



# The Dangers of Using Cq to Quantify Nucleic Acid in Biological Samples: A Lesson From COVID-19

Daniel Evans,<sup>a,b</sup> Simon Cowen,<sup>a</sup> Martin Kammel,<sup>c,d</sup> Denise M. O'Sullivan<sup>a</sup>, Graham Stewart,<sup>b</sup> Hans-Peter Grunert,<sup>e</sup> Jacob Moran-Gilad,<sup>f</sup> Jasper Verwilt,<sup>g</sup> Jiwon In,<sup>h</sup> Jo Vandesompele <sup>g,i</sup> Kathryn Harris,<sup>j</sup> Ki Ho Hong,<sup>k</sup> Nathaniel Storey,<sup>l</sup> Suzie Hingley-Wilson <sup>b</sup>, Ulf Dühring,<sup>e</sup> Young-Kyung Bae,<sup>m</sup> Carole A. Foy,<sup>a</sup> Julian Braybrook,<sup>a</sup> Heinz Zeichhardt,<sup>c,d,e</sup> and Jim F. Huggett <sup>a,b,\*</sup>

**BACKGROUND:** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA quantities, measured by reverse transcription quantitative PCR (RT-qPCR), have been proposed to stratify clinical risk or determine analytical performance targets. We investigated reproducibility and how setting diagnostic cutoffs altered the clinical sensitivity of coronavirus disease 2019 (COVID-19) testing.

**METHODS:** Quantitative SARS-CoV-2 RNA distributions [quantification cycle (Cq) and copies/mL] from more than 6000 patients from 3 clinical laboratories in United Kingdom, Belgium, and the Republic of Korea were analyzed. Impact of Cq cutoffs on clinical sensitivity was assessed. The June/July 2020 INSTAND external quality assessment scheme SARS-CoV-2 materials were used to estimate laboratory reported copies/mL and to estimate the variation in copies/mL for a given Cq.

**RESULTS:** When the WHO-suggested Cq cutoff of 25 was applied, the clinical sensitivity dropped to about 16%. Clinical sensitivity also dropped to about 27% when a simulated limit of detection of 10<sup>6</sup> copies/mL was applied. The interlaboratory variation for a given Cq value was >1000 fold in copies/mL (99% CI).

**CONCLUSION:** While RT-qPCR has been instrumental in the response to COVID-19, we recommend Cq (cycle threshold or crossing point) values not be used to set clinical cutoffs or diagnostic performance targets due to poor interlaboratory reproducibility; calibrated copy-based units (used elsewhere in virology) offer more reproducible alternatives. We also report a phenomenon

where diagnostic performance may change relative to the effective reproduction number. Our findings indicate that the disparities between patient populations across time are an important consideration when evaluating or deploying diagnostic tests. This is especially relevant to the emergency situation of an evolving pandemic.

## Introduction

The main diagnostic method for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is reverse transcription quantitative PCR (RT-qPCR) that detects viral RNA in patients. RT-qPCR is capable of high analytical sensitivity, in some cases measuring near single RNA copies per reaction. Consequently, patients who have small amounts of viral RNA, potentially as a result of mild, early, or late infection or residual RNA postinfection, will be identified alongside those with high viral titers who may represent a greater likelihood of severe disease and/or pose a more significant risk of spreading infection.

As the RT-qPCR provides a quantitative output (termed “quantification cycle” [Cq] by the MIQE guidelines (1) and the International Organization for Standardization (2), but also referred to as the cycle threshold or crossing point), there have been several groups who have reported associations with Cq and patient outcome (3–5) and proposed the Cq be used along with other factors, such as symptoms, to guide patient management (6, 7). Cq values have become a popular

<sup>a</sup>National Measurement Laboratory, LGC, Teddington, Middlesex, UK; <sup>b</sup>Department of Microbial Sciences, School of Biosciences & Medicine, Faculty of Health & Medical Science, University of Surrey, Guildford, UK; <sup>c</sup>Gesellschaft zur Foerderung der Qualitaetssicherung in Medizinischen Laboratorien e. V. (INSTAND), Dusseldorf, Germany; <sup>d</sup>IQVD GmbH, Institut fuer Qualitaetssicherung in der Virusdiagnostik, Berlin, Germany; <sup>e</sup>GBD Gesellschaft fuer Biotechnologische Diagnostik mbH, Berlin, Germany; <sup>f</sup>Health Policy and Management, School of Public Health, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel; <sup>g</sup>Department of Biomolecular Medicine, Ghent University, Ghent, Belgium; <sup>h</sup>Seoul Medical Center, Seoul, Republic of Korea; <sup>i</sup>Biogazelle, Zwijnaarde, Belgium; <sup>j</sup>Department of Virology, NHS East and South East London Pathology Partnership, Royal London Hospital, Barts Health NHS Trust,

London, UK; <sup>k</sup>Department of Laboratory Medicine, Yonsei University College of Medicine, Severance Hospital, Seoul, Republic of Korea; <sup>l</sup>Department of Microbiology, Virology and Infection Prevention and Control, Level 4 Camelia Botnar Laboratories, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK; <sup>m</sup>Center for Bioanalysis, Korea Research Institute of Standards and Science, Daejeon, Republic of Korea.

