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.

1   **Introduction**

2   In the setting of the world outbreak of Severe Acute Respiratory Syndrome-Coronavirus-2

3   (SARS-CoV-2), responsible for Coronavirus Disease-19 (COVID-19), nucleic acid testing is the

4   standard method for acute infection diagnosis. SARS-CoV-2 RT-PCR result is sometimes

5   referred to as 'viral load', whereas this term is often used in an inappropriate way.

6   Undeniably, SARS-CoV-2 RT-PCR results are expressed as cycle threshold (Ct) values, which

7   can provide a semi-quantitative estimate of viral genome levels in clinical specimens

8   However, several elements have to be considered for accurate use and interpretation of Ct

9   values in this manner. First, as for all respiratory viruses, detection relies on the quality of

10   sampling and experienced staff is required [1]. The possible joint amplification of a cellular

11   gene indicates if cells are present, but not the cell type – not all are virus target cells.

12   Constraints concerning viral inactivation prior to extraction are taken into account

13   individually in each laboratory and false negative results have been observed when using

14   thermic inactivation [2]. Next, quality of RNA extraction fluctuates according to the method

15   chosen, especially on respiratory specimens for which the viscosity may be elevated. For the

16   RT-PCR itself, analytical sensitivity for most commercial assays is similar, generally around

17   100 RNA copies/reaction. However, this limit of detection is determined on plasmid or

18   synthetic transcript sequential dilutions, not identical to extracted products from infected

19   cells potentially containing large amounts of cellular derivatives. Finally, the designation

20   'viral load' is restricted to PCR performed with standards, allowing Ct translation into

21   copies/ml [3]. Up to day many different type of standards exist, as elaboration of an

22   international calibration standard is only under progress yet [4]. A growing literature

23   describes comparisons between SARS-CoV-2 RT-PCR techniques (5-8], sometimes underlying

24   discrepancies between assays. Our aim was to assess this issue in our local laboratory

1 setting, to complete the analysis on clinical samples with quality controls and to highlight the

2 importance of considering the RT-PCR performances when interpreting the Ct value.

3

4 **Methods and Results**

5 During establishment of numerous SARS-CoV-2 RT-PCR assays in our laboratory since March

6 2020, comparative evaluations were systematically undertaken. Figure 1 summarizes

7 comparisons performed between 4 RT-PCR assays (A to D) and 2 unitary rapid one-step

8 extraction/RT-PCR assays (E and F). Techniques were chosen successively mainly according

9 to announced performances and local equipment availability – allowing automation. The 6

10 assays were A- in-house RT-PCR based on gene E amplification [9], B- Bosphore® v2 nCoV

11 assay (Anatolia geneworks), C- Allplex^{TM} nCoV assay (Seegene), D- RealTime SARS-CoV-2

12 assay on M2000 (Abbott), E- Xpert® Xpress SARS-CoV-2 assay (Cepheid) and F- Simplexa^{TM}

13 COVID-19 Direct (Diasorin molecular). All 6 assays were monitored by an internal control.

14 The initial evaluation was realized on samples selected in March and April 2020 for their

15 varied Ct results obtained by initial testing. Comparison were performed on gene E Ct result

16 for techniques A, B, C and E, on median RdRP and N Ct result for techniques C *vs* D, and on

17 median Ct result for technique C (amplifying E, N, RdRP) *vs* F (amplifying S and orf1). During

18 the evaluation period, whereas qualitative concordance was 100% on the same gene

19 (additional negative results were obtained on 43 samples, data not shown), we evidenced

20 'quantitative' discrepancies on positive results between assays. Delta Ct ranged from -27.4

21 to +7.3 on the same sample tested by 2 distinct kits (Figure 1). Such discrepancies on clinical

22 samples could reflect cell derivatives interference on viral genome amplification. However,

23 similar trends were observed when comparing techniques on quality controls (Figure 1),

24 constituted of cell culture supernatants with low cellularity.

