) (5). Thus, virus culture might be a better surrogate for transmissibility.

We attempted virus isolation in 68 specimens from 35 patients in Hong Kong. Specimens were collected at different times after symptom onset to define the kinetics of virus isolation in upper respiratory specimens. Those specimens with a viral load $\geq 5 \log_{10}$ were also examined for detection of subgenomic viral RNA (sgRNA).

The Study

The study was approved by the Research Ethics Committee of the Kowloon West Cluster (reference No. KW/EX-20-039; 144-27) of the Hospital Authority of Hong Kong. We provide methods for virus nucleoprotein (N) gene copy number quantification (6), virus culture, and sgRNA detection of RT-PCR-confirmed coronavirus disease (COVID-19) patients (Appendix, <https://wwwnc.cdc.gov/EID/article/26/11/20-3219-App1.pdf>). Virus sgRNA was tested in specimens that had $\geq 5 \log_{10}$ N gene copies/mL.

A total of 68 specimens from 35 patients were studied (Table 1; Appendix); patients with prolonged virus shedding (10 who remained virus RNA positive for >30 days) and patients readmitted because RT-PCR positivity was detected after discharge ($n = 6$) were oversampled (i.e., selected to make up a larger share of the survey sample than is performed for the patient population). Patient age ranged from 17 to 75 years (median 38 years); 23 were male and 12 female (Table 1). Specimens submitted for virus culture were nasopharyngeal aspirates and throat swab specimens ($n = 46$), nasopharyngeal aspirates ($n = 2$), nasopharyngeal swab specimens and throat swab specimens ($n = 4$), nasopharyngeal swab specimens ($n = 3$), sputum ($n = 11$), and saliva ($n = 2$). The duration after

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Table 1. Comparison of patients and clinical respiratory specimens that were positive or negative by culture for severe acute respiratory syndrome coronavirus 2 and duration of illness for patients with mild coronavirus disease, Hong Kong*

Characteristic	Culture positive, n = 16	Culture negative, n = 52	Total, n = 68	Statistical significance
Patients, n = 35				
Asymptomatic	1	2	3	ND
Median age (range), y	39 (21–73)	38 (17–75)	38	ND
Concurrent condition	5	4	9	ND
Clinical specimens				
Median log ₁₀ viral load/mL†, n = 68	7.5	3.8		p<0.0001 by Mann-Whitney test
Viral load log₁₀, range, n = 68				
7.0–9.5	12 (75)	5 (10)	17 (25)	p = 0.018 by Fisher exact test
6.0–6.99	3 (19)	8 (15)	11 (16)	
5.0–5.99	1 (6)	6 (12)	7 (10)	
<5.0	0	33 (63)	33 (49)	
Days after onset of illness when sample was collected, n = 68				
1–2	8 (53)	7 (13)	15 (22)	p = 0.00001 by Fisher exact test
3–8	8 (35)	15 (29)	23 (34)	
9–67	0	30‡ (58)	30 (44)	
Days after onset of illness when sample was collected from patients without or before antiviral treatment, n = 42				
1–2	8 (50)	7 (27)	15 (36)	p = 0.01 by Fisher exact test
3–8	8 (50)	9 (35)	17 (40)	
9–67	0	10 (38)	10 (24)	

*Values are no. (%) patients unless indicated otherwise. ND, not determined.

†Viral load below limit of detection for the assay was scored as 1 log₁₀/mL.

‡Six of these patients were readmissions because of detection of virus RNA after discharge from isolation.

onset of illness to specimen collection ranged from 1 to 67 days.

Virus was isolated from 16 specimens for 16 patients. The median age of the culture-positive patients was 39 years and of the culture-negative patients was 38 years. SARS-CoV-2 N gene copy number in the specimens overall ranged from 9.5 log₁₀ copies/mL to undetectable (limit of detection 10 copies/mL) (Figure 1). The median viral load in culture-positive samples was 7.5 log₁₀ copies/mL and in culture-negative samples was 3.8 log₁₀ copies/mL (p = 0.00001) (Table 1).

