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Viral Culture Confirmed SARS-CoV-2 Subgenomic RNA Value as a Good Surrogate Marker of Infectivity

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ABSTRACT Determining SARS-CoV-2 viral infectivity is crucial for patient clinical assessment and isolation decisions. We assessed subgenomic RNA (sgRNA) as a surrogate marker of SARS-CoV-2 infectivity in SARS-CoV-2-positive reverse transcription PCR (RT-PCR) respiratory samples (n = 105) in comparison with viral culture as the reference standard for virus replication. sgRNA and viral isolation results were concordant in 99/105 cases (94%), indicating highly significant agreement between the two techniques (Cohen's kappa coefficient 0.88, 95% confidence interval [CI] 0.78 to 0.97, $P < 0.001$). sgRNA RT-PCR showed a sensitivity of 97% and a positive predictive value of 94% to detect replication-competent virus, further supporting sgRNA as a surrogate marker of SARS-CoV-2 infectivity. sgRNA RT-PCR is an accurate, rapid, and affordable technique that can overcome culture and cycle threshold (C_T) value limitations and be routinely implemented in hospital laboratories to detect viral infectivity, which is essential for optimizing patient monitoring, the efficacy of treatments/vaccines, and work reincorporation policies, as well as for safely shortening isolation precautions.

KEYWORDS SARS-CoV-2, COVID-19, subgenomic RNA, viral culture, infectivity

Reverse transcription-PCR (RT-PCR) is the most sensitive and widely used technique for the diagnosis of COVID-19. Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) RT-PCR can remain positive in respiratory secretions for weeks or months due to the detection of viral particle debris, even in asymptomatic individuals (1). RT-PCR provides a cycle threshold (C_T) value which is inversely related to viral load, with every 3.3 increase in C_T value reflecting a 10-fold reduction of RNA (2). C_T values are highly variable depending on the swabbing technique, specimen types, assays, and platforms used for RNA extraction and amplification, limiting their utility for predicting viral loads or infectivity (3, 4). Determining the duration of active SARS-CoV-2 replication is key for the clinical management of patients, discharge from isolation and work reincorporation.

Viral isolation is the gold standard for determining virus infectivity. Several studies have isolated SARS-CoV-2, showing virus recovery in specimens collected within 1 to 9 days after symptom onset (5), after 12 days in patients with mild to moderate disease (6), and after 20 days in critically ill patients (7).

Subgenomic RNA (sgRNA) is only transcribed in infected cells and is poorly packaged into virions, indicating the presence of active replication (8). Previous studies have shown a significant association between sgRNA detection and high viral loads during the first 5 to 7 days of symptoms, when most viral transmission has been reported (9 to 11).

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sgRNA detection by RT-PCR can overcome the limitations of time-consuming culture techniques which require high-biosafety laboratories (BSL-3) and fresh samples. Only a few reports have compared SARS-CoV-2 culture and sgRNA detection (9, 12); despite using an insufficient sample size to determine agreement, these reports found moderate to complete concordance. Nonetheless, further studies are needed.

This study aimed to determine the correlation of sgRNA with viral culture to verify if sgRNA can be used as a surrogate marker of active SARS-CoV-2 replication.

(Preliminary results from this study were presented at the 31st European Congress of Clinical Microbiology and Infectious Diseases [ECCMID]; 9 to 12 July, Vienna, Austria.)

MATERIALS AND METHODS

Study design and sample collection. The study design consisted of using RNA RT-PCR to select samples that were positive for SARS-CoV-2, which were kept at 4°C for ≤48 h before inoculation for SARS-CoV-2 culture. Aliquots of each sample were stored at −80°C for sgRNA detection by RT-PCR.

A total of 105 samples (88 nasopharyngeal aspirates/swabs, 17 bronchoalveolar lavages) from the Hospital de la Santa Creu i Sant Pau (HSCSP) and the Hospital Clínic de Barcelona (HCB), collected from 6 November 2020 to 25 March 2021, were included for fulfilling the above criteria. They were tested for SARS-CoV-2 RNA using the platforms established in the respective hospitals. Each sample corresponded to an individual subject, except in the case of 1 patient from whom 3 samples were included in the study.

