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BRIEF REPORT

Prediction of Symptomatic Severe Acute Respiratory Syndrome Coronavirus 2 Infection by Quantitative Digital Polymerase Chain Reaction Normalized to International Units

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Although numerous studies have evaluated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection using cycle threshold (Ct) values as a surrogate of viral ribonucleic acid (RNA) load, few studies have used standardized, quantitative methods. We validated a quantitative SARS-CoV-2 digital polymerase chain reaction assay normalized to World Health Organization International Units and correlated viral RNA load with symptoms and disease severity.

Keywords. COVID-19; digital PCR; international units; SARS-CoV-2; viral load.

Since the discovery of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December 2019, more than 500 million individuals have been infected and more than 6 million died worldwide. In the United States alone, more than 80 million infections and more than 1 million deaths have been reported [1]. Despite the development and availability of effective vaccines, rapid viral evolution, vaccine hesitancy, and vaccine inequity have enabled the pandemic to continue [2, 3].

Although our knowledge on SARS-CoV-2 has significantly expanded, the role of viral ribonucleic acid (RNA) load in coronavirus disease 2019 (COVID-19) remains poorly understood, partly due to the widely adopted misuse and faulty interpretation of cycle threshold (Ct) values. Cycle threshold values have been used to predict disease severity, infer transmissibility, and discriminate active viral infection from viral shedding [4–6]. However, real-time reverse-transcription PCR (RT-PCR) assays are not typically calibrated with known RNA standards and are therefore suboptimal tools for accurate measurement of nucleic acid in a sample [7–9]. Accordingly, scientific societies have issued statements advising against reporting and using Ct values as a method to infer viral quantity [10].

Even when using real-time RT-PCR methods calibrated to provide results in copies/mL, assays can vary markedly in their results. Similar to other quantitative viral nucleic acid amplification tests, results among laboratories testing the same material can vary across several log units [11, 12]. In turn, this limits the utility of any quantitative findings to those using the same methodology with the same calibrators. Digital PCR (dPCR) and digital reverse-transcription PCR (RT-dPCR) have been demonstrated to provide reproducible, highly accurate results without the need for quantitative calibrators [13, 14]. Furthermore, the use of international quantitative standards has helped to markedly improve interlaboratory viral nucleic acid load agreement in other settings [11, 12]. In this study, we describe the use of a quantitative SARS-CoV-2 RT-dPCR assay to allow correlation of viral RNA load to clinical course, providing results in a standardized manner that will facilitate widespread applicability and consistent interlaboratory result interpretation.

METHODS

Human Cohort

The SJTRC (ClinicalTrials.gov Identifier NCT04362995) is a prospective, longitudinal cohort study of St. Jude Children's Research Hospital adult (≥18 years old) employees. The St. Jude Institutional Review Board approved the study. For this study, we included 114 individuals who have tested positive for SARS-CoV-2 and in whom a respiratory sample was available between March 2020 and April 2021 at a time in which Wuhan-like and B.1.1.7 variants were the predominant circulating strain locally, and before the Delta surge. Severity of illness was classified as asymptomatic, mild-moderate, severe or critical as previously described [15] (Supplementary Methods).

Patient Consent Statement

All participants consented to the study that was reviewed and approved by The St. Jude Institutional Review Board.

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Severe Acute Respiratory Syndrome Coronavirus 2 Reverse-Transcription Digital Polymerase Chain Reaction Assay

The SARS-CoV-2 RT-dPCR Test (Bio-Rad Laboratories, Inc., Hercules, CA) was used for qualitative detection of SARS-CoV-2 RNA authorized for emergency use by US Food and Drug Administration. Viral RNA loads were generated for both N1 and N2 targets and showed similar results. Quantitative results in the manuscript are for N1, whereas N2 results are shown in the Supplementary Documents. Results were normalized to the 20/146 First World Health Organization (WHO) International Standard for SARS-CoV-2 RNA (product code 20/146; National Institute for Biological Standards and Control (NIBSC), Potters Bar, Hertfordshire, UK) to produce data in $\log_{10}$ IU/mL (Supplementary Methods).

Statistical Analysis

Descriptive statistics of the sample were presented as (1) frequencies and percentages for categorical variables and (2) mean (standard deviation) or median (25th to 75th percentiles) based on data distribution for continuous variables. Values of positive controls reported in copies/mL were regressed against corresponding nominal values in IU/mL with simple linear regression. The slope and intercept derived from linear regression was used to calculate viral RNA load in copies/mL from patient samples into IU/mL units, with the regression recalibration result approximately showing 5 copies/mL equaled 1 IU/mL across the quantitative range of the assay. To assess the value of viral RNA load in predicting outcome, receiver operating characteristic (ROC) curves were built to estimate the area under curve (AUC). Optimal cut-point levels were derived using the highest Youden indices (J statistic). All analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC) and R 4.2.0 (R Core Team, 2020; R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria) (https://www.R-project.org/) (Supplementary Methods).

