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Maxwell Su April 2, 2023

Impact of Omicron sublineage, natural and vaccine-derived immunity on SARS-CoV-2 subgenomic RNA abundance

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Abstract

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SARS-CoV-2 subgenomic RNA (sgRNA) are RNA transcripts produced during viral replication that indicate active infection or allow for identification of patients with a higher likelihood of transmission. sgRNA abundance has been systematically compared between SARS-CoV-2 variants, but no differences in relative abundance have been found. However, additional Omicron sublineages have appeared, and the link between sgRNA and prior immunity derived by vaccination or infection has not yet been established. For the current study, sgRNA was quantified by rRT-PCR in 246 clinical samples from symptomatic patients collected before the rollout of the bivalent Omicron booster and 94 clinical samples collected after the rollout. sgRNA was found to correlate with total viral RNA. Similar relative sgRNA abundance was demonstrated among Omicron sublineages, individuals from differing vaccination strata, individuals with and without previous COVID infection, and individuals with and without the bivalent booster. Thus, sgRNA detection can identify individuals with active viral replication regardless of sublineage or vaccination status. These data are consistent with current guidance on isolation for symptomatic individuals and indicate that prior immunity may not play a role in mitigating SARS-CoV-2 transmission in breakthrough infections. Participants may have been immunologically imprinted with ancestral variants, and the concept of the original antigenic sin should be explored further in relation to SARS-CoV-2.

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INTRODUCTION

SARS-CoV-2, the virus that causes COVID-19 and has resulted in the ongoing global pandemic, is a single-stranded, positive sense RNA virus with a genome of around 30 kilobases [1]. SARS-CoV-2 replication progresses through the transcription of negative-sense RNA templates for both positive-sense genomic RNA and subgenomic RNAs (sgRNAs). sgRNAs are produced by discontinuous transcription that occurs as part of active replication of genes for viral structural proteins, including envelope (E), membrane (M), nucleocapsid (N), and spike (S) (**Figure 1**) [2]. sgRNAs are characterized by an untranslated leader sequence from the 5' end of the genome linked to the structural protein genes located at the 3' end of the genome [1]. As a result of this altered genomic arrangement, sgRNAs can be selectively amplified by RT-PCR and distinguished from full-length genomic RNA.

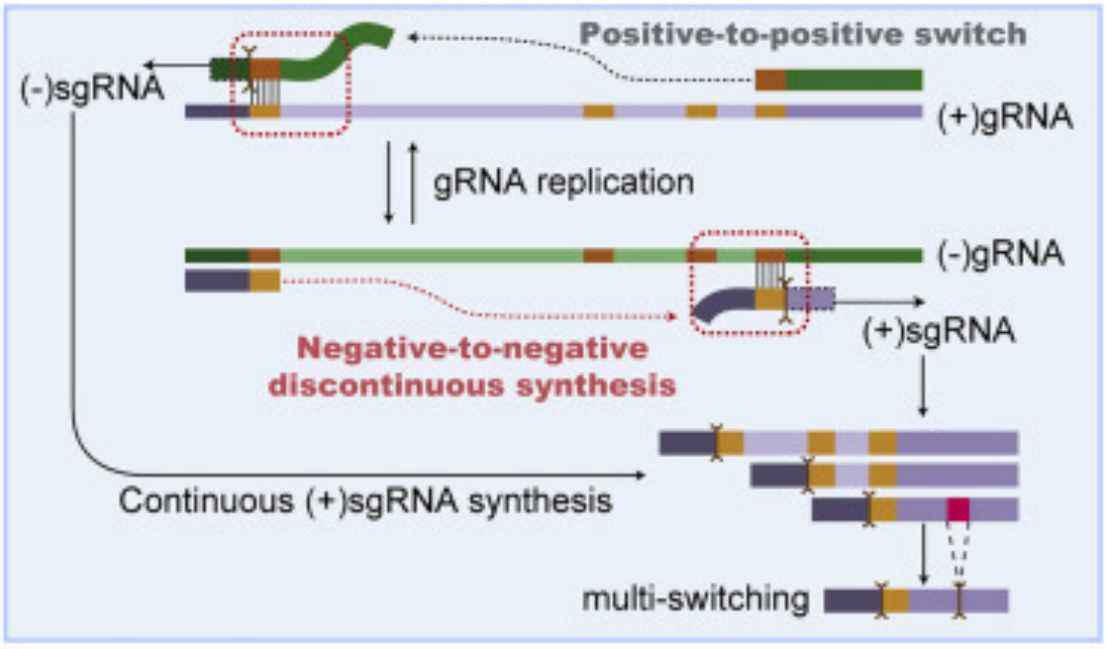

Figure 1. Depiction of SARS-CoV-2 replication and transcription of both genomic and subgenomic RNA. Strands are displayed 5' (left) to 3' (right). The dark purple block at the 5' end of each sgRNA transcript represents the leader sequence. Adapted from Wang et al. [3].