SARS-CoV-2 culture. In the BSL3 lab of the HSCSP, each sample was treated with a mixture of antibiotics (vancomycin and streptomycin) and an antifungal (amphotericin B) for 30 min. After sample treatment, 300 μl was inoculated into the VERO-E6 cells and incubated at 37°C for a maximum of 10 days (see the S1 text at https://doi.org/10.6084/m9.figshare.16802200.v1). The appearance of a cytopathic effect (CPE) was examined daily with an inverted microscope (×40). A culture was considered positive when a characteristic CPE was observed. Each CPE was confirmed as being caused by SARS-CoV-2 either by indirect immunofluorescence using a specific monoclonal antibody, anti-SARS-CoV-2 (CerTest, Spain), and/or by SARS-CoV-2 RT-PCR, where a value of ≥3 cycles lower than the original RT-PCR C_T value confirmed the presence of replicating virus. The viral culture was considered negative if CPE was absent 10 days after inoculation.

SARS-CoV-2 sgRNA RT-PCR. At the HCB, all samples were inactivated with 1:1 volume of Cobas Omni Lys (Roche, Germany), and total nucleic acid was extracted using MagNA Pure Compact (Roche, Switzerland). Respiratory samples and elutes were aliquoted and stored at −80°C.

Extracted RNAs were tested for the presence of *Envelope* (*E*) sgRNA using the leader-specific primer described by Wölfel et al. (9) as well as primers and probes targeting sequences downstream of the start codons of the *E* gene (13) (see Text S2 and Table S1 at https://doi.org/10.6084/m9.figshare.16802200.v1). RT-PCR was performed using the SuperScript III Platinum One-Step RT-PCR kit (Invitrogen) with a primer concentration of 400 nM and a probe concentration of 200 nM. The C_T cutoff for negative samples was >40.

Statistical analysis. We recorded the number and percentage of samples for categorical variables and the median (first quartile [Q1]; third quartile [Q3]) for continuous variables. The assumption of normality was checked using the Kolmogorov-Smirnov test. Categorical variables were compared with a chi-square test. Two groups of continuous variables were compared using a nonparametric Mann-Whitney U test. Cohen's kappa (14) was calculated to measure agreement between the two methods (i.e., culture and sgRNA) for nominal categorical variables. To calculate the yield of the test, we analyzed the area under the receiver operating characteristic (ROC) curve (AUC) (15, 16). We also calculated the sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios, along with the 95% confidence intervals (CI). The level of significance was set at 0.05 (2-tailed). All analyses were performed using IBM SPSS Version 26.0 (IBM Corp., Armonk, NY, USA).

Ethical approval. This study protocol was evaluated and approved by the Ethical Board HCB (HCB/2021/0024). Informed consent was waived due to the state of infectious disease emergency.

RESULTS

This study analyzed sgRNA and viral isolation of 105 RT-PCR positive SARS-CoV-2 upper and lower respiratory tract samples (Table 1). The median (Q1; Q3) C_T value of RT-PCR diagnosis was 23 (18; 29), and the median number of days after symptom onset was 4 (1; 15). Eighteen (17.1%) samples were from asymptomatic patients, and 11 (10.5%) were from patients with prolonged viral shedding (RNA positive for >30 days).

sgRNA was detected in 66 (62.9%) specimens: 13 (19.7%) from asymptomatic patients and 38 (57.6%) from patients with 1 to 7 days of symptoms. SARS-CoV-2 isolation was successful in 64 (61%) specimens: 13 (20.3%) from asymptomatic patients and 37 (57.8%) from patients in the first 7 days after symptom onset. The median number of days until characteristic CPE was observed was 3 (2; 3).

