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Pathology (August 2022) **54(5)**, pp. 623–628

VIROLOGY

Factors associated with weak positive SARS-CoV-2 diagnosis by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

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Summary

During the COVID-19 pandemic, the reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) assay has been the primary method of diagnosis of SARS-CoV-2 infection. However, RT-qPCR assay interpretation can be ambiguous with no universal absolute cut-off value to determine sample positivity, which particularly complicates the analysis of samples with high Ct values, or weak positives. Therefore, we sought to analyse factors associated with weak positive SARS-CoV-2 diagnosis.

We analysed sample data associated with all positive SARS-CoV-2 RT-qPCR diagnostic tests performed by the Victorian Infectious Diseases Reference Laboratory (VIDRL) in Melbourne, Australia, during the Victorian first wave (22 January 2020–30 May 2020). A subset of samples was screened for the presence of host DNA and RNA using qPCR assays for CCR5 and 18S, respectively. Assays targeting the viral RNA-dependent RNA polymerase (RdRp) had higher Ct values than assays targeting the viral N and E genes. Weak positives were not associated with the age or sex of individuals' samples nor with reduced levels of host DNA and RNA. We observed a relationship between Ct value and time post-SARS-CoV-2 diagnosis.

High Ct value or weak positive SARS-CoV-2 was not associated with any particular bias including poor biological sampling.

Key words: SARS-CoV-2 diagnosis; RT-qPCR; biological sampling; cycle threshold.

Received 16 November 2021, revised 12 April, accepted 29 April 2022
Available online 29 June 2022

INTRODUCTION

Undoubtedly, the reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) assay is the frontline diagnostic test for COVID-19. Even though it is extensively used, the qPCR diagnostic test has several drawbacks. Firstly, viral load decreases as the disease progresses, thus its detectability via RT-qPCR test also decreases.[1] This complicates result interpretation in later stages of the disease. Additionally, the interpretation of RT-qPCR results is ambiguous as there is no universal absolute cycle threshold (Ct) cut-off value to determine whether a sample is positive or negative. The background is assay dependent and thus the cut-off value differs between assays. A comprehensive list of SARS-CoV-2 RT-qPCR-based diagnostic assays is provided in Supplementary Table 1 (Appendix A). Therefore, different tests can interpret the same sample differently when the Ct value is high. Despite this, several studies have investigated the use of Ct values as a proxy for severity of disease and infectiousness. Correlations have been reported between Ct values (indicative of viral load) and severity of disease and mortality.[2–4]

Importantly, RT-qPCR cannot distinguish between the presence of actively replicating infectious virus particles and the non-infectious nucleic acid remnants from dead virus, complicating the interpretation of RT-qPCR results. Detecting replication-competent virus requires culturing the virus; however, the method is cumbersome and requires biosafety level III facilities which are not widely available. Therefore, alternate measures need to be considered to determine the infectious status of a person. Bullard *et al.* reported no infectious virus recovery from samples when the Ct value exceeded 24.[5] Similar studies by Hiroi *et al.* and La Scola *et al.* demonstrated that infectious virus could not be isolated from samples with Ct values exceeding 30 and 33–34, respectively.[6,7] Through their regression model, Singanayagam *et al.* demonstrated that the estimated odds ratio of infectious virus recovery decreased by a factor of 0.67 with every unit increase in Ct value and the probability decreased to about 8% when the Ct value was greater than 35.[8] These data suggest that positive tests with high Ct values (weak positives) do not necessarily indicate active viral shedding, and further understanding of the significance of such weak positive samples is required.

Another inaccuracy associated with the RT-qPCR assays is the occurrence of false negative results. In addition to the analytical sensitivity of the diagnostic assay, several biological

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2022.04.001

factors may contribute to false negatives such as the timing of sampling, infection stage, presence of PCR inhibitors, inappropriate sample type, suboptimal biological sampling, low viral load, and variability on viral shedding.[9–14] Whether these same factors play a role in weak positives remains unknown. Therefore, understanding the factors behind weak positives in SARS-CoV-2 diagnostic tests and determining the infectious status of such samples is critical for determining the significance of weak positive diagnosis. To that end, we analysed all positive diagnostic tests performed by the Victorian Infectious Diseases Reference Laboratory (VIDRL) over the period of 22 January 2020 to 30 May 2020 for the incidence of weak positives and potential factors associated with weak positive testing.

