Title

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Journal

The Journal of Infectious Diseases, 228(3)

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Publication Date

2023-08-11

DOI

10.1093/infdis/jiad061

Peer reviewed

The Journal of Infectious Diseases

MAJOR ARTICLE

Total and Subgenomic RNA Viral Load in Patients Infected With SARS-CoV-2 Alpha, Delta, and Omicron Variants

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Background. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genomic and subgenomic RNA levels are frequently used as a correlate of infectiousness. The impact of host factors and SARS-CoV-2 lineage on RNA viral load is unclear.

Methods. Total nucleocapsid (N) and subgenomic N (sgN) RNA levels were measured by quantitative reverse transcription polymerase chain reaction (RT-qPCR) in specimens from 3204 individuals hospitalized with coronavirus disease 2019 (COVID-19) at 21 hospitals. RT-qPCR cycle threshold (Ct) values were used to estimate RNA viral load. The impact of time of sampling, SARS-CoV-2 variant, age, comorbidities, vaccination, and immune status on N and sgN Ct values were evaluated using multiple linear regression.

Results. Mean Ct values at presentation for N were 24.14 (SD 4.53) for non-variants of concern, 25.15 (SD 4.33) for Alpha, 25.31 (SD 4.50) for Delta, and 26.26 (SD 4.42) for Omicron. N and sgN RNA levels varied with time since symptom onset and infecting variant but not with age, comorbidity, immune status, or vaccination. When normalized to total N RNA, sgN levels were similar across all variants.

Conclusions. RNA viral loads were similar among hospitalized adults, irrespective of infecting variant and known risk factors for severe COVID-19. Total N and subgenomic RNA N viral loads were highly correlated, suggesting that subgenomic RNA measurements add little information for the purposes of estimating infectivity.

Keywords. SARS-CoV-2; subgenomic RNA; variants of concern; viral load.

The relationships between severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral load and patient infectiousness and clinical outcomes have been areas of intense inquiry throughout the coronavirus disease 2019 (COVID-19) pandemic. Much of our understanding of SARS-CoV-2 viral load in patients relies on measurement of viral RNA from upper respiratory tract specimens amplified by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Although RNA viral load and infectivity (the ability to isolate replication competent virus from patients) are correlated, viral RNA can be detected long after virus can be cultured from nasopharyngeal specimens [1, 2]. Because detection of infectious virus requires time-consuming cell culture techniques in biosafety level 3 containment, correlates of infectivity in a clinical setting are needed.

Received 20 February 2023; editorial decision 03 March 2023; accepted 06 March 2023; published online 8 March 2023

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The Journal of Infectious Diseases® 2023;228:235–44

https://doi.org/10.1093/infdis/jiad061

During replication, coronaviruses generate subgenomic RNA transcripts from the 3′ one-third of the 30-kb positive-sense genome through a process of discontinuous transcription. This process generates transcripts with a common leader sequence from the 5′ end of the genome attached to an open reading frame (ORF) located at the 3′ end. This arrangement allows subgenomic transcripts to be distinguished from genomic transcripts by pairing a primer in the leader with a primer in a subgenomic ORF. In SARS-CoV-2, subgenomic transcripts are used for expression of the spike (S), envelope (E), nucleocapsid (N), and membrane (M) proteins, as well as nonstructural accessory proteins. The subgenomic N (sgN) RNA is the most abundant subgenomic transcript expressed [3]. Because subgenomic RNA generation only occurs during active replication, is not packaged into virions, and is found mainly in infected cells, its detection in clinical specimens may be better correlated with the presence of infectious virus than genomic RNA. Some, but not all, studies from early in the pandemic correlated subgenomic RNA viral load with duration of culture positivity [2, 4–6].

Throughout the pandemic, SARS-CoV-2 variants of concern (VOC) have emerged and are defined by factors such as evidence of increased transmissibility, increased disease severity, or reduced effectiveness of therapeutics or vaccines [7–10]. In experimental models, these phenotypes have been attributed to differences in viral replication, leading to high RNA viral loads and potentially increased transmission [11, 12]. Omicron lineages have been found to have relatively higher transmissibility but attenuated replication, lower viral load, and lower virulence relative to other VOCs [13–15]. Although most research on variants has focused on S, some data suggest that mutations in the N gene result in increased subgenomic transcripts and immune evasion associated with the Alpha variant [16].

RNA viral load in infected patients is influenced by viral and host factors. Identification of these determinants has been challenging due to the dynamics of RNA viral load and the limited sample size of early studies. While some have suggested that vaccination may decrease viral load [15, 17, 18], others have not found this same effect [19]. Even less is known about the effect of immunosuppression [20, 21]. Some studies have shown that viral RNA and culturable virus can persist for weeks in a subset of immunocompromised patients [22–25].

Here, we evaluate the association between RNA viral load, viral lineage, and patient characteristics in adults hospitalized with COVID-19 at 21 hospitals across the United States. We used RT-qPCR cycle threshold (Ct) as a proxy for the amount of total and subgenomic RNA in clinical specimens. Our goal was to examine the relationship between total and subgenomic RNA viral load to understand variables that might affect expression of these RNA transcripts in infected patients.