Positive and negative sgRNA and viral culture subsets were compared with the C_T value of the diagnostic RT-PCR, the C_T value of the sgRNA RT-PCR, and the number of days after symptom onset stratified by intervals (Table 1). Qualitative sgRNA detection

TABLE 1 Comparative association of subgenomic RNA and viral culture with categorical and continuous variables[a]

		Viral culture			sgRNA		
Parameter	Total	Negative	Positive	*P*	Negative	Positive	*P*
Total no. (%)	105 (100)	41 (39)	64 (61)		39 (37.1)	66 (62.9)	
Diagnostic C_T	23 (18.0; 29.0)	30.1 (27.3; 34.1)	18.8 (16.5; 22.8)	<0.001	30.8 (27.9; 34.5)	19.1 (16.5; 22.9)	<0.001
sgRNA C_T	29.4 (26.8; 33.8)	37.7 (35.6; 39.0)	28.8 (26.6; 33.1)	=0.003		29.4 (26.8; 33.9)	
Days of symptoms	4 (1; 15)	15 (4; 21)	2 (1; 6.5)	<0.001	14 (4; 22)	2 (1; 7)	<0.001
No. of samples							
Asymptomatic	18 (17.1)	5 (12.2)	13 (20.3)		5 (12.8)	13 (19.7)	
1 day	16 (15.2)	3 (7.3)	13 (20.3)		3 (7.7)	13 (19.7)	
2–7 days	32 (30.5)	8 (19.5)	24 (37.5)		7 (17.9)	25 (37.9)	
8–15 days	15 (14.3)	8 (19.5)	7 (10.9)		8 (20.5)	7 (10.6)	
16–30 days	13 (12.4)	12 (29.3)	1 (1.6)		11 (28.2)	2 (3.0)	
>30 days	11 (10.5)	5 (12.2)	6 (9.4)		5 (12.8)	6 (9.1)	

[a]Data are indicated as number of samples (%) or as the median (first quartile; third quartile). C_T, cycle threshold value of the RT-PCR. *P* values are indicated as <0.05 versus positive group.

was significantly associated with the diagnostic C_T value and days after symptom onset, while viral culture was significantly associated with the diagnostic C_T value, the sgRNA C_T value, and days after symptom onset.

C_T values of diagnostic and sgRNA RT-PCRs were stratified by intervals of days after symptom onset (Table 2). The sgRNA C_T value increased by 1.43 every interval change (approximately 7 days). This ratio could not be appropriately calculated for the diagnostic C_T value due to its high variability over intervals between and within days after symptom onset.

An increase of 11.35 in the diagnostic C_T value between the negative and positive culture samples is shown in Fig. 1A. No virus was isolated when the RT-PCR C_T value was >29, except in one sample from an asymptomatic patient (C_T = 31.4). Only 4 samples presented sgRNA-positive and culture-negative results (Fig. 1B), with sgRNA RT-PCR C_T values of >34 (34.4, 36.8, 38.7, 39.4).

Of the specimens with both positive culture and positive sgRNA, 89% were from <15 days after symptom onset (Fig. 1A). These results were also found in 3 specimens from beyond 240 days after symptom onset, in 3 specimens from 33 to 45 days after symptom onset, and in 1 specimen from 23 days after symptom onset. These last 7 specimens corresponded to immunosuppressed patients with hematological malignancies who had received chemotherapy and required admission to the Critical Care Unit due to severe COVID-19 complications. Seven specimens from 7 to 15 days after symptom onset were positive for both sgRNA and viral culture. These corresponded to patients who presented risk factors (1 with hematological malignancy, 1 with HIV, 3 with severe pneumonia, 1 with morbid obesity, 1 who was 102 years old) for severe COVID-19; however, the early date at which the sample was collected did not allow us to consider them as persistent (>21 days of symptoms) (17).

