Correlation of SARS-CoV-2 Subgenomic RNA with Antigen Detection in Nasal Midturbinate Swab Specimens

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Among symptomatic outpatients, subgenomic RNA of severe acute respiratory syndrome coronavirus 2 in nasal midturbinate swab specimens was concordant with antigen detection but remained detectable in 13 (82.1%) of 16 nasopharyngeal swab specimens from antigen-negative persons. Subgenomic RNA in midturbinate swab specimens might be useful for routine diagnostics to identify active virus replication.

Accurate detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is critical for patient management and infection control (*1*). Molecular diagnostics are highly sensitive in the acute phase of coronavirus diseases (COVID-19), but viral RNA remains detectable long after replicating virus can be isolated from respiratory samples (*1–5*). Antigen diagnostics, though often less sensitive, are touted as providing accurate detection during peak infectivity, thereby identifying persons most likely to transmit SARS-CoV-2 (*6*,*7*).

Prolonged SARS-CoV-2 RNA detection has led to evaluation of molecular assays to detect subgenomic RNA (sgRNA) or negative-strand RNA, which are produced during active viral replication (*2–5*,*8–10*). sgRNA detection has predominantly been studied in hospitalized adults who have COVID-19 (*2*,*3*,*5*,*8*,*9*); published reports have not compared sgRNA and antigen detection, which should be highly correlated. We compared real-time reverse transcription PCR (rRT-PCR) detection of nucleocapsid sgRNA, the most abundant sgRNA in SARS-CoV-2–infected cells (*2*), with nucleocapsid antigen detection among symptomatic outpatients who had SARS-CoV-2 infections.

The Study

We obtained 88 nasal midturbinate and 39 nasopharyngeal swab specimens (PurFlock Ultra Flocked Swabs; Puritan Medical Products, https://www.puritanmedproducts.com) from 127 persons who came to COVID-19 testing centers affiliated with Emory University and Children's Healthcare of Atlanta (Atlanta, GA, USA) during January 2021. Inclusion criteria were a symptomatic respiratory illness for ≤7 days and a positive, routine-care SARS-CoV-2 molecular test (nasopharyngeal swab specimen). The study was approved by the Emory University Institutional Review Board and Children's Healthcare of Atlanta.

We extracted total nucleic acids from 500 µL of sample and eluted them into a volume of 50 µL by using an EMAG Instrument (bioMérieux, https://www.biomerieux.com). We tested eluates side-by-side in rRT-PCRs for sgRNA and total SARS-CoV-2 RNA (genomic plus sgRNA). For sgRNA, we combined a forward primer in the leader sequence (5′-CGATCTCTTGTAGATCTGTTCTC-3′) with the nucleocapsid 2 (N2) target reverse primer and probe (*11*).

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DOI: https://doi.org/10.3201/eid2711.211135

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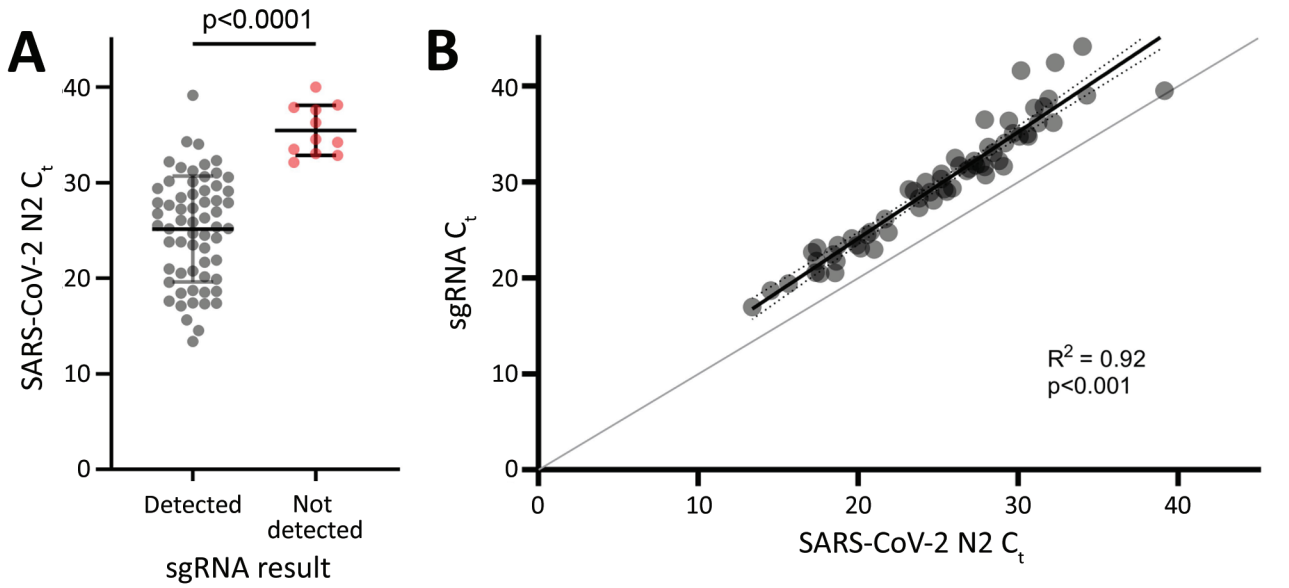