METHODS

Source of data and comparison of gene target sensitivity

COVID-19 screening data, from 22 January 2020 to 30 May 2020, were obtained from VIDRL as a large database file that outlined several aspects such as the age, sex, outcome, gene target(s) tested, Ct values and additional comments such as recent travel history. The majority of sample types were classified as 'nose and throat swabs' or 'nasopharyngeal swabs' (Supplementary Table 2, Appendix A). An in-house qRT-PCR assay was used to determine the presence of SARS-CoV-2 RdRp, N or E gene.[15] Bovine viral diarrhoea virus RNA was spiked into each sample to assess for nucleic acid extraction, reverse transcription and PCR inhibition. A sample was declared positive when two assays, testing for different gene targets (the RdRP gene target was the primary screen and the N or E gene was used as a confirmatory screen), yielded a positive result (with a Ct value of ≤45).[16]

Analysis of weak positives

For analysis of weak positive samples, three Ct cut-off values were chosen for the analysis based on the review of several RT-qPCR-based assays that have been authorised by the US Food and Drug Administration (FDA) for emergency use (Supplementary Table 1, Appendix A). These cut-offs were Ct cycle 36, 38, and 40. Based on the three Ct cut-off values, the data were sorted and evaluated for the incidence of weak positive samples and different parameters such as age, sex, and sample type (for RdRP gene target). The weak positives were defined as samples with Ct > Ct.

Longitudinal sampling

Samples were included for longitudinal sampling if a minimum of three independent tests were performed after a positive test in the same individual. Follow-up tests were only included if they were nasopharyngeal samples or nose and throat swabs.

Quantification of CCR5 and 18S copies

Sample quality was determined by quantification of host RNA and DNA through quantification of the 18S gene and CCR5 gene, respectively. Quantification of 18S RNA copies and CCR5 DNA copies was performed by qPCR as we have described previously.[17–20] All samples were run in triplicate wells and an average was taken.

Statistical analysis

Paired analysis was conducted between different gene targets (RdRP gene versus E gene, RdRP gene versus N gene, and E gene versus N gene) using Wilcoxon matched-pair signed-rank test to compare the sensitivities of these gene targets. Change in Ct value over time after the first positive test was performed via linear regression analysis. Comparison of Ct values before and after the first negative test for discordant testing was made using a paired Student's t-test. The comparison of the average number of 18S copies for weak positives and the other positive samples, for each Ct cut-off, was performed using an unpaired t-test (Welch's t-test). Mann–Whitney U test was performed to compare the average number of CCR5 copies for weak positives and the other positive samples. Spearman correlation analysis was conducted to determine the correlation between the average number of 18S and CCR5 copies with Ct value. Visualisation of data and the data analysis were performed on GraphPad Prism (Version 8.4.3, GraphPad Software, USA).

RESULTS

Analysis of SARS-CoV-2 diagnostic testing

VIDRL conducted a total of 77,650 RT-qPCR tests between 22 January and 30 May 2020. Of these, 1792 tests (2.31%) were positive for SARS-CoV-2 based on detection of either RdRP, N, or E gene wherein the RdRP RT-qPCR assay was used as the primary screen and the N or E gene assay was used as the confirmatory screen. We compared the Ct values for RdRP, E, and N genes from positive samples using a paired non-parametric test and observed that the RdRP assay had significantly higher Ct values than assays for the E gene ($p<0.0001$) and for the N gene ($p<0.0001$) (Fig. 1). The Ct values for the E and N gene assays were not statistically significantly different ($p=0.5989$, Fig. 1). These data suggest that the N gene and E gene RT-qPCRs may be more sensitive than the RdRP gene assay.