METHODS

Participants and Specimens

This work was determined to be a public health surveillance activity by all enrolling sites, Vanderbilt University Medical Center (the lead site and coordinating center), and the US Centers for Disease Control and Prevention (CDC; the funder and government sponsor). Consistent with a public health surveillance activity, written informed consent was not obtained. The work was conducted in a manner consistent with CDC policy and applicable US federal law, including: 45 C.F.R. part 46.102(l)(2), 21 C.F.R. part 56; 42 U.S.C. §241(d); 5 U.S.C. §552a; 44 U.S.C. §3501 et seq. Specimens were obtained from hospitalized patients enrolled between 11 March 2021 and 25 January 2022 in the COVID-19 surveillance program conducted by the Investigating Respiratory Viruses in the Acutely Ill (IVY) Network [26, 27]. Site staff conducted in-hospital screening of patients ≥18 years of age through daily review of electronic medical records (EMR). Hospitalized patients with COVID-19 had a clinical syndrome consistent with acute COVID-19 [28–30] and a positive molecular or antigen test within 10 days of symptom onset. A single nasal swab specimen was either collected from each patient as soon as possible after hospital arrival or a residual clinical specimen from hospital admission was used.

Patient demographics, symptom onset date, COVID-19 vaccination status, and comorbid medical conditions were captured by structured interview and EMR review. Immunocompromising conditions included: hematologic malignancy or solid organ cancer (with diagnosis or treatment in the last 6 months), human immunodeficiency virus (HIV) infection, congenital immunodeficiency, prior splenectomy, prior solid organ transplant, receipt of immunosuppressive medication, systemic lupus erythematous, rheumatoid arthritis, psoriasis, scleroderma, or inflammatory bowel disease [31]. Information on vaccination status was collected from interview, EMR, and state registries. For this study, patients were considered vaccinated if they had received ≥1 dose of a COVID-19 vaccine ≥14 days prior to illness onset. This allowed inclusion of patients who received the Ad26.COV2-S vaccine.

Nasal swab specimens were tested at Vanderbilt University Medical Center by RT-qPCR [32]. Specimens with a Ct value of ≤32 for either the N1 or N2 target were then shipped to the University of Michigan for SARS-CoV-2 lineage determination by whole-genome sequencing and RT-qPCR for total N and sgN RNA.

Measurement of Total and Subgenomic RNA Viral Load

RNA was extracted from 200 µL of specimen transport media using the MagMax Viral/Pathogen II Nucleic Acid Isolation Kit on the KingFisher Flex System and eluted in 50 µL water. Amplification of Total N RNA for N genes was performed

using amplification conditions outlined in the CDC 2019-Novel Coronavirus EUA protocol [32]. These primers amplify genomic and subgenomic targets; hence, the product is referred to as total nucleocapsid (N). Total RNA transcripts were amplified using the CDC N1 primer and probe set as follows: 2019-nCoV N1 forward primer GACCCCAAAATC AGCGAAAT; 2019-nCoV_N1 reverse primer TCTGGT TACTGCCAGTTGAATCTG; 2019-nCoV_N1 probe ACCCCGCATTACGTTTGGTGGACC. Omicron lineage viruses have a C→U mutation at position 28 311, which corresponds to the third nucleotide from the 5′ end of the N1 probe sequence. This has been found not to affect assay sensitivity or efficiency [33], and we found a high correlation between total N and Orf1ab Ct values on testing of 370 samples using both primer/probe sets (data not shown). RT-qPCR reactions were performed as previously published [34]. Briefly, 20 µL RT-qPCR reactions included 5 µL template,5 µL Taqpath one-step RT-qPCR master mix, 500 nM of each primer, and 250 nM of each probe. Reactions were run for 40 cycles on an ABI 7500FAST real-time PCR system. Run thresholds were set manually. Subgenomic N transcripts were amplified by substituting subgenomic leader sequence sgLeadSARSCoV2-F: 5′-CGATCTCTTGTAGATCTGTTCTC-3′ [2], which was combined with the N1 gene reverse primer and N1 probe from the total N1 primer-probe set [32]. Probe sequences were 6-carboxyfluorescein (FAM) labeled with Iowa Black quencher. As this study evaluated relative viral RNA loads, Ct values are presented. Absolute copy number was not determined.

Viral Sequencing

Specimens were processed for whole-genomic sequencing using the ARTIC Network protocol on a GridION instrument as previously described [35, 36]. Lineages were assigned using PANGO [37] on sequences meeting the following quality criteria: genome coverage >80%, PANGO status = passed_qc, nextclade_qcoverallstatus = "good" or "mediocre." VOC were classified based on World Health Organization assignments: Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2 and AY), and Omicron (B.1.1.529 or BA). Sequence outcomes, quality metrics, and GISAID accession numbers (for samples with >90% genome completeness) are in Supplementary Table 1.