SARS-CoV-2 sgRNA has been studied as a marker of active replication, to track antiviral response, and to identify patients at higher likelihood of viral transmission [4, 5]. Current molecular diagnostics for SARS-CoV-2 detect total viral RNA but cannot distinguish genomic from sgRNA [6]. 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 [7]. This led to the development of molecular assays for sgRNA, including a real-time RT-PCR (rRT-PCR) developed in our lab to detect nucleocapsid (N)-gene sgRNA (**Figure 2**) [8], which is the most abundant SARS-CoV-2 sgRNA [5].

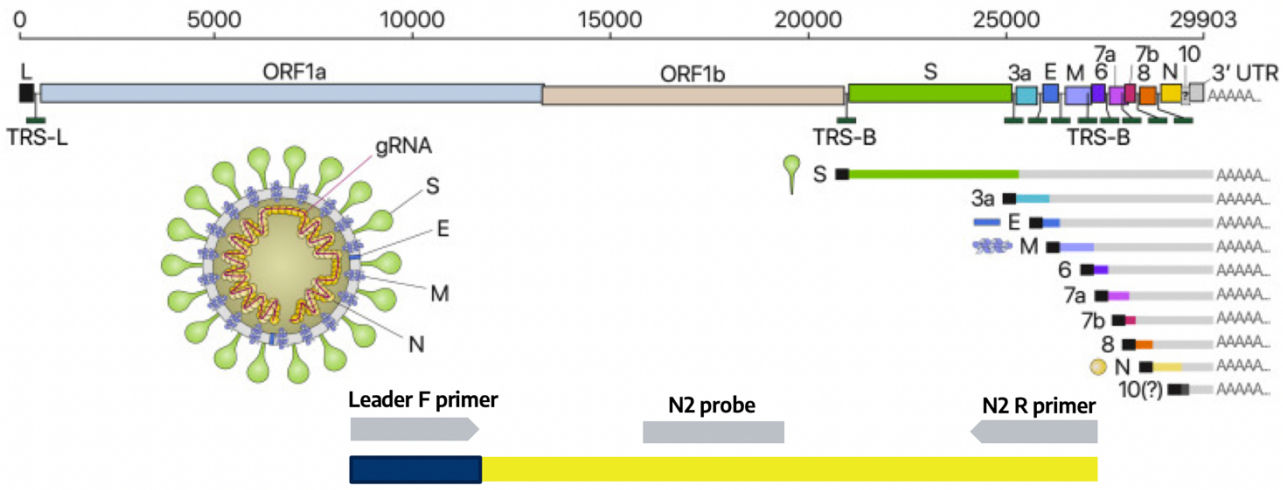

Figure 2. Representation of sgRNA rRT-PCR assay used in our lab. Our assay utilizes a forward primer for the leader sequence, which is attached to sgRNA transcripts as they are produced through discontinuous transcription. This allows us to use a probe and reverse primer for the N2 sequence without picking up a signal from genomic RNA. Adapted from Kim. et al [5].

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 [5, 7, 8]. Considering that N-gene mutations in SARS-CoV-2 variants may generate novel sgRNAs and increase sgRNA expression, possibly influencing detection, repeated evaluation of sgRNA abundance is necessary with the emergence of new variants if such assays are to be used to identify individuals with active viral replication in a variant-agnostic manner [1]. To address this in a previous study, 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. Our data demonstrated that the relative abundance of sgRNA is similar across variants (Alpha, Beta, Delta, P.2, early Omicron strains) and directly correlated with total SARS-CoV-2 RNA at the time of testing [9]. This supports the general use of sgRNA detection regardless of the infecting variant to identify individuals at a higher risk of SARS-CoV-2 transmission.

However, there are still many other factors that may influence relative sgRNA abundance in SARS-CoV-2 infections. Now that Omicron variant cases represent a majority of SARS-CoV-2 infections, it is important to determine if Omicron sublineage (BA.1, BA.2, BA.3, etc.) may affect sgRNA abundance. Additionally, there is a lack of information on how vaccination status, vaccine timing, and previous COVID may impact sgRNA abundance. The rollout of the bivalent booster, which contains mRNA from the spike protein from both ancestral strains and the BA.4/BA.5 Omicron sublineages [10], may change sgRNA abundance as these sublineages become more widespread. Gathering additional results may allow us to refine the understanding of how immunity, resulting from both vaccination and natural infection, impacts sgRNA abundance as a mechanism to control SARS-CoV-2 in acute infection and affect the likelihood of transmission.

Objectives

1. **Study the effects of previous COVID infection, Omicron sublineage, and vaccination with monovalent mRNA vaccines on relative sgRNA abundance.** Based on our previous work, we expected that these factors will not have a quantifiable effect on relative sgRNA, as the differences in infectivity between SARS-CoV-2 variants did not translate into differences in sgRNA abundance. Additionally, without bivalent booster availability at the time these samples were collected, a lack of Omicron-specific immunity would likely translate into similar relative abundance across vaccination strata.

