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Potential pitfalls in analysing a SARS-CoV-2 RT-PCR assay and how to standardise data interpretation

Melvyn Smith [a,*], Kate El Bouzidi [b], Simon Bengen [c], Aron Cohen [a,b,c], Mark Zuckerman [b]

[a] Viapath Analytics, South London Specialist Virology Centre, King's College Hospital NHS Foundation Trust, Denmark Hill, London SE5 9RS, UK
[b] South London Specialist Virology Centre, King's College Hospital NHS Foundation Trust, London, UK
[c] Diagnostics.ai, 59a Brent Street, London, UK

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ABSTRACT

The emergence of SARS-CoV-2 in December 2019 lead to the rapid implementation of assays for virus detection, with real-time RT-PCR arguably considered the gold-standard. In our laboratory Altona RealStar SARS-Cov-2 RT-PCR kits are used with Applied Biosystems QuantStudio 7 Flex thermocyclers. Real-time PCR data interpretation is potentially complex and time-consuming, particularly for SARS-CoV-2, where the laboratory handles up to 2000 samples each day. To simplify this, an automated system that rapidly interprets the curves, developed by diagnostics.ai was introduced. QuantStudio software provides two methods for interpretation, relative threshold and baseline threshold. Many of our assays are analysed using relative threshold and directly exported into pcr.ai software, however, in some rare cases the QuantStudio software assigns positive results to 'ambiguous' curves, flagged by pcr.ai, requiring manual intervention. Due to the sample numbers processed and the proportionate increase in curves flagged by pcr.ai, the two methods were investigated. An audit was carried out to determine the frequency of these curves, involving 138 samples tested during November 2020, including 97 serial samples from 38 patients and it was determined that the relative threshold method produced unreliable results in many of these cases. In addition, we present a solution to simplify the interpretation and automate the process.

1. Introduction

The emergence of SARS-CoV-2 in December 2019 resulted in an unparalleled world-wide effort to characterise the virus and the clinical disease. Coronavirus disease 2019 (COVID-19), follows a biphasic pattern, likely resulting from a combination of the early viral response phase and an inflammatory secondary phase. The typical clinical symptoms of COVID19 are broad and include malaise, fever, cough, shortness of breath, myalgia, sore throat, headache, nausea, or diarrhoea, together with loss of smell and taste (Callejon-Leblic et al., 2021). The World Health Organization (WHO) declared SARS-CoV-2 a pandemic on 11th March 2020. According to the European Centre for Disease Prevention and Control, since 31 December 2019 and as of week 2022–1, 307,373,791 cases of COVID-19 (in accordance with the applied case definitions and testing strategies in the affected countries) have been reported, including 5492,154 deaths worldwide. SARS-CoV-2 is an enveloped β-coronavirus, with a positive sense, single-stranded genome with a sequence similar to both SARS-CoV-1 (80%) and bat coronavirus RaTG13 (96.2%) (Yan et al., 2020). The genome encodes four main structural proteins: the envelope (E) protein, the spike (S) protein, which mediates host cell binding and entry via the peptidase domain of angiotensin-converting enzyme 2 (ACE 2) the nucleocapsid (N) protein and the membrane (M) protein which is organized in a 2D lattice and provides a scaffold in viral assembly, all four proteins are required to form a structurally complete virion (Artika et al., 2020).

RT-PCR is, arguably, considered the gold-standard diagnostic test and in our laboratory we use the Altona RealStar SARS-Cov-2 RT-PCR kit 1.0. This is a multiplex RT-PCR for the qualitative detection of lineage B β-coronavirus and SARS CoV-2 RNA, using two probes specific for the E gene of Beta β Coronavirus and the S gene of SARS CoV-2, together with a heterologous internal control to monitor extraction efficiency and identify inhibition. The assays are run on Applied Biosystems QuantStudio 7 Flex Real-Time PCR systems (ThermoFisher Scientific, Horsham, UK).