Figure 1. Correlation of sgRNA levels with total SARS-CoV-2 RNA in samples from study participants in Atlanta, Georgia, USA. A) N2 C_t values for samples in which sgRNA was detectable (gray dots) or not detectable (red dots). Horizontal bars indicate means, and error bars indicate SDs. B) sgRNA C_t values versus corresponding C_t values for the N2 target. Results of simple linear regression (black line) and error bars (dotted lines) are shown. Line of identity (gray line) is shown for reference. C_t, cycle threshold; N2, nucleocapsid 2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sgRNA, subgenomic RNA.

We performed the sgRNA assay in 20-µL reactions using the Luna Probe One-Step RT-qPCR Kit (New England Biolabs, https://www.neb.com) with 500 nmol/L of each primer, 250 nmol/L of probe, and 5 µL of eluate by using the following conditions: 55°C for 15 min, 95°C for 2 min, and 45 cycles of 95°C for 15 s and 60°C for 60 s. We detected total SARS-CoV-2 RNA by using a duplex N2-RNase P rRT-PCR performed as described (*12*). We obtained an anterior nares swab specimen for nucleocapsid antigen detection with the Abbott BinaxNOW COVID-19 Ag Card (swabs supplied with the BinaxNOW kit; Abbott Laboratories, https://www.abbott.com) performed per the package insert.

The first 73 participants had a midturbinate swab specimen available for molecular testing (evaluation group) and have been described (*13*). The subsequent 54 participants had dedicated midturbinate (n = 15) or residual nasopharyngeal (n = 39) swab specimens for molecular testing and available antigen test results (antigen testing group) (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/27/11/21-1135-App1.pdf).

We estimated nucleocapsid sgRNA as a percentage of total RNA by calculating copies per microliter of sgRNA and total RNA for each sample based on a standard curve for each target and then calculating the percentage of sgRNA. We used unpaired t-tests to compare continuous variables and the Fisher exact test for testing categorical variables. We performed simple linear regression to compare cycle threshold (C_t) values for sgRNA and total RNA. We conducted

Table 1. Demographic and clinical variables of study participants who had MT swab specimens in antigen-testing group analyzed for SARS-CoV-2 subgenomic RNA, Atlanta, Georgia, USA*

Variable	Overall, n = 15	Antigen positive, n = 8	Antigen negative, n = 7	p value
Mean age, y (SD)	54.35 (14.49)	53.98 (16.12)	54.78 (13.65)	0.921
Female sex	9 (60.00)	5 (62.50)	4 (57.14)	1.000
Mean days after symptom onset (SD)†	4.14 (2.44)	3.88 (2.23)	4.50 (2.88)	0.655
MT swab specimen, rRT-PCR positive	12 (80.0)	8 (100.0)	4 (57.1)	0.077
Race‡				
White	2 (14.3)	0	3 (33.3)	0.026
Black/African American	11 (78.6)	8 (100.0)	3 (50.0)	NA
Asian	11 (78.6)	8 (100.0)	3 (50.0)	NA

*Values are no. (%) unless indicated otherwise. MT, nasal midturbinate; NA, not available; rRT-PCR, real-time reverse transcription PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
†Day sample was collected.
‡One participant did not identify race; none identified as Hispanic.