\*Address correspondence to this author at: National Measurement Laboratory (NML), LGC, Queens Rd, Teddington TW11 0LY, UK. E-mail jim.huggett@lgcgroup.com. Received June 25, 2021; accepted September 20, 2021. <https://doi.org/10.1093/clinchem/hvab219>

part of the lexicon of SARS-CoV-2 test metrics within the scientific literature and popular press (8, 9), as well as when applied to in vitro diagnostic (IVD) performance targets: with the WHO proposing a Cq of 25 as the minimum level of virus required to be detected by point-of-care tests (10). Cq values are also being used by physicians to guide decisions pertaining to individual patients despite lack of convincing evidence.

While the concept of using Cq as a diagnostic threshold or cutoff for risk based on viral burden is logical, Cq has not been typically applied as such when managing other viral infections in this way. This is despite the fact that clinical virology arguably represents the medical field that most broadly applies accurate molecular quantification to manage patients based on nucleic acid quantity. It has been known for over a decade that the Cq value can vary considerably between laboratories (11), which is likely to result from several factors including choice of nucleic acid extraction, assay performance, and instrument and data-analysis settings. The same has been demonstrated for SARS-CoV-2 (12, 13) and the use of Cq for associated quantification has been challenged (14, 15). Consequently, it would seem using Cq cutoffs to quantify SARS-CoV-2 burden, stratify risk, and aid patient management would be challenging while also problematic if used to discuss broader analytical performance or as a measure to guide the development of IVDs (10).

In this work, we aimed to assess the use of Cq values as a quantitative measure for SARS-CoV-2 RNA burden. We used Cq values of SARS-CoV-2-positive patients from 3 clinical laboratories serving geographically distinct patient populations and well-characterized control panels (from international external quality assurance schemes) to estimate the viral RNA copies/mL for a given Cq for the respective laboratories. Finally, we estimated the likely variability, in copies, between laboratories that might be expected for a given Cq value and simulated the impact of differing analytical sensitivity on percentage correct diagnostic decision within the respective patient populations. Our findings provide guidance on how to apply quantitative measurements of viral burden to determine risk and highlight considerations that may be applied when using less sensitive diagnostic tests.

## Materials and Methods

### PATIENT INFORMATION

The Cq values from the first positive SARS-CoV-2 clinical results of patients diagnosed with coronavirus disease 2019 (COVID-19) were examined from clinical testing laboratories from 3 countries: Great Ormond Street Hospitals NHS Trust, UK (Cohort A), Biogazelle, Belgium (Cohort B), and Seoul Medical Center,

Republic of Korea (Cohort C). For the purpose of the study, the positive patient data were considered as true positives and Cq values were anonymized. Detailed information about patient cohorts and procedures used by the respective clinical testing laboratories are provided in the online [Supplemental Data](#).

### ASSAY INFORMATION

To reflect the real-world situation, cohorts were selected where protocols (extraction, assay, etc.) differed considerably but all assays were performed as a 1-step RT-PCR protocol using the reverse PCR primer for complementary DNA synthesis. Cohort A was assessed using an assay targeting the nucleocapsid (*N*) gene designed by Grant et al. (16), in duplex with an internal control, using One Step PrimeScript III RT-PCR mastermix (Takara Bio) on a QuantStudio 5 (ThermoFisher) platform. Primers and probes were synthesized by Integrated DNA Technologies and Biosearch, respectively. Cohort B was assessed using the envelope (*E*) assay designed by Corman et al. (17), in duplex with an internal spike-in control 'SIC', using iTaq Universal Probes One-Step Kit (Bio-Rad) on a CFX384 (Bio-Rad). Primers and probes were synthesized by Integrated DNA Technologies (*E* gene in double-quenched FAM hydrolysis probe; SIC in double-quenched HEX hydrolysis probe). Cohort C was assessed using a commercial assay provided by SD Biosensor. This comprised a triplex assay targeting the RNA dependent RNA polymerase (*RdRp*) and *E* gene and an internal control. Choice of assay did not significantly alter the conclusions. For the analysis, the *RdRp* data were used to add variation as it was a different gene target than for Cohorts A and B.

### EXTERNAL QUALITY ASSESSMENT PANELS

The three laboratories analyzed control samples of an external quality assessment (EQA) scheme of INSTAND (further details can be found in the online [Supplemental Data](#)).

The Cq values reported by the participants of the previously mentioned EQA scheme in June/July 2020 (633 laboratories from 39 countries) were also provided for data analysis in comparison to the results of the 3 laboratories having tested Cohorts A, B and C.

### DATA ANALYSIS

The Cq distributions of SARS-CoV-2 results were compared between cohorts, and the effect of setting Cq cutoffs on the percentage correct diagnostic decision were assessed. The EQA panel values [assigned by reverse transcription digital PCR (13)] were then used to determine the respective laboratory assay performance (slope and intercept) and estimate the concentration in copies/mL for a given Cq for each laboratory. The respective

distributions in copies/mL was also explored and used to determine the percentage correct diagnostic decision when a copy-based cutoff or a less analytically sensitive method (i.e., those that are unable to detect as low concentrations of viral burden as PCR) was applied. For both C<sub>q</sub> and equivalent concentration data, the relationship between percentage correct diagnostic decision and cutoff value was determined from each data set's empirical cumulative distribution function.

