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Coronavirus Pandemic

Use of qRT-PCR for SARS-CoV-2 sgRNA leader for the therapeutic plan: a preliminary report on 10 patients

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Abstract

Introduction: Duration of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) shedding is important for infection control. The presence of SARS-CoV-2 subgenomic RNA (sgRNA) leader indicates that the virus is replicative. This study examined the shedding duration of SARS-CoV-2 sgRNA leader and genomic RNA (gRNA) in diverse respiratory specimens.

Methodology: One hundred and eleven respiratory specimens collected sequentially from 10 COVID-19 patients with real-time RT-PCR SARS-CoV-2 orf1ab gene confirmed positive admitted to King Chulalongkorn Memorial Hospital were examined for SARS-CoV-2 E sgRNA leader and E gRNA by using Real-time reverse transcription PCR (qRT-PCR). These specimens included nasopharyngeal swab and throat swabs, nasal swab and throat swabs, sputum, and endotracheal aspirate, and were collected from the first day of admission until the time of orf1ab real-time RT-PCR negative of at least 2-4 consecutive days.

Results: E sgRNA leader could only be detectable in specimens with ≥ 1E+05 virus E gene copies per ml within the first 15 days after hospitalization. SARS-CoV-2 sgRNA leader was undetectable from one to 15 days earlier than that of gRNA in all patients. Re-shedding of sgRNA was evident in 2 cases, both on a single occasion after being undetectable for 3-10 days.

Conclusions: Assessment of the presence of sgRNA leader may be useful for therapeutic planning.

Key words: COVID-19; SARS-CoV-2; sgRNA leader; qRT-PCR.

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Introduction

SARS-CoV-2 virus or Coronavirus disease-2019 (COVID-19) virus is a pandemic emerging infectious virus. The treatment plan and duration of quarantine also rely on the absence of viruses assessed by real-time RT-PCR on samples primarily from respiratory specimens. Real-time RT-PCR positivity in respiratory specimens after symptom resolution can be prolonged for two or more weeks [1-8]. However, this may not necessarily reflect the replicative potential of the virus itself.

Coronaviruses have a unique mechanism, discontinuous transcription with the synthesis of sgRNAs to add a copy of the leader sequence [9]. Viral replication can be determined by the detection of the SARS-CoV-2 sgRNA leader [10-13]. The objectives of this study were to analyze the presence of SARS-CoV-2 sgRNA leader at which critical level of SARS-CoV-2 gRNA that sgRNA leader could still be detected and how long that real-time RT-PCR positivity of gRNA could be persisted despite the absence of sgRNA leader by using qRT-PCR.

Methodology

Study design

This retrospective study was performed with 111 specimens collected from 10 COVID-19 patients admitted to the King Chulalongkorn Memorial Hospital (KCMH) between March 2020 and April 2020 during COVID-19 outbreak in Thailand. Presumptive patients were diagnosed according to WHO interim guidance [14]. All patients had confirmed SARS-CoV-2 routine diagnosis by using the real-time RT-PCR for detecting orf 1 ab gene of SARS-CoV-2 (produced by BGI Biotechnology Co., Ltd, Wuhan, China). The assay was performed on the QuantStudio7 flex real-time PCR system (Applied Biosystems, Waltham, MA, USA).

Specimen collection and Viral RNA extraction

Nasopharyngeal combined with throat swabs (NT) and nasal swabs combined with throat swabs (NST) were collected using a Universal Transport Medium (UTM) kit containing a viral transport medium. Sputum (SP) and endotracheal aspirate (ETA) were collected directly into a sterile dry container. Viral RNA was extracted from 400 µL of the samples with the MagDEA Dx reagents (Precision System Science, Chiba, Japan) using a Magtration system magLEAD 12gC (Precision System Science, Chiba, Japan) is an instrument fully automated nucleic acid extraction system according to the manufacturer's instructions. Fifty-microliter eluate was kept at -20 °C for further analysis.