Virus was isolated from 12 of 17 specimens with viral loads ≥7.0 log₁₀ copies/mL, 3 of 11 specimens with viral loads 6.0–6.99 log₁₀ copies/mL, 1 of 7 specimens with viral loads 5.0–5.99 log₁₀ copies/mL, and 0 of 33 specimens viral loads <5 log₁₀ copies/mL.

The sgRNA provides evidence of replicative intermediates of the virus, rather than residual viral RNA. Detection of virus sgRNA was attempted for 33 of the 35 the clinical specimens that had viral loads ≥5.0 log₁₀ virus genome copies/mL; 2 specimens had insufficient specimen for this testing. Of 33 specimens tested for sgRNA and by virus culture, both tests showed positive results for 12 (36.4%) specimens, both tests showed negative results for 12 (36.4%), sgRNA showed positive results and culture was negative for 7 (21.2%) specimens, and culture was positive and sgRNA showed negative results for 2 (6.1%) samples (Cohen κ 0.467, p = 0.005 against κ 0) indicating a moderate agreement between virus culture and sgRNA detection. Virus

sgRNA was detectable in 18 (81.8%) of 22 specimens collected ≤8 days after symptom onset and in 1 (9.1%) of 11 specimens collected ≥9 days after onset of disease (p = 0.0003 by χ² test with Yates correction) (Figure 2). We also provide culture and sgRNA results stratified by specimen type (Table 2).

We conducted a subset analysis for 42 specimens collected from patients who did not receive antiviral

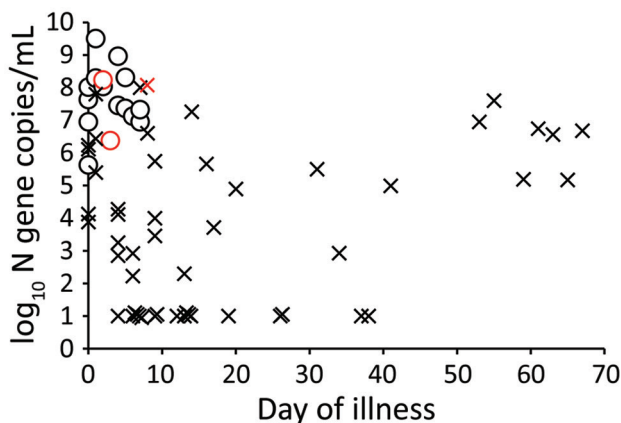


Figure 1. Severe acute respiratory syndrome coronavirus 2 RNA load, virus culture, and days after onset of illness in respiratory specimens and duration of illness for patients with mild coronavirus disease, Hong Kong. ○ indicates samples positive by virus culture and × indicates samples negative by virus culture. Red indicates 2 critically ill patients and 1 patient who died; black indicates mild, moderate, or asymptomatic infections. The limit of detection of the viral N gene RNA was 1 log₁₀ copies/mL; undetectable virus load is indicated as the limit of detection. N, nucleoprotein.

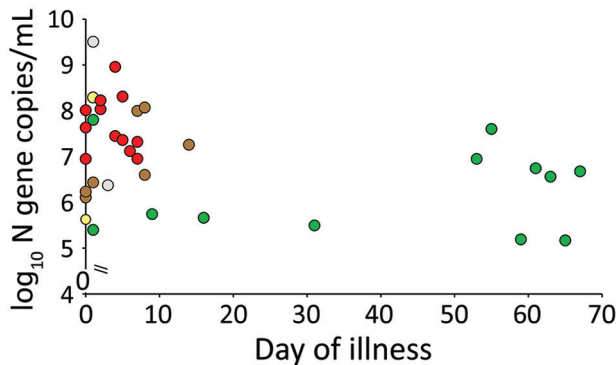


Figure 2. Severe acute respiratory syndrome coronavirus 2 viral RNA load, virus culture, and subgenomic virus RNA (sgRNA) in relation to days after onset of illness for patients with mild coronavirus disease, Hong Kong. Red indicates culture and sgRNA positive, green indicates culture and sgRNA negative, yellow indicates culture positive and sgRNA negative, brown indicates culture negative and sgRNA positive, and gray indicates culture positive and no sgRNA data (because of insufficient specimen).