2. **Determine how sgRNA varies between participants with and without the bivalent booster.** This objective leveraged both acute-phase samples and paired acute- and convalescent-phase samples from the same participant. We expected that receipt of the bivalent booster will significantly decrease sgRNA detection in both cases. We also hypothesized that acute-phase samples would have greater relative sgRNA when compared to convalescent-phase samples, due to the differences in active replication between these disease stages.

METHODS

Clinical samples

For Objective 1, 246 acute-phase nasal mid-turbinate (MT) samples, positive for Omicron variant, were selected from an ongoing study to characterize SARS-CoV-2 variants among individuals in the Atlanta area tested at the Emory/Children's Laboratory for Innovative Assay Development (ELIAD). ELIAD samples include MT swabs collected from individuals

with an acute respiratory illness of ≤7 days who present to COVID-19 testing centers affiliated with Emory University and Children's Healthcare of Atlanta. The initial sample set included 683 acute-phase samples from the ELIAD study. Thirty-one samples without detectable genomic RNA in initial testing and 11 samples without data regarding vaccination status or previous COVID infection were removed. Next, due to a low sample size and possible confounding of our analysis, we removed 23 samples from participants who received the Johnson&Johnson vaccine, 18 samples from participants who were partially vaccinated (defined as having 1 dose), and 2 samples collected by nasopharyngeal swab. For the remaining 598 samples, sublineages were defined as BA.1/BA.3, BA.2, and BA.4/BA.5, and vaccination status was defined as unvaccinated, fully vaccinated, and boosted. All participants received either Moderna or Pfizer vaccine doses, so unvaccinated status was defined as having 0 doses, fully vaccinated status was defined by having 2 doses, and boosted status was defined by having >2 doses. Samples were chosen randomly from each subcategory until a maximum of 30 were selected for each combination of sublineage and vaccination status. Because there were not enough samples of BA.1/BA.3 sublineage in all vaccination categories, we included all possible samples. All possible samples from participants with previous COVID infection were also included due to low sample size.

For Objective 2, we included 72 additional acute-phase nasal samples from the ongoing ELIAD study (n=13 with the bivalent booster) to evaluate the effects of the bivalent booster on relative sgRNA abundance in Omicron infections. These samples included all samples tested that had a sample collection date past the rollout date of the bivalent booster (September 2, 2022). We also included paired MT swabs from 11 participants (n=2 with the bivalent booster), with

one acute-phase sample collected 7 days or less after symptom onset and one convalescent-phase sample collected 14 +/- 4 days afterwards.

Sample size calculation

The target sample size was 25 participants in each sublineage/vaccination category. Sample size calculation was based on difference between genomic RNA Ct and sgRNA Ct among non-bivalent booster recipients found in our previous study (mean 5.6, standard deviation of 1.6) [9]. This provided 80% power to detect a 0.5 $\log_{10}$ difference in the relative abundance of sgRNA between comparison categories. Where possible, 30 samples were selected to account for run failures or lack of detection. Sample size was calculated with PASS software.

Molecular testing

Samples were extracted using a KingFisher Apex instrument (Thermo Fisher Scientific, Waltham, MA) from 400µL of sample into 50µL of elution buffer. All eluates were tested in the rRT-PCR Spike SNP assay including probes for 417K, 452R, 478K, 484K, and 501Y as previously described [11, 12] and compared to expected profiles of known variants to confirm Omicron lineage. Eluates were also tested in the Yale triplex rRT-PCR assay, including probes for the Δ69/70 deletion, the wild-type 69/70 region, and the wild-type ORF1a region in order to confirm Omicron sublineage [13]. Following initial testing, nucleic acid eluates were stored at -80°C until tested again for sgRNA in the current study. Samples were thawed and tested with an rRT-PCR for the detection of SARS-CoV-2 N-gene sgRNA, as previously described [8], alongside our rRT-PCR for genomic RNA (the N2 target). Paired acute and convalescent

samples from the same patient for Objective 2 were tested on the same rRT-PCR run to limit run-to-run variability.

Statistical analysis

Relative quantification of genomic RNA and sgRNA was measured by Ct value, the cycle at which signal crosses a detection threshold in rRT-PCR. Low Ct values equate to high concentrations of RNA. Relative abundance of sgRNA was measured as the difference between sgRNA and N2 Ct values. The difference between values is used due to the logarithmic relationship between Ct value and RNA concentration. Continuous variables were compared by two-sided t-test with multiple comparison corrections. Linear regression, ANOVA, and multiple linear regression were performed to evaluate whether sgRNA level differed by previous COVID status, sublineage, vaccination status, or disease timepoint. All statistical analyses were performed in GraphPad Prism version 9.3.1 (GraphPad, San Diego, CA).