In a separate analysis, the results from the EQA scheme, also using these dilutions (13), were used to investigate between-laboratory variability in both C<sub>q</sub> value and equivalent concentration obtained for the same samples. After an initial filtering process, the data set consisted of 844 sets of C<sub>q</sub> values obtained by different laboratories. During the filtering stage, only those results containing all 4 C<sub>q</sub> values were retained, with results containing missing values, clear outliers, or non-C<sub>q</sub> units being excluded. For each result, the sample concentrations and C<sub>q</sub> values were used to produce an equivalent RT-qPCR standard curve. Linear regression of C<sub>q</sub> on the log concentration was performed to obtain the slope, intercept, coefficient of determination (*r*<sup>2</sup> value), and residual SD. The regression lines could then be used to convert a given C<sub>q</sub> value to an equivalent concentration for that particular laboratory.

As well as laboratory identifiers, the EQA data contained methodological information, including extraction kit and gene region. The C<sub>q</sub> results from the 4 dilutions of EQA materials were inspected for sources of variation other than between laboratory. This included differences in extraction, assay/gene target, instrument, and user, but not specimen choice, specimen sampling, storage, storage buffer, or other steps prior to extraction. No strong grouping effects were found, other than 1 associated with kits incorporating preamplification (leading to low C<sub>q</sub> values), which was removed. The remaining data were pooled and treated as a single distribution for this analysis.

The regression lines were examined for quality of fit, and results with *r*<sup>2</sup> < 0.94 were excluded. Participant results were also removed where a preamplification step was included. The remaining data set of 732 regression lines was then used to obtain the expected C<sub>q</sub> and concentration ranges as follows: for 7 concentrations in the range 100 to 10<sup>8</sup> copies/mL, the 732 regression lines were used to produce a set of 732 equivalent C<sub>q</sub> values using the well-known relationship between C<sub>q</sub> and log concentration,

$$Cq = a + b \log_{10} c,$$

where *c* is the concentration, *a* is the intercept, and *b* is the slope.

## Results

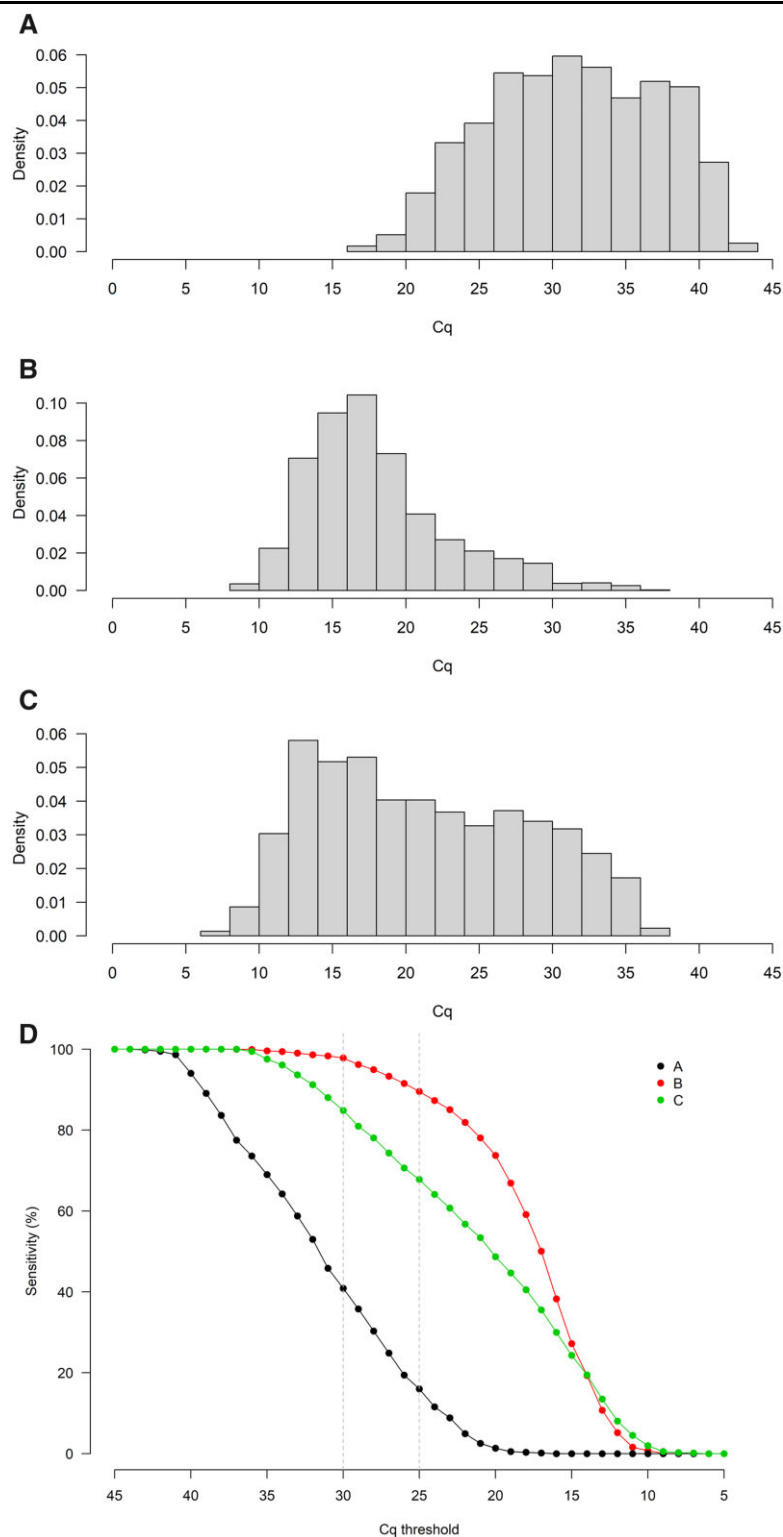
### DISTRIBUTION OF C<sub>q</sub> DATA FROM THE 3 DIFFERENT LABORATORIES AND IMPACT OF C<sub>q</sub> CUTOFFS ON REMAINING POSITIVE PATIENTS

