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# Subgenomic RNA Abundance Relative to Total Viral RNA Among Severe Acute Respiratory Syndrome Coronavirus 2 Variants

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) subgenomic RNA (sgRNA) may indicate actively replicating virus, but sgRNA abundance has not been systematically compared between SARS-CoV-2 variants. sgRNA was quantified in 169 clinical samples by real-time reverse-transcription polymerase chain reaction, demonstrating similar relative abundance among known variants. Thus, sgRNA detection can identify individuals with active viral replication regardless of variant.

**Keywords.** severe acute respiratory syndrome coronavirus 2; subgenomic RNA; variants of concern.

Subgenomic RNAs (sgRNAs) are produced by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) during active replication and have been studied as a marker of active replication, to track antiviral response, and to identify patients at higher likelihood of viral transmission [1–3]. Current molecular diagnostics for SARS-CoV-2 detect total viral RNA but cannot distinguish genomic RNA from sgRNA [4]. Genomic RNA

often remains detectable long after active viral replication ceases and symptoms have resolved, giving a potentially false impression of transmissibility or active infection [5]. This led to the development of molecular assays for sgRNA, including a real-time reverse-transcription polymerase chain reaction (rRT-PCR) assay developed in our laboratory to detect nucleocapsid (N) gene sgRNA [6], which is the most abundant SARS-CoV-2 sgRNA [3].

Compared to total viral RNA, sgRNA detection demonstrates improved correlation with isolation of live virus and antigen detection, providing a potential indicator of infectivity detectable in the diagnostic clinical sample [2, 5–7]. However, the abundance of sgRNA during infections with SARS-CoV-2 variants compared to ancestral strains is relatively unknown. Characterization of sgRNA abundance is necessary if such assays are used to identify individuals with active viral replication in a variant-agnostic manner, considering that N-gene mutations may generate novel sgRNAs and increase sgRNA expression, possibly influencing detection [8]. To address this, we leveraged a clinical specimen bank of variant and ancestral SARS-CoV-2 strains from varied patient populations to quantify sgRNA abundance relative to total viral RNA by rRT-PCR.

## METHODS

### Clinical Samples

Specimens included 169 SARS-CoV-2–positive acute-phase samples collected as part of 3 ongoing studies to characterize SARS-CoV-2 variants in distinct patient populations: (1) acute infections in Paraguay (75 samples); (2) hospitalized adults in the Emory Healthcare System as part of the ongoing RSV in Older Adults and Pregnant women Study (ROAPS, 65 samples); and (3) individuals in the Atlanta area tested at the Emory/Children's Laboratory for Innovative Assay Development (ELIAD) to confirm diagnostic test performance (29 samples). The Paraguay sample set has been described previously [9]. ROAPS samples included a mixture of nasopharyngeal swabs obtained at the time of hospitalization for standard-of-care clinical testing and nasal midturbinate swabs collected at enrollment. Samples were included in the current study from individuals who tested positive for SARS-CoV-2 by the standard-of-care clinical test. Samples were collected from both vaccinated and unvaccinated individuals. ELIAD samples included nasal midturbinate swabs collected from patients who presented with an acute respiratory illness of  $\leq 7$  days to coronavirus disease 2019 (COVID-19) testing centers affiliated with Emory University and Children's Healthcare of Atlanta.

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## Ethical Considerations

The design of the work was reviewed and approved by the Institutional Review Board of Emory University and the Scientific and Ethics Committee of the Instituto de Investigaciones en Ciencias de la Salud (IICS), Paraguay. It conforms to standards currently applied at both sites. Written informed consent, and assent where appropriate, was obtained for individuals in the ROAPS and ELIAD studies. Patient consent was waived under approval of the IICS Scientific and Ethics Committee due to all samples being de-identified, with residual samples collected for standard clinical care.

## SARS-CoV-2 Molecular Testing

Paraguay samples were extracted using an EMAG instrument (bioMérieux, Durham, North Carolina), and ROAPS/ELIAD samples were extracted using a KingFisher Apex instrument (Thermo Fisher Scientific, Waltham, Massachusetts). Nucleic acids were eluted from 400  $\mu$ L of sample into 50  $\mu$ L of elution buffer. All eluates had previously been tested in the N2RP rRT-PCR, a duplex assay targeting the N2 target in the SARS-CoV-2 genome and RNase P as an internal control [6, 9]. Each sample was also tested in 2 different rRT-PCR assays of the spike single nucleotide polymorphism (Spike SNP) assay, including probes for (1) K417, 452R, 484K, and 501Y and (2) 452Q, 478K, and 490S, as previously described [9, 10]. Variant calls shown in Table 1 were made by comparing spike SNP assay results to the expected profile of known variants. Following initial testing, nucleic acid eluates were stored at  $-80^{\circ}\text{C}$  until tested again in the current study. Samples were thawed and tested with rRT-PCR for the detection of SARS-CoV-2 N-gene sgRNA, as previously described [6]. All samples with sufficient remaining eluate ( $n = 132$ ) were concurrently retested in the N2RP assay.

