Original Article

Impact of Target Confirmatory Gene on Cycle Threshold Values in Real-Time Polymerase Chain Reaction Across Three Waves of SARS-CoV-2

Syed Arshi , Talat Masoodi , Shazia Mushtaq , Rukhsana Taj, Sheeba Bashir , Azhar Shafi.

Abstract

Introduction:

To effectively manage and control the spread of the virus, accurate and timely diagnosis is paramount. Real-time reverse transcription polymerase chain reaction (RT-PCR) has emerged as the gold standard for diagnosing SARS-CoV-2 infections due to its high sensitivity and specificity.

Aim

To analyze cycle threshold (CT) values of confirmed positive samples across three waves of SARS-CoV-2 and investigate the impact of target confirmatory genes (E, RdRp, ORF1ab, S, and N) on CT values.

Materials and Methods

The SKIMS RT-PCR laboratory received samples from April to June 2020, May 2021, and January 2022, which were analysed for the first, second, and third waves. CT values were scrutinized to assess the impact of gender, age, and confirmatory gene (RdRp/ORF/E) across all three waves. The standard RNA extraction utilized QIAGEN®'s QIAamp Viral RNA Mini Kit, and nucleic acid amplification was performed using Meril®'s COVID-19 One-step RT-PCR Kit. Thermal cycling was conducted on the Applied Biosystems™ 7500 Real-Time PCR System.

Results

CT values varied across different confirmatory genes (ORF, N, E, and RdRp). The highest CT value was observed for the ORF gene (35s), while the lowest was for the E gene (15). The mean CT values were 25.2 for the E gene, 24.7 for RdRp, 25.4 for ORF, and 25.1 for the N gene. Wave 2 had the highest mean CT score (26.4), followed by Wave 3 (22.3) and Wave 1 (24.8).

Practitioner2024;29(4):76-80.

Introduction

More than half a billion people have been infected and 6.2 million killed by the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), commonly referred to as COVID-19 [1]. This chimeric virus is a pleomorphic, positive sense, single-stranded RNA virus averaging 100 nm in diameter with the genetic material comprising around 30,000 nucleotide base pairs. The mode of entry is mainly through the angiotensin-converting enzyme 2 (ACE-2) receptor, which is present in epithelial cells [2]. Since the earliest cases were reported in late 2019, three global waves of infection, all triggered by different variants, have been observed, each wave having unique characteristics and fatality rates [3]. Several techniques are available to detect infection with the virus.

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Indexed

EMBASE, SCOPUS , IndMED, ESBCO, Google Scholar besides other national and International databases.

Cite this article as

Syed A, Masoodi T, Mushtaq S, Taj R, Bashir S, Shafi A. Impact of Target Confirmatory Gene on Cycle Threshold Values in Real-Time Polymerase Chain Reaction Across Three Waves of SARS-CoV-2.JK Pract2024;29(4):76-80.

Full length article available at **jkpractitioner.com** one month after publication

Keywords

Diagnostic testing, Viral load, Infectivity, Disease severity, Diagnostic protocols

Radio diagnostic imaging and serological and nucleic acid amplification tests have found significant applications in the past few years. Most notably, the real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) has emerged as the gold standard for SARS-CoV-2 detection [4]. Usually, specific primer/probe sets targeting unique conserved gene sequences such as the spike (S), envelope (E), nucleocapsid (N) and targeted open reading frame (ORF-1) are used to develop the assays for detection of the virus [5]. In RT-PCR, the amplification reaction is monitored in real-time via the detection of a fluorescent signal, the intensity of which increases as the products accumulate. Generally, the higher the concentration of viral RNA in the initial sample, the higher the amplification magnitude and corresponding fluorescence signal and the lower the CT value. Thus, patients with higher oropharyngeal viral loads may present with lower CT values. It is plausible that different variants with different R_0 values may have respectively analogous values [7]. Age has also been seen to affect CT values [8].