drugs or specimens that were collected before antiviral therapy (Table 1). This sample included all 16 specimens that were culture positive and 18 of 19 specimens that were sgRNA positive. The main conclusions remained unchanged. Median viral RNA load in culture-positive specimens was 7.54 \log_{10} genome copies/mL and in culture-negative specimens was 4.0 \log_{10} genome copies/mL ($p < 0.00001$ by Mann-Whitney 2-tailed U test). Of the 16 culture positive specimens, 15 (94%) had viral RNA load $\geq 6 \log_{10}$ copies/mL ($p < 0.01$ by Fisher exact test). All of them were collected within the first 8 days of illness ($p = 0.01$ by Fisher exact test) (Table 1). However, the duration of illness in this subset of specimens was limited to 31 days. Five specimens with viral load $\geq 6 \log_{10}$ virus N gene copies/mL collected ≥ 50 days after onset of illness were negative by virus culture and virus sgRNA, but all of these patients had received antiviral therapy.

Conclusions

For a cohort of patients with predominantly mild COVID-19, our findings suggest that virus isolation and sgRNA detection were positive within the first 8 days after onset of illness and mainly for specimens with $\geq 6 \log_{10}$ virus N gene copies/mL of clinical specimen. We did not carry out serologic testing in parallel with viral culture, but data on a larger cohort of patients in Hong Kong showed that most patients had detectable virus neutralizing antibodies after day 9 of illness (7). Two other studies of virus culture for mildly ill or moderately ill patients showed virus culture was only successful within the first 9 days after onset of illness (8,9). Patients who are severely ill and immunocompromised might shed infectious virus for much longer periods (J.J.A. van Kampen, Erasmus University Medical Center, pers. comm., 2020 Jun 9), and this shedding might also be prolonged by corticosteroid therapy.

The World Health Organization has recently amended its guidelines for releasing COVID-19 patients from isolation (i.e., 10 days after symptom onset and ≥ 3 additional days without symptoms), but these guidelines do not distinguish between mild and severely ill patients (10). Our findings suggest that patients with mild or moderate illness might be less contagious 8 days after symptom onset. Mildly ill patients who have clinically recovered and are not immunocompromised might be discharged from containment ≥ 9 days after symptom onset, as long as they are not being discharged into settings that contain other highly vulnerable persons (e.g., old age care homes).

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Table 2. Culture and sgRNA results stratified by specimen type for patients with mild coronavirus disease, Hong Kong*

Specimen	Investigation	Days after onset of illness, no. positive/no. tested				
		1–2	3–8	9–14	15–21	>21
NPA + TS	Culture	8/14	2/14	0/8	0/2	0/8
	sgRNA	8/11	4/4	0/1	0/0	0/4
NPA	Culture		2/2			
	sgRNA		1/1			
NPS + TS	Culture		0/2	0/2		
	sgRNA					
NPS	Culture	1/1	1/1			0/1
	sgRNA	1/1	1/1			
Sputum	Culture		0/1	0/2	0/8	
	sgRNA		1/1	1/1	0/5	
Saliva	Culture		2/2			
	sgRNA		2/2			

*Blank cells indicate that no specimens were collected at that time point. NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; sgRNA, subgenomic viral RNA; TS, throat swab.

About the Author

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SARS-CoV-2 Virus Culture and Subgenomic RNA for Respiratory Specimens from Patients with Mild Coronavirus Disease

Appendix

Methods

Patients

Patients were given a diagnosis of coronavirus disease by reverse transcription PCR (RT-PCR) at individual hospitals. Follow-up specimens were sent to the School of Public Health, The University of Hong Kong, for virus culture and determination of viral RNA load. Specimens were transported and stored at 4°C until they were processed.

Nine patients had underlying concurrent conditions. None of the patients were immunocompromised. Three patients were asymptomatic; 29 patients had mild clinical illness (mild influenza-like illness symptoms not requiring supplemental oxygen, or requiring <3 L/min); 2 patients were in critical condition (intubated, required extracorporeal membrane oxygenation, or in shock); and 1 patient died.