Statistical Analysis

We summarized participant characteristics using proportions (frequencies) and means with standard deviations or median and interquartile range (IQR). Comparisons of demographic characteristics and vaccination status were performed using χ^2 or Wilcoxon/Kruskal-Wallis tests when appropriate. The relationship between days since symptom onset and Ct value from collected specimen at enrollment was displayed with a scatter plot and best fit line using a univariate linear regression model. The y-axis of the linear regression line was a Ct value-based estimate for RNA viral load at enrollment and the slope was an estimate of the rate of decline in RNA viral load over time. The y-axis consisted of Ct values in reverse order so that increases on the y-axis represented greater viral load (lower Ct). To evaluate the relationship between days from symptom onset to specimen collection and SARS-CoV-2 RNA Ct, we also constructed a multivariable linear regression model with Ct as the dependent variable, days of symptoms as the primary independent variable, and age, sex, number of underlying medical conditions, and vaccination status covariates. A restricted cubic spline function was applied on the primary independent variable (days) with 6 knots (chosen based on the lowest Aikake information criterion (AIC)) to allow for nonlinear association. To understand differences in sgN RNA Ct values across variants, we used total N Ct values to normalize sgN Ct values by subtracting the Ct value of total N from sgN (ΔCt value). We repeated comparisons between variants using normalized sgN Ct values. All analyses were conducted using R version 4.1.3.

RESULTS

A total of 5728 patients with COVID-19 were enrolled; 3810 (66.5%) patients had nasal specimens positive for SARS-CoV-2 with an N1 or N2 Ct value ≤32, which were processed for whole-genome sequencing and RT-qPCR of total N and sgN RNA. Among these 3810 patients, 606 patients were excluded from the current analyses for the following reasons: 556 with indeterminate SARS-CoV-2 lineage, 33 who had a negative RT-qPCR reaction on repeat testing for N, 16 with incomplete clinical data, and 1 with no information on immune status. This resulted in a final analytical population of 3204 patients (Supplementary Figure 1): median age was 60 years (IQR, 48–71 years), 1493 (47%) were female, 2582 (81%) had at least 1 underlying medical condition, 1327 (41%) were admitted to the intensive care unit, and 698 (22%) had an immunocompromising condition (Table 1); 1987 (62%) patients received remdesivir and 2404 (95%) received dexamethasone near the time of sample collection (Supplementary Table 2).

Among 3204 patients, 1158 (36%) had received at least 1 dose of a COVID-19 vaccine, 1985 (62%) were unvaccinated, and 61 (2%) had unknown vaccination status (Table 1). Of vaccinated patients, 79% completed the primary series and 12% had received the primary series and at least 1 booster. Vaccinated patients, compared with unvaccinated patients, were older (mean 65.1 [SD 15.6] years vs 55.2 [SD 16.3] years, $P < .001$), had more underlying medical conditions (2.71 [SD 1.47] conditions vs 1.5 [SD 1.33] conditions, $P < .001$), and had nasal specimens collected earlier after illness onset (5.22 [SD 3.49] days vs 6.83 [SD 5.29] days, $P < .001$) (data not shown). VOCs were identified in most patients, including Delta in 2246 (70%), Omicron in 481 (15%), Alpha in 295

Table 1. Characteristics of Patients With COVID-19—IVY Network, 18 US States, 11 March 2021 to 25 January 2022

Characteristic	Immunocompetent (n = 2506)	Immunocompromised (n = 698)	Overall (n = 3204)
Age, y, median (IQR)	59 (46–72)	62 (52–70)	60 (48–71)
Female	1159 (46)	334 (48)	1493 (47)
Race and ethnicity			
Non-Hispanic white	1260 (50)	385 (55)	1645 (51)
Non-Hispanic black	571 (23)	161 (23)	732 (23)
Hispanic, any race	484 (19)	116 (17)	600 (19)
Non-Hispanic, other	136 (5)	31 (4)	167 (5)
Unknown	55 (2)	5 (1)	60 (2)
BMI, median (IQR)	31 (26–37)	28 (24–33)	30 (26–36)
Smoking history			
Not current smoker	1804 (72)	573 (82)	2377 (74)
Current smoker	244 (10)	61 (9)	305 (10)
Unknown	458 (18)	64 (9)	522 (16)
Hospital admission in last year	666 (29)	337 (51)	1003 (34)
Any underlying medical condition	1884 (75)	698 (100)	2582 (81)
Number of underlying conditions			
0	622 (25)	0 (0)	622 (19)
1	649 (26)	83 (12)	732 (23)
2	625 (25)	132 (19)	757 (24)
3	610 (24)	483 (69)	1093 (34)
Admission to ICU	1048 (42)	279 (40)	1327 (41)
Vaccination status			
≥1 dose	731 (29)	427 (61)	1158 (36)
Unvaccinated	1733 (69)	252 (36)	1985 (62)
Unknown	42 (2)	19 (3)	61 (2)
Variant			
Alpha	232 (9)	63 (9)	295 (9)
Beta	8 (0)	0 (0)	8 (0)
Delta	1793 (72)	453 (65)	2246 (70)
Gamma	43 (2)	9 (1)	52 (2)
Omicron	332 (13)	149 (21)	481 (15)
Non-variant of concern	98 (4)	24 (3)	122 (4)

Data are No. (%) except where indicated.