Blake et al. [9] analyzed RT-PCR CT values from symptomatic COVID-19 patients to assess the risk of false negatives and explore the relationship between CT values and patient age. Results indicate a low risk of false negatives in combined nasopharyngeal-oropharyngeal specimens, even near the assay's limit of detection. Additionally, no significant age-dependent difference in viral load or test sensitivity was observed. Harrison et al. [10] investigated the potential of PCR test cycle threshold (Ct) data for SARS-CoV-2 as an early indicator of epidemic growth. By analyzing daily mean Ct values in England and the UK and using iterative sequential regression, the study detected breakpoints in mean Ct values and positive test counts. Results showed that decreases in Spike (S) gene target-specific mean Ct values occurred 6–29 days before increases in positive test counts, providing an early indication of rising new variants like Delta and Omicron. This approach proved beneficial for monitoring the COVID-19 pandemic's progression and can support future infectious disease surveillance efforts.

As relatively less research has been undertaken in this area, this study aims to unravel this association between variant, age and CT value during the peak periods of the three waves in 2020-2022 by analyzing the threshold (CT) values of confirmed positive samples across three waves of SARS-CoV-2 and investigate the impact of target confirmatory genes (E, RdRp, ORF1ab, S, and N) on CT values.

Materials and Methods

This is a retrospective cross-sectional study conducted in the Indian Council of Medical Research (ICMR) designated COVID-19 virology laboratory of the Department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences (SKIMS) Medical College and Hospital, Bemina, Srinagar.

The SKIMS RT-PCR laboratory, serving as a pivotal testing centre for nearby districts, routinely receives samples from the hospital. It operates as a nodal testing facility, contributing significantly to the region's COVID-19 testing efforts. CT values were meticulously scrutinized to analyze positive samples coinciding with the peak periods of the three COVID-19 waves.

The study encompassed sample data from distinct phases of each wave: April to June 2020 for the first wave, May 2021 for the second wave, and January 2022 for the third wave, aligning with the infection peaks reported by Reuters in 2022. A minimum of 500 positive samples from each wave underwent thorough analysis. The examination of CT values explored potential correlations with demographic factors such as gender and age, alongside the influence of specific confirmatory genes (RdRp, ORF, and E) across all three waves.

The laboratory employed QIAGEN®'s QIAamp Viral RNA Mini Kit for RNA extraction and Meril®'s COVID-19 One-step RT-PCR Kit for nucleic acid amplification to ensure standardized procedures. Thermal cycling was conducted using the Applied Biosystems™ 7500 Real-Time PCR System, maintaining consistency and reliability throughout the testing process.

Time Period – Two (02) months

Inclusion Criteria- All samples declared positive within the defined time periods

Exclusion Criteria- All negative samples or those with CT values above 35 or where data is inconclusive/missing.

Results and Discussion

The study group comprised of 1803 participants. The distribution of the subjects in Wave 1,2 and 3 was 86(4.8%), 1248 (69.2%) and 469 (26%), respectively. Out of the 1803 individuals, 1007 (55.9%) were males, and 796 (44.1%) were females. The participants ranged between the ages of 0.07 to 82 years. The mean age among subjects was 36.8 years, with the median and interquartile range of 35 and 26.47, respectively. The standard deviation in age was found to be 15.55 (in years). This demographic analysis provides insight into the study's participant variety and distribution. The significant representation of subjects in Wave 2 reflects a larger cohort recruited during this phase, which could indicate enhanced availability or recruitment efforts

during that time period. The virtually equal ratio of male and female participants demonstrates a balanced gender representation in the study population. Furthermore, the extensive age range and fluctuation, as evidenced by the mean, median, and interquartile range, highlight the different age demographics represented in the study population. The estimated standard deviation reveals the dispersion of ages around the mean, demonstrating the degree of variability in the age distribution of participants.

Table 1 Description of various CT scores.

