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Caution in interpretation of SARS-CoV-2 quantification based on RT-PCR cycle threshold value

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ARTICLE INFO

Article history:
Received 8 December 2020
Revised in revised form 15 February 2021
Accepted 27 February 2021
Available online 4 March 2021

ABSTRACT

RT-PCR is the reference method for diagnosis of a Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) infection. During the setting up of 6 SARS-CoV-2 RT-PCR assays in our laboratory, comparative evaluations were systematically undertaken and allowed to evidence major discrepancies on cycle threshold RT-PCR results between techniques. These tendencies were confirmed in routine application when analyzing sequential samples from the same patients. Our aim was to examine the impact of the technique among factors influencing RT-PCR result, a far surrogate of ‘viral load’ in the heterogeneous environment of respiratory specimens.

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1. Introduction

In the setting of the world outbreak of Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2), responsible for Coronavirus Disease-19 (COVID-19), nucleic acid testing is the standard method for acute infection diagnosis. SARS-CoV-2 RT-PCR result is sometimes referred to as “viral load,” whereas this term is often used in an inappropriate way. Undeniably, SARS-CoV-2 RT-PCR results are expressed as cycle threshold (Ct) values, which can provide a semi-quantitative estimate of viral genome levels in clinical specimens. However, several elements have to be considered for accurate use and interpretation of Ct values in this manner. First, as for all respiratory viruses, detection relies on the quality of sampling and experienced staff is required (Piras et al., 2020). The possible joint amplification of a cellular gene indicates if cells are present, but not the cell type – not all are virus target cells. Constraints concerning viral inactivation prior to extraction are taken into account individually in each laboratory and false negative results have been observed when using thermal inactivation (Pan et al., 2020). Next, quality of RNA extraction fluctuates according to the method chosen, especially on respiratory specimens for which the viscosity may be elevated. For the RT-PCR itself, analytical sensitivity for most commercial assays is similar, generally around 100 RNA copies/reaction. However, this limit of detection is determined on plasmid or synthetic transcript sequential dilutions, not identical to extracted products from infected cells potentially containing large amounts of cellular derivatives. Finally, the designation “viral load” is restricted to PCR performed with standards, allowing Ct translation into copies/ml (Han et al., 2020). Up to day many different type of standards exist, as elaboration of an international calibration standard is only under progress yet (Vogels et al., 2020). A growing literature describes comparisons between SARS-CoV-2 RT-PCR techniques (Dust et al., 2020; Kasteren et al., 2020; Procop et al., 2020; Zhen et al., April 27, 2020), sometimes underlying discrepancies between assays. Our aim was to assess this issue in our local laboratory setting, to complete the analysis on clinical samples with quality controls and to highlight the importance of considering the RT-PCR performances when interpreting the Ct value.

2. Methods and results

During establishment of numerous SARS-CoV-2 RT-PCR assays in our laboratory since March 2020, comparative evaluations were systematically undertaken. Fig. 1 summarizes comparisons performed between 4 RT-PCR assays (A to D) and 2 unitary rapid one-step extraction/RT-PCR assays (E and F). Techniques were chosen successively mainly according to announced performances and local equipment availability – allowing automation. The 6 assays were A- inhous RT-PCR based on gene E amplification (Corman et al., 2020), B- Bosphore\textsuperscript{v2} nCoV assay (Anatolia geneworks), C- Allplex\textsuperscript{Tm} nCoV assay (Seegene), D- RealTime SARS-CoV-2 assay on M2000 (Abbott), E- Xpert\textsuperscript{X} Xpress SARS-CoV-2 assay (Cepheid) and F- Simplexa\textsuperscript{Tm}
COVID-19 Direct (Diasorin molecular). All 6 assays were monitored by an internal control. The initial evaluation was realized on samples selected in March and April 2020 for their varied Ct results obtained by initial testing. Comparison were performed on gene E Ct result for techniques A, B, C and E, on median RdRP and N Ct result for techniques C vs D, and on median Ct result for technique C (amplifying E, N, RdRP) vs F (amplifying S and orf1). During the evaluation period, whereas qualitative concordance was 100% on the same gene (additional negative results were obtained on 43 samples, data not shown), we evidenced ‘quantitative’ discrepancies on positive results.

