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Original article

Viable SARS-CoV-2 in various specimens from COVID-19 patients

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ARTICLE INFO

Article history:

Received 21 April 2020

Received in revised form

13 July 2020

Accepted 14 July 2020

Available online 23 July 2020

Editor: M. Cevik

Keywords:

Saliva

SARS-CoV-2

Shedding

Stool

Urine

ABSTRACT

Objectives: The aim was to determine whether various clinical specimens obtained from COVID-19 patients contain the infectious virus.

Methods: To demonstrate whether various clinical specimens contain the viable virus, we collected naso/oropharyngeal swabs and saliva, urine and stool samples from five COVID-19 patients and performed a quantitative polymerase chain reaction (qPCR) to assess viral load. Specimens positive with qPCR were subjected to virus isolation in Vero cells. We also used urine and stool samples to intranasally inoculate ferrets and evaluated the virus titres in nasal washes on 2, 4, 6 and 8 days post infection.

Results: SARS-CoV-2 RNA was detected in all naso/oropharyngeal swabs and saliva, urine and stool samples collected between days 8 and 30 of the clinical course. Notably, viral loads in urine, saliva and stool samples were almost equal to or higher than those in naso/oropharyngeal swabs (urine $1.08 \pm 0.16 - 2.09 \pm 0.85 \log_{10}$ copies/mL, saliva $1.07 \pm 0.34 - 1.65 \pm 0.46 \log_{10}$ copies/mL, stool $1.17 \pm 0.32 \log_{10}$ copies/mL, naso/oropharyngeal swabs $1.18 \pm 0.12 - 1.34 \pm 0.30 \log_{10}$ copies/mL). Further, viable SARS-CoV-2 was isolated from naso/oropharyngeal swabs and saliva of COVID-19 patients, as well as nasal washes of ferrets inoculated with patient urine or stool.

Discussion: Viable SARS-CoV-2 was demonstrated in saliva, urine and stool samples from COVID-19 patients up to days 11–15 of the clinical course. This result suggests that viable SARS-CoV-2 can be secreted in various clinical samples and respiratory specimens. **Hye Won Jeong, Clin Microbiol Infect 2020;26:1520**

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Introduction

In December 2019, a novel coronavirus disease (COVID-19) was identified in Wuhan, Hubei Province of China, and a previously unknown *Betacoronavirus* was subsequently isolated from patients with pneumonia [1]. The infectious agent was identified as a novel coronavirus (2019-nCoV), which was named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) due to its marked similarity to SARS-CoV, that could cause various levels of disease

severity ranging from asymptomatic infection (1%) [2,3] to severe respiratory illness (20%) and fatality in 2–3% of infected patients [4]. In order to effectively control the COVID-19 outbreak, information regarding the period and routes of infectious virus shedding in patients is essential. In general, droplets and close contact with infected respiratory secretions are considered the main transmission routes of the SARS-CoV-2; however, faecal–oral transmission has also been suggested since some COVID-19 patients have gastrointestinal symptoms, and viral RNA has been detected in oral and anal swabs from patients [5–9]. In addition, SARS-CoV-2 has been detected in the saliva of infected patients [10], suggesting saliva may also be a source of human-to-human transmission.

In our previous study, we established a ferret model of SARS-CoV-2 infection [11] and found a relatively high amount of viral RNA in saliva, urine and faecal specimens from SARS-CoV-2 infected animals, suggesting various clinical specimens from infected

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hosts could be transmission sources. The aim of this study is to examine the viral load in various clinical specimens from acute or recovery phase patients and to investigate the viability of the detected virus.

Methods

Patient sample collection

Five laboratory-confirmed COVID-19 patients hospitalized in the Chungbuk National University Hospital from 25 February 2020 to 5 March 2020 were enrolled in this study. We collected naso/oropharyngeal swabs, saliva, urine and faecal specimens from enrolled patients at days 8, 11, 13, 15 and 30 of the clinical course. We also collected serum samples at the same time points for serological tests.