Table 2. Correlation of SARS-CoV-2 subgenomic RNA with nucleocapsid detection in NP specimens from study participants analyzed for SARS-CoV-2 subgenomic RNA, Atlanta, Georgia, USA *

Variable	Overall, n = 39	Antigen positive, n = 20	Antigen negative, n = 19	p value
Mean age, y (SD)	8.6 (5.8)	9.8 (5.6)	7.4 (5.8)	0.148
Female sex	16 (41.0)	8 (40.0)	8 (42.1)	0.894
Mean days after symptom onset (SD)†	3.7 (2.2)	3.0 (1.4)	4.5 (2.7)	0.227
Repeat NP swab specimen, rRT-PCR positive	36 (92.3)	20 (100.0)	16 (84.2)	0.106
Race				
White	26 (66.67)	13 (65.0)	13 (68.42)	0.077
Black/African American	6 (15.38)	3 (15.0)	3 (15.79)	NA
Asian	4 (10.26)	4 (20.0)	0	NA
Biracial	3 (7.69)	0	3 (15.79)	NA
Hispanic ethnicity	19 (48.72)	6 (30.0)	13 (68.42)	0.016

*Values are no. (%) unless indicated otherwise. NA, not available; NP, nasopharyngeal; rRT-PCR, real-time reverse transcription PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
†Day sample was collected. One participant was asymptomatic (did not report symptoms in the past 14 days).

analyses by using GraphPad version 9.02 (https://www.graphpad.com) and SAS version 9.4 (https://support.sas.com).

The evaluation group included midturbinate swab specimens from 36 adults and 37 children. All samples (73/73) were positive for SARS-CoV-2 by rRT-PCR. Samples with detectable sgRNA (62/73, 84.9%) had significantly lower C_t values, indicative of higher viral loads, than samples without detectable sgRNA (mean C_t 25.1, SD 5.5, vs. mean C_t 35.5, SD 2.6; p<0.0001) (Figure 1, panel A). sgRNA was detectable in all samples (49/49) that had N2 C_t values <30 compared with 13 (54.2%) of 24 samples that had C_t values ≥30 (p<0.0001). Although sgRNA rRT-PCR amplification efficiency was slightly lower than that for the N2 assay, there was a strong linear correlation between sgRNA and N2 C_t values (Figure 1, panel B), and the assay provided linear sgRNA detection across the range of N2 C_t values observed in this study (Appendix Figure 2). sgRNA C_t values were a mean of 4.8 (SD 1.8) cycles higher than corresponding N2 C_t values, and nucleocapsid gene sgRNA accounted for a mean of 1.4% (SD 1.1%) of SARS-CoV-2 RNA. Samples from children had higher viral loads than samples from adults, although the relative amount of sgRNA did not differ (Appendix Figure 3).

We complied characteristics of participants in the antigen-testing group who had midturbinate (n = 15) swab specimens (Table 1) and nasopharyngeal (n = 39) swab specimens (Table 2). All midturbinate swab specimens from participants who had detectable antigen (n = 8) were also positive for sgRNA, whereas 0/4 samples from antigen-negative persons were positive (κ 1.0). Samples that had detectable sgRNA had significantly lower C_t values (mean 25.8, SD 2.7) than samples that did not have detectable sgRNA (mean 36.3, SD 1.8; p = 0.002) (Figure 2).

All (20/20) nasopharyngeal swab specimens from antigen-positive participants were positive for sgRNA. N2 C_t values were significantly lower among antigen-positive participants (mean 18.2, SD 5.0) than antigen-negative participants (mean 27.8, SD 4.5; p<0.0001) (Figure 2). sgRNA was detectable in 13 (81.2%) of 16 nasopharyngeal swab specimens from antigen-negative persons. Days after symptom onset (when the sample was collected) did not differ significantly between antigen-positive and sgRNA-positive/antigen-negative participants (mean 3.4, SD 1.9 days, vs. mean 3.8, SD 2.4 days; p = 0.6).