Table 2. Total and Subgenomic N RT-qPCR Cycle Threshold Values in Nasal Swabs Collected at Enrollment—IVY Network, 18 US States, 11 March 2021 to 25 January 2022

	Non-VOC	Alpha	Beta	Gamma	Delta	Omicron	Combined	*P* Value[c]
All patients								
No. of patients	122	295	8	52	2246	481	3204	
Total RNA Ct	24.14 ± 4.53	25.15 ± 4.33	23.97 ± 2.69	25.15 ± 3.93	25.31 ± 4.50	26.26 ± 4.42	25.39 ± 4.48	<.001
Subgenomic N Ct[b]	27.46 ± 4.74	28.18 ± 4.63	27.04 ± 2.49	28.51 ± 4.18	28.62 ± 4.83	29.67 ± 5.03	28.69 ± 4.85	<.001
Vaccinated patients[a]								
No. of patients	34	45	2	10	767	300	1158	
Total RNA Ct	23.53 ± 4.86	25.12 ± 3.54	22.53 ± 3.18	23.74 ± 5.70	24.79 ± 4.67	26.18 ± 4.42	25.11 ± 4.62	<.001
Subgenomic N Ct[b]	26.71 ± 5.01	28.28 ± 3.97	25.35 ± 2.67	26.79 ± 5.82	27.81 ± 4.76	29.07 ± 4.69	28.10 ± 4.76	.002
Unvaccinated patients								
No. of patients	88	248	6	40	1439	164	1985	
Total RNA Ct	24.38 ± 4.40	25.08 ± 4.38	24.45 ± 2.65	25.39 ± 3.42	25.57 ± 4.36	26.21 ± 4.42	25.50 ± 4.36	.022
Subgenomic N Ct[b]	27.46 ± 4.29	27.86 ± 4.44	27.61 ± 2.40	28.83 ± 3.70	28.61 ± 4.40	29.22 ± 4.52	28.52 ± 4.41	.008

Cycle threshold values are mean ± standard deviation. Abbreviations: COVID-19, coronavirus disease 2019; Ct, cycle threshold; N, nucleocapsid; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

[a] ≥ 1 dose of any COVID-19 vaccine >14 days prior.

[b] Specimens with undetectable RNA were not included.

[c] Kruskal-Wallis test.

(9%), Gamma in 52 (2%), and Beta in 8 (< 1%). Non-VOC viruses were identified in 122 (4%) patients (Table 1).

RNA viral load in samples at hospital presentation was not significantly different in vaccinated versus unvaccinated patients for either total N RNA (25.11 [SD 4.62] vs 25.50 [SD 4.36], $P = .053$; Table 2) or sgN RNA (28.10 [SD 4.76] vs 28.52 [SD 4.41], $P = .056$). Viral load on presentation did differ by SARS-CoV-2 lineage. Total N viral load was lower (ie, a higher Ct) in those infected with the Omicron variant (26.26 [SD 4.42]) compared with Alpha (25.15 [SD 4.33]) and Delta (25.31 [SD 4.50]) ($P < .001$).

There was a strong correlation between time elapsed since symptom onset and lower total N and sgN viral load based on the slope of the best fit linear regression line (Figure 1). Furthermore, total N and sgN rates of decline were highly correlated within each variant. For instance, among immunocompetent patients infected with Delta lineages, the slope of decline for sgN was 0.11 and for total N was 0.12; for the Omicron lineages slope for sgN was 0.23 and total N RNA was 0.23 (Figure 1). Minor differences in the rate of viral RNA decline were observed by variant. The rates of decline for total N as reflected by the slopes of the regressions were similar between