Using Ct values at 36, 38, and 40 that are within the range of the suggested cut-off for several different FDA-authorised COVID-19 RT-qPCR assays (Supplementary Table 1, Appendix A), we determined that the RdRP gene assay

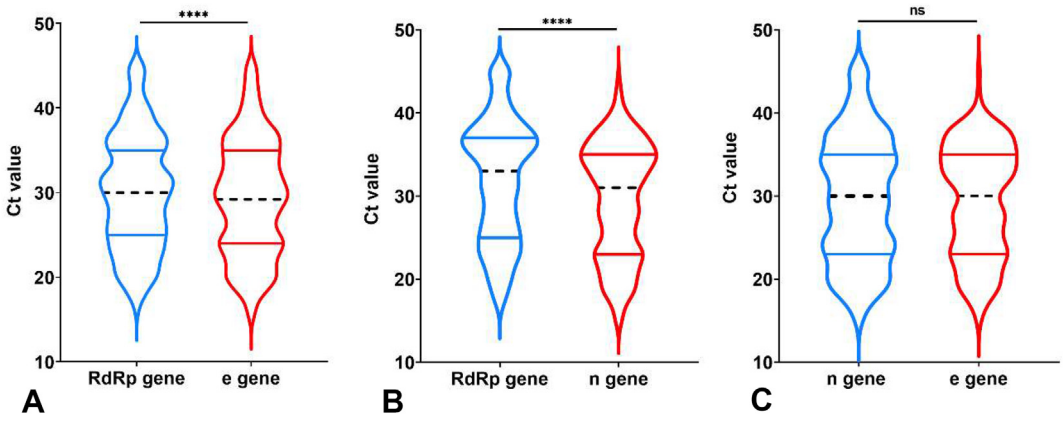

Fig. 1 Different SARS-CoV-2 gene targets lead to different Ct values in diagnostic assays. Comparisons between Ct values for (A) RdRP gene and E gene, (B) RdRP gene and N gene, (C) N gene and E gene. Data are represented by a violin plot, wherein the dotted horizontal bar represents the median Ct value, and the solid horizontal bars represent the interquartile range. Comparisons were made by Wilcoxon matched-pair signed-rank test. ns, p>0.05; **** p≤0.0001.

showed the highest number of available samples with weak positives (observed Ct > Ct cut-off values) compared to the N and E genes (Table 1). Therefore, further analysis on weak positive samples was focused on the RdRP gene to maximise the largest available sample size of the three genes.

Factors associated with high Ct values (weak positives)

Based on the three Ct cut-off values, the data for weak positive samples was evaluated for a range of different parameters. When we compared weak positive samples and the other positive samples, we did not detect any significant differences in sex (Supplementary Fig. 1, Appendix A) or age (Supplementary Fig. 2, Appendix A) of the individuals tested, suggesting these factors were not relevant to weak positives. We then sought to determine whether the quality of the sample would be associated with a weak positive. We utilised host gene DNA and RNA quantities as a measure of the sampling depth.[12] The amount of host RNA was determined by ribosomal RNA 18S quantification, and the amount of host DNA was determined by CCR5 quantification.[15,21] We observed that there were significantly more 18S copies in the weak positives compared to the other positive samples for each Ct cut-off value ($p<0.05$) (Fig. 2A–C). We observed no difference between weak positives and other positive samples in terms of the amount of CCR5 copies for each Ct cut-off value (Fig. 2D–F). Furthermore, we observed no association between the quantity of 18S copies and Ct value (Fig. 2G), or between the quantity of CCR5 copies and Ct value (Fig. 2H). Taken together, these results suggest that poor sampling is not a factor in whether a positive sample has a low or high Ct value.

Longitudinal sampling

The COVID-19 screening data had several instances where the same individual (n=42 individuals) was tested multiple times over a period of days. We used this data set to determine if the time since the first positive test was a factor in the Ct value of a positive test. We indeed found a relationship between time post first positive test and Ct value, with the Ct value increasing over time (p=0.001 and r^2=0.6550, Fig. 3).

In addition, in a subset of individuals (n=11/42, 26%), we observed 'blipping' of positivity, where two positive tests were separated by a negative test (Supplementary Fig. 3, Appendix A).[21] The Ct values of the positive tests that bracketed the negative were typically high (Supplementary Fig. 4, Appendix A). Prior to the negative test, the Ct value of the positive test tended to be comparatively lower (mean=36.08, SD=2.906) than after the negative test (mean=38.17, SD=3.070). However, the increase was not statistically significant (p=0.1529) (Supplementary Fig. 4, Appendix A). These data suggest that time and likely viral clearance contributes to high Ct value positive tests.