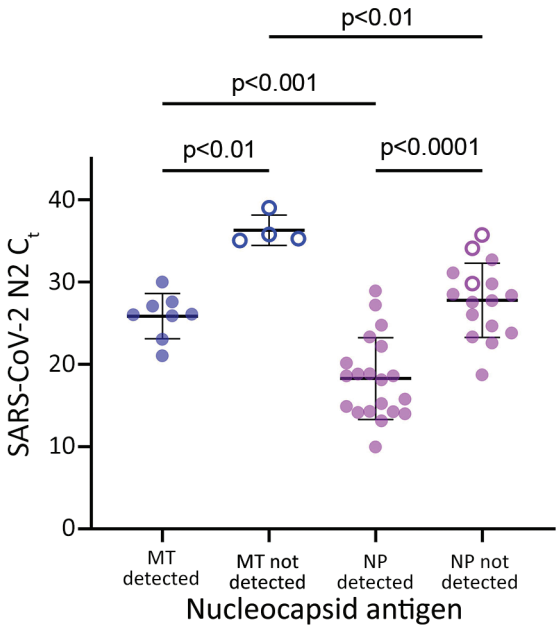

Figure 2. Concordance of SARS-CoV-2 sgRNA with nucleocapsid antigen detection in MT swab specimens, but not NP swab specimens, from study participants in Atlanta, Georgia, USA. sgRNA remains detectable in NP swab specimens for persons who showed negative results for nucleocapsid antigen. Symbols represent MT (blue) and NP (purple) swab specimens for persons with (filled circles) and without (open circles) detectable sgRNA. Horizontal bars indicate means, and error bars indicate SDs. C_t, cycle threshold; MT, nasal midturbinate; NP, nasopharyngeal; N2, nucleocapsid 2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sgRNA, subgenomic RNA.

Nucleocapsid gene sgRNA accounted for a smaller percentage of total SARS-CoV-2 RNA in antigen-negative participants (mean 0.6%, SD 0.4%) vs. antigen-positive participants (mean 1.0%, SD 0.5%; p = 0.012) (Appendix Figure 4). Compared with midturbinate swab specimens, nasopharyngeal swab specimens had lower C_t values for RNase P (Appendix Figure 5).

Conclusions

SARS-CoV-2 sgRNA was detected in all samples from antigen-positive participants (28/28 total), consistent with identification of active viral replication and potential shedding (*4*,*5*,*8*). However, among antigen-negative participants, sgRNA detection varied between SARS-CoV-2 RNA-positive midturbinate (0/4) and nasopharyngeal (13/16) swab specimens. Although nasopharyngeal swab specimens are expected to have higher viral loads (*14*), this difference did not appear to be the sole explanation. sgRNA represented a smaller proportion of total SARS-CoV-2 RNA in discordant nasopharyngeal swab specimens, and overall, nasopharyngeal swab specimens had higher amounts of human cellular material (lower RNase P C_t values) than midturbinate swab specimens. Therefore, discordant sgRNA and antigen results in nasopharyngeal swab specimens probably resulted from persistent detection of waning SARS-CoV-2 infections with low levels of detectable sgRNA, which is only found in infected cells but insufficient viral replication to yield detectable nucleocapsid antigen in the anterior nares.

Nucleocapsid antigen was detected by using the widely available BinaxNOW COVID-19 Ag Card. This card demonstrates similar performance to other rapid antigen tests, which commonly detect nucleocapsid protein, and maintains analytical sensitivity against SARS-CoV-2 variants (*7*). Therefore, it provided a useful and relevant comparator for sgRNA detection.

Limitations of our study include a relatively small number of midturbinate swab specimens tested in the antigen-testing group, which was affected by the need for multiple swab specimens at a single time point. The race/ethnicity makeup of groups that had midturbinate and nasopharyngeal swab specimens differed (Tables 1, 2), although this limitation is not expected to have affected our findings (*15*).