RESULTS

The 246 samples for Objective 1 had the following distribution: BA.1/BA.3 (n=66), BA.2 (n=90), and BA.4/BA.5 (n=90) Omicron sublineages as determined by initial testing; unvaccinated (n=75), fully vaccinated (n=83), and boosted (n=88) individuals; and individuals with (n=71) and without (n=175) previous COVID infection (**Table 1**). SARS-CoV-2 genomic RNA was detectable in 238 (96.7%) of samples, while sgRNA was detectable in 166/246 (67.5%) samples. sgRNA was detected in a significantly higher proportion (by chi-squared test) of BA.2 samples vs. both BA.1/BA.3 (p=0.003) and BA.4/BA.5 (p=0.020) samples, as well as in a significantly higher portion of samples from boosted vs. fully vaccinated individuals (p=0.038)

(**Table 2**). Female participants were more likely than males to have detectable sgRNA (p=0.003), and participants without previous COVID infection were also more likely to have sgRNA detection than those with previous infection (p=0.032) (**Table 3**). Age and symptomatic illness were also analyzed, but no significant association was found.

		Sublineage			
Vaccination Status		**BA.1/BA.3**[a]	**BA.2**	**BA.4/BA.5**[a]	**Grand Total**
Total		**66**	**90**	**90**	**246**
Unvaccinated		**28** (42.4)	**30** (33.3)	**30** (33.3)	**88 (35.8)**
Previous COVID, yes (%)		5 (17.9)	4 (13.3)	12 (40.0)	21 (23.9)
Fully Vaccinated		**23** (34.8)	**30** (33.3)	**30** (33.3)	**83 (33.7)**
Previous COVID, yes (%)		3 (13.0)	8 (26.7)	14 (46.7)	25 (30.1)
Boosted		**15** (22.7)	**30** (33.3)	**30** (33.3)	**75 (30.5)**
Previous COVID, yes (%)		0 (0)	7 (23.3)	18 (60.0)	25 (33.3)

Table 1. Distribution of ELIAD samples by sublineage/vaccination status/previous COVID infection. All data presented as n (%).

[a]Sublineage stratification based on Yale triplex assay results – BA.1 is indistinguishable from BA.3, BA.4 from BA.5

	Boosted	Fully Vaccinated	Unvaccinated	Grand Total
BA.1/BA.3[a]	9/12 (75.0)	10/22 (45.5)	16/26 (61.5)	**35/60 (58.3)**
BA.2[b]	25/30 (83.3)	22/30 (73.3)	25/29 (86.2)	**72/89 (80.9)**
BA.4/BA.5[c]	22/30 (73.3)	17/30 (56.7)	20/30 (66.7)	**59/90 (65.6)**
Grand Total	**56/75 (74.7)**	**49/83 (59.0)**	**61/88 (69.3)**	**166/239 (69.5)**

Table 2. Qualitative detection of sgRNA by rRT-PCR by Omicron sublineage and vaccination status. All entries formatted as sgRNA+/N2+ (%).

[a]6 samples negative in N2 (2 unvaccinated, 1 fully vaccinated, 3 boosted)

[b]1 sample (unvaccinated) negative in N2

[c]All samples positive in N2

	Previous COVID+	Previous COVID-	Grand Total
Female	29/42 (69.0)	78/97 (80.4)	**107/139 (77.0)**
Male	12/27 (44.4)	47/73 (64.4)	**59/100 (59.0)**
Grand Total	**41/69 (59.4)**	**125/170 (73.5)**	**166/239 (69.5)**

Table 3. Qualitative detection of sgRNA by rRT-PCR by gender and previous COVID status. All entries formatted as sgRNA+/N2+ (%).

N2 and sgRNA Ct distributions are shown by vaccination status (**Figure 3**), Omicron sublineage (**Figure 4**), and previous COVID infection (**Figure 5**). sgRNA Ct distributions include samples with detectable sgRNA (n=166). Fully vaccinated individuals had significantly higher N2 Cts (less SARS-CoV-2 RNA) when compared to unvaccinated individuals, and had

higher N2 Cts approaching significance compared to boosted individuals (p=0.056) (Figure 3). Additionally, individuals with previous COVID infection had higher N2 Ct values than those who did not (Figure 5), but these differences did not translate into significant changes in sgRNA abundance.