Fig. 1. SARS-CoV-2 RT-PCR cycle thresholds differences (delta Ct) between techniques on the same sample during comparative evaluation of 6 assays. A- in-house RT-PCR based on gene E amplification (Corman et al., January 23, 2020), B- Bosphore v2 nCoV assay (Anatolia geneworks), C- Allplex nCoV assay (Seegene), D- RealTime SARS-CoV-2 assay (Abbott), E- Xpert Xpress SARS-CoV-2 assay (Cepheid), and F- Simplexa COVID-19 Direct (Diasorin molecular). Techniques E and F are unitary rapid one-step extraction/RT-PCR assays. Open circles: clinical samples, selected in March and April 2020 for varied initial Ct value; open triangles: quality controls, composed of Qnostics (Randox laboratories) and/or QCMD 2020 panel for external quality assessment. Error bars show medians and interquartile ranges (GraphPad Prism v9 software).

Fig. 2. Bland-Altman representations of comparisons of RT-PCR cycle thresholds (Ct) obtained on the same sample by 2 comparative assays (GraphPad Prism v9 software). Technique A- in-house RT-PCR based on gene E amplification (Corman et al., January 23, 2020), B- Bosphore v2 nCoV assay (Anatolia geneworks), C- Allplex nCoV assay (Seegene), D- RealTime SARS-CoV-2 assay (Abbott), E- Xpert Xpress SARS-CoV-2 assay (Cepheid), F- Simplexa COVID-19 Direct (Diasorin molecular). Similar or higher numbers of negative specimens were also tested for evaluation and found negative by both techniques (n = 43, data not shown). Solid lines indicate bias and horizontal dotted lines indicate 95% limits of agreement. Black dotted lines indicate simple linear regression.
Table 1.
Technical characteristics of the 6 SARS-CoV-2 RT-PCR assays (A to F).

| Technique | LOD (copies/ml) | Sample volume as input | Fraction of elution used as PCR input | Volume equivalent analyzed |
|-----------|-----------------|------------------------|--------------------------------------|---------------------------|
| A (in-house) | nd | 200 μL | 10% | 20 μL |
| B (Bosphore) | 625 | 200 μL | 20% | 40 μL |
| C (Allplex) | 100 | 300 μL | 8% | 24 μL |
| D (M2000) | 100 | 500 μL | 50% | 250 μL |
| E (Xpert) | 250 | 300 μL | one-step, 300 μL analyzed | 300 μL |
| F (Simplexa) | 242 | 50 μL | one-step, 10 μL analyzed | 10 μL |

LOD = limit of detection; nd = not determined.

between assays. Delta Ct ranged from -27.4 to +7.3 on the same sample tested by 2 distinct kits (Fig. 1). Such discrepancies on clinical samples could reflect cell derivatives interference on viral genome amplification. However, similar trends were observed when comparing techniques on quality controls (Fig. 1), constituted of cell culture supernatants with low cellularity.

Ct values between assays were correlated, with no significant drift depending on the genome quantity, especially when comparing techniques B (Bosphore), C (Allplex), D (M2000) and E (Xpert) (Fig. 2). However, whereas Ct values could be considered as equivalent (+/- 3 Ct for a majority of samples) by techniques A (in-house), B (Bosphore), C (Allplex) and E (Xpert), Ct values were constantly earlier with techniques D (M2000) (median -10.8 vs technique C) and F (Simplexa) (median -3.0 vs technique C) (Fig. 1 and 2). These discrepancies could not be solely related to technical parameters of sample input and fraction of nucleic acid elution used as input in the PCR (Table 1), as it is commonly admitted that a variation of a factor 10 in the genome quantity measurement is reflected by a variation of approximately 3 in the Ct value. Interestingly, when testing serial dilutions of both a clinical sample and a quality control sample (Table 2), a similar detection cut-off was measured, in accordance of findings earlier for other respiratory viruses (Wishaupt et al., 2017).