One of the issues around real-time PCR analyses is related to the interpretation process, which is complex and time-consuming, particularly so in the case of SARS-CoV-2, where the laboratory typically handles up to 2000 samples each day. To simplify this stage an automated

* Corresponding author.
E-mail address: melvyn.smith@kcl.ac.uk (M. Smith).

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system that rapidly interprets real-time PCR curves from the thermocycler's data, exports the results to the LIMS and monitors all the QC data for quality assurance purposes, called pcr.ai (www.pcr.ai) developed by diagnostics.ai (59a Brent Street, London, UK; www.diagnostics.ai) was introduced. Further developments are underway to apply the software to analyse all the in-house molecular diagnostic tests. The system employs proprietary algorithms to analyse the raw fluorescence thermocycler output data, and holds-up result export to the laboratory information management system (LIMS) if there is any ambiguity in this data.

The interpretation of the various PCR curves could be carried out by either the relative threshold (RT) or baseline threshold (BT) methods. With the baseline method all the amplification curves for a specific target are considered in determining the threshold and hence the Ct values for each sample. The relative method sets a threshold for each individual amplification curve, based on its shape and disregards the height and variability of the curve during early baseline fluorescence. This method uses a model of reaction efficiency to estimate the amplification curve, which is then used to determine the relative threshold and the Ct values derived from it, which are approximately in the middle of the exponential growth region (ThermoFisher application note, 2016). While most of our assays can be analysed using the RT method, with very little manual intervention before exporting into the pcr.ai software, there are some rare cases where the QuantStudio software assigns a positive result to ambiguous curves, flagged by pcr.ai, that require manual intervention to determine their true status. Given a rise in the number of curves being flagged for review by pcr.ai and due to the sample numbers being processed each day by the Altona assay, the two methods were investigated further to determine the most effective approach to analyse the samples and provide the correct data for the machine learning processes.

A clinical audit was carried out to determine, whilst testing over 1000 samples a day, the frequency of finding PCR curves that were difficult to interpret, involving 138 samples tested during November 2020 that included 40 serial samples. The latter were of interest as we also wished to look at the samples with results that were positive at the limit of detection of the assay as there had been a discussion about their clinical significance. In addition, we present a solution to simplify interpretation and automate the process.

2. Materials and methods

Samples were extracted on the KingFisher automated extraction and purification system with the MagMaxCore Extraction kit with 300 μl of sample eluted in 120 μl of elution buffer as described by the manufacturer (ThermoFisher Scientific, Horsham, UK). Twelve microliters of provided internal control material was added to the lysis buffer per sample. The Altona RealStar assay was carried out according to the manufacturer's instructions using 10 μl of extracted sample in a total reaction volume of 30 μl. The assay uses a probe specific for B-βCoV targeting the E gene and a probe specific for SARS-CoV-2 targeting the S gene, also included are primers and a probe specific for a heterologous internal control. Reaction conditions were as per manufacturer's instructions, with a $>$ 45 cycle cut-off (denoting a not detected result for each gene target) and threshold levels set automatically for both methods. The positive at limit of detection (PLOD) value as judged by running both the Qnostics (Glasgow, Scotland) analytical panel and the NIBSC (Hertfordshire, UK) panel was a Ct of $>$ 37 for both E and S gene targets. The manufacturer's guidelines were followed when analysing the results. Briefly, if both the E- and S-genes were positive, the sample is considered SARS-CoV-2 positive; if the E-gene only is positive, the samples is considered a presumptive positive for SARS-CoV-2; if only the S-gene is positive, the sample is considered SARS-CoV-2 positive. If both targets are negative (Ct $>$45; and the IC is positive) the sample is negative. Results were further interpreted as positive at the limit of detection (PLOD) when one, or both targets gave a Ct value $>$ 37 but $<$ 45; a Ct $<$ /= 36 was interpreted as positive.