## Statistical Analysis

Relative abundance of sgRNA was measured as the difference between sgRNA and N2 cycle threshold (Ct) values. Continuous variables were compared by 2-sided  $t$  test with multiple comparison corrections. Linear regression, analysis of variance, and multiple linear regression were performed to evaluate whether sgRNA level differed by variant and swab type. All statistical analyses were performed in GraphPad Prism version 9.3.1 software (GraphPad, San Diego, California).

## RESULTS

The 169 samples included ancestral SARS-CoV-2 (31 [18.3%], all containing D614G) plus the following variants: 22 Alpha (13.0%), 12 Beta (7.1%), 30 Gamma (17.8%), 14 Zeta/P.2 (8.3%), 31 Delta (18.3%), and 29 Omicron (17.2%). Demographic and clinical information, spike SNP results, and qualitative sgRNA detection are shown in Table 1. N2 and sgRNA Ct values and distributions by variant are shown

**Table 1. Demographic and Clinical Information for 169 Samples Tested for Severe Acute Respiratory Syndrome Coronavirus 2 Subgenomic RNA by Variant, as Defined by Specific Mutations Detected in the Spike SNP Assay**

Category <sup>a</sup>	Total <sup>b</sup>	Ancestral	Alpha	Beta	Gamma	Zeta (P.2)	Delta	Omicron
Spike SNP result	Any (100)	K417	501Y	417var, 484K, 501Y	417var, 484K, 501Y	484K	452R, 478K	417var, 478K, 501Y
No. (%)	169 (100)	31	22	12	30	14	31	29
Sex, female, No. (%)	92 (55.4)	16 (55.2)	10 (45.6)	6 (54.6)	16 (53.3)	8 (57.1)	18 (58.1)	18 (62.1)
Age, y	41.0 $\pm$ 17.2	38.2 $\pm$ 15.2	51.1 $\pm$ 9.4	50.3 $\pm$ 9.8	47.6 $\pm$ 17.2	37.8 $\pm$ 15.7	47.6 $\pm$ 11.2	20.2 $\pm$ 29 <sup>c</sup>
Day of illness <sup>d</sup>	5.7 $\pm$ 3.7	4.8 $\pm$ 2.8	7.6 $\pm$ 5.4 <sup>e</sup>	7.5 $\pm$ 2.7	4.6 $\pm$ 2.6	4.7 $\pm$ 3.0	8.1 $\pm$ 3.1 <sup>e</sup>	3.0 $\pm$ 1.2
Vaccinated, No. (%)	34 (20.2)	0 (0)	3 (13.6)	3 (27.3)	0 (0)	0 (0)	12 (38.7)	16 (55.2)
N2 Ct values	21.7 $\pm$ 4.9	21.8 $\pm$ 4.2	22.8 $\pm$ 5.3	26.8 $\pm$ 4.2	18.4 $\pm$ 4.0	21.4 $\pm$ 5.0	23.1 $\pm$ 4.0	21.0 $\pm$ 5.0
sgRNA <sup>a</sup>	165 (97.6)	30 (96.8)	22 (100)	10 (83.3)	30 (100)	14 (100)	31 (100)	28 (96.6)

Abbreviations: Ct, cycle threshold; N2, severe acute respiratory syndrome coronavirus 2 nucleocapsid real-time reverse-transcription polymerase chain reaction target; sgRNA<sup>a</sup>, subgenomic RNA detected by real-time reverse-transcription polymerase chain reaction; SNP, single nucleotide polymorphism.