Quantitation of SARS-CoV-2 gRNA and sgRNA leader

To detect SARS-CoV-2 gRNA and sgRNA leader were performed using two designed monoplex qRT-PCR as shown in Table 1. SuperScript III One-Step RT-PCR system with Invitrogen Platinum Taq DNA polymerase kit (Invitrogen, Waltham, MA, USA) was performed according to the manufacturer's protocol on the Rotor-Gene Q instrument (QIAGEN, Hilden, Germany). Briefly, each 25-µL reaction contained 5 µL of nucleic acid, 12.5 µL of 2× reaction mix, 1 µL of enzyme, 0.4 µL of 50 mM $MgSO_4$, and 3 µL of 10 µM primer/probe mix. Cycling conditions were as followed: 55 °C for 20 minutes, 95 °C for 3 minutes, then 45 cycles of 95 °C for 15 seconds, 58 °C for 30 seconds, and 72 °C for 5 seconds. The threshold was set at 0.02. The amplification curve of any samples showed a typical S-shaped with a cycle threshold (Ct) < 40 was considered to be detected. To quantitate viral load, were synthetic oligonucleotide of 131 base pairs and 171 base pairs (gBlocks Gene Fragments, Integrated DNA Technologies, IA, USA) for SARS-CoV-2 gRNA and sgRNA leader are standard control and cut to standard curves. Agarose gel electrophoresis followed by direct sequencing was performed for SARS-CoV-2 sgRNA leader-positive qRT-PCR products. The specimens were confirmed as SARS-CoV-2 sgRNA leader only when the sequences included SARS-CoV-2 leader sequence and were consistent with ≥ 98% SARS-CoV-2 genome by using Basic Local Alignment Search Tool (BLAST) program version BLASTN 2.11.0.

Statistical analysis

Analysis of trends in virus concentrations and detection of sgRNA leader were performed using the Pearson correlation. Mann Whitney on GraphPad Prism 8 Software was used for comparison of specimens with or without detectable sgRNA leader. Only a *p*-value less than 0.05 was considered statistically significant.

Ethics statement

The experimental procedures used in this study were approved by the Ethics Committee, the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA No. 972/2020).

Results

One hundred and eleven respiratory specimens acquired from 10 COVID-19 patients admitted to KCMH were included in this study: 19 NT, 8 NST, 77 SP, and 7 ETA. To quantitate both SARS-CoV-2 gRNA and sgRNA leader simultaneously, qRT-PCR was performed. The coefficient of determination (R^2) value of SARS-CoV-2 gRNA and sgRNA leader were 0.99971 and 0.99955, respectively. The cut-off value of Ct for the target genes and internal reference is 40, limit of detection of both SARS-CoV-2 gRNA and sgRNA leader is equal to 1E + 03 copies/µL. The specificity of SARS-CoV-2 gRNA and sgRNA leader were described previously [12,15]. Duration of viral shedding of sgRNA leader varied from 4 to 15 days while gRNA remained up to 24 days. Detailed experimental results were described in the supplementary data.

A similar trend was observed between gRNA and sgRNA in terms of the magnitude of viral copies.

sgRNA leader, representing virus actively replicating index, can be detected in this study, correlates with virus concentrations of ≥ 1E + 05 copies per ml. During the initial period of infection, SARS-CoV-2 sgRNA leader strongly correlated with gRNA

Table 1. Primer and probe used in this study.

Primers	Sequence (5'- 3')	Length	Product size	Targets	References
SgLead–F	CGATCTCTTGTAGATCTGTTCTC	23 bp.	171 bp.	Sub-gRNA leader	[12]
Probe-E	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	26 bp.			[15]
E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	22 bp.			
E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	26 bp.	113 bp.	gRNA	[15]
Probe-E	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	26 bp.			
E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	22 bp.			

(Pearson correlation coefficient = 0.836). There was a statistically significant difference in the level of SARS-CoV-2 gRNA viral load between samples with and without detectable sgRNA leader ($p < 0.0001$). Higher SARS-CoV-2 gRNA viral load was detected in the specimens with detectable sgRNA leader than in those without (Figure 1). In samples with gRNA less than 1E + 05, sgRNA would be absent. Samples from 8 of 10 patients assayed to the last qRT-PCR positive of SARS-CoV-2 gRNA showed that gRNA continued to persist for up to 14 days once sgRNA leaders were qRT-PCR negative.