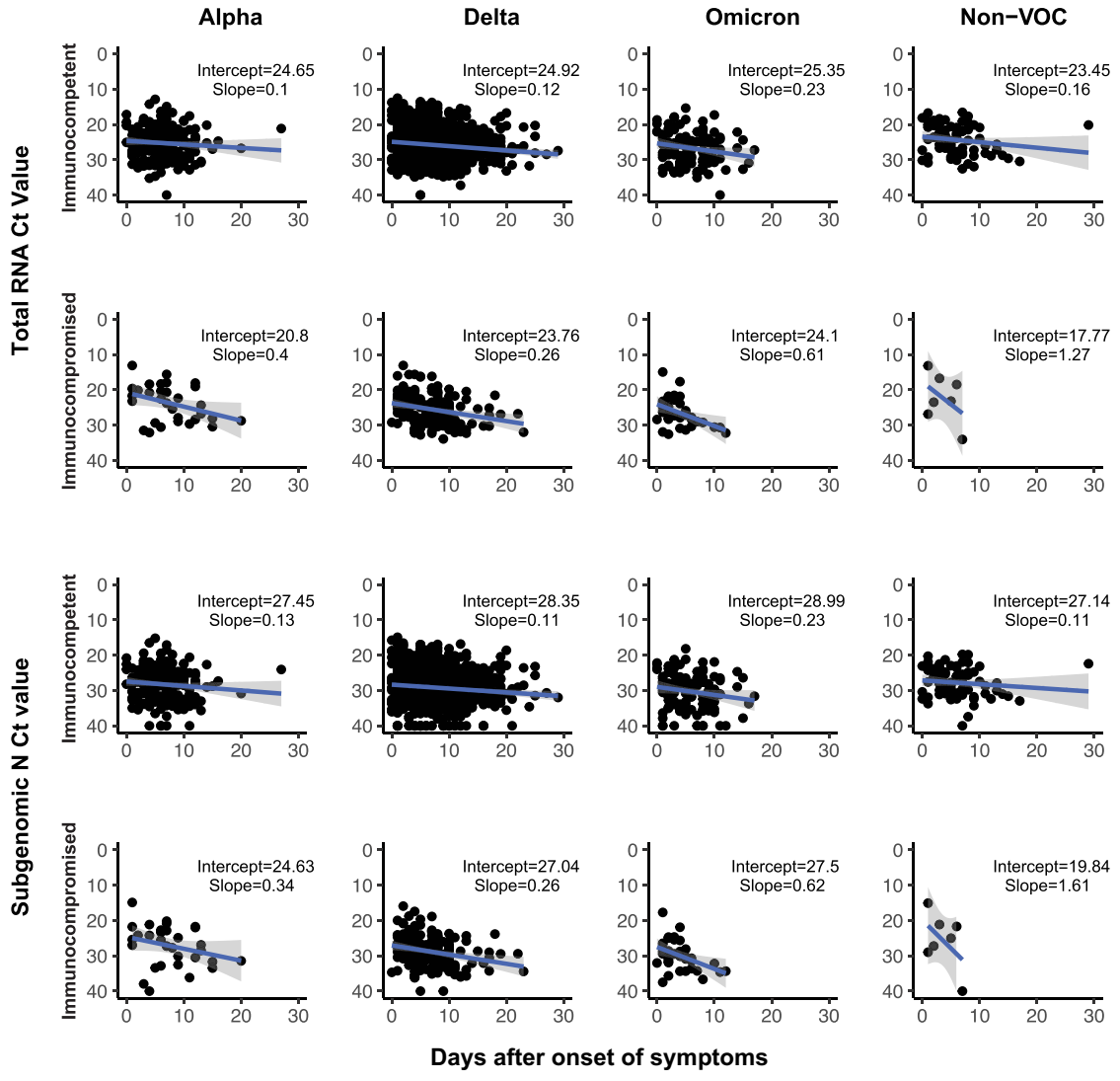

Figure 1. Total and subgenomic N RNA viral load by SARS-CoV-2 variant and immune status—IVY Network, 18 US states, 11 March 2021 to 25 January 2022. Total (top two rows) and subgenomic (bottom two rows) N Ct values are plotted relative to days after onset of symptoms for Alpha, Delta, Omicron, and non-VOC variants in unvaccinated immunocompetent (first and third rows) and immunocompromised (second and fourth rows) patients. Linear regression was performed with slope and intercept of each line as indicated. The y-axis is inverted because higher cycle thresholds correspond to lower RNA copy number. The y-axis of the linear regression line estimates viral load at the onset of symptoms and the slope estimates decline of viral load over time. The shaded areas represent the 95% confidence interval for the regression. Abbreviations: Ct, cycle threshold; N, nucleocapsid; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VOC, variant of concern.

Alpha and Delta (slope = 0.1 and slope 0.12, respectively) but faster for Omicron lineage specimens (slope 0.23).

The rate of decline in sgN and total N RNA levels was greater in immunocompromised relative to immunocompetent patients infected with all variants in a simple regression (Figure 1). This is particularly apparent for Omicron, where the slope in immunocompromised patients was 0.62 for sgN and 0.61 for total N RNA compared to a slope of 0.23 for both sgN and total N RNA in immunocompetent patients.

Compared with unvaccinated immunocompetent patients, vaccinated immunocompetent patients had faster clearance of viral RNA (slope of 0.26 vs 0.13 increase in Ct value per day; Figure 2). We found no difference in the rate of decline for either sgN or total N RNA based on vaccination status in immunocompromised patients (Figure 2).