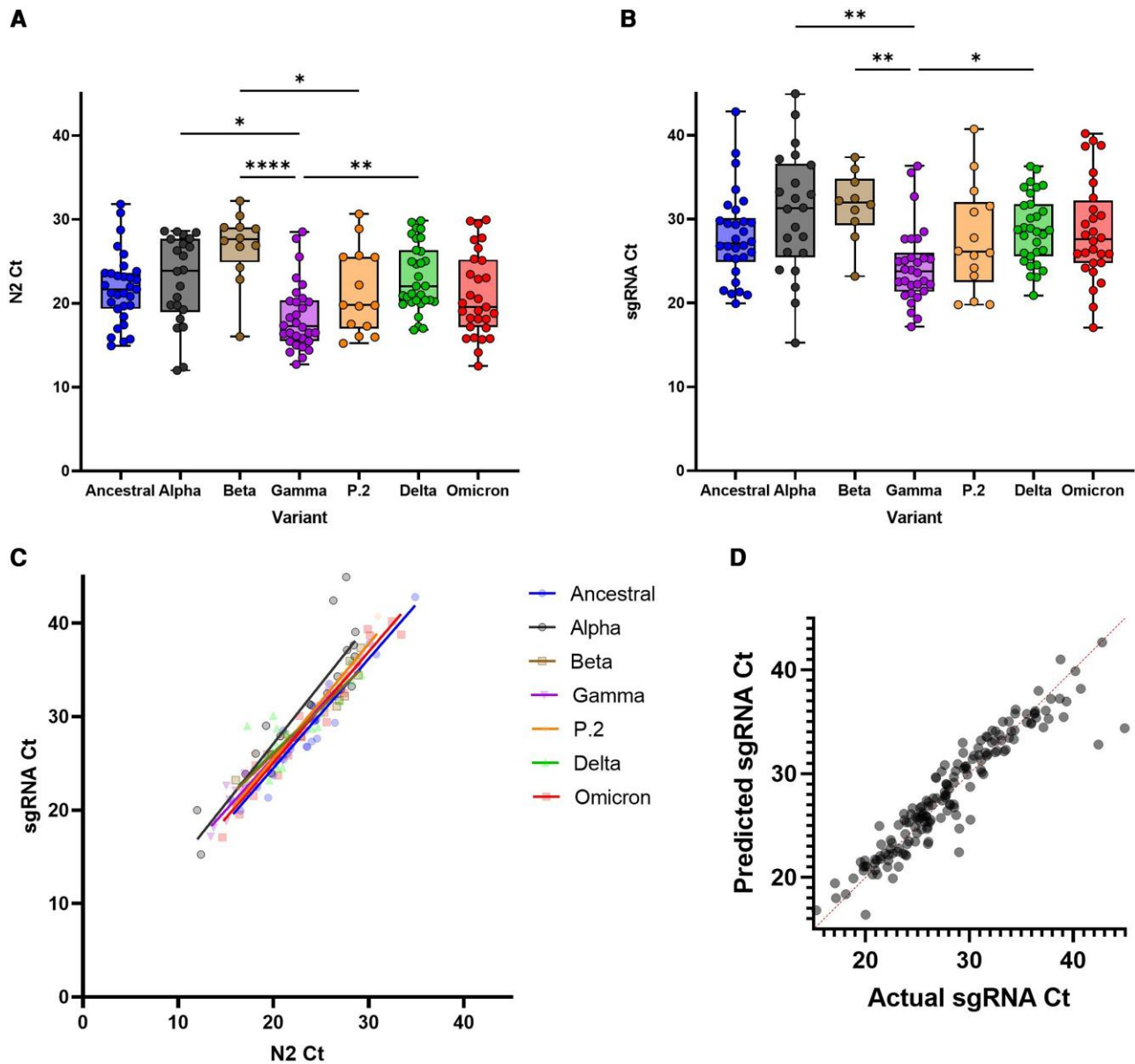
<sup>a</sup>Presented as mean  $\pm$  standard deviation unless otherwise noted.

<sup>b</sup>Three participants missing sex, 5 missing age, 1 missing vaccine status.

<sup>c</sup> $P < .01$  compared to all other variants (Dunnett multiple comparison test).

<sup>d</sup>Day of illness defined as days after symptom onset with day 1 as the day on which symptoms began; 8 of 169 (4.7%) individuals, all infected with Gamma variant, were asymptomatic.

<sup>e</sup> $P < .05$  compared to ancestral, Gamma, and Omicron; for Delta only,  $P < .05$  compared to Zeta (Dunnett multiple comparison test).



**Figure 1.** Box-and-whisker plots of severe acute respiratory syndrome coronavirus 2 nucleocapsid (N2) cycle threshold (Ct) (A) and subgenomic RNA (sgRNA) Ct (B), by variant. Significant pairwise comparisons are shown: \* $P < .05$ ; \*\* $P < .01$ ; \*\*\*\* $P < .001$ . C, Linear regression of sgRNA Ct against N2 Ct, by variant. D, Predicted vs actual plot showing fit of the multiple linear regression evaluating sgRNA Ct as a function of N2 Ct after dropout of the variant term ( $R^2 = 0.91$ ).

in Figure 1A and 1B. For 132 samples (78.1%), sufficient material for N2RP retesting was available. Original and repeat N2 Ct values did not differ significantly ( $P = .2$ , Supplementary Figure 1). Original N2 Ct values were used for all primary analyses for consistency.

