Virology

Evaluation of PCR cycle threshold values by patient population with the quidel lyra SARS-CoV-2 assay

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A R T I C L E I N F O

A B S T R A C T

The Lyra SARS-CoV-2 assay was the primary method for molecular testing performed at Barnes-Jewish Healthcare System in St. Louis, Missouri during the initial COVID-19 surge from mid-March to late-April 2020. We performed a retrospective analysis of 1,043 positive Lyra SARS-CoV-2 results during these 36 days to investigate associations between cycle threshold (C_T) value and patient characteristics. Total RNA were extracted from NP or OP swabs using either the EasyMag or KingFisher automated extraction systems and quantified with RotorGene Q (Qiagen) or Applied Biosystems 7500 Fast Dx thermocyclers respectively. Notably, we found lower a significant median lower C_T for samples tested on the KingFisher-ABI 7500 fastDX (KF/ABI) system compared to the EasyMag/RotorGene (EM/RGQ) platform. Since 77.5% of our tests were ran on the EM/RGQ pipeline we then perform additional analysis on these values and found that C_T values in outpatient care settings compared to samples obtained in the emergency department or inpatient had significantly lower C_T values. These collective findings suggests a difference in viral load amongst various patient populations.

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1. Introduction

Molecular detection of SARS-CoV-2 RNA is the primary method used to identify viral presence in patients with suspected COVID-19 (Ransom et al., 2020). Upper respiratory specimens, most commonly nasopharyngeal (NP) and oropharyngeal swabs (OP), are utilized for testing (Han et al., 2020; Ransom et al., 2020). Currently, molecular platforms range from sample-to-answer assays that require minimal hands-on time or technical expertise to systems requiring separate instruments for RNA extraction and real time RT-PCR systems (Han et al., 2020; Ransom et al., 2020). The Lyra SARS-CoV-2 Assay (Quidel) is a RT-PCR assay for detection of SARS-CoV-2 presence using fluorescent probes that bind to non-structural polyprotein (pp1ab)/ Orf1ab RNA within the SARS-CoV-2 genome. The analysis is preceded by total RNA extraction and the assay uses a separate extraction system and amplification/detection system (Ransom et al., 2020).

The Barnes-Jewish Healthcare System, a network of hospitals in the St. Louis metropolitan area (Missouri, USA) implemented the Lyra

SARS-CoV-2 Assay as one of our initial tests for molecular identification of SARS-CoV-2. Here we report on the experience with the assay during March and April 2020, using two different extraction and quantification platforms. Samples were sent to either the EasyMag (bioMerieux)/RotorGene Q (Qiagen) or KingFisher (ThermoFisher)/ Applied Biosystems 7500 Fast Dx (ThermoFisher) extraction/amplification systems. We have recently demonstrated that the KingFisher/ 7500 Fast Dx system results in a statistically significant -1 cycle threshold (C_T) difference compared to the EasyMag/RotorGeneQ system but was not likely to be biologically meaningful (Ransom et al., 2020). We performed a retrospective analysis of the C_T values from 1,043 valid positive clinical results (from a total of 7,411 samples t during this time frame) to identify differences between platform and patient demographic information.

2. Materials & methods

2.1. Molecular detection of SARS-CoV-2

Patients symptomatic for COVID-19 or with known sick contacts had mucosal sites sampled using NP or OP swabs (including BD, Copan, Puritan, and Hardy) as part of routine clinical management. The encounter types occurred primarily in outpatient (i.e.