DISCUSSION

The instances of weakly positive samples with high Ct values are an important consideration in the diagnosis of SARS-CoV-2 via PCR. To assess factors associated with weak positive tests we analysed SARS-CoV-2 diagnostic testing performed by VIDRL. The proportion of weak positive results (based on arbitrary Ct cut-off values) ranged from 5.44% to 19.61%, 2.54%–24.71%, and 4.57%–14.46%, for the RdRP, N, and E genes, respectively. Aside from an association between higher Ct value and increased time from the first positive test, we were unable to find any factors associated with weak positive SARS-CoV-2 diagnosis.

We observed no correlation between cellular RNA or DNA input and the Ct value of the SARS-CoV-2 diagnostic assay. This indicates that sub-optimal biological sampling or amount of cellular material in the sample does not dictate Ct value and therefore is not a determinant of weak positive results. Interestingly, this is in contrast with another study that showed sub-optimal sampling may contribute to false-negative assays.[12] Further, our data are inconsistent with other studies that have seen associations between the Ct value of an internal control (p30 subunit of ribonuclease P, RPP30) and Ct value for SARS-CoV-2 gene targets.[22,23] This may reflect differences in host gene expression in the respiratory tract and warrants further study. The majority of diagnostic kits use RPP30 as the host internal control (Supplementary Table 1, Appendix A), although others have used 18S, suggesting its utility in assessing biological sampling of the respiratory tract.[24,25] It remains unclear whether markers of host DNA or RNA are surrogate measures of sampling quality, as the transcriptome in the nose and throat may change during a SARS-CoV-2 infection. Several studies have reported differential expression of some host genes in the nasopharyngeal swabs of COVID-19 positive individuals compared to healthy individuals.[26–29] Amati *et al.* analysed the expression profiles of several SARS-CoV-2 host invasion genes and found overexpression of several host genes specifically ACE2 and DPP4 in the nasopharyngeal and oropharyngeal swabs.[29] In another study, Biji *et al.* reported a consistent upregulation of S100 family genes (S100A6, S100A8, S100A9, and S100P) in nasal swabs known to be involved in the differentiation of myeloid cells to dendritic cells and macrophages.[28,30] Studies have also shown differential expression of some host genes across infection status, age, sex, and the type of sample.[26,27] This indicates the potential of such host genes to be used as

Table 1 Distribution of weak positives for different Ct value cut-offs

Ct cut-off value		RdRP gene	E gene	N gene
36	Ct≤36	1316 (80.39%)	899 (85.54%)	326 (75.29%)
	Ct>36	321 (19.61%)	152 (14.46%)	107 (24.71%)
38	Ct≤38	1460 (89.19%)	967 (92.00%)	400 (92.38%)
	Ct>38	177 (10.81%)	84 (8.00%)	33 (7.62%)
40	Ct≤40	1548 (94.56%)	1003 (95.43%)	422 (97.46%)
	Ct>40	89 (5.44%)	48 (4.57%)	11 (2.54%)

Weak positives: Ct values > $Ct_{cut\text{-}off}$.