In conclusion, sgRNA detection in midturbinate swab specimens correlates with nucleocapsid antigen and could be implemented as a molecular test to evaluate infectivity. Given the strong correlation between sgRNA, nucleocapsid antigen, and total SARS-CoV-2 RNA, these data also support use of antigen testing or establishment of rRT-PCR C_t values as markers of active replication.

Acknowledgments

We thank the staff of the Atlanta Center for Microsystems Engineered Point-of-Care Technologies for their contributions and efforts, and study participants and family members for providing samples and clinical information.

This study was supported by the National Institutes of Health/National Institute of Biomedical Imaging and Bioengineering (grants U54 EB027690 02S1 and UL1 TR002378).

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At the time of this study, Ms. Immergluck was a research specialist in the Division of Infectious Diseases, Emory University School of Medicine, Atlanta, GA. She is currently completing her final year at Pomona College, Claremont, CA. Her research interest is development of new molecular methods for the detection of RNA viruses.

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etymologia

Prototheca [pro″to-the′kə]

Rüdiger D. Ollhoff, Fábio P. Sellera, Fabio C. Pogliani

From the Greek *proto-* (first) + *thēkē* (sheath), *Prototheca* is a genus of variably shaped spherical cells of achloric algae in the family *Chlorellaceae*. Wilhelm Krüger, a German expert in plant physiology and sugar production, reported *Prototheca* microorganisms in 1894, shortly after spending 7 years in Java studying sugarcane. He isolated *Prototheca* species from the sap of 3 tree species. Krüger named these organisms as *P. moriformis* and *P. zopfii*, the second name as a tribute to Friedrich Wilhelm Zopf, a renowned botanist, mycologist, and lichenologist.

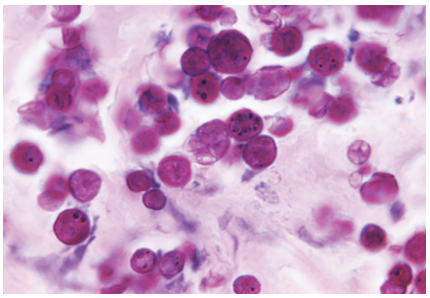

Figure 1. Periodic acid–Schiff–stained tissue sample from a case-patient who had protothecosis, showing several sphere-like cells of *Prototheca* spp. Source: Dr. Jerrold Kaplan, Centers for Disease Control, 1971.

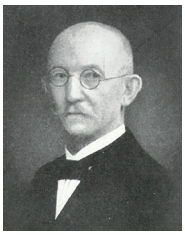

Figure 2. Wilhelm Krüger (1857–1947). Source: Institute for Sugar Beet Research (http://www.ifz-goettingen.de).

Protothecosis affects humans and wild and domestic animals, primarily causing mastitis in cows. Human protothecosis was reported in 1964 from a skin lesion in a farmer from Sierra Leone. There are increasing reports of infections in immunocompromised patients. Debates regarding *Prototheca* taxonomy persist.

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DOI: https://doi.org/10.3201/eid2801.211554