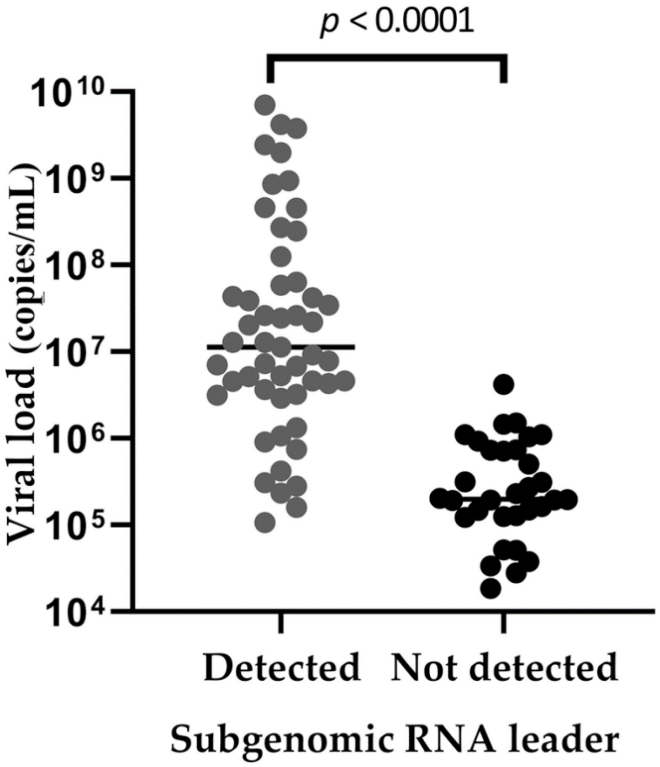
sgRNA leader reemerged in 2 patients. In patient no.7, sgRNA was detected until day 11 and it became positive again only on day 15. sgRNA leader remained undetectable through the end of the observation while gRNA of this patient lasted for 18 days.

sgRNA leader of patient No.8 was positive until day 4 and remained undetectable throughout the observation except for day 14. gRNA of this patient was negative since day 16 (Supplementary Table 1).

Discussion and Conclusions

Our study in 10 patients with an assay on retrospective samples suggested that SARS-CoV-2 sgRNA leader could be demonstrable during the first 15 days of hospitalization. All samples with ≥ 1E + 05 virus E gene copies per ml, except six samples with viral load ≥ 1E + 06 virus E gene copies per mL, had sgRNA leader gene co-undetected. It may be possible that viral load itself was not predictive of the presence of an infectious virus or sgRNA leader as reported by Perera *et al*. [11]

Figure 1. The SARS-CoV-2 gRNA titers in the specimens with sgRNA leader detected (in grey) versus where sgRNS leader was not detected (in black).



We here demonstrated that once gRNA was less than 1E + 05, sgRNA could not be detected in the subsequent sampling. Whether the presence of sgRNA leader represented the infectious state or degree of infectivity cannot be concluded in this study due to lack of culture proof. However, its absence may hint the virological success in therapeutic management. There have been studies of SARS-CoV-2 virus culture that showed a correlation between sgRNA leader and infectivity. They found that the sgRNA leaders are abundant in the infectious cells. As this is generally considered to be the hallmark of active replication, it may provide sufficient proof that the virus is indeed actively replicating in the cultures [10]. A similar finding from Wolfel *et al.* showed that live virus could be successfully isolated from throat swabs given that sgRNA-transcribe cells in throat swabs contained peak concentrations of up to 5E + 05 copies per swab, particularly during the first 5 days of symptoms [12].

Although it has been unclear whether only gRNA can be infective or not, early discharge with ensuing home isolation for patients who are beyond day 10 of mild symptoms with negative sgRNA leader and less than 1E + 05 viral RNA copies per ml has been proposed [12]. In this case, self-quarantine for at least 14 additional days and retesting for sgRNA leader at the end should be performed.

Despite small numbers of subjects without clinical data, it has been recognized that the treatment outcome depends on several factors other than the virus itself, for example, clinical-grade upon initiation of treatment, comorbidities and health status of the patients, degree of accentuated or aberrant immune responses or cytokine storm and quality of care.

One peculiar finding, sgRNA leader emerged again (see text and supplementary data) in two patients. It may be possible that the virus is sequestered but then temporarily evades the immune system.