Three distinctive distributions were observed from the different laboratories (Fig. 1, A–C). For Cohort A, there was a broad distribution with the lowest C<sub>q</sub> around cycle 15 with a sharp drop around cycle 40 (Fig. 1, A). For Cohort B, the distribution was different from Cohort A (Fig. 1, B) with a much higher representation of low C<sub>q</sub> values with few >30 cycles. The data for Cohort C appeared to contain parts of the 2 other distributions with a peak at low C<sub>q</sub> and a considerable number of patients with lower C<sub>q</sub> values and a sharp drop around cycle 35 (Fig. 1, C), which represents the approximate limit of detection (LOD) of this protocol. The timing of results for the 3 cohorts differed both in terms of duration, calendar, and incidence (Supplemental Fig. 1). If C<sub>q</sub> cutoffs are applied to these data, then the proportion of RNA-positive patients who would remain positive differs with the patient population (Fig. 1, D). When a C<sub>q</sub> cutoff of 25 was applied, a clinical sensitivity of about 16% or about 90% was observed for Cohorts A and B, respectively.

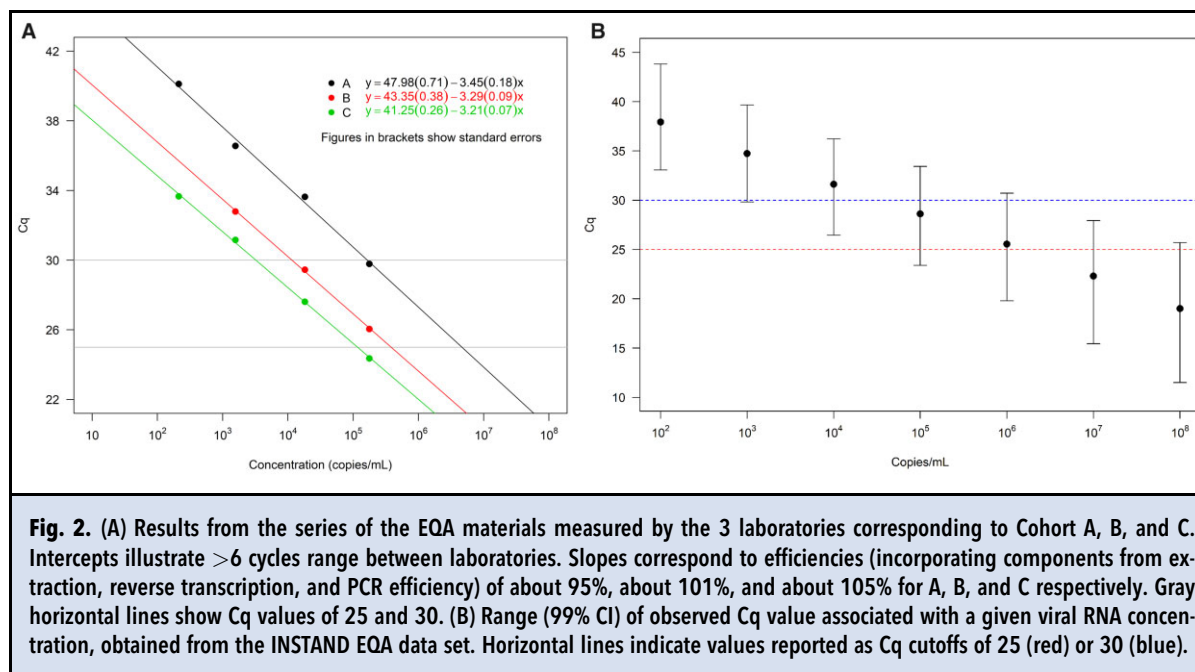
### EVALUATION OF C<sub>q</sub> DIFFERENCE BETWEEN COHORT LABS AND COPIES/ML USING EQA DILUTION SERIES AND ASSESSMENT OF THE VARIATION ASSOCIATED WITH A GIVEN C<sub>q</sub> VALUE WHEN COMPARING THE EQA PARTICIPANT RESULTS