Detection of the viral genome by reverse Transcription-PCR and sequencing

In order to detect SARS-CoV-2 RNA in clinical specimens, we performed a reverse transcription polymerase chain reaction as previously described [11]. Briefly, viral RNA was extracted from clinical specimens of COVID-19 patients using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) and cDNAs were generated by reverse transcription using QuantiTect Reverse Transcription (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Two primers for SARS-CoV-2, S region (forward (5'-3') ATCAAGACTCACTTCTCCACA, reverse (5'-3') TGTTTAAAGCTTGTGCATTTGGTTGACC) were used to detect SARS-CoV-2-specific RNA. Thermal cycling was performed with the following conditions: initial denaturation at 95°C for 3 min and then 40 cycles of 95°C for 15 s, 60°C for 15 s and elongation at 72°C for 30 s, and a final elongation step at 72°C for 5 min. To quantify the viral RNA copy number, quantitative real-time PCR (qRT-PCR) was performed using the SYBR Green kit (iQ™ SYBR Green supermix kit, Bio-Rad, Hercules, CA, USA). The number of viral RNA copies was calculated as log₁₀ copies/mL with identified qRT-PCR Ct values and compared with the number of copies of the standard control [12]. The limit of viral RNA detection of qRT-PCR is 0.3 log₁₀ copies/mL per reaction.

Isolation of infectious virus from clinical specimens

Clinical specimens were used to inoculate African green monkey kidney Vero cells (Vero cell, ATCC, CCL-81) for virus isolation. Vero cells were cultured in Eagle's minimal essential medium (Lonza) with 8% heat-inactivated fetal bovine serum (FBS) (Gibco) and antibiotics. The infection of Vero cells with each specimen was carried out in phosphate-buffered saline containing 50 µg/mL DEAE dextran and 2% FBS as previously described [13]. Cells were monitored daily for 4 days to examine the cytopathic effects. To confirm virus isolation, we performed RT-PCR on supernatants from infected cell cultures using S gene-specific primer sets (forward (5'-3'): AGGGCAAAGTGGAAAGATTGCTGA, reverse (5'-3'): TCTGTG CAGTTAACATCCTGATAAAGAAC). Thermal cycling was performed using the following conditions: initial denaturation at 95°C for 3 min and then 35 cycles of 95°C for 30 s, 56°C for 30 s and elongation at 72°C for 40 s, and final elongation step at 72°C for 5 min. All PCR products regardless of whether ferret or human clinical specimens were sequenced and compared with the SARS-CoV-2 reference genome (GISAID accession ID: EPI_ISL_407193). All sequenced-confirmed specimens were considered positive for SARS-CoV-2 infection and used for further study.

Immunofluorescence antibody (IFA) assay

To evaluate the IgG antibody response against SARS-CoV-2 infection, we performed an IFA test in Vero cells with a clinical SARS-CoV-2 isolate. Briefly, Vero cells on 96-well plates were infected with a CBNU-nCoV01 strain for 48 hr and fixed with 80% acetone. Serially diluted serum samples were incubated with fixed cells for 3 hr at 37°C and 30 min for fluorescein-labelled antibody against human IgG (H + L) (Seracare, MA, USA). The examination for fluorescent staining was performed under a fluorescence microscope.

Infection of ferrets with clinical specimens from COVID-19 patients

Groups of ferrets ($n = 2$) were inoculated through the intranasal route with 500 µL of qPCR-positive clinical specimens (urine and faecal specimens) from COVID-19 patients. Faecal specimens were diluted tenfold in 2 mL of phosphate-buffered saline and then centrifuged at 12 000 rpm for 20 min to remove debris. Nasal washes were collected from anesthetized ferrets at 2-day intervals until 8 days post infection (dpi) to determine the viral load by qRT-PCR. A portion of each nasal wash was used to inoculate Vero cells for virus isolation. Virus isolation was confirmed by RT-PCR and sequencing with an ABI 373 XL DNA sequencer (Perkin-Elmer, Foster City, CA) by Bionix (Seoul, Korea).

