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Virus Research 306 (2021) 198604

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Normalization of SARS-CoV-2 viral load via RT-qPCR provides higher-resolution data for comparison across time and between patients

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ARTICLE INFO

Keywords:
COVID-19
reference gene
normalization
viral concentration
viral dynamic

ABSTRACT

The 2020 pandemic has transformed the world and elicited thousands of studies to better understand the SARS-CoV-2 virus. Viral load has been a common measure to monitor treatment therapies and associate viral dynamics with patient outcomes; however, methods associated with viral load have varied across studies. These variations have the potential to sacrifice the accuracy of findings as they often do not account for inter-assay variation or variation across samples. In a retrospective study of nasopharyngeal samples, we found a significant amount of variation within the DNA and RNA targets; for example, across time within a single patient, there was an average of a 32-fold change. Further, we explore the impacts of host normalization on 94 clinical samples using the TGen Quantitative SARS-CoV-2 assay, finding that without host normalization samples with the same viral concentration can have up to 100-fold variation in the viral load.

Text

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has transformed virtually every aspect of human life and the widespread impact of this pandemic will continue for years. Some of the largest and most visible impacts have occurred because of the response from the scientific community. Just beyond a year after the first SARS-CoV-2 genome was published, three vaccines and several treatment interventions had been approved for emergency use for coronavirus disease 2019 (COVID-19) by the U.S. Food and Drug Administration (U.S. Food and Drug Administration, 2021). In parallel, COVID-19 has spurred thousands of studies that have used a variety of patient outcome measures, many of which focus on time to recovery, blood tests, or viral load dynamics (Beigel et al., 2020; Gottlieb et al., 2021; The RECOVERY Collaborative Group, 2021).

Viral load has been a common measure within clinical trials and scientific investigations primarily focusing on identifying COVID-19 treatments and how disease processes relate to patient outcomes. However, the methods for viral load determination have varied across studies. Viral load has commonly been assessed using SARS-CoV-2 reverse transcriptase polymerase chain reaction (RT-PCR) threshold cycle (Ct) values (Piubelli et al., 2021; Shen et al., 2020; Zhou et al., 2020). A technique that could prove dangerous, as some studies do not consider inter-assay variation (Han et al., 2021) or variation within collections (Dahdouh et al., 2020). Another common method applies interpolation across a linear range of a SARS-CoV-2 RT-qPCR assay with a standard curve to calculate the number of viral particles within a sample (Caillard et al., 2020; Cao et al., 2020; Gottlieb et al., 2021; Pan et al., 2020). This method provides a simple means to estimate viral particle quantity within a sample; however, it does not account for potential variation in sample collections across time or among patients, an aspect that could add significant variation when attempting to make statistical comparisons across time or between patients. Alternatively, investigators have used a viral RT-qPCR assay and a second RT-qPCR assay to target human nucleic acids with a delta Ct analysis (Dahdouh et al., 2020; Liu et al., 2020; Miranda et al., 2021) to account for variations in collections within a patient. One benefit of this method is that it is simple and accounts for sample variation (Dahdouh et al., 2020; Miranda et al., 2021; Schmittgen and Livak, 2008); however, RNA expression across time, cells, and individuals can add variability into these analyses (Jacob et al., 2013; Kozera and Rapacz, 2013; Schmittgen and Livak, 2008).

To further investigate these potential sources of variation, we performed a retrospective analysis of nasopharyngeal samples submitted for SARS-CoV-2 testing to our clinical laboratory. In addition to SARS-CoV-2 detection, our clinical test utilized the CDC RNase P RT-PCR assay to assess sample quality (Lu et al., 2020). This retrospective analysis was limited to patients who had been tested across at least

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https://doi.org/10.1016/j.virusres.2021.198604
Received 2 September 2021; Accepted 8 October 2021
Available online 16 October 2021