Cohen's kappa was calculated to measure the agreement between the two methods: culture and sgRNA (Table 3). Over the 105 specimens tested, both methods showed positive results for 62 (59%) specimens, both tests showed negative results for 37 (35.2%) specimens, positive culture and negative sgRNA results in 2 (1.9%) specimens, and positive sgRNA and

TABLE 2 Cycle threshold values of diagnostic and subgenomic RNA RT-PCRs according to days after symptom onset

	C_T value[a]	
Days of symptoms	Diagnosis	sgRNA
Asymptomatic	20.5 (16.5; 29.9)	28.6 (26.8; 33.1)
1 day	19.3 (17.5; 23.6)	27.9 (25.7; 29.4)
2–7 days	22.3 (17.3; 25.7)	29.4 (27.1; 33.1)
8–15 days	26.1 (19.7; 31.0)	31.1 (27.0; 34.4)
16–30 days	34.1 (26.9; 35.4)	32.5 (25.6; 39.4)
>30 days	23.1 (12.2; 32.8)	33.6 (28.6; 35.4)

[a]Data are indicated as median (first quartile; third quartile).

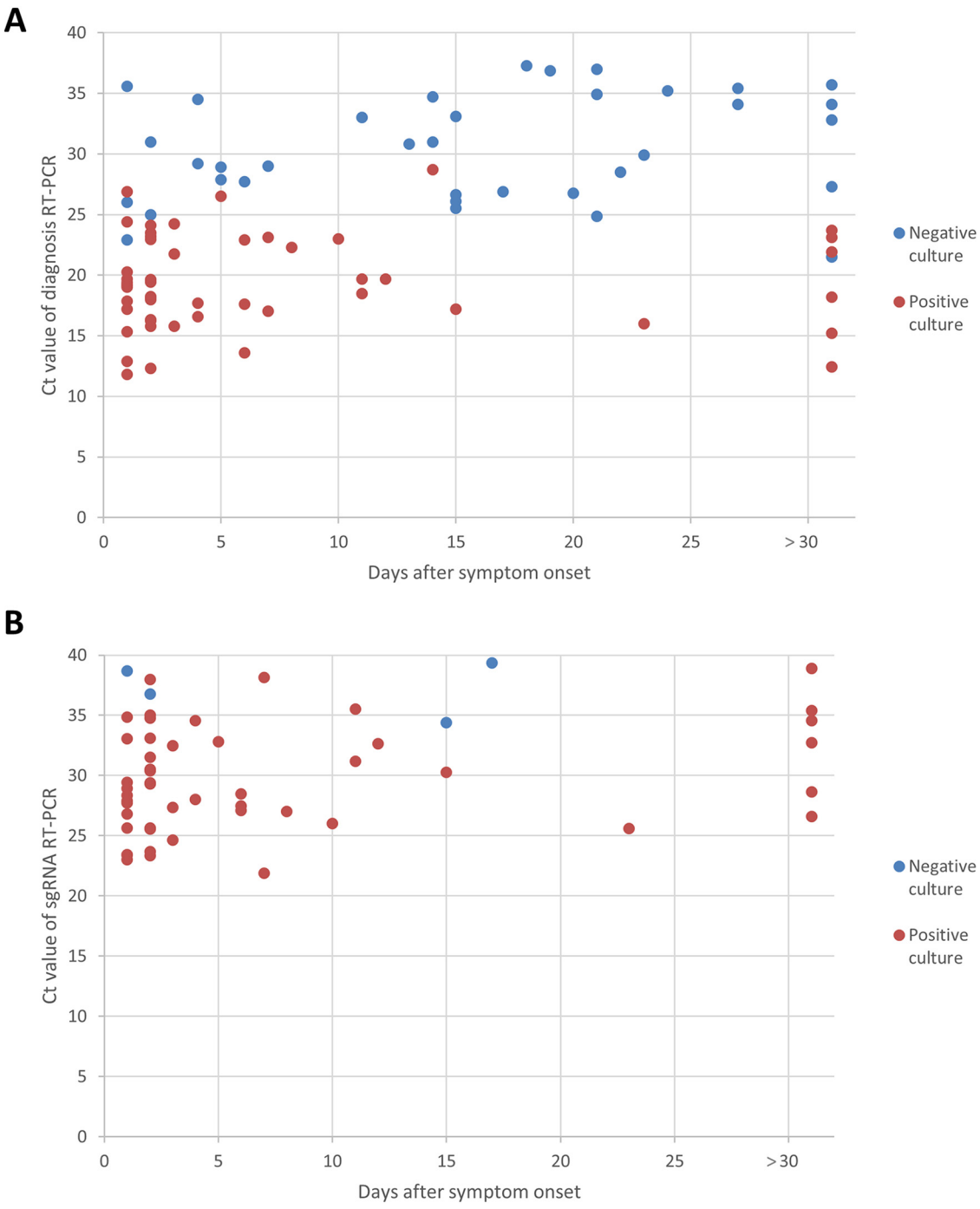

FIG 1 Viral culture results plotted by days after symptom onset and diagnostic C_T value of RT-PCR (A) and sgRNA RT-PCR (B).

negative culture results in 4 (3.8%) specimens (Cohen's kappa 0.88, 95% CI 0.78 to 0.97, $P < 0.001$), indicating a strong significant agreement between viral culture and sgRNA detection.

The predictive performance of sgRNA compared to viral culture is shown in Table 4. The sensitivity, specificity, positive and negative predictive values were 97%, 90%, 94%, and 95% respectively, with positive and negative likelihood ratios of 9.93 and 0.03, respectively. This result indicates that the probability of infection significantly increased with positive sgRNA results and significantly decreased with negative sgRNA results.

TABLE 3 Results of the distribution of samples by sgRNA and viral culture methods

	No. of sgRNA samples		
Culture	Negative	Positive	Total
Negative	37	4	41
Positive	2	62	64
Total	39	66	105

DISCUSSION