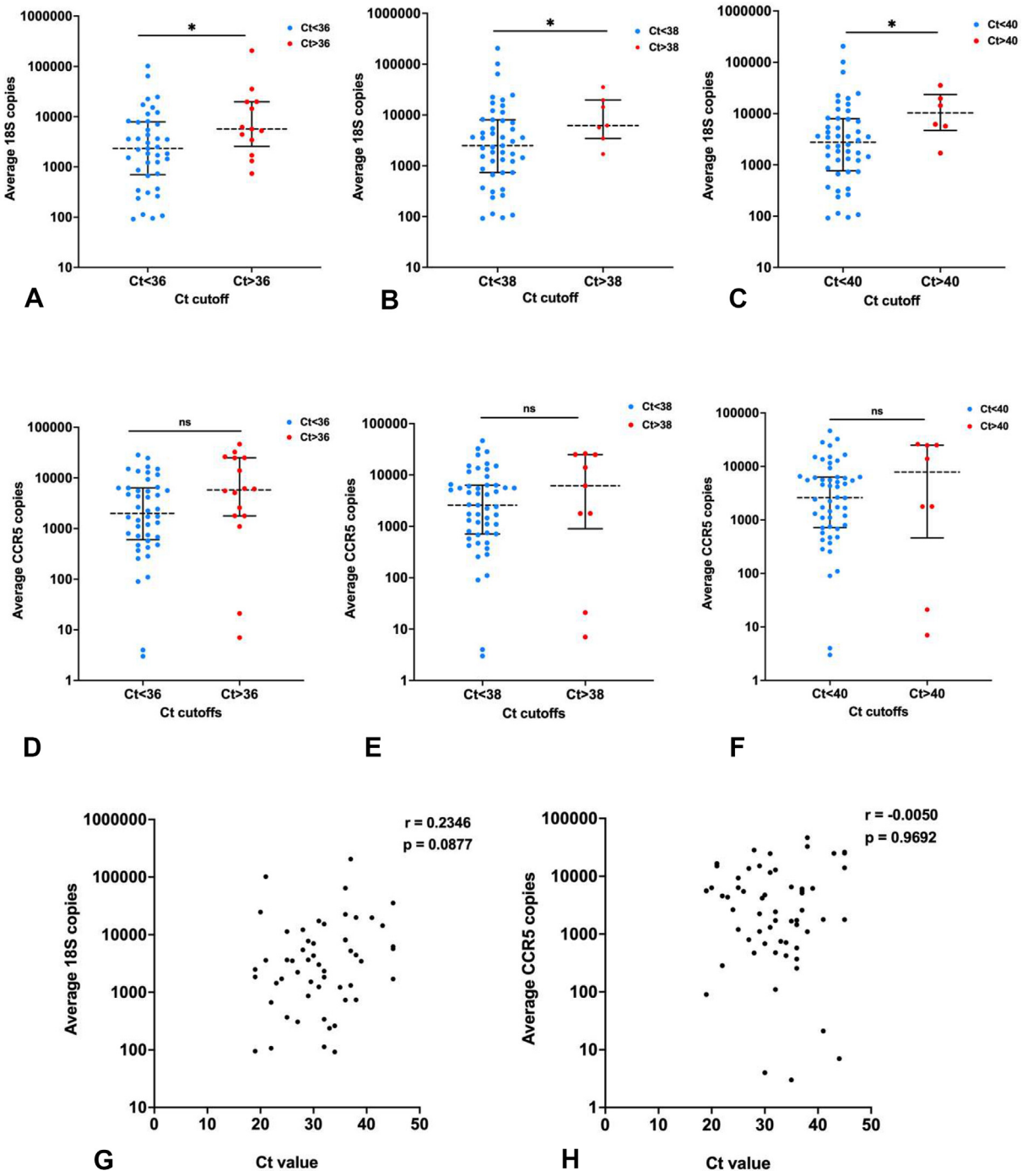

Fig. 2 High Ct values in a SARS-CoV-2 diagnostic assay are not associated with poor biological sampling. Average number of 18S RNA copies at (A) Ct cut-off=36, (B) Ct cut-off=38, (C) Ct cut-off= 40. Average number of CCR5 copies at (D) Ct cut-off=36, (E) Ct cut-off=38, (F) Ct cut-off= 40. The dotted horizontal bar represents the median and the error bars represent the interquartile range. (G) Association between the average number of 18S RNA copies and Ct value, and (H) average number of CCR5 copies and Ct value. Dataset for the average number of 18S copies was transformed and then comparison was made using an unpaired t-test (Welch's t-test). Comparison of the average number of CCR5 copies was made using the Mann–Whitney U test. Associations were made using a Spearman correlation. ns, $p>0.05$, *$p<0.01$.

surrogate markers to distinguish between individuals with SARS-CoV-2 infection and healthy individuals. Interestingly, we observed more 18S RNA in weakly positive samples which may indicate increased host cell turnover following a resolving infection or may also be a result of immune cell infiltration.