1   Ct values between assays were correlated, with no significant drift depending on the

2   genome quantity, especially when comparing techniques B (Bosphore), C (Allplex), D

3   (M2000) and E (Xpert) (Figure 2). However, whereas Ct values could be considered as

4   equivalent (+/- 3 Ct for a majority of samples) by techniques A (in-house), B (Bosphore), C

5   (Allplex) and E (Xpert), Ct values were constantly earlier with techniques D (M2000) (median

6   -10.8 *vs* technique C) and F (Simplexa) (median -3.0 *vs* technique C) (Figure 1 and 2). These

7   discrepancies could not be solely related to technical parameters of sample input and

8   fraction of nucleic acid elution used as input in the PCR (Table 1), as it is commonly admitted

9   that a variation of a factor 10 in the genome quantity measurement is reflected by a

10   variation of approximately 3 in the Ct value. Interestingly, when testing serial dilutions of

11   both a clinical sample and a quality control sample (table 2), a similar detection cut-off was

12   measured, in accordance of limit of detection data announced by manufacturers and

13   reported in Table 1. The earlier Ct values obtained by technique D (M2000), and to a lesser

14   extend F (Simplexa), were then not related to a better sensitivity. Additionally, reliability of

15   the techniques over time was verified through the regular testing of an independent quality

16   control (Qnostics©, Randox laboratories). Results on this quality control on a 6 months

17   period allowed calculation of coefficients of variation of 3.5% (orf1) and 6.6% (gene E) for

18   technique B (Bosphore); 4.0% (gene E), 3.5% (gene N) and 3.4% (gene RdRP) for technique C

19   (Allplex); 1.7% (gene RdRP+N) for technique D (M2000). Coefficients of variation mainly

20   below 5% attest to a satisfactory stability of the tests in time.

21   Moreover, in routine application during a 2-month study period (March – April 2020)

22   with over 8000 RT-PCRs performed, we analyzed sequential samples from 833 patients and

23   observed various temporal profiles. Apart from the sample collection issue, variations may

24   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 Figure 3. This selection consisted mostly of severe

cases presenting prolonged viral excretion, as it has been previously described [10]. 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.

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 [11]. 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 [5-8]. 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 [12]. 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,

1 several techniques being even sometimes implemented in the same laboratory due to

2 various local constraints, RT-PCR Ct values require informed interpretation. Such thresholds

3 also remain restricted to sample type and sampling site. Furthermore, interpretation has to

4 take into account the differential situation of a result on a single diagnosis sample and on

5 iterative sampling from more severe patients. Any longitudinal monitoring should be based

6 on the same technique in the same experimental conditions. In the absence of normalization

7 of the SARS-CoV-2 RT-PCR Ct, decisions on the management of PCR positive patients remain

8 challenging based on this data alone.

9

10 Word count 1303

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1 **Figure legends**

2 **Figure 1:**

3 SARS-CoV-2 RT-PCR cycle thresholds differences (delta Ct) between techniques on the same

4 sample during comparative evaluation of 6 assays. A- in-house RT-PCR based on gene E

5 amplification [9], B- Bosphore® v2 nCoV assay (Anatolia geneworks), C- Allplex[TM] nCoV assay

6 (Seegene), D- RealTime SARS-CoV-2 assay (Abbott), E- Xpert® Xpress SARS-CoV-2 assay

7 (Cepheid) and F- Simplexa[TM] COVID-19 Direct (Diasorin molecular). Techniques E and F are

8 unitary rapid one-step extraction/RT-PCR assays. Open circles: clinical samples, selected in

9 March and April 2020 for varied initial Ct value; open triangles: quality controls, composed of

10 Qnostics (Randox laboratories) and/or QCMD 2020 panel for external quality assessment.

11 Error bars show medians and interquartile ranges (GraphPad Prism v9 software).

12 **Figure 2:**

13 Bland-Altman representations of comparisons of RT-PCR cycle thresholds (Ct) obtained on

14 the same sample by 2 comparative assays (GraphPad Prism v9 software). Technique A- in-

15 house RT-PCR based on gene E amplification [9], B- Bosphore v2 nCoV assay (Anatolia

16 geneworks), C- Allplex nCoV assay (Seegene), D- RealTime SARS-CoV-2 assay (Abbott), E-

17 Xpert Xpress SARS-CoV-2 assay (Cepheid), F- Simplexa COVID-19 Direct (Diasorin molecular).

18 Similar or higher numbers of negative specimens were also tested for evaluation and found

19 negative by both techniques (n=43, data not shown). Solid lines indicate bias and horizontal

20 dotted lines indicate 95% limits of agreement. Black dotted lines indicate simple linear

21 regression.