This study compared the results of *E* sgRNA with viral culture to evaluate the ability of *E* sgRNA to detect SARS-CoV-2 infectivity. Our findings show that sgRNA was able to detect replication-competent virus with a sensitivity of 97% and a positive predictive value of 94%; with a coefficient of agreement of 0.88 in relation to viral culture as the reference standard. These results suggest that *E* sgRNA could be used as a surrogate marker of active viral replication.

RT-PCR detects the presence of viral genomic RNA but is not able to distinguish whether an infectious virus is present. To determine whether there is active virus replication and, therefore, potential risk of person-to-person transmission, it is necessary to perform a viral culture. However, virus isolation is labor-intensive and has some limitations which make it unsuitable for many laboratories. The growth and identification of virus in culture requires specialized facilities and expertise. Nevertheless, viral cultures should be maintained in experienced laboratories as a reference method to validate new molecular techniques (18).

Several reports support the use of sgRNA as a surrogate marker of infectivity in the context of challenged primate models (19, 20), patients who persistently test positive for SARS-CoV-2 total RNA (17), and clinical isolates with comparative viral culture data (9, 12). In addition, to demonstrate a statistical association between sgRNA and viral culture, our study included a considerable sample size ($n > 100$) and the confirmation of positive culture results with indirect immunofluorescence and/or RT-PCR.

In contrast, some studies have postulated that sgRNA is not a good indicator of infectivity, suggesting that the loss of sgRNA detection is due to the lower overall RNA transcript concentration compared with genomic RNA (11, 21). However, they agree with the previously reported significant association of sgRNA with normalized viral loads and days after symptom onset (10). A significant limitation of these studies was the absence of correlative viral culture data.