Quantification of Virus Nucleoprotein Gene Copy Number

RNA was extracted from specimens by using the QIAamp Viral RNA Extraction Kit (QIAGEN, <https://www.qiagen.com>) according to the instructions of the manufacturer and tested by using an RT-quantitative PCR targeting the nucleoprotein (N) gene of severe acute respiratory syndrome coronavirus 2 (*SARS-CoV-2*). Absolute copy number was quantitated. A cloned plasmid DNA extract carrying a DNA insert of the N gene was prepared and used for determination of DNA copy number. The copy number control plasmid DNA with serial dilutions were included in each RT-quantitative PCR to construct a standard curve to correlate cycle threshold values and gene copy number of samples. Virus gene copy number per milliliter was calculated by adjusting for the dilution effect of nucleic acid extraction.

Virus Culture

Vero E6 cells (ATCC-ORL-1586) were seeded at a cell count of 150,000 cells/well into 24-well tissue culture plates (TPP Techno Plastic Products, <https://www.tpp.ch>) sufficient to give a subconfluent cell monolayer after incubation for 24 hours in a CO₂ incubator. The culture medium was removed and 125 µL of the clinical specimen in virus transport medium diluted 1:1 in Dulbecco modified Eagle medium (GIBCO, <https://www.thermofisher.com>) containing 2% fetal calf serum (GIBCO) was inoculated in into 2 wells. After 2 hours incubation in a CO₂ incubator at 37°C, 1 mL of Dulbecco modified Eagle medium containing 2% fetal calf serum was added the wells. The plates were incubated at 37°C in a CO₂ incubator. A sample (100 µL) of supernatant was sampled for a quantitative real-time RT-PCR at 0 and 72 hours postinoculation. At 72 hours, cells were scraped into the supernatant and transferred onto fresh cells in 24-well plates, followed by refeeding the cells with fresh culture medium and monitoring for an additional 72 hours. A final aliquot was collected for quantitative real-time RT-PCR. Cells were observed for cytopathic effect daily and harvested for passage if 25%–50% of cells showed a cytopathic effect.

Detection of Subgenomic mRNA by RT-PCR

RNA was extracted from samples by using the QIAamp Viral RNA Mini Kit (QIAGEN). The method was modified from published methods to detect coronavirus subgenomic mRNA (2). Purified RNA was reverse transcribed by using SuperScript II (ThermoFisher Scientific, <https://www.thermofisher.com>) and a severe acute respiratory syndrome coronavirus 2–specific primer (WHS-29950R: 5'-TCTCCTAAGAAGCTATTTAAAT-3'). The complementary DNA obtained was subjected to PCR (40 cycles for 94°C for 30 s, 56°C for 30 s, and 72°C for 1.5 min. a condition optimized for amplifying small subgenomic mRNA) and AmpliTaq Gold DNA Polymerase (ThermoFisher Scientific) with primers (WHS-00025F: 5'-CCAACCAACTTTTCGATCTCTTGTA-3' and WHS-29925R: 5'-ATGGGGATAGCACTACTAAAATTA-3'). The PCR products were then subjected to 1% agarose gel electrophoresis.

Results

Patient concurrent conditions included diabetes mellitus, hypertension, ischemic heart disease, atrial fibrillation, chronic obstructive airways disease, carcinoma of the lung, mild

renal dysfunction, and chronic hepatitis B. For 3 asymptomatic patients, days after onset was estimated as days after first detection by RT-PCR. A total of 14 patients were sampled sequentially: 2–6 samples were collected from each patient at 3–4 day intervals. A total of 12 patients received no antiviral therapy, and 23 received lopinavir/ritonavir alone (n = 1) or in combination with ribavirin (n = 9), ribavirin and interferon β (n = 10), interferon β (n = 2), and ribavirin, β interferon, and tocilizumab (n = 1). Doses and regimens have been described (3). Duration of treatment was ≤ 14 days. Thus, antiviral drugs were not in use beyond day 30 of illness.

Of the 11 lower respiratory tract samples (sputa) collected, none of them yielded virus isolates, although 6 of them had viral N gene copies $>6.0 \log_{10}/\text{mL}$. Two them were collected ≤ 8 days of illness onset.

Corticosteroid therapy was used for 5 patients overall; 3 had critical conditions or died. Virus was isolated from 4 of these patients, and sgRNA was detected in 3 of them. For all patients, specimens were collected before commencement of corticosteroid therapy.

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