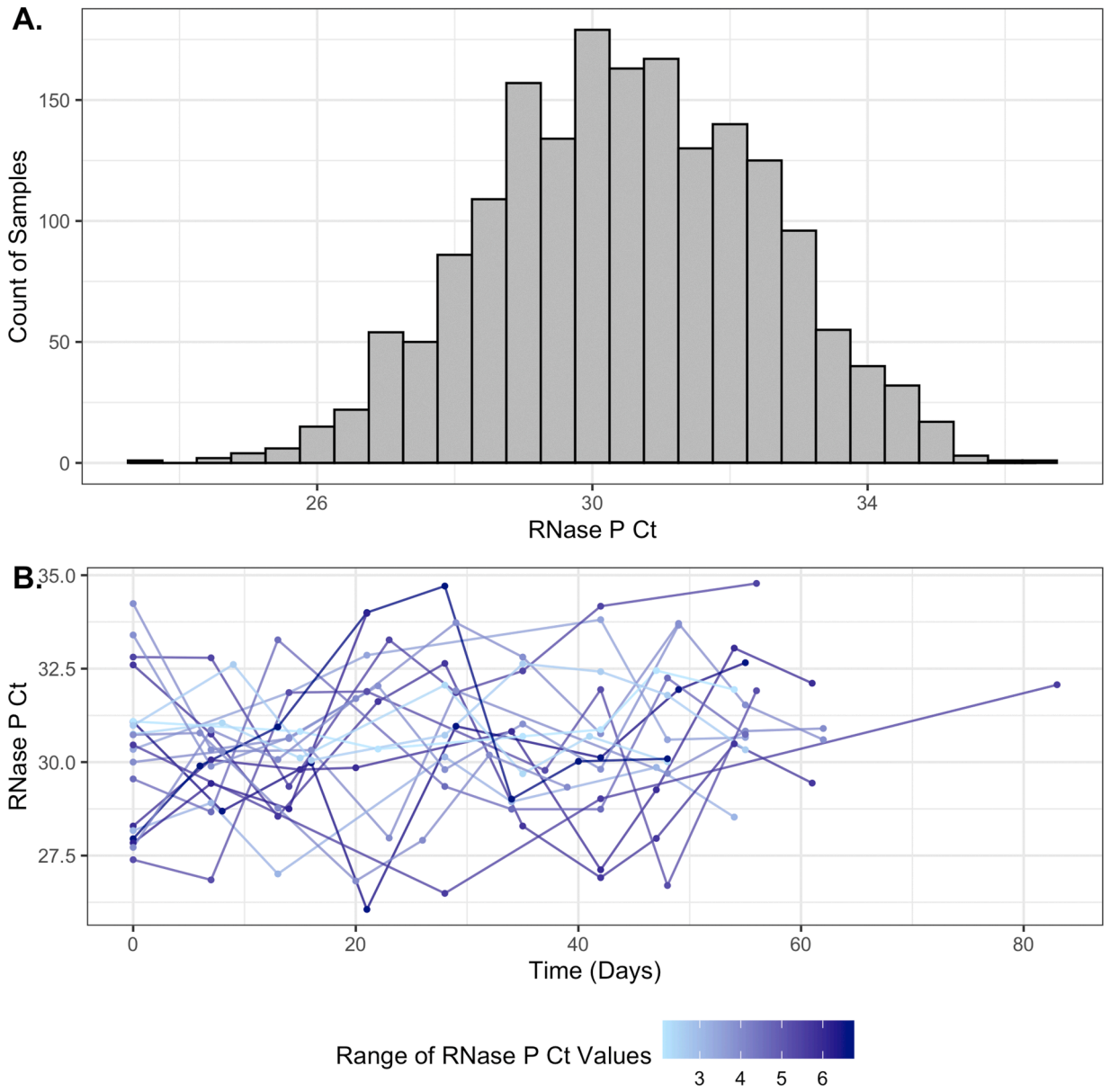

Fig. 1. Distribution of RNase *P* values across all 1789 samples (A) and trends of RNase *P* within 20 random individuals across time (B). The color of each line represents the range of RNase *P* Cts within that sample.
Fig. 1 Greyscale in text.