A total of 138 samples from 79 patients, including 97 serial samples from 38 patients were analysed by the routine RT method and further evaluated by the BT method with the results for both imported and re-analysed by the pcr.ai software.

3. Results

Supplementary Table S1 shows the Ct values and status of the 138 samples analysed by pcr.ai software using data from both the RT and BT methods. Table 1 shows the overall positive and negative results. The RT analyses produced 90/138 (65.2%) positive samples compared to 48/138 (34.8%) using the BT method; we also found 19/138 (13.8%) presumptive positives by RT compared with 6/138 (4.3%) by BT; there were 15/138 (10.9%) PLOD samples by BT analysis, and none by RT. The 15 PLOD samples were all positive by RT (see Table 2), with average E- and S-gene Ct values of 36 and 33 respectively (range 28->45 and 26–34). This compares with average BT values of 36 and 40 (ranges 29->45 and 37->45) for the E- and S-genes. Forty-one individual samples were followed up together with 25 pairs, 9 sets of three, two sets of four and two sets of six over the duration of four weeks. Further comparisons of RT versus BT methods showed that RT analysis would have resulted in lower Ct values (i.e., more positive samples) for both the E- and S-genes in 60 patients, 10 of which were PLOD.

Forty-eight positive samples were in agreement between the two methods, where the Ct values for the E-and S-genes were similar for both methods: 28 and 27 for RT and 29 and 27 for BT respectively. For the 31 presumptive PLOD and PLOD samples by BT the average Ct values were 38 and 43 for the E- and S-genes respectively, this compares with 35 and 33 by RT analysis for the E- and S-genes. There were 13 RT positive samples with E- and S-gene Ct average values of 28 and 27, compared with BT Cts of $>$ 45 (not detected) for both gene targets. There were 110 positives by RT (including positive, presumptive positive and presumptive PLOD samples), of these 98 were overestimated by the RT method, with E- and S-gene average Cts of 27 and 25 (range 17–36 and 16–35)) respectively compared with 31 and 27 for BT analysis (range 18->45 and 17->45 respectively). Fifteen discrepant PLOD by BT samples were positive by RT analysis.

Fig. 1 illustrates the differences between RT and BT analyses when imported into pcr.ai prior to retraining of the learning machine (discussed below). The amplification curves for sample 1a are poor, with low delta Rn values, and RT analysis assigns inappropriate Ct values to the E- and S-genes and no user warning to the status of the sample (Fig. 1a). Whilst there is an observable sigmoidal shape, the delta Rn values are very low for both gene targets; 0.184 for the S-gene and 0.182 for E-gene, this compares with a delta Rn of 0.624 for the IC. This is not at all accounted for in the Ct values assigned by the RT analysis. The BT method however provides a more realistic assessment of the sample together with the appropriate warning message (Fig. 1b).

3.1. Serial samples

3.1.1. Patient 1

Over a three-day period, two samples were collected from patient 1, whom had been admitted to hospital 7 days previously with a COVID

Table 1
Summary of results from Table S1.

Status	RT results	BT results
Positive	90	48
Negative	28	53
PLOD	0	15
Presumptive positive	19	6
Presumptive PLOD	1	16
Total	138	138

Table 2
details of the 15 discrepant PLOD results. *All runs were carried out within 12 h of sample collection.