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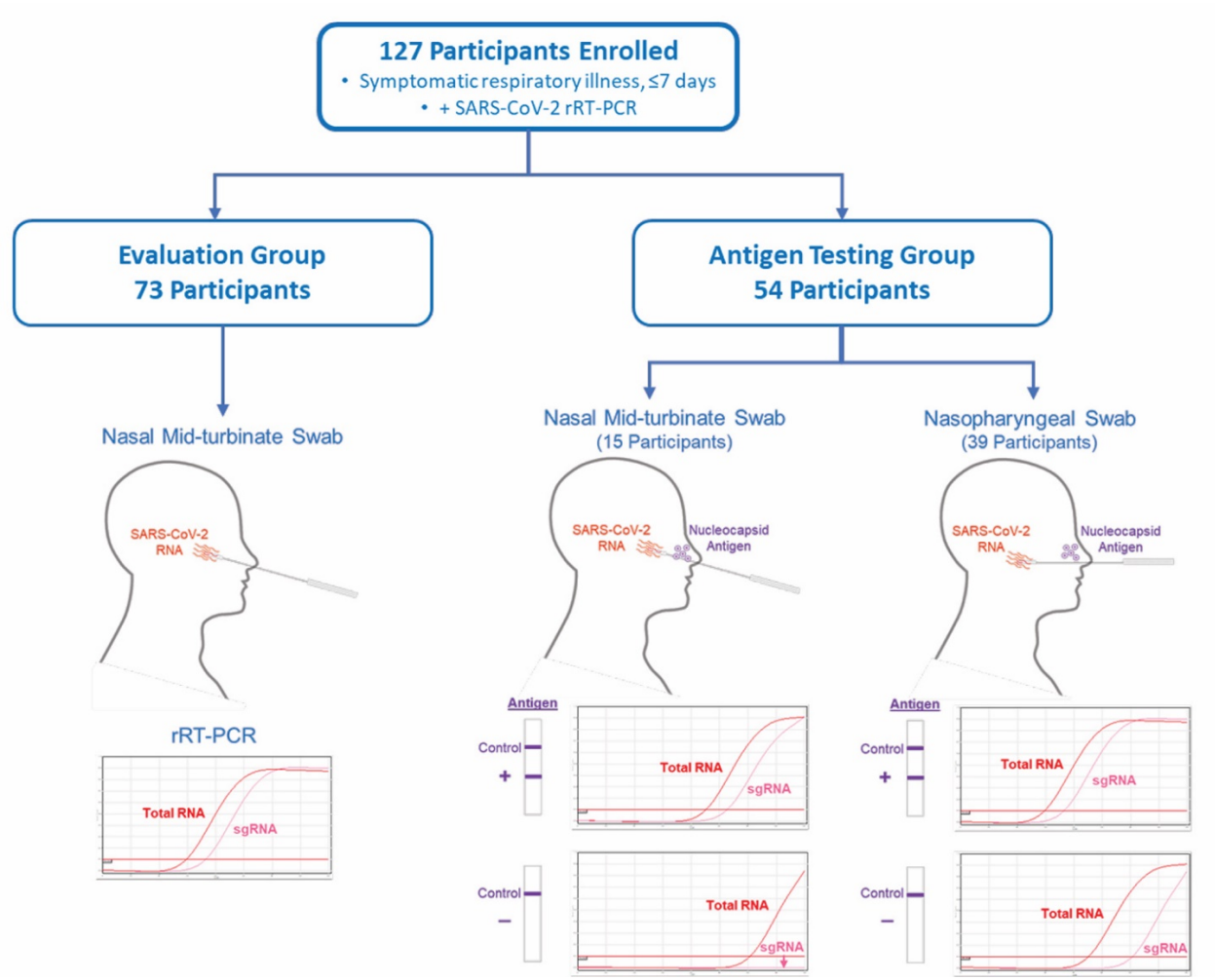
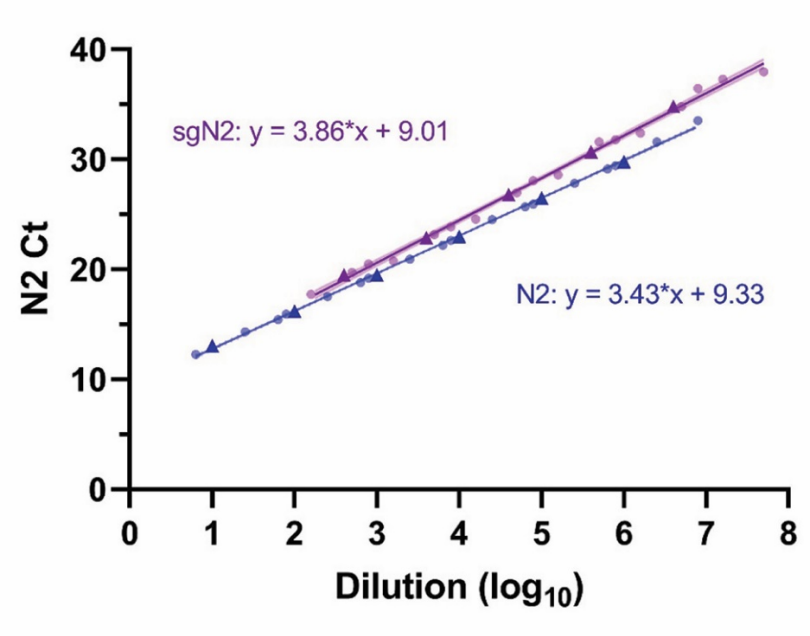
Article DOI: https://doi.org/10.3201/eid2711.211135