sgRNA was detectable in 165 of 169 samples (97.6%). sgRNA Ct averages and distributions by variant are shown in Figure 1B and Supplementary Table 1. Associations between swab type, N2 Ct, and the difference in sgRNA and N2 Ct were evaluated. N2 Ct values were significantly lower with nasopharyngeal ( $n = 134$ ; mean  $\pm$  standard deviation,  $21.65 \pm 4.83$ ) and nasal

midturbinate ( $n = 29$ ;  $20.95 \pm 4.99$ ) swabs compared to anterior nasal swabs ( $n = 6$ ;  $27.31 \pm 1.09$ ) ( $P = .014$  and  $P = .010$ , respectively). However, relative abundance of sgRNA did not differ significantly by swab type ( $P \geq .4$  for all comparisons; Supplementary Figure 2). N2 Ct increased with day of illness at sample collection but with poor goodness-of-fit for the linear regression ( $R^2 = 0.05$ ,  $P = .006$ , Supplementary Figure 3A). Relative abundance of sgRNA did not differ significantly by day of illness ( $P = .07$ ; Supplementary Figure 3B). N2 Ct values and sgRNA did not differ significantly between vaccinated and unvaccinated individuals ( $P = .8$ , Supplementary Figure 4).

The distribution of N2 and sgRNA Ct values exhibited similar patterns by variant (Figure 1A and 1B); to evaluate how sgRNA varied in relation to genomic RNA, simple linear regression analysis of sgRNA versus N2 Ct was performed for each variant. All variants showed robust linear trends ( $R^2 \geq 0.89$ ) with slopes that varied only slightly (Figure 1C). Multiple linear regression was then performed to determine if there was effect modification on the relationship between N2 and sgRNA Ct by variant type, using ancestral strains as reference. In the final model, sgRNA Ct increased 1.24 cycles for each cycle increase in N2 (95% confidence interval [CI], 1.08–1.39;  $P < .001$ ) in the ancestral strain. For the Delta variant, sgRNA increased less relative to N2 Ct compared to ancestral strains, indicating delayed clearance of sgRNA for this variant (average,  $-0.32$  cycles [95% CI,  $-.54$  to  $-.10$ ];  $P = .005$ ). No other variants differed significantly from the ancestral strain. The final model had an  $R^2 = 0.91$  (Figure 1D, Supplementary Table 2). Final model results did not change when performed using only samples with sufficient volume for repeat N2RP testing or if day of illness was included.

## DISCUSSION

Abundance of sgRNA among SARS-CoV-2 variants is consistently predicted by levels of genomic RNA when compared to ancestral strains with D614G. Whereas previous studies of sgRNA either predated the widespread emergence of variants of concern or were not designed to compare sgRNA levels between variants [2, 5, 6, 11], the current study included acute-phase samples of SARS-CoV-2 variants of concern collected across varied patient populations using different swab types. In our analysis, Delta variant samples demonstrated lower sgRNA Ct values (higher levels of sgRNA) than predicted by N2 Ct alone. Although the estimate was small per unit increase in N2 Ct ( $-0.32$  cycles), this is consistent with a single published study that observed prolonged detection of sgRNA among individuals infected with the Delta variant compared to infections with other variants [12]. Taken together, these data demonstrate that the relative abundance of sgRNA is similar across variants and directly correlates with total SARS-CoV-2 RNA at the time of testing. This supports the general use of sgRNA detection to identify individuals with active viral replication and therefore at higher risk of SARS-CoV-2 transmission.

Antigen diagnostics for SARS-CoV-2 N protein correlate with sgRNA level in certain compartments and may better predict SARS-CoV-2 viability in clinical samples compared to rRT-PCR [6, 7]. Although antigen diagnostics come in convenient, rapid-test formats, there is concern that detection will be deleteriously affected by new variants and tests may be impacted differently based on the targeted epitopes [13]. In contrast, the sgRNA rRT-PCR assay utilized in the current study targets

highly conserved regions of the leader sequence and N-gene [6], and our data show that variant confirmation would not be necessary for variants identified to date. This is notable as the infecting variant cannot be immediately confirmed [14]. In addition, sgRNA detection can be performed with the original standard-of-care sample or residual RNA that was frozen following clinical testing.

Limitations of the current study include a small sample size for the Beta variant, which was not prevalent in areas from which samples were collected; inclusion of 3 different specimen types, though no difference in relative sgRNA abundance was observed across specimens; and low vaccine coverage among participants, limiting the evaluation of vaccination on sgRNA levels.

In conclusion, SARS-CoV-2 sgRNA strongly correlates with total SARS-CoV-2 RNA in clinical samples, and the relative abundance of sgRNA is similar among known variants. Assays for sgRNA could be implemented to identify individuals with active viral replication irrespective of the infecting SARS-CoV-2 strain.

## Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

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