Ethics statement

This study was conducted following the study protocol approved by the Institutional Review Board of Chungbuk National University Hospital (IRB no. 2020-03-036). Written informed consent was obtained from all patients or their guardians. All experiments were carried out in a BSL3 facility at Chungbuk National University. (approval number CBNUA-1352-20-02).

Results

Demographics and clinical features of COVID-19 patients

The median age of the five enrolled patients was 63 years and three were male. Their underlying diseases, COVID-19 severity and treatment courses are summarized in Table 1. Briefly, patient 1 had a mild clinical course without pneumonia, and patients 2, 3 and 5 were categorized as severe disease cases based on a previous study (pneumonia needing oxygen supply) [4]. Patient 4 was in a critical condition with respiratory failure and septic shock [4]. At the time of sampling, patients 1, 2, 3 and 5 were on days 8, 13, 11 and 30 of illness, respectively, and their clinical symptoms had resolved completely. However, patient 4 was on day 15 of illness with a ventilator and extracorporeal membrane oxygenation support. Serum IgG antibody responses of all COVID-19 patients are described in Table 2.

Viral genome detection by reverse Transcription-PCR and sequencing

All clinical specimens collected from the five patients were positive for the SARS-CoV-2 spike gene using qPCR (Table 2), even though four of the patients no longer displayed clinical symptoms. These symptom-recovered COVID-19 patients (patients 1, 2, 3 and 5) showed positive qPCR results in their urine specimens with viral RNA titres ranging from 1.08 ± 0.16 to 2.09 ± 0.85 log₁₀ copies/mL. Further, in faecal specimens from patients 2, 4 and 5 ($n = 3$) SARS-CoV-2 RNA was detected at 1.17 ± 0.32 , 2.18 ± 0.11 log₁₀ copies/mL and 2.01 ± 0.28 log₁₀ copies/mL, respectively. Four symptom-

Table 1
Patient characteristics

Patient number	Age	Sex	Underlying diseases	Clinical severity	Treatment
1	51	M	Ischaemic heart disease	Pneumonia (–) mild case	—
2	64	M	Schizophrenia	Pneumonia (+) severe case	LPV/r ¹
3	62	F	Schizophrenia	Pneumonia (+) severe case	LPV/r ¹
4	63	M	None	Pneumonia (+) critical case	LPV/r ¹ , HCQ ² , steroid,
5	63	F	None	Pneumonia (+) severe case	LPV/r ¹

The demographic and clinical characteristics of COVID-19 patients enrolled in this study are summarized. Patients with non-pneumonia or mild pneumonia are classified as mild. Patients with dyspnoea (respiratory frequency ≥ 30 /min, blood oxygen saturation $\leq 93\%$, the partial pressure of arterial oxygen to fraction of inspired oxygen ratio < 300 and/or lung infiltration $> 50\%$ within 24–48 hr) are classified as severe. Patients with respiratory failure or multiple organ dysfunction are classified as critical (5). LPV/r¹, lopinavir 400 mg/ritonavir 100 mg twice daily; HCQ², hydroxychloroquine 400 mg once daily.

Table 2
Clinical courses of patients, qRT-PCR of clinical specimens and virus isolations

Patient number	Patient's disease state on sampling time (illness day)	Clinical sample	Viral quantitation (log ₁₀ copies/mL)	Virus isolation in Vero cell	IgG titre (GMT \pm SD)
1	Recovery stage (illness day 8)	Naso/oropharyngeal swab	1.19 \pm 0.10	—	80 \pm 0
		Urine	1.08 \pm 0.16	ND	
2	Recovery stage (illness day 13)	Naso/oropharyngeal swab	1.18 \pm 0.12	—	320 \pm 0
		Urine	1.51 \pm 0.19	ND	
		Saliva	1.07 \pm 0.34	—	
		Stool	1.17 \pm 0.32	ND	
3	Recovery stage (illness day 11)	Naso/oropharyngeal swab	1.34 \pm 0.30	+	640 \pm 0
		Urine	2.09 \pm 0.85	ND	
		Saliva	1.65 \pm 0.46	+	
4	Acute stage (illness day 15)	Naso/oropharyngeal swab	1.15 \pm 0.03	—	640 \pm 0
		Urine	0.59 \pm 0.35	ND	
		Saliva	1.44 \pm 0.40	+	
		stool	2.18 \pm 0.11	ND	
5	Recovery stage (illness day 30)	Naso/oropharyngeal swab	1.43 \pm 0.42	ND	507.9 \pm 184.7
		Urine	2.02 \pm 0.49	ND	
		Saliva	0.96 \pm 0.31	ND	
		Stool	2.01 \pm 0.28	ND	