RESULTS

The RT-dPCR assay was confirmed to have a limit of detection (LOD) of 3.84 $\log_{10}$ IU/mL for N1 and 3.82 $\log_{10}$ IU/mL for N2, matching the manufacturer's LOD. The quantitative linearity was demonstrated across a wide dynamic range, with lower limits of quantitation (LOQ) of 3.84 $\log_{10}$ IU/mL and 3.82 $\log_{10}$ IU/mL for N1 and N2, respectively, and upper LOQ of 7.86 $\log_{10}$ IU/mL and 7.88 $\log_{10}$ IU/mL for both N1 and N2, respectively. The assay showed a high degree of reproducibility (coefficient of variation% = 8% and 7% for N1 and N2); and when WHO standards were tested, regression of results against nominal values showed a slope of 1.0060 and a y-intercept of (1.0199) for N1 and a slope of 1.0131 and a y-intercept of (0.9878) for N2.

During the study period, 114 individuals tested positive for SARS-CoV-2 and contributed 125 samples. The median age was 42 years (interquartile range [IQR], 33–53), 76.3% were female, and most were non-Hispanic and White. Fourteen (12.3%) were asymptomatic at the time of positive test and never developed symptoms. Among those who had symptoms, fever, headaches, rhinorrhea, and loss of smell and/or taste were most commonly reported (Supplementary Table 1). Most cases were mild, with 26 (22.8%) seeking medical care and only 2 individuals needing hospital admission. The median SARS-CoV-2 viral RNA load was 6.45 $\log_{10}$ IU/mL (IQR, 34.13–7.98). Median viral RNA load in symptomatic individuals (6.96 $\log_{10}$ IU/mL; IQR, 4.46–8.32) was higher when compared to those without symptoms (3.53 $\log_{10}$ IU/mL; IQR, 3.53–4.31) with $P < .001$ (Figure 1, Supplementary Table 2), and viral RNA load significantly correlated with the severity of symptoms (correlation coefficient 0.33, $P < .001$). However, viral RNA load upon symptom onset did not significantly correlate with duration of symptoms among those who reported being sick.

Viral RNA load from the initial sample discriminated and predicted individuals with and without symptoms (odds ratio [OR] = 2.26; 95% confidence interval [CI], 1.41–3.61) in univariate analysis (Supplementary Table 3). These results did not change in a multivariate stepwise logistic regression model adjusted for age, gender, and race (OR = 2.26; 95% CI, 1.41–3.61) (Supplementary Table 4). An ROC curve analysis determined that a viral RNA load of 5.68 $\log_{10}$ IU/mL discriminated asymptomatic from symptomatic infection with a sensitivity of 71.7% and specificity of 92.9% (AUC = 0.84) (Supplementary Table 5, Supplementary Figure 1). In addition, viral RNA load predicted development of symptoms in those individuals who were asymptomatic at the time of diagnosis irrespective of age, gender, and race (OR = 2.33; 95% CI, 1.22–4.44) (Supplementary Tables 6 and 7). A similar ROC curve analysis showed that a viral RNA load of 4.86 $\log_{10}$ IU/mL upon initially asymptomatic presentation predicted subsequent development of symptoms with a sensitivity of 70.6% and specificity of 85.7% (AUC = 0.81) (Supplementary Table 8, Supplementary Figure 2).

DISCUSSION

Although much work has been done in correlating viral RNA load with disease severity, most studies have used Ct values, with relatively few using quantitative methods and international quantitative standards. In this study, we provided details and results of a standardized quantitative SARS-CoV-2 RT-dPCR assay, normalized to the first WHO International Standard for SARS-CoV-2 RNA.

As reported by others, we observed a correlation between RNA quantity and presence of symptoms. Although we also noted an increase in viral RNA load in those with more severe