Parameter	E gene	RdRp	ORF	N	Average CT Score
Number	86	86	1803	1717	1803
Minimum	15.0	15.0	13.0	14.0	13.5
Maximum	34.0	33.0	35.0	35.0	35.0
Mean	25.0	24.7	25.4	25.1	25.2
Std. Deviation	5.3	5.2	5.3	5.3	5.3
Median	25.0	25.0	26.0	25.0	25.5
IQR	21-29	21-29	21-30	21-29	21-29.5

As per Table 1, the CT values for ORF were 1803 (maximum), and for N 1717, the E gene, and RdRp, they were 86 (minimum). For the E gene, the maximum CT score was 34, and the minimum was 15. Similarly, the maximum CT score for RdRp was 33, and the minimum was 15. The maximum CT score for ORF was 35, and the minimum was 13. The max and min CT scores for N protein were 35 and 14, respectively. The average CT score for all four parameters was 1803, with the maximum average CT values of 35 and min 13.5. The mean for the E gene, RdRp, ORF and N are 25, 24.7, 25.4 and 25.1, respectively, with the average mean CT value of 25.2. Similarly, the std deviation is 5.3 for the E gene, ORF and N. While it is 5.2 for RdRp with an average of 5.3 for all four parameters. The median ranges from 25 in e gene, RdRp, n to 26 in ORF, with an average median CT score of 25.5. In the same manner, IQR for e gene, RdRp and n protein is 21-29, while for ORF, it is 21-30. The average IQR of CT values for all four parameters is 21-29.5. In Wave I, out of the total 86 participants, 55.8 % (48) were males, and 44.2% (38) were females. In the 2nd wave, 55.8% (697 of 1248) were males and 44.2 % (551 of 1248) were females. Similarly, 3rd wave consisted of 55.9% (262 of 469) males and 44.1% 207 of 469) females.

Table 2. shows a consistent gender distribution pattern throughout all three waves.

Table 3. compares the age of study participants and average CT scores across the genders of the participants.

Table 74.shows the mean CT score across the waves. The mean of the average CT score in wave 1 was 24.8, with a std deviation of 5.2. In 2nd wave, the CT score mean was 26.4 with a std deviation of 5. In 3rd wave, the mean of average CT scores was 22.3 with a std deviation of 4.9. The p-value of the CT score for all three waves remains 0. The mean age in years for wave 1 is 39.4, with a std deviation of 15.8. For wave

Table 2. Comparison between gender and waves.

Wave	Gender		Total
	Female	Male	
Wave I	38 (44.2%)	48 (55.8%)	86 (100%)
Wave II	551 (44.2%)	697 (55.8%)	1248 (100%)
Wave III	207 (44.1%)	262 (55.9%)	469 (100%)
Total	796 (44.1%)	1007 (55.9%)	1803 (100%)

P Value: 1.0

Table 3. Comparison of age of study participants and average CT score across gender of study participants.

Parameter	Gender	N	Mean	Std. Deviation	Mean Difference	p Value
Age in Years	Female	796	35.68	14.86	-2.03	.01
	Male	1007	37.71	16.02		
Average CT Score	Female	796	25.09	5.31	-.26	.29
	Male	1007	25.35	5.23		

2, the mean age is 36.6 with a std deviation 15.6. In wave 3, the mean age in years is 36.9, and the standard deviation is 15.3. The p-value for age in years across all waves is 0.27. Furthermore, the mean age varied between waves, with Wave 1 having a mean age of 39.4 years, Wave 2 having a mean age of 36.6 years, and Wave 3 having a mean age of 36.9 years. However, the p-value for age across all waves was 0.27, indicating that there was no statistically significant difference in the age distribution of individuals across waves. The data is illustrated with a box plot in Figure 1.

Table 4. Comparison of age of study participants and average CT score across three COVID-19 waves.