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symptomatic healthcare worker testing), inpatient (e.g. intensive care unit,) or emergency department (ED) settings at various locations in the Barnes-Jewish Healthcare System in the St. Louis region. All samples were submitted to the Barnes-Jewish Hospital Molecular Infectious Disease (BJH-MID) Lab for SARS-CoV-2 testing. Due to potential challenges with procurement of reagents, the BJH-MID Laboratory established two different testing pipelines for detection of SARS-CoV-2 RNA (Ransom et al., 2020). Total RNA was extracted from samples by trained clinical laboratory staff using either the NUCLISENS® Easy-Mag (bioMerieux) or KingFisher (Thermo Fisher) instruments. For extraction, 20 μL of the Lyra Processing Control was added to 180 μL aliquots of the VTM or UTM. The KingFisher system elutes in 50 μL while the EasyMag system elutes in 100 μL. To perform the PCR reaction, 135 μL of the reagent solvent is added to lyophilized master mix and 15 μL of reconstituted solution is used with 5 μL of input RNA. This method is in accordance with the manufacturer's instructions for use required for compliance with the emergency use authorization granted by the FDA. Samples extracted on the EasyMag were paired with amplification/detection on the RotorGene Q (Qiagen) thermocycler and samples from KingFisher were detected on the Applied Biosystems 7500 Fast Dx (ThermoFisher). Samples were deemed valid if the Lyra processing control (added to every specimen during the extraction phase) had a detectable C_T value and runs were deemed valid if both the Lyra positive and negative SARS-CoV-2 control samples, provided in the test kit, had detectable processing control and the SARS-CoV-2 target was respectively detected or not detected. C_T values were manually recorded.

2.2. Patient demographic information

Health information pertaining to each patient sampled for SARS-CoV-2 presence was recorded and retrieved from the Epic electronic medical record from mid-March to late-April 2020 after approval from the Washington University in St. Louis Medical Center Human Research Protection Office (IRB #202005002). Encounter type was clustered into three categories, inpatient, outpatient, and ED. Other non-identifying medical information procured for the purpose of our study was sample type, gender, and age.

2.3. Statistics

Pairwise comparison of C_T values for the different conditions was analyzed in Prism v8.4.3 using an unpaired parametric t-test.

One-way ANOVA was used to determine significance in C_T values for comparisons with >2 groups.

3. Results

### 3.1. Overall trends in test positivity

Using the first positive sample per patient, we found a total percent positivity of 14.3% (1043/7257). The highest proportion of positive tests came from the ED (20.9%, 497/2369) compared to outpatient (13.2%, 472/3579) and inpatient (5.6%, 74/1309) facilities. When examining positivity by age we observed a stepwise increase between larger age bins. The 20-29 and 30-39 groups had the lowest positivity rate (12.8% and 12.1%) which increased in the 40-49 and 50-59 groups (16.6% and 17.6%) and again in the 60-69 and 70-79 (20.0% and 20.62%) before rising in the 80-89 and 90+ groups (30.3% and 37.2%). The major trend observed for test order setting was a higher count of outpatient tests compared to ED for 20-29 (73 vs 35 tests), 30-39 (75 vs 46 tests), and 40-49 (76 vs 57 tests) groups but an inverse of this for 50-59 (80 vs 96 tests), 60-69 (71 vs 108 tests), 70-79 (44 vs 73 tests), and 80-89 (37 vs 52 tests). Contingency analysis within the inpatient group was underpowered for further statistical evaluation.