The 3 cohort laboratories performed repeat measures for the EQA with 1 of the repeats for Cohort C dilution series failing. Laboratories who provided Cohorts A and C measured all 4 dilutions with Cohort B, not detecting the lowest dilution of approximately 200 copies/mL (Fig. 2, A). This was explained by their protocol, which added an effective volume (after correcting for extraction eluate volume) of 20 μL of clinical sample, corresponding to about 4 copies per reaction for the lowest dilution (assuming no loss during extraction). The intercepts differed between the 3 laboratories with the highest (Cohort A) measuring approximately 6 cycles higher than the lowest (Cohort C) (Fig. 2, A). The range of slopes, which included contributions from RNA extraction, reverse transcription, and PCR efficiency, resulting in efficiency between 95% and 105%.

These differences between the 3 cohort laboratories were compared with those present in the EQA data set (Supplemental Table 3). The latter used to estimate the expected concentration range in copies/mL for a given C<sub>q</sub>. Figure 2, B, shows the confidence interval of observed C<sub>q</sub> values between 100 and 10<sup>8</sup> copies/mL. The mean residual SD associated with the regression



**Fig. 1.** (A-C) Histograms showing measured Cq values for the 3 different cohorts used in the study. (D) impact of applying different Cq cutoff on sensitivity. The plot shows for each cohort the proportion of positive samples that lie below a given cutoff. Vertical dotted lines indicate the sensitivity at Cq 25 and 30 (see [Supplemental Table 1](#)).



lines was 0.51 Cq. This level of variability and the concentration range covered by the EQA dilution series (13) was insufficient to account for the size of the error bars in Fig. 2, B. Therefore, the between-laboratory differences in gradient and intercept are responsible for almost 90% of the variability seen. It should be noted that Fig. 2, B, includes only the variability in the observed relationships between Cq and concentration (the regression lines) and that measurement variability is an additional source of uncertainty. At 99% confidence (i.e., for 99 out of 100 laboratories), the difference in measured concentration for a given Cq measuring the same sample can be up to 1000-fold or more (Fig. 2, B).

**EVALUATION OF THE DISTRIBUTION OF COPIES/ML AND IMPACT OF THE SIMULATED REDUCTION IN LOD ON THE REMAINING POSITIVE PATIENTS**

The distribution of the copies/mL mirrors that of the Cq for the respective cohorts although the differences in position between cohorts is less distinct (Fig. 3). The impact of this difference can be seen with a Cq 25 corresponding to about 10<sup>7</sup> copies/mL for Cohort A and about 10<sup>5</sup> for Cohort B (Fig. 3). Most of Cohort A was estimated to be of lower viral burden (Fig. 3, A). Cohort B showed a peak at about 10<sup>8</sup> copies/mL with patients included having viral burdens over 10<sup>10</sup> copies/mL; few patients contained RNA copies of <10<sup>6</sup>/mL. As with Cq, copy distribution in Cohort C appeared to contain components of Cohorts A and B with viral burdens spanning over 8 orders of magnitude. The copies/mL

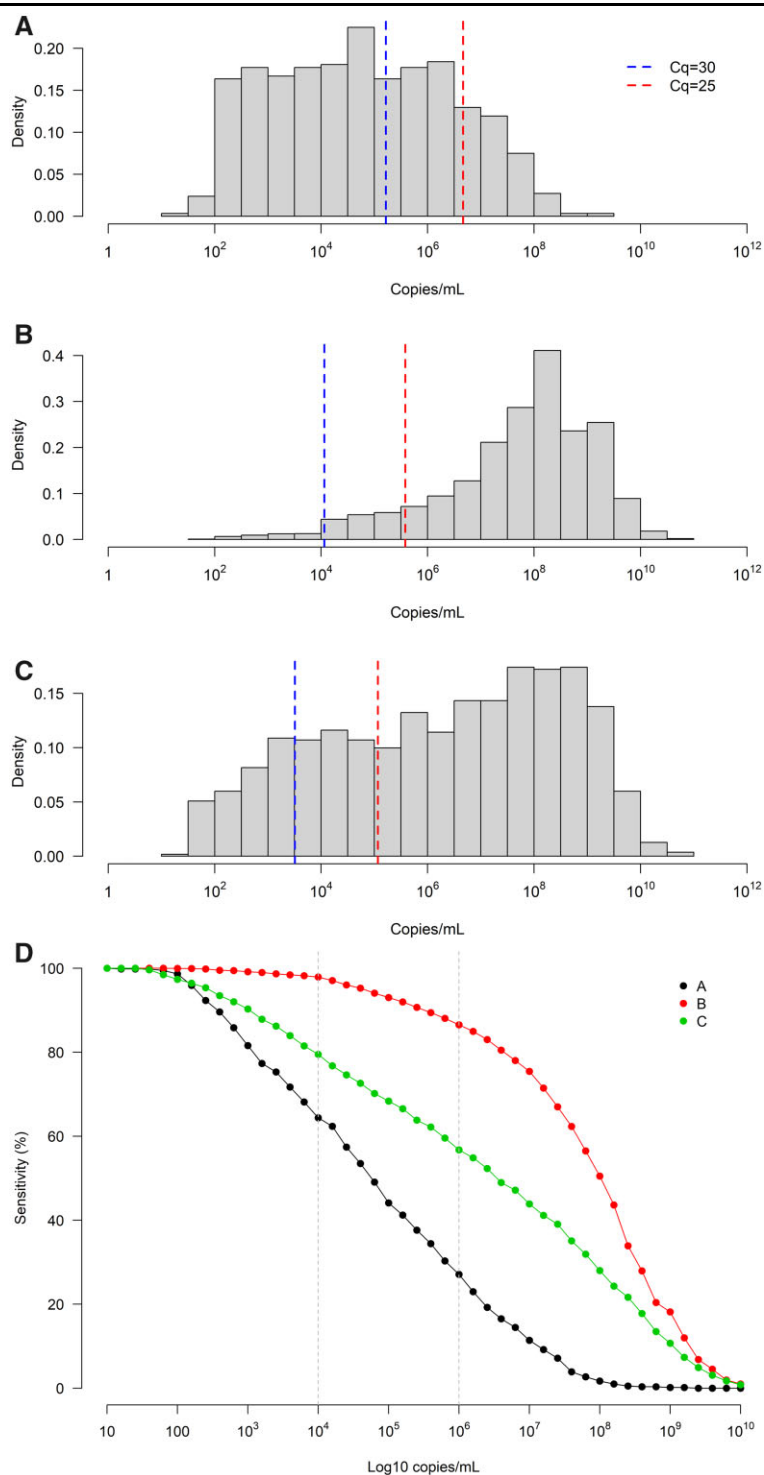
metric also better demonstrates that the sharp drop in viral burden at about 100 copies/mL for Cohorts A and C is artificial due to the LOD of the respective methods (Fig. 3, A and C).