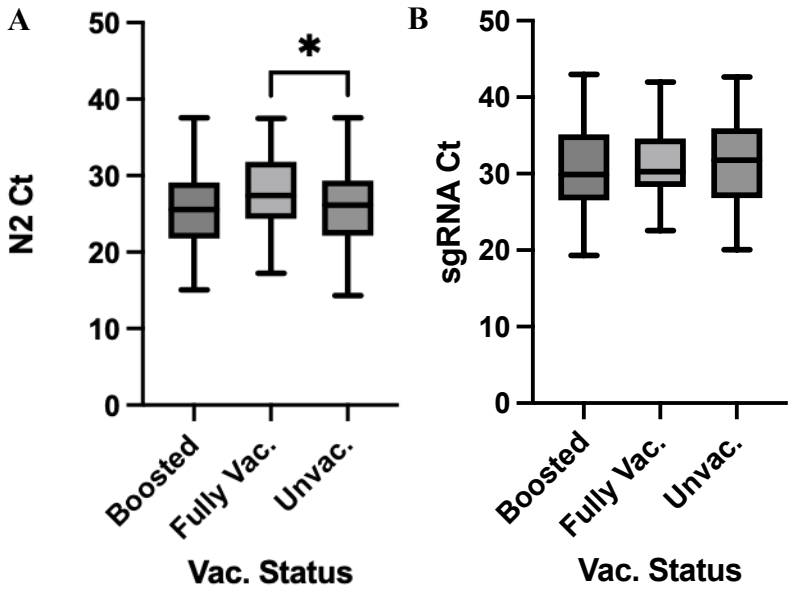

Figure 3. Box-and-whisker plots of N2 Ct (A) and sgRNA Ct (B) by vaccination status. Fully vaccinated individuals were found to have significantly higher (p=0.046) N2 Cts when compared to unvaccinated individuals by Tukey multiple comparison test. No other significant statistical differences were found (Boosted vs. Fully Vac. p=0.056, Boosted vs. Unvac. p=1.0 (A), p=0.725 by one-way ANOVA (B)).

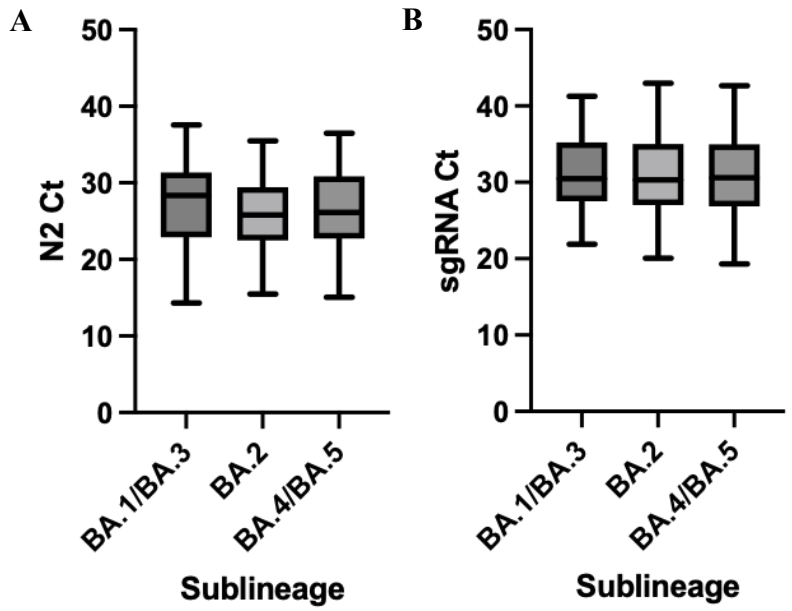

Figure 4. Box-and-whisker plots of N2 Ct (A) and sgRNA Ct (B) by Omicron sublineage. No significant statistical differences were found (p=0.119 (A), p=0.975 (B)).

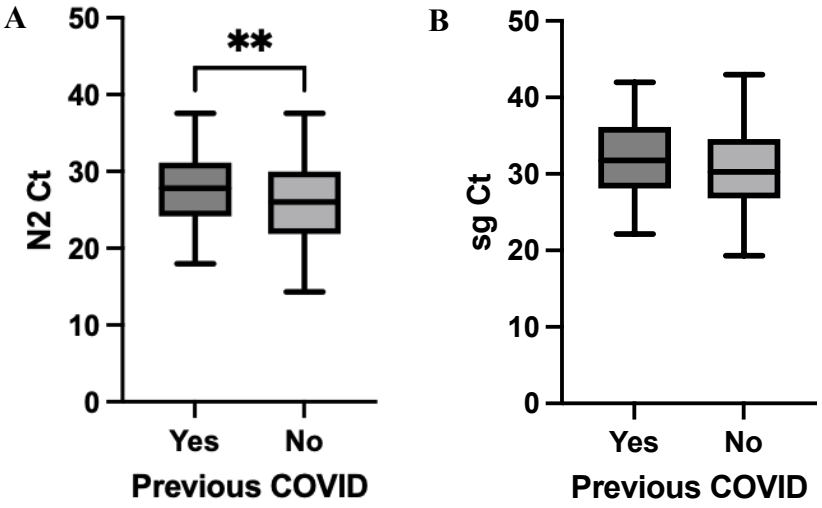

Figure 5. Box-and-whisker plots of N2 Ct (A) and sgRNA Ct (B) by previous COVID infection. Individuals with previous COVID infection were found to have significantly higher (p=0.009) N2 Cts when compared to individuals without previous infection by unpaired t-test. No difference was found in sgRNA Ct (p=0.2119).