To identify the impact of various host and viral factors on viral load, we performed multiple linear regression including SARS-CoV-2 variant, days from symptom onset, age, sex, number of comorbid conditions, vaccination, and immunosuppression status. The number of days since onset of symptoms was a major predictor of total N and sgN Ct value ($P < .001$) with an estimated change of 2.52 Ct of total N comparing 14 days after symptom onset with day 1 after symptom onset controlling other covariates as constant (Supplementary Table 3). In our analysis, total N viral load was significantly lower for Omicron lineages, but not for other lineages, relative to the Alpha variant (1.95 [SD 0.34] Ct relative to Alpha, $P < .001$; Supplementary Table 4). The sgN viral load was significantly lower for the Omicron lineages (2.01 [SD 0.36] Ct, $P < .001$) and the Delta lineages (0.60 [SD 0.29] Ct, $P = .038$) compared to Alpha. In this analysis we identified no significant effect of immune or vaccination status on Ct value. We also found no effect of medical comorbidity, sex, or age on Ct values for subgenomic or total N RNA in this population (Supplementary Table 4).

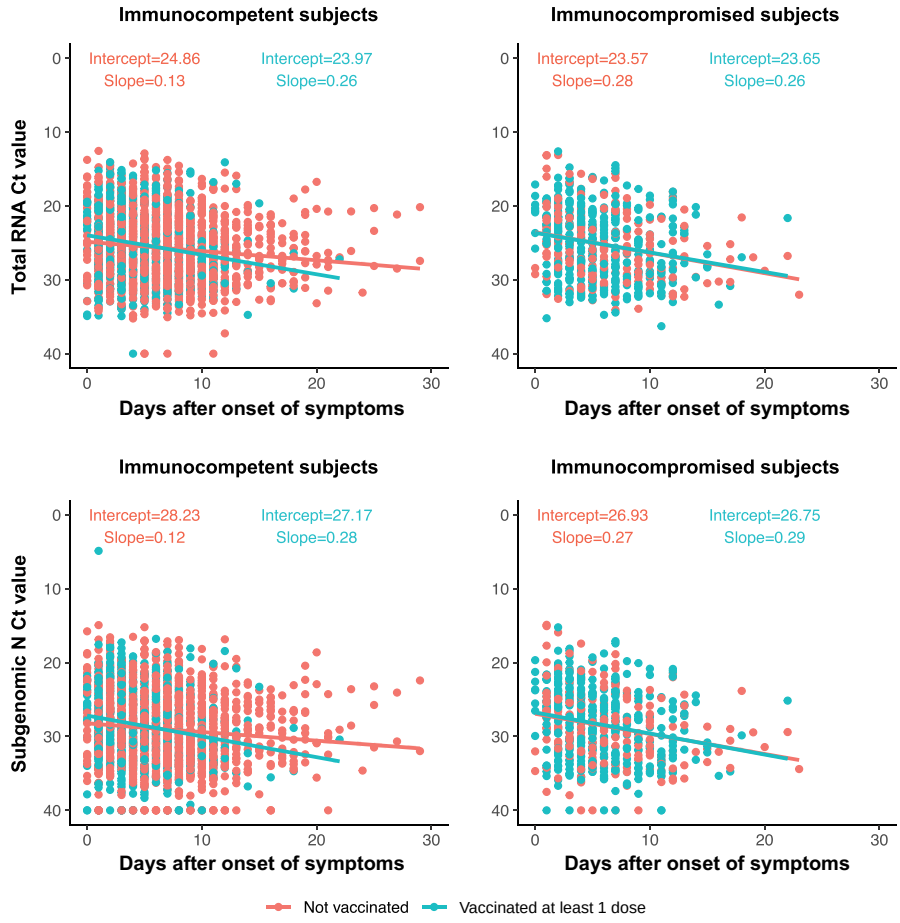

Figure 2. Total and subgenomic N RNA viral load by COVID-19 vaccination and immune status—IVY Network, 18 US states, 11 March 2021 to 25 January 2022. Total and subgenomic N Ct values plotted against days after symptom onset in immunocompetent or immunocompromised patients who were either vaccinated or not vaccinated. Abbreviations: COVID-19, coronavirus disease 2019; Ct, cycle threshold; N, nucleocapsid.

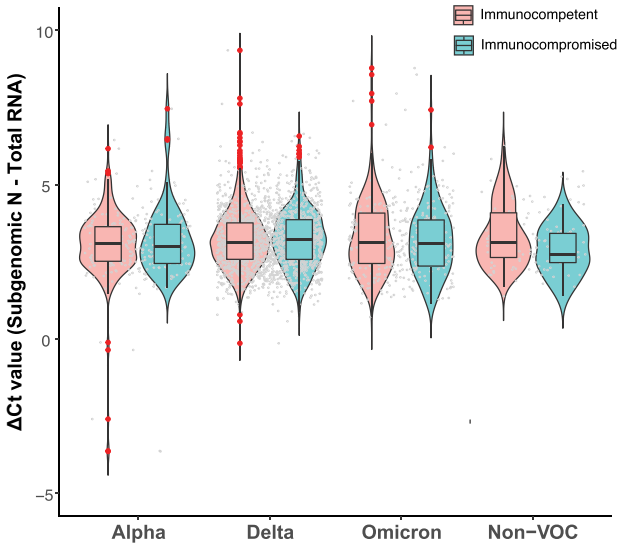

Figure 3. Relationship of subgenomic N to total N RNA by variant and immune status—IVY Network, 18 US States, 11 March 2021 to 25 January 2022. The ΔCt value was calculated by subtracting Ct value for total N from sgN. The average ΔCt was 2.98 for Alpha, 3.21 for Delta, 3.23 for Omicron, and 3.25 for non-VOCs. There was no significant difference in the ΔCt between variants. Boxplots show median (horizontal line), interquartile range (box), and 1.5 times the interquartile range (whiskers), and outliers (large points). Abbreviations: Ct, cycle threshold; N, nucleocapsid; sgN, subgenomic N; VOC, variant of concern.

