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

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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 thermic 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- in-house RT-PCR based on gene E amplification (Corman et al., 2020), B- Bosphore® v2 nCoV assay (Anatolia geneworks), C- Allplex™ nCoV assay (Seegene), D- RealTime SARS-CoV-2 assay on M2000 (Abbott), E- Xpert® Xpress SARS-CoV-2 assay (Cepheid) and F- Simplexa™

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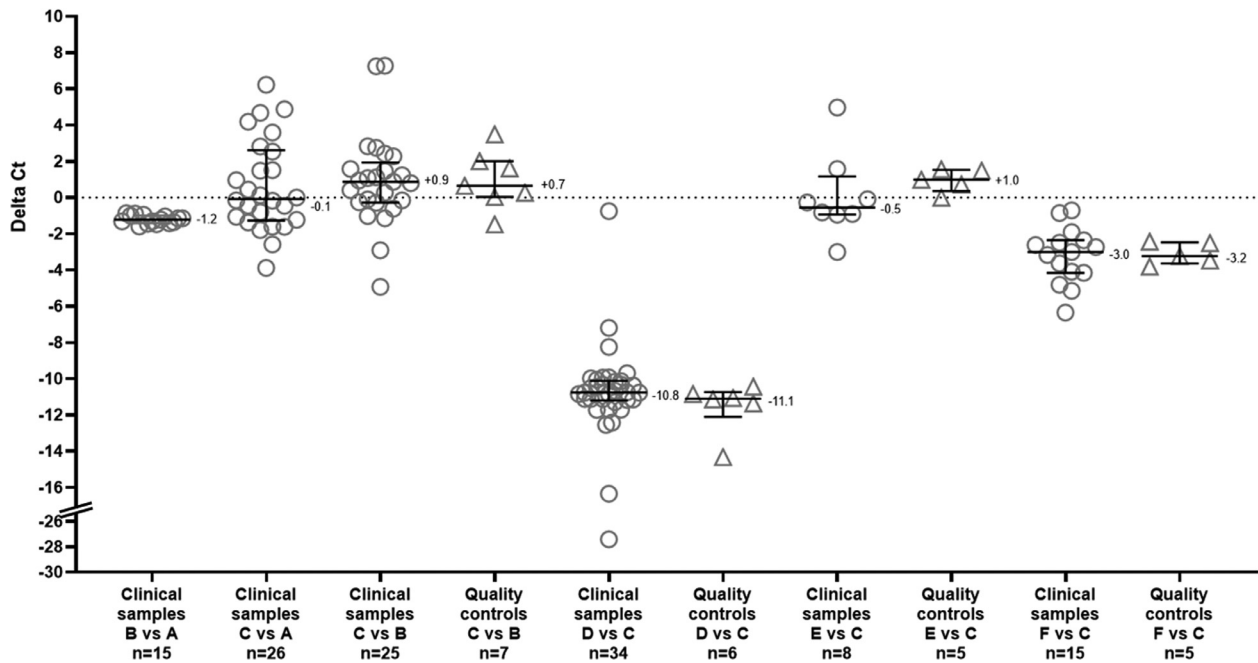


Fig. 1. SARS-CoV-2 RT-PCR cycle thresholds differences (delta Ct) between techniques on the same sample during comparative evaluation of 6 assays. A- in-house RT-PCR based on gene E amplification (Corman et al., January 23, 2020), B- Bosphore® v2 nCoV assay (Anatolia geneworks), C- Allplex™ nCoV assay (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).