In previous studies, sgRNA demonstrated a direct linear relationship with genomic RNA. Multiple linear regression was performed with the current data set to determine if there was effect modification on the relationship between N2 and sgRNA Ct by vaccination status, Omicron sublineage, previous COVID infection, and other demographic and clinical information including gender and time since last vaccination dose. Unvaccinated status was used as the reference level for vaccination status analysis, and BA.1/BA.3 strains were used as the reference level for sublineage analysis. All variables dropped out of the model except for N2 Ct ($p<0.0001$), with the final model having a high goodness-of-fit (Adjusted $R^2=0.803$, **Figure 6**). In the final model, sgRNA Ct increased 1.225 cycles for each cycle increase in N2 (95% confidence interval 1.130-1.320; $p<0.0001$). Because all samples without detectable sgRNA drop out of the analysis, multiple linear regression was re-run after assigning a high Ct value (44.0) to samples with undetectable sgRNA in order to confirm model results when all samples with an N2 Ct were included. This had no impact on the overall model ($R^2=0.809$). The change in sgRNA for each cycle increase in N2 increased minimally (1.307), but all other variables still dropped out of the model.

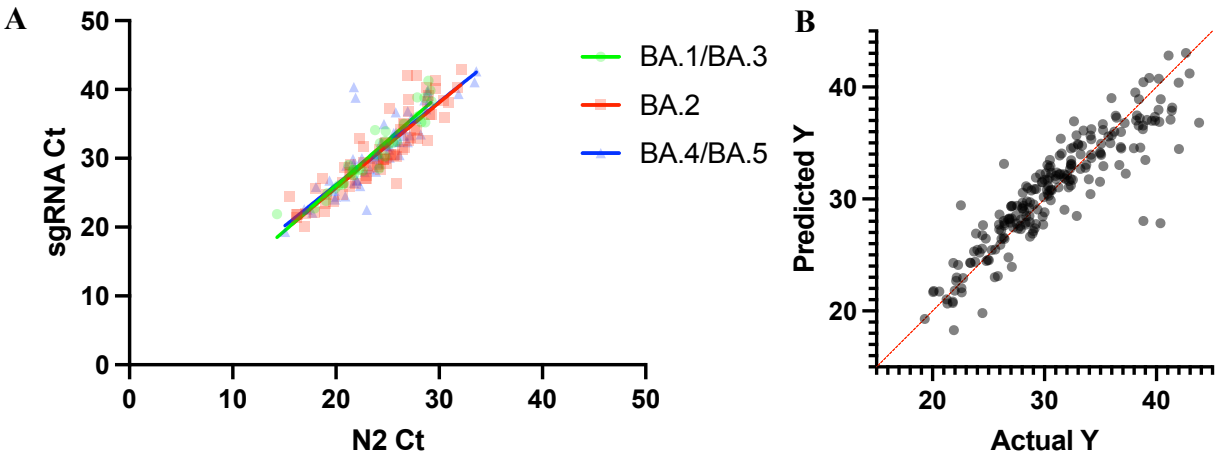

Figure 6. **A)** Linear regression plot of sgRNA Ct vs. N2 Ct by sublineage. Slopes were found to be non-significantly different between the sublineages (p=0.732). **B)** Predicted vs. actual plot showing fit of the multiple linear regression evaluating sgRNA Ct as a function of N2 Ct and symptomatic illness after dropout of the sublineage, vaccination status, previous COVID infection, age, and gender terms (Adjusted $R^2 = 0.803$).

Time since last vaccination dose was available for 156 individuals (63.4%). Including this variable in the multiple linear regression did not change the final model. However, we considered the use of sgRNA as a possible qualitative detector of increased active replication or transmissibility. Samples with high N2 Cts (>30) typically no longer have detectable sgRNA. However, a subset of samples retained sgRNA detection in this range (i.e., at low viral loads). sgRNA positive samples with N2 Cts >30 (n=5) exhibited a significantly longer time since last vaccine dose (p=0.014) than sgRNA negative samples (n=41) (**Figure 7**).

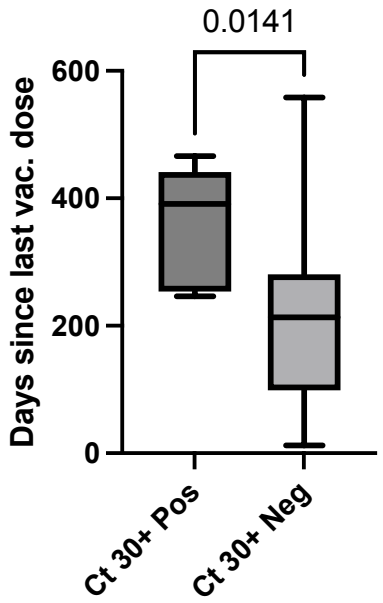

Figure 7. Box-and-whisker plot of the time in days since last vaccination dose of sgRNA positive and negative samples with an N2 Ct > 30. sgRNA+ samples (n=5) had a significantly longer time since dose (p=0.0141) than sgRNA- samples (n=41).