Lab No.	RT E-gene Ct	BT E-gene Ct	RT S-gene Ct	BT S-gene Ct	Sample date*	RT interpretation	RT SARS-CoV-2 results	BT interpretation	BT SARS-CoV-2 results
4a	34.60	37.85	32.94	40.96	05/11/2020	BOTH GENES POSITIVE	POSITIVE	BOTH GENES PLOD	PLOD
4b	31.07	37.37	29.54	41.29	05/11/2020	BOTH GENES POSITIVE	POSITIVE	BOTH GENES PLOD	PLOD
13d	31.71	32.20	27.60	42.74	01/12/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE POSITIVE/S-GENE PLOD	PLOD
19b	34.82	34.61	33.92	38.03	26/11/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE POSITIVE/S-GENE PLOD	PLOD
20b	33.97	38.15	32.32	41.31	28/11/2020	BOTH GENES POSITIVE	POSITIVE	BOTH GENES PLOD	PLOD
33a	28.38	29.30	27.88	41.62	04/11/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE POSITIVE/S-GENE PLOD	PLOD
41a	32.54	> 45.00	30.96	39.95	09/11/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE NEGATIVE/S-GENE PLOD	PLOD
45a	34.93	35.09	34.38	37.32	14/11/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE POSITIVE/S-GENE PLOD	PLOD
47a	31.07	33.26	29.82	43.07	16/11/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE POSITIVE/S-GENE PLOD	PLOD
51a	> 45.00	> 45.00	32.23	39.09	17/11/2020	E-GENE NEGATIVE/S-GENE POSITIVE	POSITIVE	E-GENE NEGATIVE/S-GENE PLOD	PLOD
59a	28.54	33.68	27.11	37.84	21/11/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE POSITIVE/S-GENE PLOD	PLOD
60a	34.87	37.28	33.92	41.12	21/11/2020	BOTH GENES POSITIVE	POSITIVE	BOTH GENES PLOD	PLOD
64b	31.73	35.66	30.44	37.46	23/11/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE POSITIVE/S-GENE PLOD	PLOD
66a	27.66	32.92	26.30	44.82	25/11/2020	BOTH GENES POSITIVE	POSITIVE	E-GENE POSITIVE/S-GENE PLOD	PLOD
73b	34.47	38.24	32.48	38.57	26/11/2020	BOTH GENES POSITIVE	POSITIVE	BOTH GENES PLOD	PLOD

diagnosis in the community and increasing dyspnoea. Patient 1 had been transferred to the intensive care unit the next day where the first swab had been collected, which was presumptive PLOD by BT but positive by RT analysis, (Ct 29 for both E and S genes). The following sample had Cts of 24 and 23 for the E and S genes respectively by both RT and BT analysis.

3.1.2. Patient 5

Patient 5 was immunocompromised and had an asymptomatic COVID infection. Four samples tested, the first of which was positive by RT but negative by BT. Twenty-five days later, two samples were tested on the same day, the first was positive by both RT and BT methods; the second was negative by both methods. Although the Ct values were low at around 32/33, the first sample showed good amplification. While the negative results may be due to a poorly taken sample, the following day a further sample tested negative by both methods.

3.1.3. Patient 11

Patient 11 was also asymptomatic and was tested for SARS-CoV-2 as part of work screening. Six samples were tested over a period of seven days, with results alternating between positive and negative and agreement between RT and BT methods for all samples. Samples 11c and 11d were both tested on 18/11/20, with sample 11c a clear positive and sample 11d a clear negative. Samples 11e and 11 f were tested on the same run (20/11/20), with sample 11e a clear negative and sample 11 f positive but with a low delta Rn for both targets and Cts of 35 (BT), this equates to approximately 10–100 copies per reaction and the differences between these two samples may be due to stochastic effects at low copy numbers.

3.1.4. Patient 12

Patient 12 had multiple comorbidities and had been admitted to hospital with abdominal pain. Three samples were tested, where the first, collected at admission, was assigned E- and S-gene Ct values of 31 and 30 respectively by RT, but > 45 (not detected) for both genes by BT, with the second sample, five days later becoming S-gene negative by RT and E-gene PLOD by BT. The last sample five days later was positive for both genes by the two methods.

3.1.5. Patient 13

Patient 13 had been admitted to hospital as a trauma call. Four samples sent from intensive care were tested over a period of nine days. The first sample was E-gene positive by RT (Ct 33.66), PLOD by BT (Ct 43.27) and S-gene negative by both methods. The results for the second sample collected six days later showed that both genes were positive by both methods, with Ct values around 28 cycles. The next sample, two days later, became negative for both genes and both methods, with all Ct values at > 45, (not detected) the following day, the sample was positive by RT and PLOD by BT.