Correlation of SARS-CoV-2 Subgenomic RNA with Antigen Detection in Nasal Midturbinate Swab Specimens

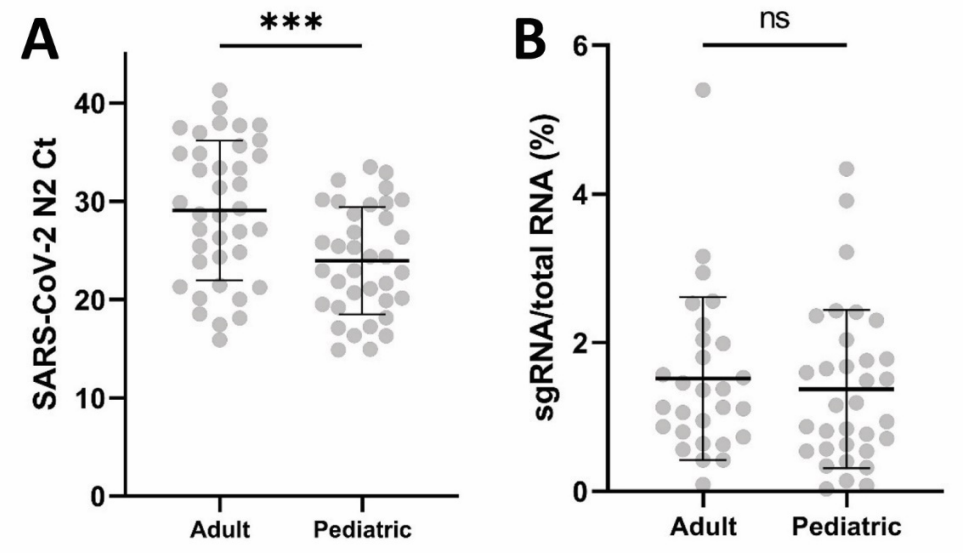
Appendix



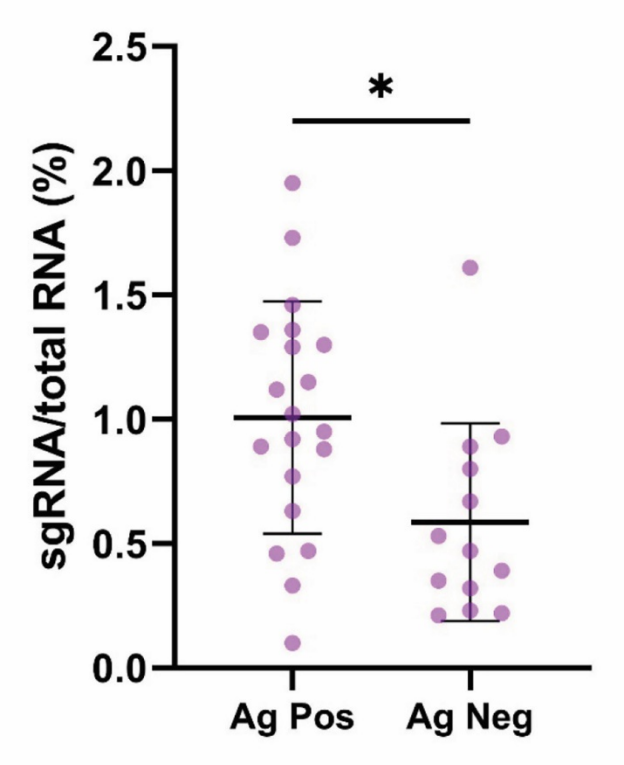
Appendix Figure 1. Study flowchart for 127 enrolled participants. Those in the evaluation group had a nasal midturbinate swab tested for total SARS-CoV-2 RNA and N gene containing sgRNA. Participants in the antigen-testing group had nucleocapsid antigen detection in the anterior nares compared with SARS-CoV-2 total RNA and sgRNA detected in nasal midturbinate or nasopharyngeal swab specimens. rRT-PCR, real-time reverse transcription PCR; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sgRNA, subgenomic RNA.