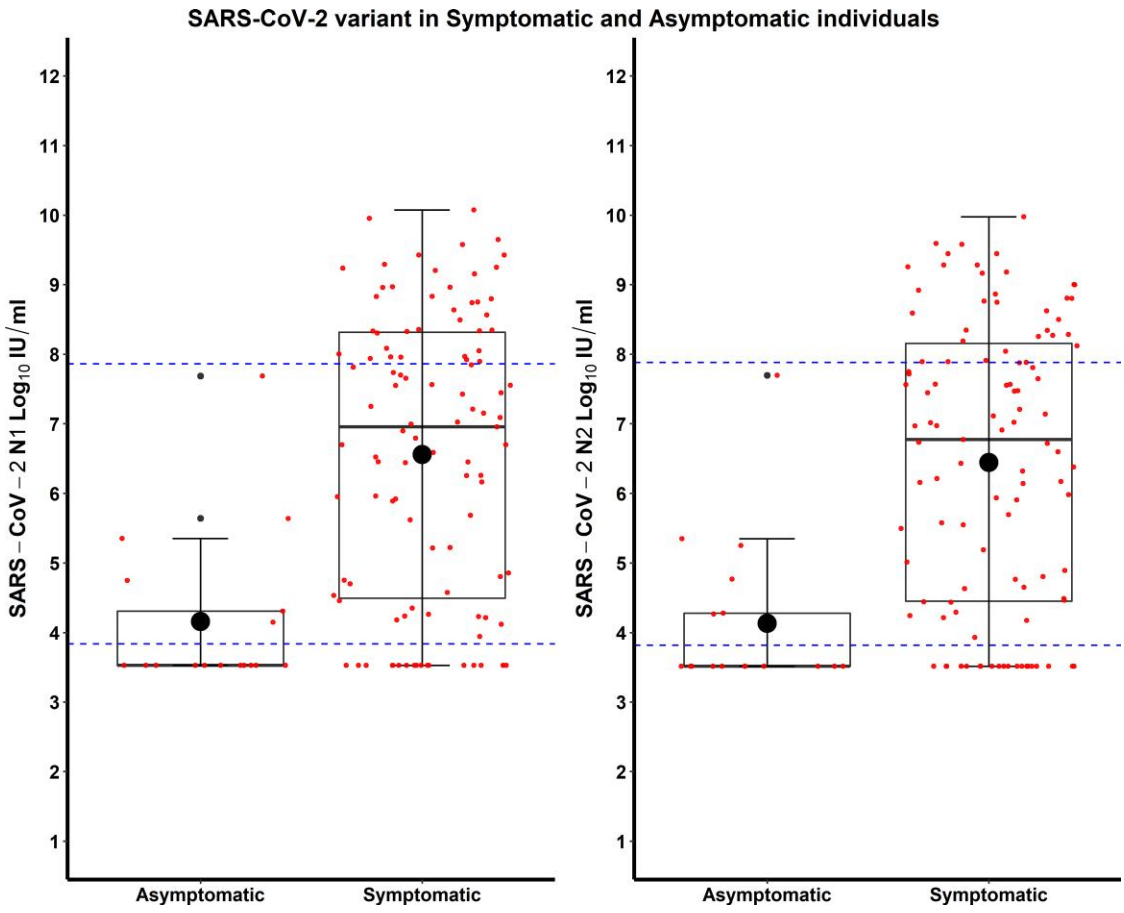

Figure 1. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral load (N1 and N2 $\log_{10}$ IU/mL) in asymptomatic and symptomatic individuals. Dotted lines represent the demonstrated upper and lower limits of the assay analytical measurement range.

symptoms, few participants had critical illness to be able to correlate viral RNA load with poor clinical outcomes. Only those 14 cases who never developed symptoms were considered "asymptomatic" for all models. Although we were able to distinguish individuals who develop symptoms from those who remained asymptomatic, results should be taken with caution given the small numbers in our cohort. If this is confirmed by others, initial viral RNA load could be considered an additional variable when prioritizing access to early treatment to prevent progression of COVID-19. This emphasizes the need to establish reliable quantitative methods that are standardized and can be replicated, potentially allowing the development of consensus thresholds for treatment.

There are many limitations in using RT-PCR Ct values as a surrogate of RNA concentration. First, most Ct values are not normalized to standardized controls of known concentration. In addition, such assays may not have a linear relationship between the Ct values and the amount of nucleic acid across the full range of detected RNA loads. This is particularly important when working with specimens with high or low viral load [7]. In general, Ct values have not been well evaluated for analytical measurement range (linear range of quantitation), within- or between-run reproducibility, or collinearity using differing viral strains. The Ct values cannot be compared across different platforms or laboratories, making the generalization or portability of results unfeasible. In fact, the College of American Pathologists found variability of up to 4000-fold across different emergency use authorization platforms using same controls and up to 10-fold differences in the reproducibility of the control using the same instrument [8]. Although reporting viral quantity is clearly more useful than Ct values, in the absence of normalization to a common quantitative standard, results remain hard to interpret and replicate, and establishment of consensus treatment thresholds remains elusive.

This study has several limitations. The samples are limited to infections with ancestral strain virus and B.1.1.7. Viral RNA load among different variants and stratified by vaccination status could not be performed. In addition, the study was underpowered to evaluate an association between viral RNA load and disease severity, and very few individuals had more than 1 sample to be able to perform those analysis. Although it is difficult to guarantee that sample quality had no impact on results, a

wide variety of clinical samples were tested during assay validation to demonstrate consistent performance across patients. In addition, all tested samples included an internal positive control that had to be detectable at a certain level to demonstrate lack of assay inhibition. Finally, staff underwent training on proper sample collection techniques and competency assessment before collecting samples for testing.

CONCLUSIONS

This work was performed with commercially available reagents, on a widely available digital PCR platform. Such testing would therefore likely be feasible for implementation in any laboratory running high-complexity molecular testing for clinical purposes. This may largely limit the initial use of such methods to referral laboratories and academic centers. Nonetheless, this work represents a substantial advance in providing a correlation between clinical course and standardized quantitative results. Our results will facilitate reproducibility of these data in other settings, comparison to results in other centers, and open the door to the establishment of common interpretive thresholds. These potentially marked advantages suggest that this work can serve as a guide for future work related to quantitative detection of SARS-CoV-2 and of other pathogens of clinical and public health interest.

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.

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Potential conflicts of interest. All authors: No reported conflicts of interest.

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