3.2. C_T values differ between testing platform and are not normally distributed

We retrieved the C_T values for 1043 positive Lyra SARS-CoV-2 assays (from 7,411 samples tested) from mid-March to late-April 2020 at Barnes-Jewish Healthcare system. Since the Lyra assay does not include the first 10 cycles in the report out of C_T from the instrument, we added 10 to each value for more consistent comparison with other molecular assays (Ransom et al., 2020). Given supply procurement challenges and difficulty obtaining reagents necessary for SARS-CoV-2 molecular testing, we used two separate extraction and detection platforms. Both methods were used interchangeably through the course of testing, with decisions for use based upon the reagent availability or test batch size. Initially we split the 1,043 positive samples by their respective test platform and found a significant (p=.0012) difference between the C_T values produced by EM/RGQ versus KF/ABI (Fig. 1.A). The median for EM/RGQ was 24.96 while the median for KF/ABI was 23.25. Despite having fewer samples, the KF/ABI values had a slightly larger standard deviation (6.8) compared to the EM/RGQ (6.1) values. Given that the EM/RGQ represents 77.5% (809/1043) of positive tests and because of the difference in C_T value
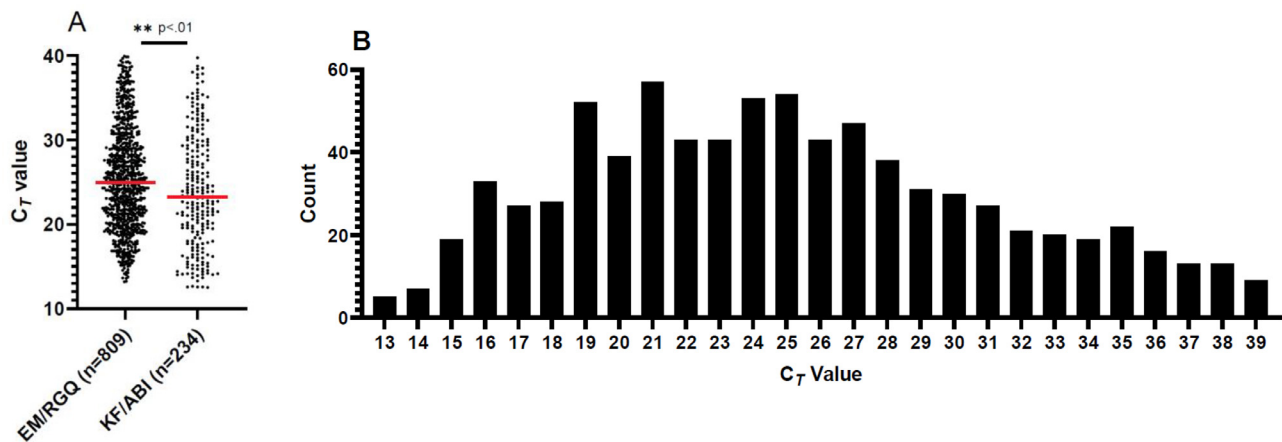
Fig. 1. Comparison of C_T values for both testing platforms and distribution of EM/RGQ Lyra SARS-CoV-2 test C_T values from March-April, 2020. (A) Scatter dots depict each positive C_T value as a point. Red line depicts the median C_T value. We find differences in C_T values obtained between platform (unpaired parametric t-test p value .0012). (B) Histogram depicting number of samples binned to their lowest whole number for EM/RGQ samples. Distribution of C_T values indicates our data is not normally distributed as the Shapiro-Wilk test (p value <.0001) did not pass normality.
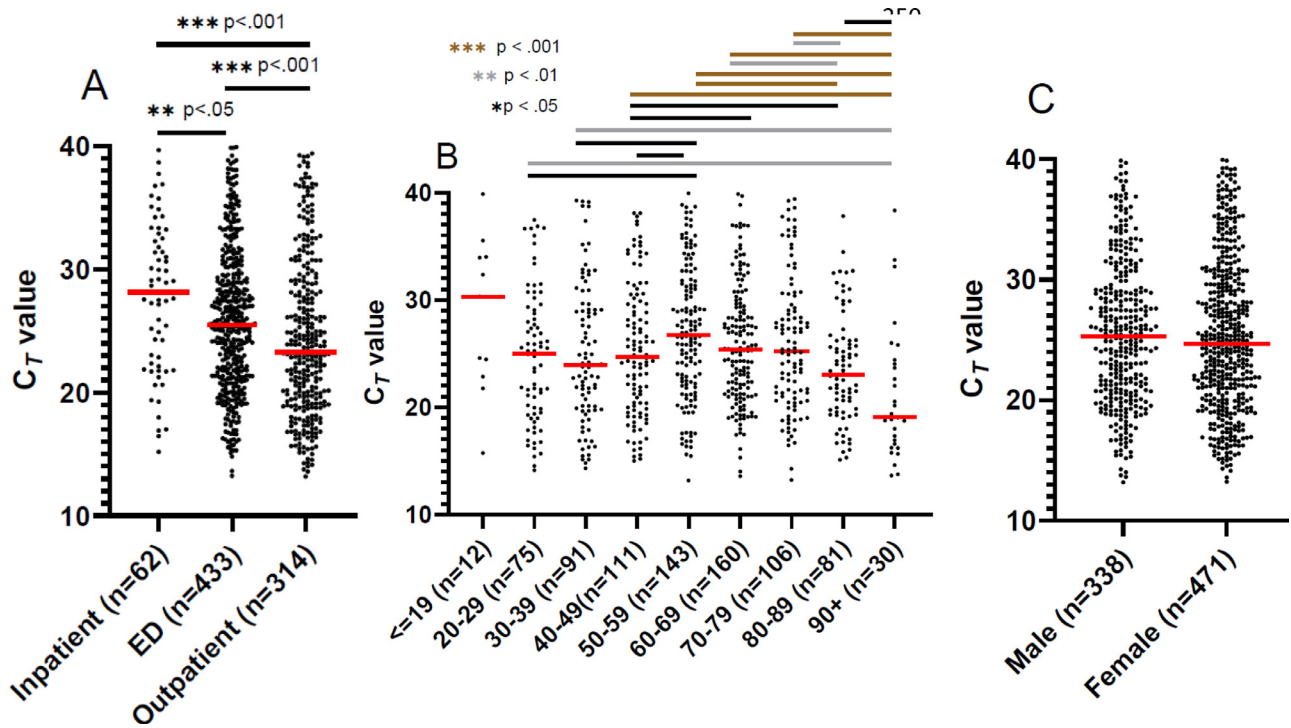
Fig. 2. C_T values by age, setting, and gender. Dotplot for EM/RGQ C_T values depicting depict each positive C_T value as a point for test order setting (A),age (B), and gender (C). Red line depicts the median C_T value. Overall difference (ANOVA p <0.0001 in C_T for age (A) and test order setting (B) but not gender (C). Individual pairwise comparisons significant by unpaired parametric t-test denoted in figure.