There is some controversy regarding the choice of which sgRNA species to measure, as it may have a significant impact on detection and result interpretation. Nucleocapsid sgRNA is more abundant than *E* sgRNA and persists for longer periods (11, 22). This difference between sgRNA species could be explained by the enclosure of RNA transcripts in double-membrane vesicles and/or extracellular vesicles, allowing longer persistence of specific sgRNA species (23); however, there are no clear data as to whether *E* gene transcripts are more rapidly degraded by ribonucleases or whether they better reflect recent transcription. Our study demonstrates that *E* sgRNA has a better coefficient of agreement according to viral culture (Cohen's kappa coefficient 0.88) than that previously obtained using *Nucleocapsid*

TABLE 4 Predictive performance of subgenomic RNA compared to viral culture as the reference standard[a]

Parameter	Value	95% CI
Sensitivity (%)	96.9	91.8–100
Specificity (%)	90.2	79.9–100
Positive predictive value (%)	93.9	87.4–100
Negative predictive value (%)	94.9	86.7–100
Positive likelihood ratio	9.93	3.91–25.22
Negative likelihood ratio	0.03	0.01–0.14
AUC	0.94	0.88–0.99

[a]CI, confidence interval; AUC, area under the ROC curve.

sgRNA (Cohen's kappa coefficient 0.467) (12), suggesting that *E* sgRNA of SARS-CoV-2 is the best option to detect infectivity thus far.

In agreement with the literature, we found that virus isolation was unsuccessful in samples with diagnostic C_T values >29, except in 1 case (24). However, C_T values are no longer recommended as an indicator of infectivity due to their high variability between and within methods and their lack of standardization (4, 24). This study demonstrates that sgRNA detection can overcome the limitations of C_T values, as we compensated for the diagnostic C_T values bias by including diverse platforms from two different hospitals.

The 73% for successful virus isolation was achieved in samples from symptomatic patients within the first 7 days of symptoms, as reported previously (6). These samples were also sgRNA-positive in our study. Prolonged viral replication (>21 days of symptoms) (17), as detected by culture and sgRNA test, was demonstrated in 7 severely immunosuppressed patients who required hospital admission. Virus infectivity was also detected in 13 out of 18 asymptomatic subjects. Therefore, it is key to identify infectious individuals in order to control the spread of infection. sgRNA could be used both to detect asymptomatic infectious individuals and to better characterize the shedding of replicant-competent virus longitudinally from people with moderate to mild disease, as well as severely ill and immunocompromised patients, to define the timing of infectivity.

Our study was limited by the impracticability of repeating viral isolation, since these samples were stored at −20°C, and by the incapacity to quantify sgRNA due to the absence of an approved WHO standard for sgRNA species; therefore, this technique is meant to be qualitatively implemented. Four samples were sgRNA-positive, culture-negative, with sgRNA C_T values close to 40: this suggests that the higher sensitivity of sgRNA RT-PCR found compared to viral culture provides a beneficial overestimation of infectivity, which is crucial for preventing the risk of prematurely releasing patients from isolation or treatment discontinuation.

Overall, sgRNA provides rapid and robust determination of SARS-CoV-2 infectivity in upper and lower respiratory tract samples from patients with different clinical outcomes, including immunosuppressed subjects and individuals with persistent COVID-19 related symptoms. The sgRNA test overcomes both the limitations of C_T values, and the relative insensitivity and need for specialized facilities for viral culture, as it is detected by simple RT-PCR which can be easily implemented in hospital practice. Predicting SARS-CoV-2 infectivity is essential for optimizing patient monitoring, work reincorporation policies, and the efficacy of treatments/vaccines as well as for safely shortening isolation precautions.

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Marta Santos Bravo and Carla Berengua selected the samples included in the study, performed the majority of the experimental techniques, and wrote the manuscript, figures, and tables. Marta Santos Bravo designed and optimized the protocol for the SARS-CoV-2 sgRNA assay and performed it with the help of Genoveva Cuesta. Carla Berengua and Núria Rabella designed and optimized the protocol for SARS-CoV-2 isolation and identification in cell culture. Cell cultures were maintained by Montserrat Esteban with the help of Cristina Rodriguez. Pilar Marín inoculated the clinical samples and performed the immunofluorescence assay. Núria Rabella and María Ángeles Marcos were the promoters of the project and major proofreaders of the manuscript. Sonsoles Sánchez-Palomino, Mar Mosquera, Jordi Vila, Margarita del Cuerpo, and Elisenda Miró reviewed and corrected the manuscript.

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