When a copies/mL-based cutoff (and a simulation of using analytically less sensitive tests) was applied, Cohort A resulted in a considerable decrease in the percentage correct diagnostic decision. A simulated LOD of 10<sup>6</sup> RNA copies/mL resulted in clinical sensitivities of approximately 27%, 57%, and 87% for Cohorts A, C, and B, respectively (Fig. 3, D).

**Discussion**

This work compared the distributions of quantitative measures (Cq and copies/mL) of SARS-CoV-2 RNA from nasal/oral pharyngeal specimens from 3 different COVID-19-positive patient populations using diagnostic RT-qPCR. The study did not evaluate the clinical performance of the RT-qPCR diagnostic protocols but treated them as gold standard (100% clinical sensitivity) for the purpose of the analyses. We explored how setting Cq or copy-based cutoffs would impact the actual percentage correct diagnostic decision (ultimately affecting clinical sensitivity) for the respective cohorts and used the June/July INSTAND Corona virus EQA scheme (13) to define the interlaboratory differences in copies/mL for a given Cq value.

When the Cq cutoff of 25, advocated by WHO for an acceptable LOD of point-of-care tests for suspected



**Fig. 3.** (A–C) Histograms showing measured equivalent concentrations (copies/mL) associated with the Cq values in Fig. 1 obtained using the INSTAND dilution panel. (D) Impact of applying different copy/mL cutoff on sensitivity. Gray vertical lines indicate sensitivities (see Supplemental Table 2) at  $10^6$  (red) or  $10^4$  (blue) copies/mL. The plot shows for each cohort the proportion of positive samples which lie below a given copy/mL cutoff (or simulated LOD).

COVID-19 cases and their close contacts (10), was applied to the cohorts, the clinical sensitivity reduced; varying from about 16% to about 90% depending on cohort (Fig. 1, D, Supplemental Table 1). The reasons for the discrepancies were 2-fold. First, Cohort A, and to a lesser extent Cohort C, data sets contained considerable proportions of patients with higher Cq. Second, the magnitude (in copies) a given Cq value corresponded to differed between the 3 laboratories by up to 6 cycles (Fig. 2, A), corresponding to approximately 100-fold. Furthermore, the EQA data sets (incorporating 732 laboratories) demonstrated that this difference was not abnormal, with an individual Cq value differing by over 1000 copies/mL (99% CI) between laboratories. This is due to differences in RNA extraction, assay performance, and instrument and quantification threshold settings. However, as this approach [using cultured virus dilutions employed for the EQA (12)] did not capture additional sources of error (such as specimen choice, sampling, etc.), the reported difference represents a conservative estimate of the true variation in copies/mL associated with a given Cq.

Our findings suggest Cq values should not be used for cutoff setting to stratify risk or guide analytical performance, such as for target product profiles. For example, the WHO target product profiles for priority diagnostics to support response to the COVID-19 pandemic v.1.0 [published in September 2020 (10)] stipulates that point-of-care tests (including those that measure viral proteins) must be able to detect SARS-CoV-2 quantities of Cq 25 to 30, reported to correspond to about  $10^6$  copies/mL. While we agree that about  $10^6$  copies/mL can be measured in the range of Cq 25 to 30, we also report that Cq 25 can correspond to over  $10^8$  copies/mL and Cq 30 to as little as  $10^3$  copies/mL (Fig. 2, B). Such a recommendation means that an IVD manufacturer will find meeting this requirement considerably easier or more difficult depending on the choice of RT-qPCR used to provide the reference value.