3.1.6. Patient 16

Patient 16 had been admitted with acute pancreatitis and COVID-19. 28 days after admission and whilst being treated in intensive care, 3 samples were tested over a period of 19 days, the first being positive for both genes by both methods, with similar Ct values. The second sample 15 days later had similarly low level positive values (negative for the S-gene by both methods), with the third sample becoming positive with Ct values of 30 for both gene and both methods.

3.1.7. Patient 24

Patient 24 was screened for COVID before having a surgical procedure. This patient had X-linked agammaglobulinaemia and had had COVID-19 earlier in the year and SARS-CoV-2 RNA had been detected in saliva and combined nose and throat swab samples for the previous 6 months. Two samples were tested on the same day, the first with RT Ct values of 25 for both genes and > 45 (not detected) for both genes by BT analysis. The second sample returned not detected results for both genes by both methods.

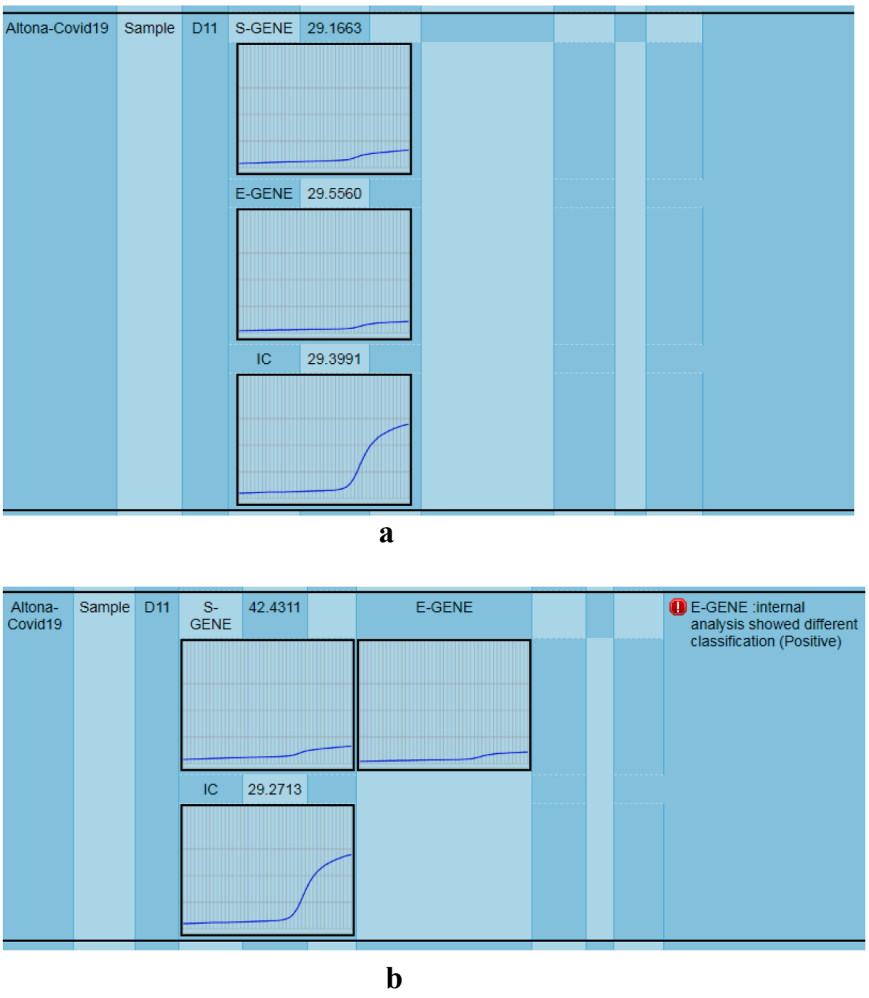

Fig. 1. a and 1b showing the results of re-analysing sample 1a using pcr.ai and data from RT and BT methods. The Ct values are as shown Table S1. The fluorescence levels of the curves are all normalised to the highest target in the run file to allow easy comparison between wells.