four-time points (mean: 7.3, median: 8), and in total included 265 patients and 1,789 nasopharyngeal sample collections. Across all samples, a range of 13.2 Ct was observed in the RNase P signal, suggesting a ~9,500-fold range in the concentration of host DNA and RNA across samples (Fig. 1A). However, within a single patient and across time, variation was lower, with a mean range of 5.0 (median: 4.8) Ct values, translating to a ~32-fold range (Fig. 1B). These data reveal drastic differences in the amount of host DNA and RNA collected between and within individuals across time; for example, some patients experience a > 1,000-fold (10 Ct) change in DNA/RNA concentration across time with most patients experiencing a 10-fold to 100-fold change across time. These data suggest that variation in sampling could impact results of viral concentration (viral copies / μL transport media) and normalization of viral load to the amount of human DNA present (*i.e.*, viral copies/ng human DNA) within the sample would provide a valuable increase in the accuracy of viral load estimates.

As a solution, we introduce the TGen Quantitative SARS-CoV-2 assay, which pairs an RT-qPCR and qPCR assay together. First, the assay utilizes parallel RNA and DNA extractions to quantify the concentration of SARS-CoV-2 using RT-qPCR and human DNA using qPCR within a sample, allowing for viral normalization to the amount of host DNA collected in a specimen (Supplemental File 1). This approach allows for normalization across sample collections to appropriately compare viral load (measured as viral copies/ng human DNA) across and between patients, without relying on stable RNA expression among patients, a common challenge in RT-PCR experiments. With this assay, we investigated the importance of sample collection, sample normalization in SARS-CoV-2-positive nasopharyngeal samples, and compared viral concentration (SARS-CoV-2 target copies per μL transport media) to normalized viral load (SARS-CoV-2 target copies per ng human DNA).

To investigate the impacts of normalization 94 SARS-CoV-2 positive samples were quantified using the TGen Quantitative SARS-CoV-2 assay, which pairs a quantitative SARS-CoV-2 N gene assay (RNA) with a quantitative human RNase *P* assay (DNA). By pairing these quantitative assays, the investigator captures the viral concentration (viral copies / μL transport media) using the SARS-CoV-2 N gene assay and the amount