Converting the 3 cohort data sets to copies/mL (using the EQA dilution series) reduced the difference between them (Fig. 3, A–C), offering a more analytically reproducible alternative (such as routinely applied in clinical virology for over a decade). Limitations to this approach are that the standard curves were not conducted in the same run as the >6000 samples, the dilution series in question does not cover the upper range of measured quantities, and specimen choice, sampling, storage, and transport are not controlled for. This means our estimation is not as accurate as other examples of clinical viral quantification (e.g., HIV viral load determination). However, the distinct distributions are real, and the assumptions included lead to a conservative estimation:

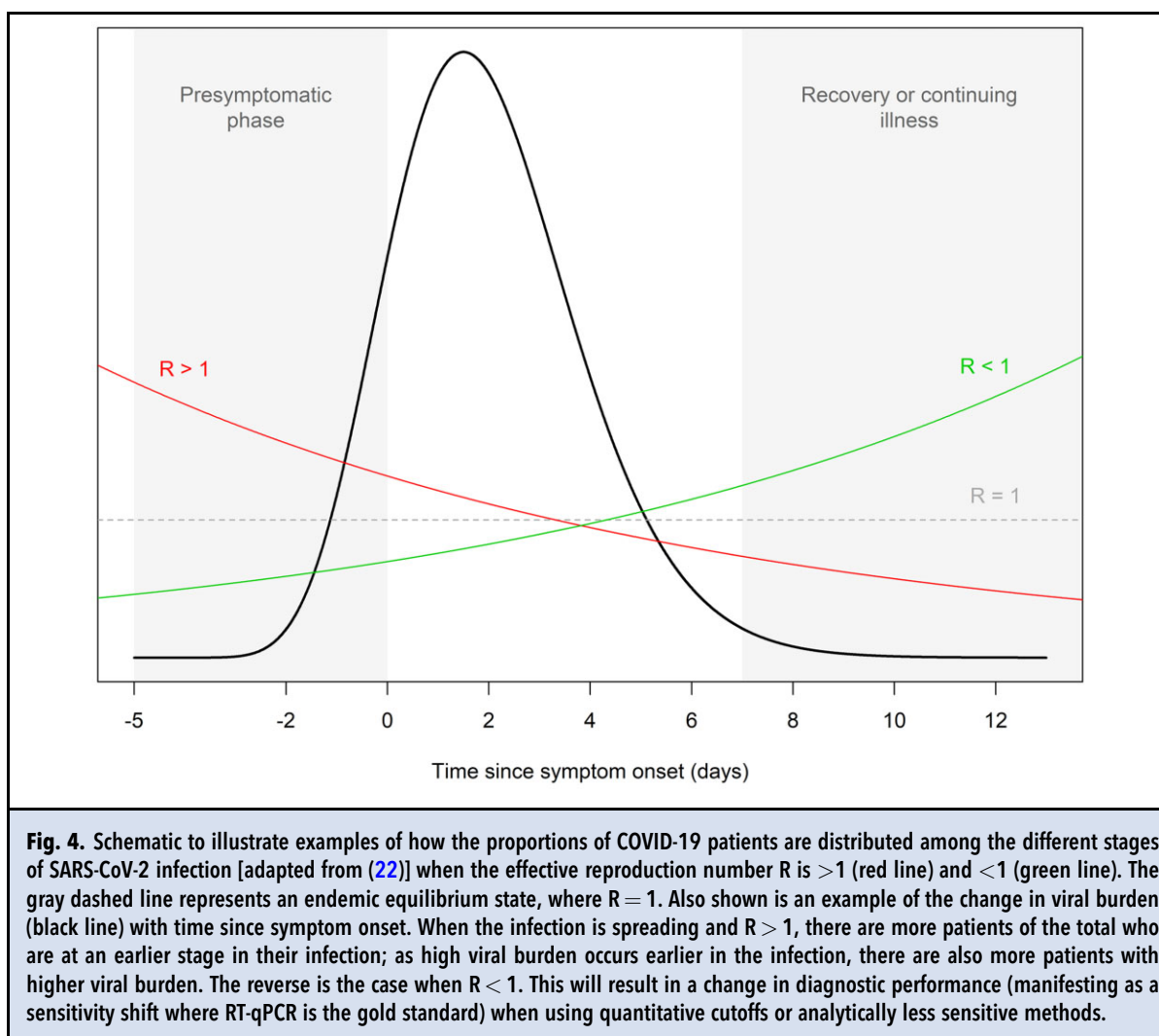
addressing the previously outlined limitations will add to the error associated with a given Cq.

When the copies/mL data were used to explore setting a quantitative cutoff (or simulate the use of an IVD with a LOD) of  $10^6$  copies/mL on clinical sensitivity, the result was approximately 27% for Cohort A but about 87% with Cohort B (Fig. 3, D, Supplemental Table 2). While this was an improvement on the discrepancy when using Cq, a considerable difference in percentage correct diagnostic decision remained with most positive patients predicted to test negative for Cohort A (Fig. 3, D).