4. Discussion

There are two key aspects to this study, i we wanted to determine the most accurate method (RT or BT) to report the results requiring minimal manual intervention of the thermal cycler's analytical software and ii) to fully automate the interpretation and reporting process by directly importing the data from the thermal cycler into pcr.ai software. To enable the pcr.ai system to function, the software is calibrated through a machine learning process by analysing around 1000 curves consisting of at least 250 positive, negative, and equivocal results. This process is further refined by comparing results from prospective runs analysed manually and by the pcr.ai software.

4.1. Relative threshold and baseline threshold analyses

The machine learning process was initially accomplished by using the RT analysis method on the QS7, a method used routinely on all our laboratory developed tests (LDTs). However, it became apparent early on that a number of samples were being assigned low Ct values, representing high copy numbers of the two gene targets, but associated with curves that showed low, or no real evidence of amplification. The runs were then re-analysed using the BT method which assigned Ct values more in keeping with the amplification curves.

ThermoFisher suggest in their application note that the two methods give comparable results across a large dynamic range, although the RT method may suffer from stochastic effects at low copy numbers of target (ThermoFisher application Note). We found similar discrepancies between the two methods in terms of copy numbers of gene target. While the application note refers to very low volume (nanolitre) reactions, the differences between the RT and BT methods discussed in the application note are interesting and may also be relevant to larger PCR reaction volumes as we found.

By testing the NIBSC and Qnostics quantitative panels, the assay's PLOD threshold was determined to be 10 copies per reaction for both E- and S-genes with Ct values of 37. Where there was agreement between both analytical methods for the 48 positive samples the E- and S-gene average Ct values were similar: RT 30 and 29; BT 32 and 31 respectively, well below the PLOD cut-off Ct value of 37. This contrasts with the 15 BT-determined PLOD samples with average E- and S- gene Ct values of 36 and 40 compared with the RT-assigned positive Ct values of 33 and 31 for the same samples.

Looking at the results, by using the RT analysis, samples collected from 90/138 (65.2%) patients would have been interpreted as positive compared with 48/138 (34.8%) by BT analysis. For the 90 positive samples, the RT median Cts were 31 and 30 for the E and S gene (ranges E-gene 17->45 and 16–35 S-gene), whereas the median Cts were 36 and 37 for the E- and S-gene respectively (ranges 18->45 E-gene and 17->45 S-gene) using the BT analysis. We were interested to determine the optimal way of interpreting both the shape of the curve as well as producing a Ct value that more accurately reflects the true positive or negative status of the sample to assist in management of patients in the hospital.

There are a number of variables that can affect the Ct value. However, since we are only comparing two data analysis methods on the same samples tested on the same assay, we only need to consider the importance of the sample. A sample that has not been well collected can result in insufficient cellular material and a sub-optimal sample. The timing of sample collection is also a factor to be considered; was it taken at the start of an infection or towards the end? Both could lead to a low level result, but with significantly different outcomes. The impact of poorly taken samples on potentially false-negative results can be monitored by using assays that incorporate a human cellular gene target, such as RNAse P as an extraction control (Vogels et al., 2020). The Altona assay does contain an internal control, used to monitor the efficiency of extraction and inhibition, but details of the nature of the target are not disclosed. Our data show that the only stochastic, or low copy number random sampling effect is related to these low-level positive or potentially negative samples where the RT method produces less reliable results that do not match the overall characteristics of the curve. Overall, our results demonstrate that with the Altona SARS-CoV-2 RealStar assay curves must be analysed by the BT method to avoid the possibility of overcalling results leading to false positive reporting.