To further understand differences in sgN RNA Ct values across SARS-CoV-2 variants, we normalized sgN Ct values by subtracting the Ct value of total N from sgN. After normalization, we found that there were no statistically significant differences in sgN among variants (Figure 3).

DISCUSSION

We measured total and subgenomic RNA nucleocapsid viral loads in adults hospitalized with severe COVID-19 who were infected with different SARS-CoV-2 VOCs to evaluate the association of these viral loads with patient and virologic factors. Using RT-qPCR Ct values from specimens collected at the time of enrollment, we found small differences in total N and sgN viral RNA and the rate of viral RNA decline among variants. When accounting for multiple clinical variables, including time of specimen collection, we found little difference in RNA viral load based on immune or vaccine status. When normalized to total N, sgN RNA levels were similar across variants. We found a strong correlation between subgenomic and total N, which was seen across variants, suggesting subgenomic N RNA viral load adds no additional utility in predicting infectivity compared with RT-PCR against typical genomic RNA targets.

Early studies postulated that RNA viral load was higher in some VOCs, contributing to their more rapid spread [38–40]. The Alpha variant was shown to have a 10-fold higher RNA viral load than non-VOC variants; studies of the Delta variant suggested that its RNA viral load was 10 to 1000 times higher when compared to earlier variants. Here we found small, but statistically significant differences in Ct values at presentation for total N and sgN across variants. The magnitude of these differences between any pair of variants (2- to 4-fold) was not nearly as great as previous studies and is of unclear significance. A potential reason for the discrepancy is that, unlike many other studies, we evaluated RNA viral load based on time since symptom onset. Our sample size also allowed us to compare RNA viral load across variants, prospectively, using a common protocol.

Duration of infectiousness has been a critical issue for public health guidance. Because total RNA viral load was thought to differ among VOCs, there was some concern that isolation precautions may need to change based on the properties of the circulating variant. To the extent that RNA viral load correlates with infectivity, our results suggest that there are only small differences in viral load and little difference in viral RNA decline across lineages in immunocompetent hosts. Interestingly, the Omicron variant, which exhibited lower viral loads and a faster rate of decline, was the variant consistently different in both linear and multiple regression analysis. This would suggest that elevated shedding might be a relatively minor contributor to the spread of Omicron.

The more rapid decline in subgenomic and total N RNA in unvaccinated immunocompromised patients compared to immunocompetent patients was an unexpected finding that likely reflects the complexities of evaluating RNA viral load in a clinical setting. When we applied a more robust model that evaluated multiple variables, we found that immune status did not influence RNA viral load or rates of decline. This stands in contrast to a number of case series, which suggest that some immunocompromised patients remain persistently infected for several months [22–24]. We suspect that our results may be influenced by our broad definition of immunocompromising conditions, which included a significant number of patients with nonhematologic malignancies and mild-to-moderate rheumatologic disease but is similar to the CDC definition [41]. Further stratification by type of immunocompromising condition and assessment of infectivity by cell culture would be helpful in subsequent studies.

There has been significant interest in whether vaccination reduces transmission by reducing RNA viral load. In immunocompetent vaccinated patients, we found a slightly more rapid decay of RNA viral load over time compared to unvaccinated patients but no difference between vaccinated and unvaccinated immunocompromised patients. These findings are subtle and require further investigation, especially given that the host immune responses may neutralize the shed virus, altering the relationship between viral RNA and viral infectivity.

Recent studies have suggested that some SARS-CoV-2 variants express more subgenomic RNA than others [16, 42–45]. These studies focused on the Alpha variant compared to ancestral variants and showed increased expression of several subgenomic transcripts as assessed by next-generation sequencing [16, 44]. We did see small differences in the abundance of sgN RNA across variants, which was most pronounced for Omicron. Despite these findings, when sgN RNA was normalized to total N RNA, we found no differences in subgenomic RNA across variants. Differences in technical approach, such as RT-qPCR versus sequencing, or normalization method, might contribute to different conclusions about variant-dependent patterns in subgenomic RNA. The current study is consistent with prior work, which has identified a strong correlation between total and subgenomic RNA levels [34, 46], and expands this finding to VOCs.