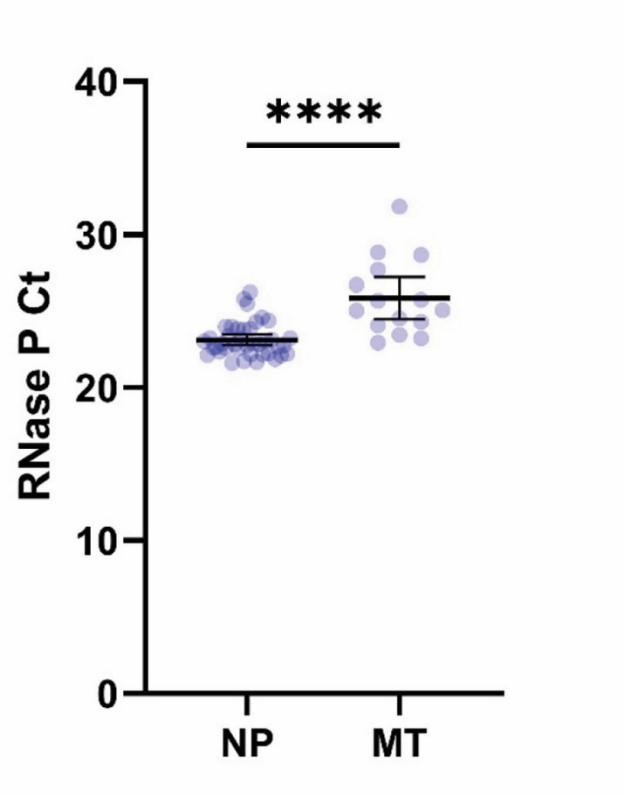
Appendix Figure 2. Linear regression of rRT-PCR results for sgRNA (sgN2, purples line) and total severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 RNA) (N2, blue line) using a 10-fold dilution series of 4 nasal midturbinate samples from the evaluation group. A standard curve was generated by using sgRNA and total RNA C_t values from a single sample (purple and blue triangles, respectively), accounting for sgRNA abundance relative to total RNA when plotting the first dilution. Series from the remaining samples were normalized to these standard curves and all data was incorporated in a linear regression. Plot displays the linear regression line and 95% CI (shaded region). Efficiency of rRT-PCRs based on this regression are 95.6% for N2 and 81.3% for sgN2. C_t, cycle threshold; rRT-PCR, real-time reverse transcription PCR; sgRNA, subgenomic RNA.



Appendix Figure 3. Distribution of SARS-CoV-2 rRT-PCR, real-time reverse transcription PCR (rRT-PCR) C_t, values (N2 target) in adult and pediatric nasal midturbinate swab specimens from A) the evaluation group and B) comparison of the percentage of total SARS-CoV-2 RNA accounted for by sgRNA. Horizontal bars indicated means, and error bars indicate SDs. C_t, cycle threshold; ns, not significant; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sgRNA, subgenomic RNA. ***$p<0.001$.



Appendix Figure 4. Percentage of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) total RNA accounted for by sgRNA in nasopharyngeal swab specimens from participants who were positive or negative for nucleocapsid antigen. Horizontal bars indicate means, and error bars indicate SDs. Ag, antigen; Neg, negative; Pos, positive; sgRNA, subgenomic RNA. *p<0.05.



Appendix Figure 5. RNase P C_t values for NP and MT swab specimens in the antigen-testing group. NP swab specimens had lower C_t values (mean 23.1, SD 1.1) than s MT swab specimens (mean 25.85, SD 2.5). Horizontal bars indicate means, and error bars indicated SDs. C_t, cycle threshold; MT, nasal midturbinate; NP, nasopharyngeal. ****$p<0.0001$.