The Ct values for samples tested for two different genes were compared, and our data suggest that the E gene and N gene assays were more sensitive than the frontline RdRP assay, while the N and E gene assays had comparable sensitivities. This finding is supported by several studies that reported a higher sensitivity of N and E gene targets over the RdRP gene.[31,32] Vogels *et al.* evaluated analytical sensitivities of several primer-probe sets including those from the China Centre for Disease Control (CDC), USA CDC, Hong Kong University (HKU), and Charité Institute of Virology.[33] They reported that the sensitivities for these primer-probe sets were comparable; however, the analytical sensitivity for the

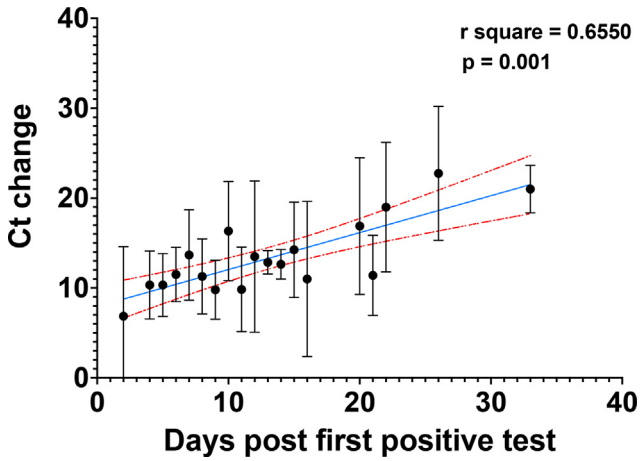

Fig. 3 Change in Ct value is associated with time since the first positive test. In individuals with longitudinal samples, the change in Ct value and time since the first positive test was plotted. Data points were only included where a minimum of three tests (including the first positive result) from different individuals (only including nasopharyngeal and nose and throat swabs) were performed. The blue line represents the linear regression line and the red dotted lines represent the 95% confidence intervals.

N gene was higher than for an ORF1 gene target, for the HKU and China CDC primer-probe sets and the sensitivity of the E gene was significantly higher than the RdRP gene for the Charité primer-probe set.

In several individuals with multiple tests, we observed viral 'blipping' where a negative test was sandwiched between positive tests. The Ct value of the positive tests on both sides of the negative test tended to be high (Ct>36), indicating that the RT-qPCR test might be detecting the remnants of dead viruses or cleared virally infected cells rather than viral recrudescence. Wolfel *et al.* demonstrated that in the case of swabs, the viral RNA load decreased significantly after day 5 of symptoms. Furthermore, they also reported that the viral sub-genomic mRNAs, found only in the infected cells, were detectable only up to day 5 post the onset of symptoms.[21] In a study by Sohn *et al.*, patient samples (nasopharyngeal swabs and saliva) with prolonged positive RT-qPCR results and rebound Ct values were analysed to determine the presence of actively replicating SARS-CoV-2. For these samples, they reported a mean Ct value of >30 and failed to isolate actively replicating virus.[34] In another study, Manzulli *et al.* analysed 84 patient samples for the presence of SARS-CoV-2 using an RT-qPCR assay at the time of hospitalisation and three days after resolution of symptoms, followed by *in vitro* cell culture.[35] All patients were reported positive for the SARS-CoV-2 N gene after the clearance of symptoms. However, 83 of the 84 patients returned a negative cell culture result, indicating the lack of viable virus. These studies further support our finding that in individuals where 'blipping' is observed, the RT-qPCR assay is unlikely to be an indication of viral recrudescence. Perhaps RT-qPCR assays that detect sub-genomic species of SARS-CoV-2 may better distinguish individuals who are shedding virus, providing greater clarity to when individuals are infectious. The importance of understanding weakly positive SARS-CoV-2 diagnosis is further emphasised in the era of pre-existing immunity as studies have shown that breakthrough infections in vaccinated individuals and individuals with prior infection had higher Ct values compared to primary infections.[36,37] Further, other studies have shown associations between Ct value and markers of immunity including neutralising antibody titres.[36,38]

Our study is limited by the fact that samples that tested as negative were not retested, this may confound our study particularly in the case of viral blipping where positive tests tended to have high Cts near the limit of detection. It is possible some of these negatives may have become positive with multiple replicate tests or use of other gene targets.

CONCLUSIONS

A range of tests have been used for the detection of SARS-CoV-2 and amongst them, RT-qPCR has been the most extensively used. The occurrence and factors behind high Ct value or weak positives are of relevance to diagnosis by PCR. Our data suggest that weak positives are not the result of any particular bias including poor biological sampling.

Acknowledgements: We thank Dr Katherine Gibney for helpful discussions.

Conflicts of interest and sources of funding: SRL is supported by an NHMRC practitioner fellowship. The authors state that there are no conflicts of interest to disclose

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2022.04.001.

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