Virus Spike RNA gene detection limit and viral titre limit were 0.3 log₁₀ copies/mL, respectively. ND, virus isolation was not determined due to cell toxicity; GMT, geometric mean titre; SD, standard deviation.

recovered patients (patients 1, 2, 3 and 5) displayed viral RNA copy numbers in urine and faecal samples that were almost equal to or higher than that of naso/oropharyngeal swabs (Table 2). The viral load in the stool of the patient in critical condition (patient 4) was higher than that of the naso/oropharyngeal swab (2.18 \pm 0.11 log₁₀ copies/mL in stool vs. 1.15 \pm 0.03 copies/mL in naso/oropharyngeal swab, $p < 0.0001$).

Virus isolation from clinical specimens using cell culture

Virus was isolated from one naso/oropharyngeal swab and two saliva specimens, as demonstrated in Vero cells (Table 2). The rest of the samples, five urine, two saliva, four naso/oropharyngeal and three stool, were negative for virus isolation. In addition, we failed to demonstrate the presence of the viable virus in urine and faecal specimens using Vero cells, although some specimens (two urine and one stool specimen) had viral loads higher than 1.30 log₁₀ copies/mL.

Infection of ferrets with urine and faecal specimens from COVID-19 patients

To confirm the presence of infectious SARS-CoV-2 in urine and faecal specimens, two urine specimens (patient 2, day 13 of illness, viral load 1.51 \pm 0.19 log₁₀ copies/mL, patient 3, day 11 of illness, viral load 2.09 \pm 0.85 log₁₀ copies/mL) and one faecal specimen (patient 4, day 15 of illness, viral load 2.18 \pm 0.11 log₁₀ copies/mL) were selected for ferret infection experiments. All urine and stool specimen-treated ferrets showed moderate increases in body

temperature, rhinorrhoea and decreased activity at 4 dpi which persisted until 6 dpi. SARS-CoV-2 viral RNA was detected in nasal washes from urine specimen-treated ferrets at 2 dpi, although titres were as low as 0.92 \pm 0.38 log₁₀ copies/mL, and peaked at 4 dpi (3.24 \pm 1.01 log₁₀ copies/mL). Further, viral RNA was detected in nasal wash specimens from stool specimen-treated ferrets as early as 2 dpi and persisted until 6 dpi (Table 3). Notably, SARS-CoV-2 was isolated from the nasal washes of the two urine specimen-treated ferrets and one stool specimen-treated ferret (Table 3).

Discussion

In this study, we found that viable SARS-CoV-2 can be shed through various clinical specimens, such as saliva, stool and urine, of COVID-19 patients even at 15 days after onset of clinical symptoms. In addition, we demonstrated the infectivity of saliva, urine and stool specimens from COVID-19 patients using cell culture and an animal model. We detected SARS-CoV-2 RNA in the saliva of four patients and isolated viable virus from two saliva specimens taken at 11 and 15 days of illness. Given the consistent detection and isolation of SARS-CoV-2 RNA from saliva in another study [14], saliva could be an important non-invasive specimen for diagnosis and virus isolation in COVID-19 patients. Viral RNA shedding does not equate to the viable virus, but because of detection of SARS-CoV-2 RNA in stool samples many studies have suggested the possibility of faecal–oral or faecal–respiratory transmission of SARS-CoV-2 [7,8,15–17]. However, despite several attempts [18–20], only one study thus far has demonstrated the presence of the infectious virus in faecal specimens from COVID-19 patients