At present, length of quarantine stay for real-time RT-PCR positive individuals requires a minimum of time in isolation until the resolution of clinical illness and virus free by 2 real-time RT-PCR testing on 2 consecutive days [16]. The new emergence of virus or sgRNA leader after the qRT-PCR negativity may

support a stricter quarantine, home-based, for another 14 days.

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Annex – Supplementary Items

Supplementary Table 1. Quantitative assessment of SARS-CoV-2 sgRNA leader compared with gRNA by following up specimens. Individual patient charts with qRT-PCR results (copies/ml) in relation to the duration of infection.

Days (specimens)	ORF 1ab gene	gRNA (E gene)		sgRNA leader (E gene)	
	Ct	Ct	Copies/mL	Ct	Copies/mL
Case #1					
01 (SP)	20.47	22.53	2.71E+08	31.76	5.13E+05
02 (SP)	20.47	22.71	2.46E+08	30.71	9.69E+05
03 (SP)	21.33	27.99	1.29E+07	37.91	1.25E+04
05 (SP)	33.16	35.43	2.04E+05	Not detected	Not detected
06 (SP)	29.59	Not detected	Not detected	Not detected	Not detected
07 (NT)	Not detected	Not detected	Not detected	Not detected	Not detected
08 (NT)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #2					
01 (SP)	23.5	26.77	2.20E+07	30.5	1.45E+06
04 (SP)	26.92	29.61	4.54E+06	33.88	1.89E+05
05 (SP)	26.39	26.46	2.63E+07	34.72	1.14E+05
08 (SP)	26.65	28.82	7.05E+06	37.71	1.86E+04
09 (SP)	31.79	35.61	1.60E+05	36.02	5.18E+04
10 (SP)	28.08	32.24	1.04E+06	Not detected	Not detected
11 (SP)	33.25	35.27	1.94E+05	Not detected	Not detected
13 (SP)	30.38	35.56	1.64E+05	Not detected	Not detected
14 (SP)	31.47	35.26	1.94E+05	Not detected	Not detected
18 (SP)	31.22	35.75	1.48E+05	Not detected	Not detected
23 (SP)	34.23	37.64	5.15E+04	Not detected	Not detected
25 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
26 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
27 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
28 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #3					
01 (NT)	12.89	14.96	7.02E+09	22.64	4.02E+08
08 (NT)	19.01	23.53	5.87E+07	32.2	1.25E+06
10 (ETA)	25.78	27.15	7.81E+06	34.72	2.73E+05
12 (SP)	24.88	26.86	9.21E+06	36.92	7.21E+04
13 (SP)	25.57	27.89	5.16E+06	37.66	4.61E+04
14 (SP)	30.42	34.84	1.07E+05	37.63	4.70E+04
15 (SP)	30.43	30.72	1.07E+06	38.62	2.59E+04
17 (SP)	32.09	34.56	1.25E+05	Not detected	Not detected
18 (SP)	28.53	30.66	1.11E+06	Not detected	Not detected
20 (SP)	29.48	31.41	7.29E+05	Not detected	Not detected
21 (SP)	31.71	36.72	3.76E+04	Not detected	Not detected
22 (SP)	22.9	28.27	4.18E+06	Not detected	Not detected
23 (SP)	26.19	30.98	9.24E+05	Not detected	Not detected
24 (SP)	28.41	30.12	1.50E+06	Not detected	Not detected
25 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
26 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
28 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #4					
01 (NST)	27.6	31.65	4.20E+05	34.86	1.05E+05
07 (NT)	26.8	32.7	2.34E+05	35.82	5.84E+04
08 (ETA)	27.57	32.2	3.09E+05	38.47	1.18E+04
09 (SP)	29.01	30.61	7.48E+05	39.57	6.06E+03
10 (SP)	31.06	32.43	2.71E+05	Not detected	Not detected
11 (SP)	33.71	Not detected	Not detected	Not detected	Not detected
12 (SP)	35.7	Not detected	Not detected	Not detected	Not detected
13 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
14 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #5					
01 (SP)	15.16	19.29	9.43E+08	25.83	3.40E+07
02 (SP)	11.89	16.8	3.78E+09	24.33	8.41E+07
03 (NT)	18.79	22.91	1.25E+08	29.15	4.56E+06
04 (SP)	20.22	28.83	4.61E+06	36.93	4.16E+04
06 (NT)	34.97	27.21	1.13E+07	35.11	1.25E+05