Out of the 72 samples selected to evaluate the effect of the bivalent booster on relative sgRNA abundance of Omicron variant infections as part of Objective 2, 12/13 (92.3%) samples from individuals with the booster had detectable genomic RNA by rRT-PCR and 10/13 (76.9%) of those samples had detectable sgRNA. Out of the 56 individuals in this set without the bivalent booster, 21 were unvaccinated, 13 were fully vaccinated, and 34 were boosted. 15 had a previous COVID infection, while 41 had not. 50/56 (89.3%) of samples from these individuals had detectable genomic RNA and 31/56 (55.4%) of those samples had detectable sgRNA (p=0.21 versus bivalent booster group). There was no significant difference found in sgRNA Ct – N2 Ct between boosted and non-boosted (unvaccinated, fully vaccinated, and boosted without the bivalent booster) individuals (**Figure 8**).

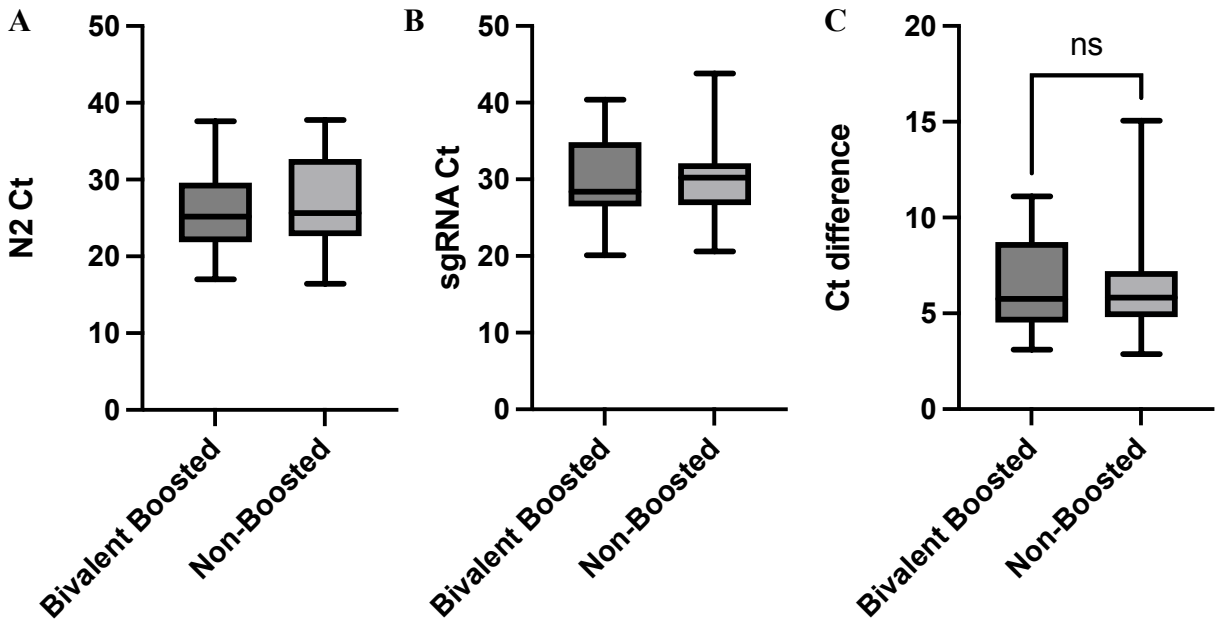

Figure 8. Box-and-whisker plot of **A)** N2 Cts, **B)** sgRNA Cts, and **C)** Ct difference (sgRNA Ct – N2 Ct) in individuals who either received or did not receive the bivalent booster. No significant differences were found (**A)** p=0.420, **B)** p=0.882, **C)** p=0.993).

Qualitative genomic and subgenomic material detection for both the acute time-point (day 0) samples and the convalescent time-point (day 14±4) are displayed in Table 4. Acute samples were more likely to contain both detectable genomic RNA (10/11, 90.9%) and sgRNA (7/11, 63.6%) when compared to convalescent samples. The 2 convalescent samples positive for N2 and the convalescent sample positive for sgRNA were from participants who had received the bivalent booster.

Qualitative Detection	Acute (Day 0)	Convalescent (Day 14±4)
N2+	10/11 (90.9%)	2/11 (18.2%)
sgRNA+	7/11 (63.6%)	1/11 (9.1%)

Table 4. Qualitative genomic and subgenomic RNA detection of paired samples by rRT-PCR.