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

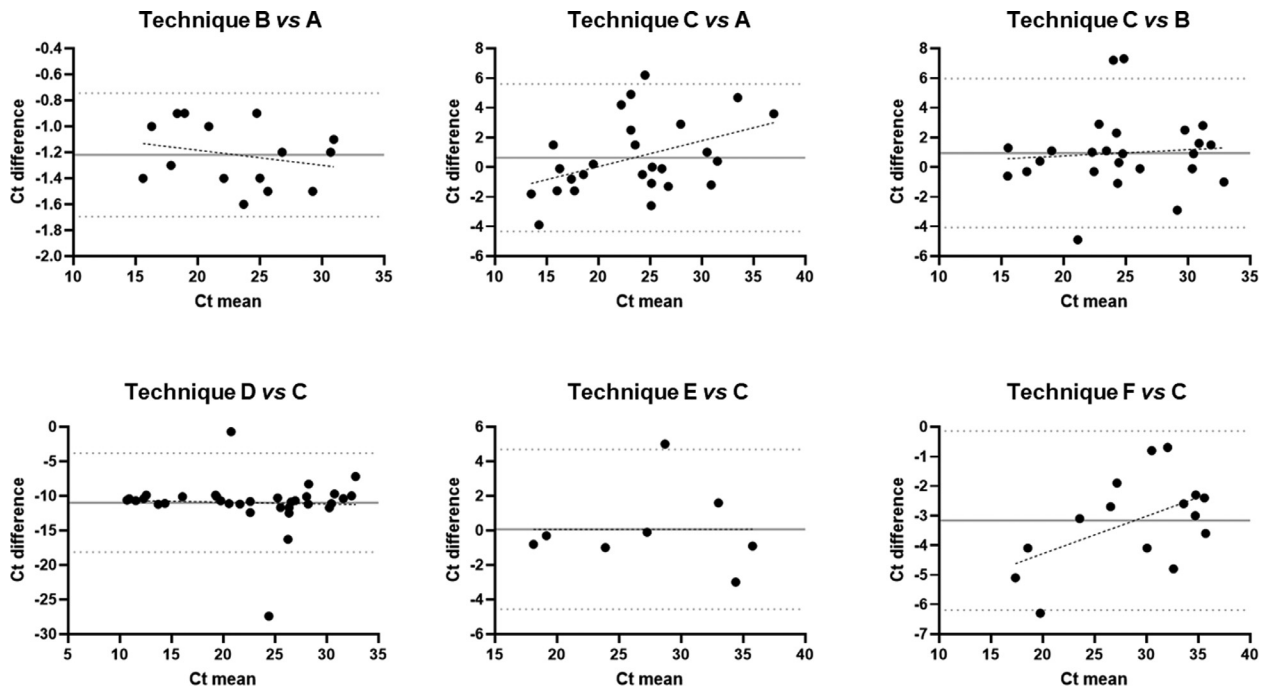


Fig. 2. Bland-Altman representations of comparisons of RT-PCR cycle thresholds (Ct) obtained on the same sample by 2 comparative assays (GraphPad Prism v9 software). Technique A- in-house RT-PCR based on gene E amplification (Corman et al., January 23, 2020), B- Bosphore v2 nCoV assay (Anatolia geneworks), 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 μ L	10%	20 μ L
B (Bosphore)	625	200 μ L	20%	40 μ L
C (Allplex)	100	300 μ L	8%	24 μ L
D (M2000)	100	500 μ L	50%	250 μ L
E (Xpert)	250	300 μ L	one-step, 300 μ L analyzed	300 μ L
F (Simplexa)	242	50 μ L	one-step, 10 μ L analyzed	10 μ L

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 limit of detection data announced by manufacturers and reported in Table 1. The earlier Ct values obtained by technique D (M2000), and to a lesser extend F (Simplexa), were then not related to a better sensitivity. Additionally, reliability of the techniques over time was verified through the regular testing of an independent quality control (Qnostics®, Randox laboratories). Results on this quality control on a 6 months period allowed calculation of coefficients of variation of 3.5% (orf1) and 6.6% (gene E) for technique B (Bosphore); 4.0% (gene

E), 3.5% (gene N) and 3.4% (gene RdRP) for technique C (Allplex); 1.7% (gene RdRP+N) for technique D (M2000). Coefficients of variation mainly below 5% attest to a satisfactory stability of the tests in time.

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 quantitative 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's 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 C*			Ct/test D N/RdRP gene	Ct/test E		Ct/test F	
	Ct/test B E gene	orf1	E gene	N gene	RdRP gene		E gene	N gene	S gene	orf1
none	24,6	24,5	pos	pos	pos	17,2	ND	ND	22,3	22,8
10-1	28,6	29,0	pos	pos	pos	19,8	ND	ND	29,4	29,1
10-2	33,0	32,6	pos	>45	>45	24,3	ND	ND	31,3	31,2
10-3	35,1	>40	>45	>45	>45	27,7	39,2	40,8	33,4	34,0
10-4	>40	>40	>45	>45	>45	>40	>45	>45	>40	>40
10-5	>40	>40	>45	>45	>45	>40	ND	ND	>40	>40
B Dilution	Quality control		Ct/test C*			Ct/test D N/RdRP gene	Ct/test E		Ct/test F	
	Ct/test B E gene	orf1	E gene	N gene	RdRP gene		E gene	N gene	S gene	orf1
none	27,5	28,6	pos	pos	pos	20,3	ND	ND	26,1	25,7
10-1	31,1	31,8	pos	>45	pos	23,6	ND	ND	31,3	30,1
10-2	35,0	35,7	>45	>45	>45	26,9	35,6	40,1	31,7	32,5
10-3	>40	>40	>45	>45	>45	>40	>45	>45	>40	>40
10-4	>40	>40	>45	>45	>45	>40	ND	ND	>40	>40
10-5	>40	>40	>45	>45	>45	>40	ND	ND	>40	>40