Moreover, in routine application during a 2-month study period (March – April 2020) with over 8000 RT-PCRs performed, we analyzed sequential samples from 833 patients and observed various temporal profiles. Apart from the sample collection issue, variations may reflect numerous factors including infection kinetics, clinical severity, immune response and potential treatments. We also evidence in this study the variation induced by the RT-PCR technique itself. Six representative patients, for whom longitudinal samples were processed by 2 different assays, are presented in Fig. 3. This selection consisted mostly of severe cases presenting prolonged viral excretion, as it has been previously described (Sethuraman et al., 2020). We observed the same individual evolution profiles by both assays, but confirmed qualitative Ct result differences with delta Ct of up to 15 on the same sample, and even a few qualitative discrepancies with samples found positive with one technique and negative with the other.

3. Discussion and conclusion

SARS-CoV-2 RT-PCR is the gold standard diagnosis method with high sensitivity. Numeric result of the RT-PCR, given as a Ct value, is assuredly informative about the level of genome quantity in the analyzed sample. Such information can be very useful for patient management, especially during follow-up of severe infections. However, respiratory specimens represent heterogeneous environments, as described earlier for other respiratory viruses (Wishaupt et al., 2017).

Table 2.
Cycle thresholds (Ct) obtained by 5 SARS-CoV-2 RT-PCR assays (test B to test F) on serial dilutions of a selected clinical specimen (A) and a Qnostics quality control (B).

| A Dilution | Clinical sample | Ct/test B | Ct/test C | Ct/test D | Ct/test E | Ct/test F |
|------------|-----------------|------------|------------|------------|------------|------------|
| E gene     | E gene          | E gene     | E gene     | E gene     | E gene     | E gene     |
| none       | 24.6            | 24.5       | pos        | pos        | 17.2       | ND         |
| 10-1       | 28.6            | 29.0       | pos        | pos        | 19.8       | ND         |
| 10-2       | 33.0            | 32.6       | pos        | pos        | 24.3       | ND         |
| 10-3       | 35.1            | >40        | >45        | >45        | 27.7       | 39.2       |
| 10-4       | >40             | >45        | >45        | >45        | >40        | >40        |
| 10-5       | >40             | >45        | >45        | >45        | >40        | >40        |

| B Dilution | Quality control | Ct/test B | Ct/test C | Ct/test D | Ct/test E | Ct/test F |
|------------|-----------------|------------|------------|------------|------------|------------|
| E gene     | E gene          | E gene     | E gene     | E gene     | E gene     | E gene     |
| none       | 27.5            | 28.6       | pos        | pos        | 20.3       | ND         |
| 10-1       | 31.1            | 31.8       | pos        | pos        | 23.6       | ND         |
| 10-2       | 35.0            | 35.7       | pos        | pos        | 26.9       | 35.6       |
| 10-3       | >40             | >45        | >45        | >45        | >40        | >40        |
| 10-4       | >40             | >45        | >45        | >45        | >40        | >40        |
| 10-5       | >40             | >45        | >45        | >45        | >40        | >40        |

ND = not done by test E (Xpert); pos = positive result.
* Results by test C (Allplex) only qualitative due to kit version modification.
SARS-CoV-2 RT-PCR in respiratory samples with varied volume and cellularity, differ largely from standardized and repeatable viral loads in blood. SARS-CoV-2 RT-PCR Ct values only indicate a semi-quantitative evaluation of genome quantity and are influenced by many factors. Among those factors, our study and others highlight the importance of the RT-PCR method used (Dust et al., 2020; Kasteren et al., 2020; Procop et al., 2020; Zhen et al., 2020). The potential patient contagiousness, evaluated in the foreground on clinical criteria and time from symptoms onset, cannot be determined on the sole RT-PCR Ct result and can also be partly estimated after inoculation in cell culture (Walsh et al., 2020). If PCR thresholds are given beyond which viral excretion can be estimated as low, they will remain strictly dependent on the technique used. As numerous kits are available worldwide, several techniques being even sometimes implemented in the same laboratory due to various local constraints, RT-PCR Ct values require informed interpretation. Such thresholds also remain restricted to sample type and sampling site. Furthermore, interpretation has to take into account the differential situation of a result on a single diagnosis sample and on iterative sampling from more severe patients. Any longitudinal monitoring should be based on the same technique in the same experimental conditions. In the absence of normalization of the SARS-CoV-2 RT-PCR Ct, decisions on the management of PCR positive patients remain challenging based on this data alone.

Acknowledgments

The authors would like to acknowledge the staff at the virology laboratory for their strong commitment to this new coronavirus diagnosis activity for our patients.

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