22 **Figure 3:**

23 Temporal profiles of SARS-CoV-2 RT-PCR cycle thresholds (Ct) for six representative patients.

24 Samples (nasopharyngeal swabs) were processed by 2 comparative assays: B- Bosphore v2

1   nCoV assay (Anatolia geneworks) or C- Allplex nCoV assay (Seegene), *vs* D- RealTime SARS-

2   CoV-2 assay (Abbott) or F- Simplexa COVID-19 Direct (Diasorin molecular).

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	200µl	10%	20µl
B (Bosphore)	625	200µl	20%	40µl
C (Allplex)	100	300µl	8%	24µl
D (M2000)	100	500µl	50%	250µl
E (Xpert)	250	300µl	one-step, 300µl analyzed	300µl
F (Simplexa)	242	50µl	one-step, 10µl analyzed	10µl

LOD: limit of detection; nd: not determined.

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	Clinical sample								
	Ct/test B		Ct/test C*			Ct/test D	Ct/test E		Ct/test F
Dilution					RdRP	N/RdRP			
	E gene	orf1	E gene	N gene	gene	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	Quality control								
	Ct/test B		Ct/test C*			Ct/test D	Ct/test E		Ct/test F
Dilution					RdRP	N/RdRP			
	E gene	orf1	E gene	N gene	gene	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.
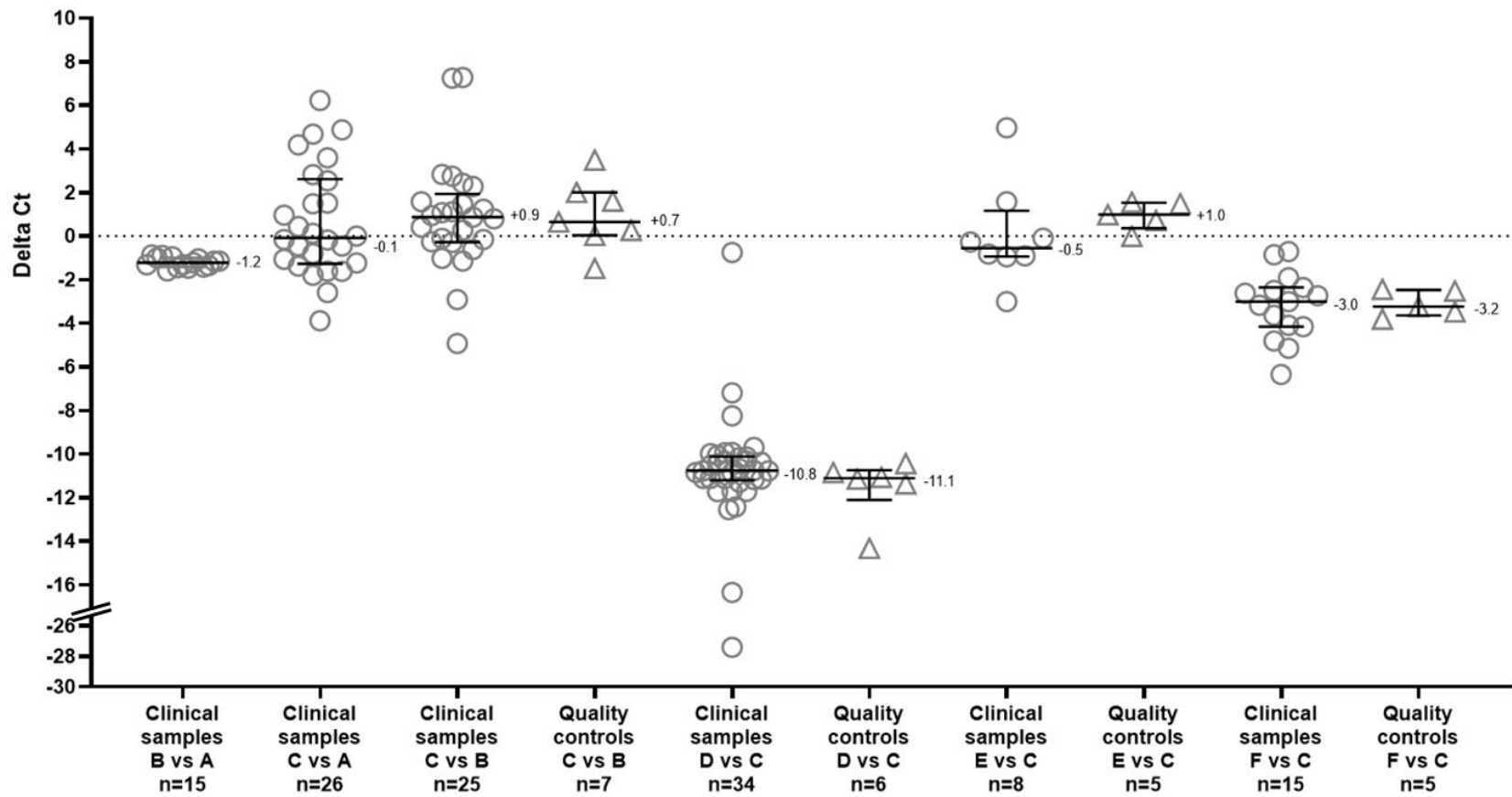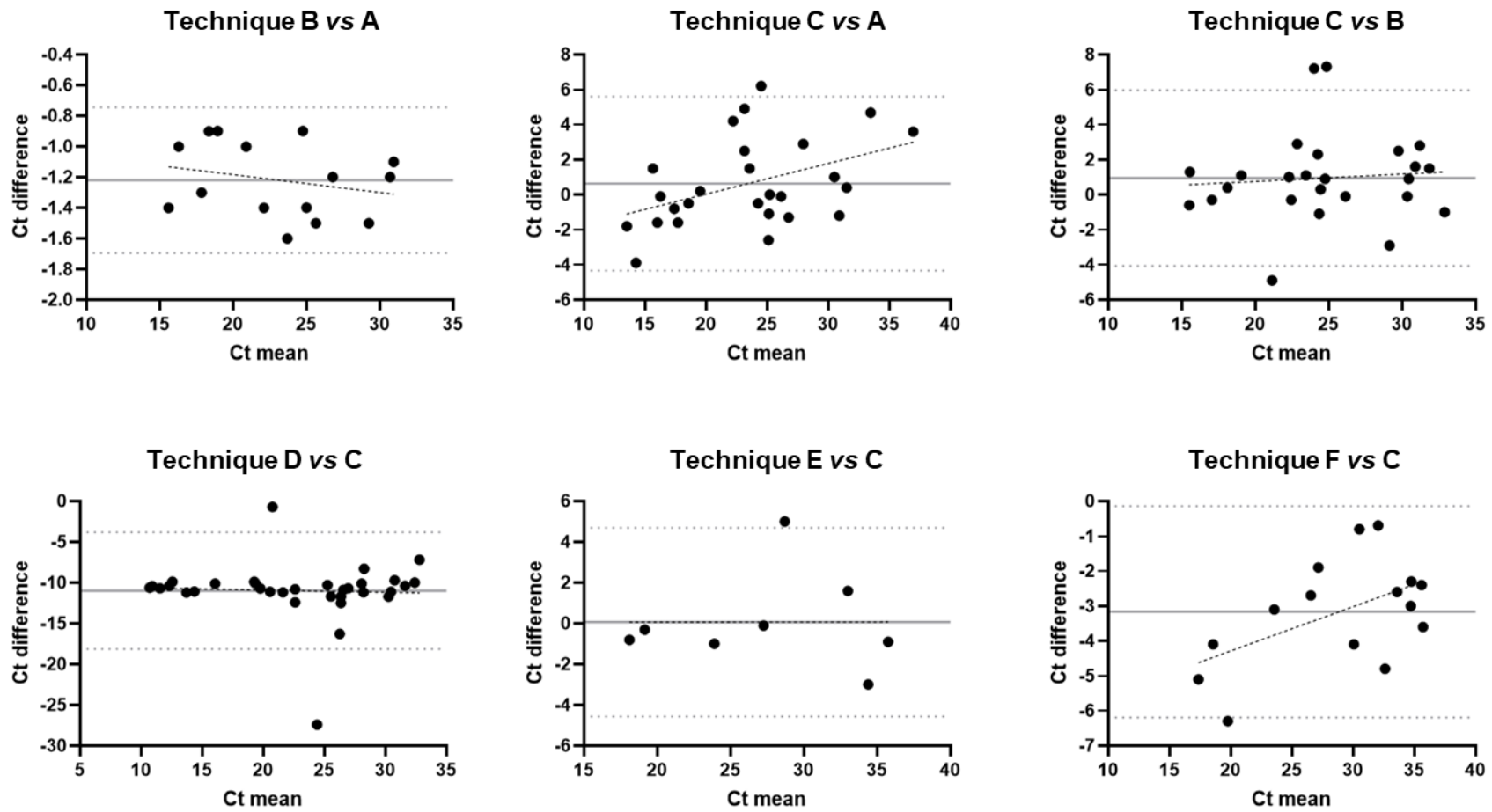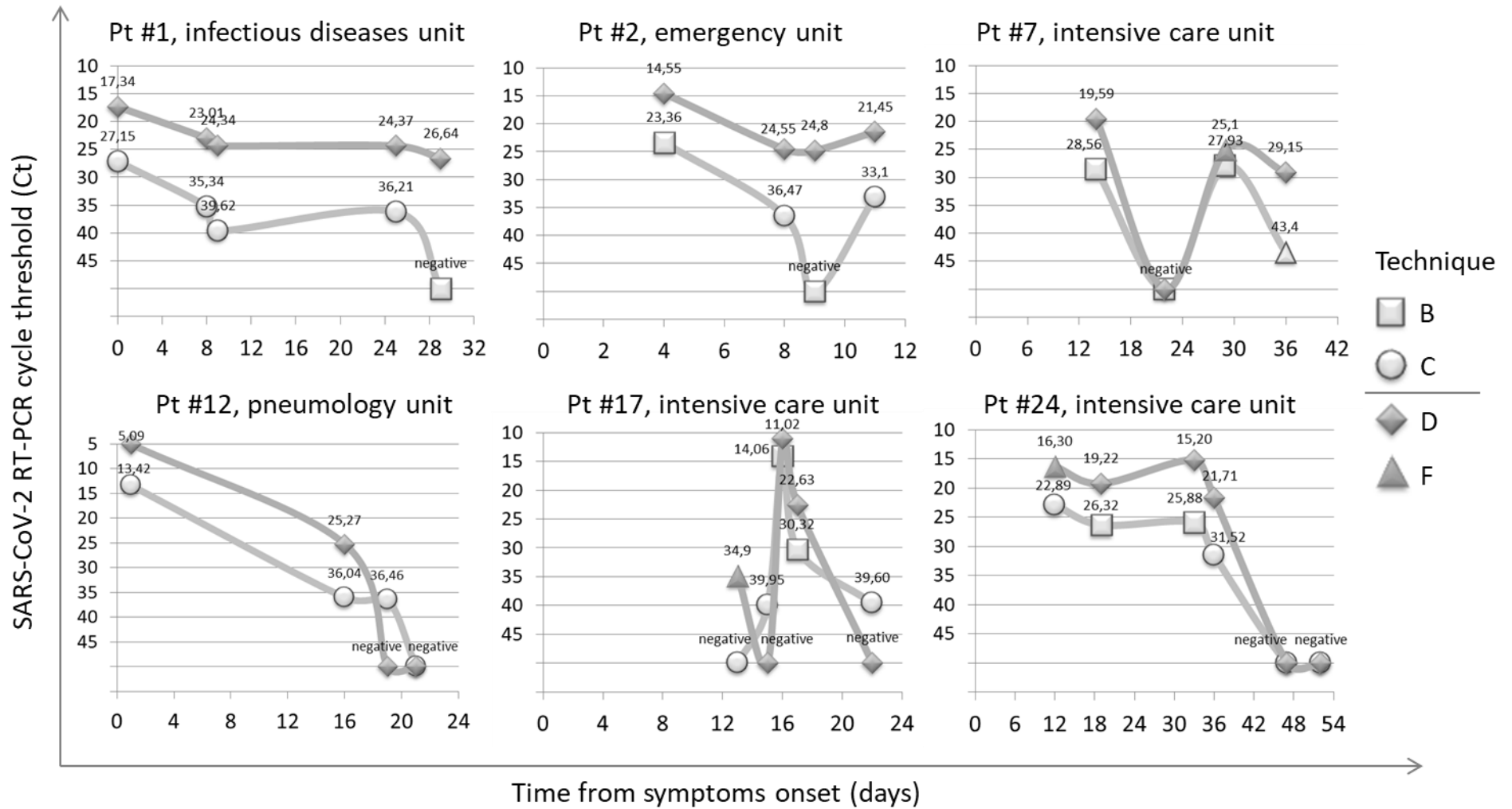
Figure 1

Figure 2

Figure 3