07 (SP)	22.52	29.67	2.89E+06	38.14	2.00E+04
08 (SP)	24.87	29.25	3.64E+06	39.74	7.61E+03
10 (SP)	24.07	32.11	7.36E+05	Not detected	Not detected
11 (SP)	30.14	35.34	1.22E+05	Not detected	Not detected
12 (SP)	28.99	32.18	7.11E+05	Not detected	Not detected
17 (NT)	31.67	38.72	1.85E+04	Not detected	Not detected
19 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
21 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
22 (NT)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #6					
01 (NT)	26.28	29.7	3.15E+06	37.02	1.43E+05
03 (ETA)	21.5	24.33	6.31E+07	33.4	1.26E+06
04 (SP)	16.5	29.15	4.30E+06	38.27	3.64E+04
05 (SP)	23.94	29.02	4.61E+06	37.8	2.04E+05
09 (NT)	31.7	34.69	1.95E+05	Not detected	Not detected
13 (NT)	Not detected	Not detected	Not detected	Not detected	Not detected
14 (NT)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #7					
01 (NST)	13.81	17.35	1.98E+09	23.89	5.18E+07
06 (NST)	20.18	24.21	4.33E+07	31.57	7.13E+05
11 (NST)	22.56	26.4	1.28E+07	35.6	7.50E+04
13 (NST)	29.34	33.03	3.15E+05	Not detected	Not detected
14 (NST)	28.29	32.18	5.06E+05	Not detected	Not detected
15 (NT)	21.84	25.24	2.43E+07	33.72	2.15E+05
18 (NT)	36.28	Not detected	Not detected	Not detected	Not detected
21 (NST)	Not detected	Not detected	Not detected	Not detected	Not detected
23 (NT)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #8					
01 (SP)	17.13	18.29	2.45E+09	27.34	8.91E+06
02 (ETA)	14.74	17.33	4.19E+09	26.51	1.48E+07
03 (SP)	18.22	25.95	3.43E+07	36.27	4.05E+04
04 (SP)	18.53	21.29	4.61E+08	30.97	9.99E+05
05 (SP)	28.83	31.62	1.45E+06	Not detected	Not detected
06 (SP)	27.62	32.11	1.10E+06	Not detected	Not detected
07 (SP)	32.67	38.68	2.81E+04	Not detected	Not detected
08 (SP)	30.86	35.7	1.49E+05	Not detected	Not detected
10 (SP)	30.32	37.61	5.10E+04	Not detected	Not detected
12 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
14 (SP)	22.93	28.84	6.80E+06	38.45	1.09E+04
16 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #9					
01 (SP)	18.36	21.17	4.56E+08	33.77	2.18E+05
04 (ETA)	20.08	25.6	3.85E+07	36.14	5.21E+04
05 (SP)	21.12	26.29	2.61E+07	36.17	5.11E+04
06 (SP)	20.82	25.44	4.21E+07	35.26	8.88E+04
08 (ETA)	27.59	34.44	2.79E+05	35.07	9.95E+04
10 (ETA)	25.64	29.15	5.30E+06	37.42	2.41E+04
13 (NT)	29.74	35.1	1.91E+05	Not detected	Not detected
18 (SP)	32.54	34.77	2.30E+05	Not detected	Not detected
24 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
26 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
Case #10					
01 (SP)	21.67	26.27	2.03E+07	32.35	6.06E+05
02 (SP)	30.74	33.76	3.10E+05	Not detected	Not detected
03 (SP)	27.68	31.85	9.05E+05	38.99	1.10E+04
04 (SP)	27.01	31.16	1.33E+06	38.64	1.36E+04
06 (SP)	22.07	28.1	7.30E+06	34.27	1.90E+05
08 (NST)	15.93	19.56	8.56E+08	27.89	8.99E+06
10 (SP)	24.84	29.57	3.23E+06	34.96	1.25E+05
12 (SP)	30.65	35.36	1.28E+05	Not detected	Not detected
15 (NT)	34.93	37.74	3.38E+04	Not detected	Not detected
19 (SP)	Not detected	Not detected	Not detected	Not detected	Not detected
21 (NT)	Not detected	Not detected	Not detected	Not detected	Not detected