Table 3
Viral quantitation in ferrets infected with clinical samples

Patient number	Clinical sample	Viral RNA quantitation (log ₁₀ copies/mL)			
		2 dpi	4 dpi	6 dpi	8 dpi
2	Urine	0.92 ± 0.38 (0/2)	1.68 ± 0.42 (1/2) ^a	0.35 ± 0.14 (0/2)	— (0/2)
3	Urine	1.22 ± 0.13 (0/2)	3.24 ± 1.01 (2/2) ^a	— (0/2)	— (0/2)
4	Stool	0.73 ± 0.38 (0/2)	2.52 ± 1.28 (2/2) ^a	— (0/2)	— (0/2)

^a SARS-CoV-2 virus isolation in Vero cells using nasal washes from specimen-treated ferrets (number of virus particles isolated from ferrets/clinical specimens inoculated).

during the reviewing process for this study [18]. Further, there are some discrepancies regarding the detection of viral RNA in urine specimens. Guan et al. [21] and Peng et al. [22] reported the detection of SARS-CoV-2 RNA in urine specimens while Lo et al. [23] and Wang et al. [24] reported that none of the urine samples they tested were positive for SARS-CoV-2 by RT-PCR assay. In this study, we detected viral RNA in urine specimens from all five patients and demonstrated the presence of the viable virus in two urine specimens using the ferret model [11]. This apparent discrepancy in results could be due to clinical stage-dependent viral shedding by various shedding routes.

Moreover, we found that patients after symptom resolution shed the viable virus in their saliva and urine up to day 15 of illness. Although we failed to directly demonstrate the presence of the viable virus in urine and faecal specimens using cell culture isolation, when we used a stool sample from a COVID-19 patient to inoculate naive ferrets we could isolate SARS-CoV-2 from the animals (two out of two ferrets). Thus, this demonstrates the presence of the viable virus in the stool of this COVID-19 patient. In general, due to the cytotoxicity of urine and faecal specimens, performing cell culture with these specimens is technically challenging. In other studies, dialysis has been utilized to remove cytotoxic materials from samples; however, in this study dialysis was not used because of the potential loss of viral particles. These findings suggest that in addition to respiratory secretions, SARS-CoV-2 may be transmitted via various routes such as through faecal–oral contact, saliva droplets and urine. However, it is unclear whether the same virus inoculum used in ferrets could be sufficient to cause clinical illness in humans.

This study has some limitations. Because we could not collect serial specimens from COVID-19 patients, this study does not provide information regarding dynamic changes in viral loads of the clinical specimens. Also, this study used a limited number of patients and a limited number of samples, making it difficult to fully understand the detailed shedding kinetics of viable viruses in various COVID-19 clinical specimens. Thus, to determine the detailed kinetics of virus shedding in COVID-19 patients, further studies are needed with serial clinical specimens collected from a large number of COVID-19 patients.

In this study, we demonstrate the presence of viable SARS-CoV-2 in various clinical specimens from symptom-recovered COVID-19 patients. Notably, by using a ferret model highly susceptible to SARS-CoV-2, we identified, for the first time, the presence of infectious SARS-CoV-2 in faecal and urine specimens. However, it should be noted that the infectious viral load that can infect a ferret might be different from humans. Taken together, our study shows that symptom-recovered COVID-19 patients shed infectious SARS-CoV-2 in various clinical specimens, including urine and stool, as well as in respiratory secretions.

Author contributions

H.W.J. and Y.K.C. were involved in the design of this study. H.W.J., H.S.K., J.H.K., J.Y.C., S.H.K. and H.K. recruited patients and

collected data. S.M.K., Y.I.K., S.G.K., S.J.P., E.H.K. and Y.K.C., performed experiments. H.W.J. and Y.K.C. were responsible for supervision of the study. All authors were involved in writing the paper and have approved the final version.

Transparency declaration

We declare that we have no conflicts of interest.

Acknowledgements

This work was supported by the Korea Research Institute of Bioscience and Biotechnology (KRIBB) Research Initiative Program (KGM9942011) and conducted during the research year of Chungbuk National University in 2018 for Young Ki Choi.

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