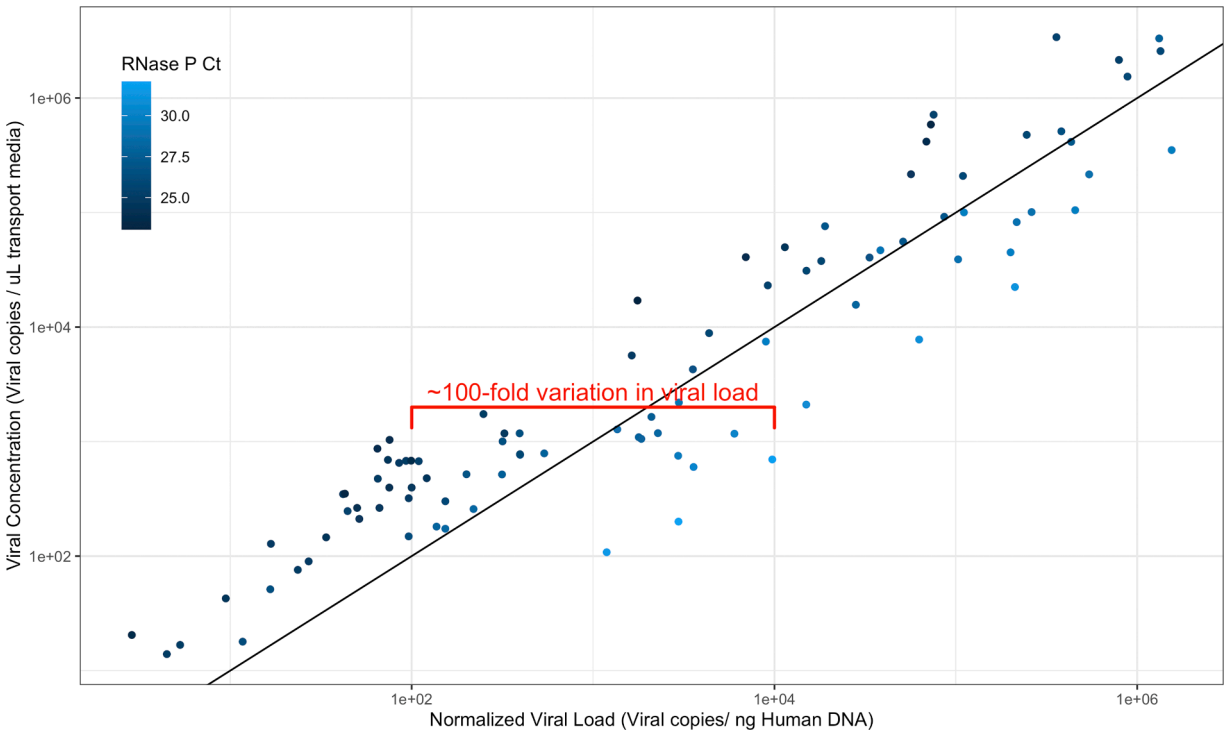

Fig. 2. Linear relationship between viral concentration and normalized viral load (Pearson = 0.75, Spearman = 0.92).
Fig. 2 Greyscale in text.

of human DNA (ng / μL) within a sample using the RNase *P* assay. Viral concentration was normalized to the amount of human DNA in a sample, producing a normalized viral load (viral copies/ng human DNA). The original viral concentration was then compared to the normalized viral load. Overall, a strong linear relationship (Pearson Correlation = 0.75, Spearman Correlation = 0.92) was observed between the measures (Fig. 1A) and viral concentration explained 56% of the variability within the viral load. These results suggest that viral concentration is highly correlated to the normalized viral load at broad scales. However, at finer scales, a high amount of variability is seen between viral concentration and viral load. For example, samples with a viral concentration of ~1×10^3 copies per μL had a normalized viral load that ranged from ~1×10^2 to 1×10^4 copies per ng Human DNA, translating to a 100-fold variation within the viral load (Fig. 2).

Molecular-based quantification methods do have some challenges. For example, these methods cannot differentiate between viral RNA within a cell, cell-free (virion) viral RNA, and subgenomic viral RNA (Alexandersen et al., 2020; Mendoza et al., 2020). These challenges need to be considered when comparing molecular-based viral load estimates to estimates produced from infectious unit estimates in tissue culture (e.g., $TCID_{50}$). Due to cell-associated RNA and possible subgenomic RNA, molecular quantification estimates commonly produce higher estimates than quantification through tissue culture (Sender et al., 2021). However, tissue culture methods also have challenges, including differing susceptibilities in cell lines and susceptibility differences between in vitro and in vivo models (Sonnleitner et al., 2021). Even with these challenges to molecular-based quantification, it has been commonly used to quantify SARS-CoV-2 viral loads throughout the pandemic.

The TGen Quantitative SARS-CoV-2 assay expands on previous work and introduces a unique method for DNA normalization. These data emphasize the importance of normalizing results with a host-specific assay in studies that utilize viral load to investigate SARS-CoV-2 and other viral infections. While quantitative normalization may not be worth the higher cost when the goal of a study is to characterize drastic differences in viral concentrations (although there are alternative methods that allow for inexpensive host normalization that should be considered (Dahdouh et al., 2020; Miranda et al., 2021)), when high-resolution data are needed to quantify differences across treatments or viral dynamics across individuals, normalization would play a critical role in resolving differences between groups. For example, across individuals' samples with the same viral concentration corresponded to a 100-fold range of normalized viral loads, an impact that would increase the variance within experiments and impact the statistical interpretations of the study. Importantly, reduction in the variance within experiments influences sample size calculations, potentially reducing the number of samples required, a critical component as COVID-19 cases fall and recruitment and sample acquisition becomes more complex, which can in turn reduce associated costs. Overall, the emergence of SARS-CoV-2 has challenged the scientific community to develop better diagnostics, vaccines, and treatments in record time. As SARS-CoV-2 vaccinations increase and COVID-19 cases decrease, it is important to begin translating the scientific advancements that were made as a result of the pandemic to other diseases to take advantage of the hard-won technological achievements of the COVID-19 pandemic.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Heather Centner, Parker Montfort, Gabriel Fitzwater, and the TGen North Clinical Laboratory team for their support and assistance.

Funding

Funding was provided by the non-profit Translational Genomics Research Institute and its many supporters.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2021.198604.

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