Parameter	Wave Type	N	Mean	Std. Deviation	p Value
Average CT Score	Wave I	86	24.8	5.2	0.00
	Wave II	1248	26.4	5.0	
	Wave III	469	22.3	4.9	
Age in Years	Wave I	86	39.4	15.8	0.27
	Wave II	1248	36.6	15.6	
	Wave III	469	36.9	15.3	

Conclusions

In conclusion, this study offers comprehensive insights into the dynamics of SARS-CoV-2 infection across three waves, elucidating the relationship between demographic factors and cycle threshold (CT) values. The analysis of 1803 participants revealed consistent gender distribution and a wide age range, reflecting the diverse nature of the study population. CT values varied across confirmatory genes, with the ORF gene exhibiting the highest variability. Despite significant age differences between genders, no statistically significant disparity in mean CT scores indicated similar viral loads. Furthermore, the comparison of CT scores across waves highlighted fluctuations in viral load over time. These findings underscore the importance of considering demographic factors and confirmatory genes in interpreting RT-PCR results for COVID-19 diagnosis and monitoring. Such insights contribute to refining diagnostic protocols and informing public health strategies to mitigate the pandemic's impact.

References

1. Worldometers.info, "COVID-19 coronavirus pandemic statistics," https://www.worldometers.info/coronavirus/, 2022.
2. A. Aleem, A. B. Akbar Samad, and A. K. Slenker, "Emerging variants of SARS-CoV-2 and novel therapeutics against coronavirus (COVID-19)," 2022.
3. J. Machhi et al., "The natural history, pathobiology, and clinical manifestations of SARS-CoV-2 infections," Journal of Neuroimmune Pharmacology: The Official Journal of the Society on NeuroImmune Pharmacology, vol. 15, no. 3, pp. 359–386, 2020, doi: 10.1007/s11481-020-09944-5.
4. J. Mikuła-Pietrasik and K. Książek, "Aging correlates with lower threshold cycle values for RdRP/RdRP+S genes during molecular detection of SARS-CoV-2," Experimental Gerontology, vol.

150, p. 111361, 2021, doi: 10.1016/j.exger.2021.111361.

5. B. Mishra et al., "High proportion of low cycle threshold value as an early indicator of COVID-19 surge," Journal of Medical Virology, vol. 94, no. 1, pp. 240–245, 2022, doi: 10.1002/jmv.27307.
6. M. Pradhan et al., "COVID-19: Clinical presentation and detection methods," Journal of Immunoassay & Immunochemistry, vol. 43, no. 1, p. 1951291, 2022, doi: 10.1080/15321819.2021.1951291.
7. Reuters, "COVID-19 Tracker (MoHFW)," https://graphics.reuters.com/world-coronavirus-tracker-and-maps/countries-and-territories/india/, 2022.
8. K. Shirato et al., "Detection of the ORF1 gene is an indicator of the possible isolation of severe acute respiratory syndrome coronavirus 2," Pathogens (Basel, Switzerland), vol. 11, no. 3, p. 302, 2022, doi: 10.3390/pathogens11030302.
9. Blake W Buchan, Jessica S Hoff, Cameron G Gmehlin, Adriana Perez, Matthew L Faron, L Silvia Munoz-Price, Nathan A Ledeboer, Distribution of SARS-CoV-2 PCR Cycle Threshold Values Provide Practical Insight Into Overall and Target-Specific Sensitivity Among Symptomatic Patients, American Journal of Clinical Pathology, Volume 154, Issue 4, October 2020, Pages 479–485, https://doi.org/10.1093/ajcp/aqaa133
10. Harrison RE, Hamada A, Haswell N, Groves A, Vihta KD, Cella K, Garner S, Walker AS, Seale AC. Cycle Threshold Values as Indication of Increasing SARS-CoV-2 New Variants, England, 2020-2022. Emerg Infect Dis. 2023 Oct;29(10):2024-2031. doi: 10.3201/eid2910.230030. Epub 2023 Sep 7. PMID: 37678158; PMCID: PMC10521603.