Our study has limitations. First, specimens were obtained only once for each patient instead of serially. This cross-sectional analysis can only reveal the impact of host and viral factors on viral load across the study population. We cannot reliably infer the trajectories in specific individuals. Second, we limited this study to Ct values of less than 32 on initial testing to prioritize specimens that were more likely to be successfully sequenced. This could bias our analysis toward higher viral loads and potentially earlier time points. This may have the most dramatic effect on immunocompromised patients who could shed virus for longer periods of time. Third, when evaluating differences in viral load across variants, we were not able to control for RNA integrity and swab technique, which could affect the measured viral load. However, our findings should be robust to any untoward effects of specimen compromise given the large number of enrollment sites and sample size. Fourth, we normalized sgN RNA to total N RNA (which includes sgN) to avoid issues related to varying primer and probe efficiency in RT-qPCR. The reported ratios may differ when normalized to a purely genomic RNA target. However, an analysis of a subset of samples with an Orf1ab target suggest that any differences would be minor (<1 Ct; Supplementary Figure 2). Fifth, our definitions of immunocompromising condition and vaccine status (≥1 dose) may be overly broad, and we do not account for the influence of prior infection, all of which may obscure important differences. Finally, our findings in this hospitalized population may not generalize to patients in other settings.

Overall, total N and subgenomic RNA virus load are similar across SARS-CoV-2 variants and deviate only modestly by immune and vaccination status. As levels of these 2 types of viral RNA are highly correlated, sgN RNA offers no clear advantage over total RNA as a marker of infectious virus in clinical specimens. Future studies in a variety of care settings are needed to determine which patient-level factors contribute to prolonged shedding of infectious virus.

Supplementary Data

Supplementary materials are available at *The Journal of 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

Financial support. This work was supported by the US Centers for Disease Control and Prevention (grant number 75D30121F00002 to W. H. S.). Scientists from the US CDC participated in all aspects of this study, including its design, analysis, interpretation of data, writing the report, and the decision to submit the article for publication. The REDCap data tool was supported by the National Center for Advancing Translational Sciences, National Institutes of Health (grant number UL1 TR002243 Clinical and Translational Science Award).

Potential conflicts of interest. J. Ca. reports grants from National Institutes of Health (NIH) and Department of Defense (DoD), outside the submitted work. J. Ch. reports grants from the NIH, DoD, and Dolly Parton COVID-19 Research Fund, outside the submitted work. D. D. reports a grant from NIH, outside the submitted work. A. D. reports grants from the NIH and National Heart, Lung, and Blood Institute (NHLBI) for the ACTIV platform and PETAL network, respectively; and consulting fees for Alung Technologies, outside the submitted work. M. E. reports grants from NIH and Regeneron; personal funds for speaking at the ASPEN conference for Abbott Labs; and payment for testimony from Medical Legal Expert Witness, outside the submitted work. A. F. reports grants from NIH, outside the submitted work. M. G. reports grants from CDC, CDC-Abt Associates, and CDC-Westat; and served as cochair of the Infectious Diseases and Immunization Committee for the Texas Pediatric Society, outside the submitted work. K. G. reports grants from NIH and DoD; and support for MHSRS 2022 travel from the DoD, outside the submitted work. A. G. reports grants from NIH, DoD, AbbVie, and Faron Pharmaceuticals, outside the submitted work. M. N. G. reports grants from NHLBI, CDC, and Agency for Healthcare Research and Quality; speaking at medicine grand rounds at New York Medical College; travel support for the American Thoracic Society (ATS) executive meeting and serving as ATS Chair Critical Care Assembly; data and safety monitoring board (DSMB) membership fees from Regeneron; and participating on the scientific advisory panel for Endpoint, outside the submitted work. C. G. reports grants from NIH, CDC, AHRQ, FDA, and Campbell Alliance/Syneos Health; and consulting fees from and participating on a DSMB for Merck, outside the submitted work. D. H. reports grants from NHLBI, outside the submitted work. N. H. reports grants from Sandofi and Quidel; and a Genentech educational

grant to give a lecture, outside the submitted work. A. K. reports grants from United Therapeutics, Johnson & Johnson, 4D Medical, Eli Lily, Dompe Pharmaceuticals, and GlaxoSmithKline; and serves on the guidelines committee for Chest, outside the submitted work. A. L. reports grants from CDC, National Institute of Allergy and Infectious Diseases, and Burroughs Wellcome Fund; and consulting fees from Sanofi and Roche for consulting on oseltamivir and baloxavir, respectively, outside the submitted work. E. M. reports grants from Flu Lab, Merck, and NIH outside the submitted work. T. M. reports a grant from CDC; a one-time payment for participating as a virtual webinar panelist for Clinical Updates in Heart Failure; and being a Practice Management Committee member for Society of Hospital Medicine, outside the submitted work. I. D. P. reports grants from NIH and Janssen Pharmaceuticals; and institutional support from Asahi Kasei Pharma and Regeneron, outside the submitted work. W. B. S. reports grants and support for meetings/travel from the NIH/NHLBI, outside the submitted work. J. W. reports payment for the American College of Emergency Physicians speaker honorarium and participating on the American Board of Internal Medicine Critical Care Exam Committee, outside the submitted work. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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