by platform we chose to analyze the 809 EM/RGQ samples. The 25%-75% interquartile range of for the EM/RGQ data was 20.76 − 29.58. The minimum and maximum C_T values obtained for this assay was 13.20 and 39.94, respectively (Fig. 1B). Interestingly, the data was not normally distributed as the Shapiro-Wilk test (p value <.0001) did not pass normality. Only 1.12% (9/809) of samples had C_T values of 39, near the limit of detection in this assay.

3.3. Differences in C_T value between test order setting and age

Given that our samples originated from a variety of patient care settings, we stratified positive results into inpatient, emergency department (ED), and outpatient bins to examine C_T value trends. Interestingly we found significant separation amongst all groups using ANOVA (p < .001) and amongst all possible pairwise comparisons (Fig. 2A). Outpatient tests had the lowest median C_T value (23.28) compared to ED (25.5) and inpatient (28.16) positive tests.

Since age has been indicated as a major risk factor for development of severe respiratory problems during SARS-CoV-2 infection, we performed an analysis of our institutional data by stratifying patients into 10-year age brackets. ANOVA found significant (p <0.0001) separation amongst all groups (Fig. 2B). We excluded positive values from patients <= 19 years of age given the smaller sample size from that population. We observed that the highest median C_T values were detected in patients 50-59, 60-69, and 70-79 (26.77, 25.3, and 25.24 respectively). The 20-29 age bracket had significant differences in median C_T values compared to the 50-59 and 90+ groups. While the middle ages had the highest median C_T values, the 30-39, 80-89, and 90+ age ranges have the lowest median C_T values (23.93, 23.00, and 19.07 respectively).We did not find a significant difference for other patient demographic factors such as gender (p=.23) (Fig. 2C).