4.2. Pcr.ai

Due to the high sample numbers being tested for SARS-CoV-2 during the pandemic, together with the laboratory's routine diagnostic workload, we implemented the pcr.ai automated analysis software. Using this fully automated approach has a number of advantages when dealing with up to 2000 tests each day. It can significantly reduce the time associated with manually interpreting and exporting results into the LIMS, reducing potential errors and improving turnaround times (TATs) (MacLean and Gunson, 2019). In addition, the automated analysis enables real-time QA monitoring of user-defined Levey-Jennings and Westgard rules so that any issues with a particular assay are highlighted and can be dealt with in a timely manner and reviewed by senior staff and approved for release into the LIMS.

Working with the developers at pcr.ai we set up the system with minimum intervention by the software to provide the most accurate analytical method directly from the thermal cycler. The initial design approach used data directly from the QS7 RT analytical method, one that the laboratory routinely used for all the assays since it provided the most consistent analyses over a wide dynamic range and reduced the effect of "noise" particularly in quantitative multiplex assays. However, in our hands, the RT method of analysing data from the Altona assay proved susceptible to misreading the Ct values associated with amplification curves that exhibited very low delta Rn values by estimating low Ct values that would have affected the management of the patient. pcr.ai flagged a number of these results as ambiguous and we worked with their team to recalibrate the learning machine for the assay with a view to finding further examples. The pcr.ai system allows for various visualisations and reports on data as well as employing search functionality that make it much easier to find results based on curve shape or other laboratory information. We were able to use the pcr.ai platform to quickly and easily access historic results and track down various results of interest.

Whilst the clinical significance and the period of infectivity are closely related to the discussion on PCR Ct values, actually determining relevant cut-off values is still somewhat contentious. A higher Ct value correlates to a lower viral load in a given sample and this has been used as marker of infectivity (Platten et al., 2021). Studies by a number of workers have shown that culturable virus can be obtained from samples with Ct values > 30 and > 35 (Singanayagam et al., 2020; Arons et al., 2020 respectively). These finding of low viral loads in samples where a patient could be potentially infectious are particularly relevant with the VOCs which have been shown to be more transmissible (Platten et al., 2021). While validating the Altona assay for use in our laboratory we established PLOD Ct values for the E- and S-genes of 37, although the actual LOD for the assay was lower for both genes with Ct values of 40 and 38 for the E- and S-genes respectively, representing 10 copies per reaction of the NIBSC control. We set the PLOD at a more conservative Ct value of 37 for both genes in order to avoid the significant consequence to patients by overcalling the number of positive results, a problem that would have been compounded by the use of the RT analysis software.

In summary, the clinical audit demonstrated that only 138 PCR curves were difficult to interpret out of nearly 30,000 samples tested in November. None of the 138 was at the limit of detection by BT analysis but 10.9% would have been by RT analysis. Furthermore, 42 samples could have been reported as positive using the RT method, which would have had ramifications in terms of discharging patients from hospital, or if they were isolated samples, could have led to the patient having to quarantine unnecessarily. All samples were collected from individuals who were symptomatic or asymptomatic and so it is difficult to provide clinical information proving that samples were either false positive or false negative. However, the curve shape (low delta Rn) and low Ct values associated with some samples analysed by the RT method suggests that this could lead to false positive results. Finally, comparisons of RT versus BT methods showed that RT analysis would have produced lower Ct values (suggesting higher viral loads) for both E- and S-genes in 60 patients, 10 of which were PLOD. Software analysis of PCR curves is a critical part of the diagnostic process, and more attention should be focused on this area of analysis.

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CRediT authorship contribution statement

Melvyn Smith: Conceptualisation, Methodology, Experimental work, Original draft preparation. **Mark Zuckerman:** Conceptualisation, Methodology, Writing – review & editing. **Kate Bouzidi:** Conceptualisation, Data curation. **Simon Bengen:** Software development, Writing – review & editing. **Aron Cohen:** Software development, Writing – review & editing.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2022.114589.

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