ND = not done by test E (Xpert); pos = positive result.

* Results by test C (Allplex) only qualitative due to kit version modification.

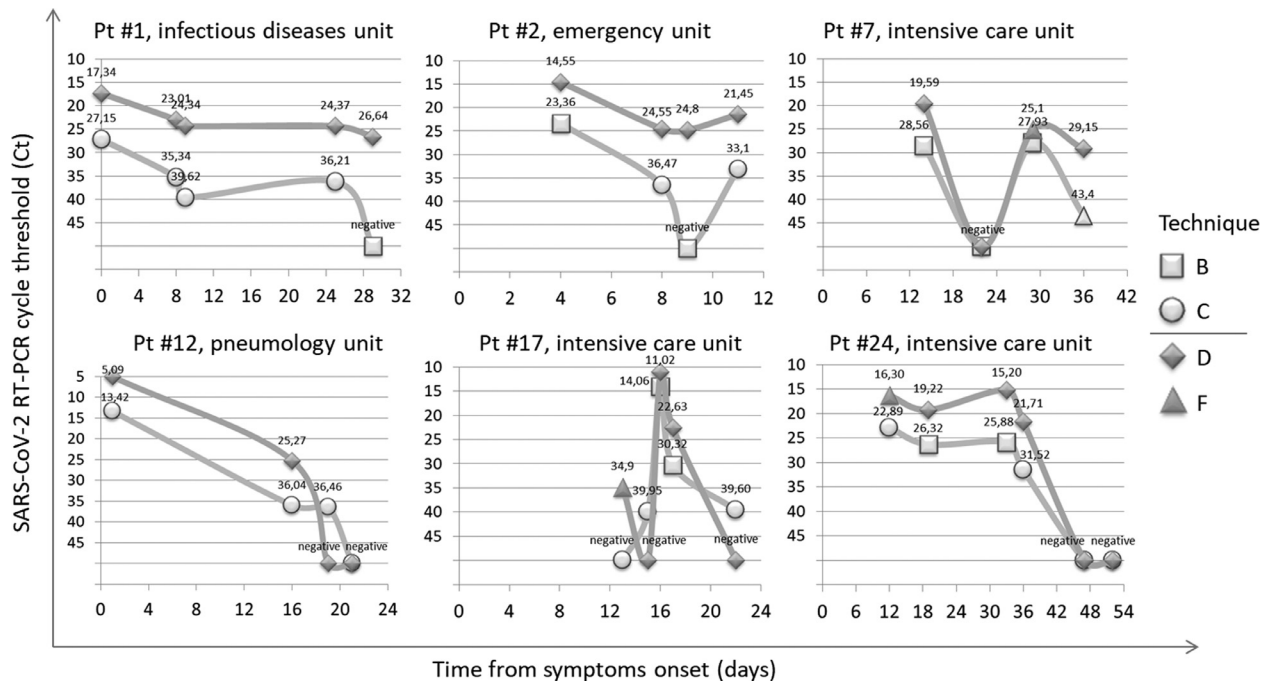


Fig. 3. Temporal profiles of SARS-CoV-2 RT-PCR cycle thresholds (Ct) for six representative patients. Samples (nasopharyngeal swabs) were processed by 2 comparative assays: B-Bosphore v2 nCoV assay (Anatolia geneworks) or C- Allplex nCoV assay (Seegene), vs D- RealTime SARS-CoV-2 assay (Abbott) or F- Simplexa COVID-19 Direct (Diasorin molecular).

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.

4. Author contributions statement

Aurélien Schnuriger and Laurence Morand-Joubert conceptualized the study. Aurélien Schnuriger performed the data curation and wrote the original draft. Marine Perrier and Laurence Morand-Joubert reviewed the draft. Valérie Marinho performed the technical work. All authors collected the data and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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