3.4. Consecutive repeat positive C_T values

During the period evaluated, 527 patients had multiple tests performed. Of these tests, we had 18 occurrences of two consecutive positive Lyra SARS-CoV-2 assay results using the EM/RGQ system for 18 patients (Fig. 3). These additional 18 C_T values were excluded from all previously mentioned analysis. We were interested in comparing the ΔC_T value (C_{T1}- C_{T2}) as a function of time between tests that were consecutively positive. We found that the median 10.5 days between positive tests with 2/18 (11.1%) actually retested on the same day and a median of -5.7 ΔC_T value. 3/18 (16.67%) instances had a positive ΔC_T indicating that the level of viral burden increased between testing. In our study cohort, covering a 36-day time frame, the greatest time difference between two positive tests was 23 days. 5 individuals were both tested outpatient, 6 were initially tested in the ED then were admitted and tested as an inpatient. 5 were tested consecutively while inpatient and 2 were tested twice in the ED.
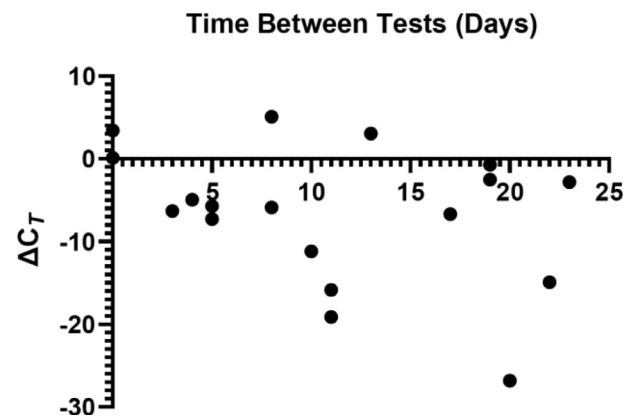
Fig. 3. Association of repeat C_T values in unique patients and the time between results. Repeat XY plot showing the EM/RGQ ΔC_T (C_{T1}-C_{T2}) as a function of days elapsed between the two tests for patients who consecutively tested positive for SARS-CoV-2. A negative ΔC_T value indicates a decrease in viral load between tests.

4. Discussion

While molecular RT-PCR tests have become the primary means for reliable detection of SARS-CoV-2 RNA for diagnostic determination of COVID-19, there exists a relative dearth of knowledge on the relationship between C_T values, patient information, and outcome. True quantification of viral levels for kinetics relies on a standard curve, but further investigation of C_T values could yield clinical insight (Han et al., 2020; Tom and Mina, 2020). This highlights the need for integration of C_T values as a proxy for relative viral load with studies that include patient demographic and clinical factors. Overall, our study found that testing platform, age, and patient setting are factors that can result in different C_T values. Our result on the difference in C_T values between the two extraction/quantification system used fits with a separate investigation by our group comparing extraction platforms which found around a 1 C_T lower C_T in KingFisher compared to EasyMag extraction, likely due to elution of RNA in a smaller volume (Ransom et al., 2020). Both methods showed exceptional sensitivity when known positive samples were diluted and had RNA extracted (Ransom et al., 2020).

One recent investigation has found associations between death, intubation and high viral loads ($C_T < 25$) compared to medium (C_T 25-30) and low viral loads ($C_T >30$) (Magleby et al., 2020). This study used the Cobas 6800 RT-PCR system (Roche) which detects the Orf1ab and the E gene within the SARS-CoV-2 genome (Magleby et al., 2020). Given that the Lyra SARS-CoV-2 Assay also analyses Orf1ab levels, there is opportunity to compare our findings with the findings of this group. Using their criteria, our EM/RGQ results had 50.2% (406/809) in the high viral load, 30.0% (243/809) with medium viral load, and 19.8% (160/809) with low viral loads. As a laboratory serving a large healthcare system, we were able to categorize tests as being ordered from an inpatient, ED, or outpatient setting and found significant differences in the C_T values between all pairwise groups. The median for inpatient and ED settings were both in the medium viral load range but the median for our outpatient tests was in the high viral load category. The observation that outpatient setting tests had lower C_T values compared to patients presenting at the ED or tested from an inpatient hospital floor was unexpected but could possibly be due to greater levels of aerosol exposure as these patients are predominantly healthcare workers. One investigation of influenza burden in children came to a similar conclusion to our own observation that C_T values in outpatients tend to be lower than inpatients (Fuller et al., 2013). One explanation given by these authors is that outpatients present earlier for testing than inpatients when viral loads are likely to be higher (Fuller et al., 2013). It has been demonstrated that C_T values can be similar between asymptomatic patients and those with COVID-19 disease (Zou et al., 2020). When stratified for age (excluding the low n <19 group) we found that the age intervals with the highest median C_T value (20-29, 50-59, 60-69, and 70-79) were in the medium viral load but all other groups had medians in the high viral load category. Further work is therefore warranted to investigate if indicators of severe disease were also more prevalent in these groups. This trend of lower C_T values amongst the youngest population and oldest adults but higher C_T values amongst the middle age has also been documented for norovirus (Shioda et al., 2017). Shioda et al hypothesized that this is likely due to age related senescence in the elderly and lack of prior norovirus exposure to the youngest. A group from Germany found no meaningful differences amongst age range and viral load, however they did not use C_T values but rather a viral load estimation using standard curve for their two different platforms (Jones et al., 2020). Additionally, these authors included lower respiratory samples although they make up a small (~3%) of the total volume. Differences between molecular target, master mix efficiency, and threshold cutoffs may make interpretation of C_T values between assays difficult. For instance, the US CDC assay detects the nucleocapsid gene and not the Orf1ab gene (the target of the Lyra assay). This could explain why an analysis of the first 12 US COVID-19 patients found a higher mean C_T value than our analysis (Anonymous, 2020).