DISCUSSION

Among participants with COVID-19 and enrolled before or after release of the bivalent SARS-CoV-2 booster, relative abundance of sgRNA did not differ due to Omicron sublineage, vaccination status, or previous COVID infection, but directly correlated with SARS-CoV-2 genomic RNA. Previous studies have documented the correlation between sgRNA and genomic RNA before the widespread emergence of variants [4, 7, 8, 14], which has remained consistent across variants of concern [9], and our study now extends these findings to SARS-CoV-2 Omicron sublineages in a varied patient population with mixed immunity from natural infection and vaccination before and after the rollout of bivalent boosters. Additionally, the cycle increase of sgRNA Ct due to N2 Ct in our model was very similar to the increase in sgRNA Ct due to N2 Ct (1.24 cycles) in our previous paper examining relative abundance among SARS-CoV-2 variants of concern [9]. This continues to support the general use of sgRNA detection to identify individuals with active viral replication, and therefore at a higher risk of SARS-CoV-2 transmission.

When looking at the qualitative detection of sgRNA in patients with lower levels of genomic RNA (high N2 Ct), sgRNA positive samples exhibited a higher time period after a patient's last vaccine dose than sgRNA negative samples. Previous evaluations of waning

vaccine immunity stop at 6 months post-dose [15]. Our results suggest that a significant effect on sgRNA detection happens in the time period between 6 months and 1 year after a vaccine dose is administered. This supports a further examination of vaccine effectiveness from 6 months-1 year, as waning immunity within this time period may allow prolonged active replication and a longer duration of transmissibility in newly infected patients.

We did note a number of differences in the qualitative detection of sgRNA among subcategories of patients, including by sublineage, vaccination status, previous COVID infection, and gender; however, these results must be placed in the context of SARS-CoV-2 genomic RNA. Although these secondary analyses may suggest differences in sgRNA abundance based on certain characteristics, these all fell out of the multiple linear regression model. However, sgRNA was only detected in 69.7% of samples with detectable genomic RNA, which resulted in 30.3% of cases initially being removed from the model due to missing data. The model was re-run with a high Ct value for sgRNA and with and without time since last vaccine dose, but this had no impact on results of the final model. Therefore, associations between patient characteristics and qualitative sgRNA detection need to account for genomic RNA and should not be used in isolation to determine risk of SARS-CoV-2 infectivity in a given population.

We did not find a difference in relative sgRNA abundance due to the bivalent Omicron booster among symptomatic individuals. One possible reason for this finding could be that prior immunity, be it from vaccine or natural infection, does not alter active viral replication or transmissibility among symptomatic cases. While the total number of cases may be reduced post-vaccine, an infection that breaks through previous adaptive immunity confers the same level of infectivity as an infection in someone with no prior immunity [16]. Another potential reason for our findings could be that adaptive immunity may impact transmissibility, but that participants

receiving the booster dose had already gone through immunological imprinting from their first exposure to SARS-CoV-2. While the concept of the original antigenic sin has not yet been fully explored in the context of the COVID-19 pandemic, one study found that SARS-CoV-2 cross-reactive but non-neutralizing antibodies were present in approximately 20% of people exposed to hCoVs prior to the start of the SARS-CoV-2 outbreak and theorized that memory responses to ancestral SARS-CoV-2 antigens may hinder the production of neutralizing antibodies to SARS-CoV-2 variants [17]. Additionally, a recent study found that antibodies generated by SARS-CoV-2 infection or vaccination can accelerate the clearance of additional vaccine antigen from the body [18]. Taken together, our findings from Objectives 1 and 2 support current CDC policies for isolation/return-to-work that do not differentiate based on vaccination status for symptomatic cases [19] and indicate that risk of transmission by symptomatic cases is not impacted by booster vaccination even with genetically similar component strains.

Limitations for this study included the enrollment of predominantly symptomatic cases, which prevented detailed analyses of differences in sgRNA abundance in subclinical infections compared to symptomatic cases. Analyses were also limited by inclusion of fewer than 25 individuals with BA.1/BA.3 infection who were fully vaccinated and boosted and only being able to collect 11 paired samples for Objective 2. Additionally, we hoped to characterize humoral immunity by utilizing serum samples from prospectively enrolled participants. However, participants were not willing to have their blood drawn, and this part of the study had to be removed. In the future, if possible, this data would allow us to identify specific antibodies and titers that impact SARS-CoV-2 replication.

In conclusion, SARS-CoV-2 sgRNA strongly correlates with total SARS-CoV-2 RNA in clinical acute-phase samples from symptomatic patients, and the relative abundance of sgRNA

remains similar irrespective of Omicron sublineage or prior immunity, even in the case of the bivalent booster. Assays for sgRNA could be implemented to identify individuals with active viral replication regardless of prior immunity or infecting sublineage.

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