These findings indicate the distribution of the viral RNA within the cohorts remained the predominant factor influencing clinical performance when setting cutoffs. It also highlights that the performance of analytically less sensitive methods would differ when used with different cohorts due to the distinct distributions of viral RNA quantities observed. This raises the question whether the higher Cq/lower viral quantities are clinically relevant as it is possible that Cohorts A and C contained large numbers of patients at later stages of disease. However, Cohort A was from symptomatic individuals, most of whom tested within 10 days of symptom onset [as stipulated by the WHO as a typical period of SARS-CoV-2 infection (18)] (Supplemental Fig. 2). Cohort A also presented a similar distribution to previously reported patterns of COVID-19–positive patients (19, 20), suggesting they were clinically accurate in terms of identifying relevant SARS-CoV-2 infection.

This leads to the question of why the 3 cohorts differed so distinctly and how viral distribution might be better understood to improve the application of different diagnostic solutions. Hay et al. have reported that measured SARS-CoV-2 viral RNA quantitative distribution within a population is linked to the effective reproduction number (R) with more individuals with newer infections where  $R > 1$  and thus more with higher a viral burden (21). This may contribute to the distinct shapes of the cohorts explored here: Cohort B was collected during September 2020 when Belgium was experiencing its second wave of exponential growth (Supplemental Fig. 1, B) whereas Cohort A was collected when the United Kingdom was in the first lockdown and incidence was greatly reduced (Supplemental Fig. 1, A).

We propose that the diagnostic performance changes at the population level with varying R. This is due to the R-associated differences in the proportion of individuals at different stages of infection (21) and corresponding differences in viral burden (Fig. 4). As viral burden is what is being quantified when setting cutoffs (and influences IVD LOD), this would manifest as a sensitivity shift when deploying quantitative cutoffs



**Fig. 4.** Schematic to illustrate examples of how the proportions of COVID-19 patients are distributed among the different stages of SARS-CoV-2 infection [adapted from (22)] when the effective reproduction number  $R$  is  $>1$  (red line) and  $<1$  (green line). The gray dashed line represents an endemic equilibrium state, where  $R = 1$ . Also shown is an example of the change in viral burden (black line) with time since symptom onset. When the infection is spreading and  $R > 1$ , there are more patients of the total who are at an earlier stage in their infection; as high viral burden occurs earlier in the infection, there are also more patients with higher viral burden. The reverse is the case when  $R < 1$ . This will result in a change in diagnostic performance (manifesting as a sensitivity shift where RT-qPCR is the gold standard) when using quantitative cutoffs or analytically less sensitive methods.

(or less analytically sensitive methods). While this phenomenon may be most acute when testing is deployed independent of symptoms, such as for population screening, it may be further enhanced for behavioral reasons if a new outbreak leads to heightened public awareness and increased diligence associated with the testing post symptom onset.

This suggests that deploying copy-based cutoffs or using analytically less sensitive methods may be more important in supporting the response to a new outbreak. However, they may be less useful when  $R < 1$  or when disease is endemic. This sensitivity shift may apply to testing of other conditions that occur in waves, such as seasonal infections. In that sense, actual test performance observed during routine testing may differ from that evident during development and validation stages, depending on the distributions of viral burden across time owing to the epidemiological situation.

## Conclusions

While Cq values may be useful in COVID-19 for epidemiological assessments of populations, they should be avoided as a quantitative measure for individual patient stratification or target product profiles. If quantification is to be performed, copy-based units calibrated to appropriate standards should be explored as applied in other areas of clinical virology. When dealing with a new pathogen, this fact is hampered by an initial absence of appropriate standards to calibrate the copy-based units; consequently, their rapid production should be an important part of diagnostic response plan to a new epidemic. A range of reference standards for SARS-CoV-2 are now available for calibrating Cq values to copy-based units (23–26). However, even when using calibrated copy-based units, SARS-CoV-2 RNA quantitative distributions can differ in populations of infected patients,

possibly as a result of differences in R. This manifested as a sensitivity shift, suggesting that analytically less sensitive methods may be of more value at the early stages of an outbreak. Due to the wide differences in distributions of SARS-CoV-2 RNA quantities in clinical samples observed by us and others, our findings suggest that molecular-based methods with high analytical sensitivity remain a vital tool for managing all the stages of the pandemic. Nevertheless, their development, validation, routine application, and quality control should take into consideration the disparities between patient populations across time, especially in emergency situations.

## Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

**Nonstandard Abbreviations:** SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RT-qPCR, reverse transcription quantitative PCR; Cq, quantification cycle; IVD, in vitro diagnostic; EQA, external quality assessment; LOD, limit of detection.

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J. Huggett was responsible for conceptualization, supervision, funding acquisition, data analysis and writing the original draft of the manuscript; D. Evans conducted data curation, analysis and was responsible for visualization and manuscript drafting; S. Cowen conducted investigation, validation, visualization, data curation and manuscript drafting; D. O'Sullivan, G. Stewart and S. Hingley-Wilson conducted supervision and manuscript drafting; K. Harris, J. Vandesompele, K. Hong, J. Verwilt, J. In and N. Storey con-

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