Importantly, the Lyra SARS-CoV-2 Assay and other molecular tests detect presence of viral RNA and not infectious virions. A recent study investigated the relationship between C_T value and growth in Vero cells, finding that no growth occurred in NP samples with C_T value >24 cycles (Bullard et al., 2020). C_T values were obtained using real time RT-PCR for a 122 bp region within the E gene. In line with this, another group found that patients with C_T values >33 cycles are non-infectious and according to their model can be discharged (La Scola et al., 2020). Considerable interest exists for the use of repeat PCR testing to diagnose patients with suspected COVID-19 but are PCR negative, as well as monitor viral levels in patients with resolving symptoms (Fisher et al., 2020). Previous investigations have shown that C_T values generally spike 5-6 days following symptom onset and gradually decline overtime, in some cases taking 6 weeks before becoming negative (Xiao et al., 2020). We therefore were interested in investigating instances where patients tested positive consecutively using the Lyra SARS-CoV-2 assay and found that most have higher C_T values in the second test indicating a decreased viral burden.

In this study we retrospectively analyzed 809 positive Lyra SARS-CoV-2 Assay tests to investigate C_T value overall distribution and differences in C_T values and positive tests as it relates to test ordering setting and patient demographics. One of the limitations of our study is that given the short timeframe of our study, patients could test persistently positive longer than the time range we investigated. Another limitation is that we are unable to ascertain an accurate day of symptom onset for our patients, if they were symptom-free at the time of testing, or whether they developed symptoms at a later date. This makes it difficult to use our data to track natural progression of SARS-CoV-2 levels within patients. Additionally, we have not integrated clinical factors with our laboratory data to ascertain which patients had severe COVID-19 disease. These correlative findings could be valid only during the initial surge of cases and may not reflect the C_T distribution in the timeframe following the initial surge, therefore continued monitoring of C_T values is ongoing. Variability in purity or quality between samples of extracted nucleic acid may be partially responsible for different C_T values. Finally there could be a difference between C_T values from NP and OP swab samples but we are underpowered given the low number of OP swabs analyzed to address this.

Our investigation has many strengths. We were able to evaluate a large number of samples from a variety of patient encounter-types. We used the Lyra Assay as our testing platform and thus we were able to obtain a C_T value for quantification which is not possible on all testing platforms used clinically. An advantage of our investigation is that as the central laboratory for a multi-hospital system we were able to achieve a high number of positive samples quickly for analysis and could stratify those tests into relevant bins. Additionally, our testing population includes healthcare workers and local residents. We found that within these groups there were significant differences between C_T values by test setting and age bins. We have also demonstrated that patients can remain consecutively positive for greater than 10 days but further work is warranted to investigate the infectivity of patients with high C_T values. In conclusion, Lyra Assay C_T values can be used within our system to identify differences in viral load between patients within different age brackets and from testing sites.

Author' contributions

RFP and BA were responsible for data curation, writing, revising, and editing. CSE, CDB, NWA, BAP, were involved in writing, revising, and editing.

Declaration of competing interest

There are no conflicts of interest.

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