



Correlation between Cycle Threshold (Ct) Value and IL-6 and D-Dimer in Chip-based RT-PCR Positive COVID-19 Cases: Study from a Stand-alone Laboratory

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: Novel Coronavirus (SARS-COV-2) is a leading cause of morbidity and mortality since the beginning of 2020 leading to range of symptoms from mild flu to respiratory distress, which is called COVID-19. RTPCR being the main diagnostic test can confirm the presence of the virus in the clinical samples, while various studies have defined Interleukin-6 and D-dimer as potent biomarker for severity. In this study, we have attempted to correlate the severity of COVID-19 with the presence of IL-6 and D-dimer and the Cycle threshold (Ct value) as determined by chip based RTPCR.

Aim: The study aims to correlate the Cycle threshold value obtained after chip-based RT-PCR with markers such as IL-6 and D-Dimers.

Methodology: It is a retrospective, observational study done in 799 subjects in a span of three months (August 2020 to October 2020) at R V Metropolis Diagnostic and Healthcare Pvt Ltd. All symptomatic patients who tested positive in the Laboratory for COVID-19 by chip-based RT-PCR

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were included. Chip based RTPCR or Truenat test was performed on Nasopharyngeal swabs of the suspected subjects. Interleukin-6 was determined by Electrochemiluminescence assay while D-dimer was done on the principle of Chemiluminescence.

Statistical Analysis Used: SPSS 12.0 version.

Results: Total number of subjects enrolled were 799, with mean age of the subjects being 46.80 ± 17.55 years. In the study, males were found to be affected by COVID-19 more than females with ratio of male to female being 1.65:1. 498 (62.3%) of males presented with COVID-19 while it was observed in 301 (37.6%) females. Out of 799 subjects, 289 (36.2%) were symptomatic and out of 289 subjects, 140 (17.5% of total subjects) required hospitalisation. Cycle threshold values of both screening as well as confirmatory genes were determined separately in the cases of symptomatic and asymptomatic cases and there was no significant difference between the Ct values in cases of symptomatic and asymptomatic patients. Symptomatic patients were subcategorised under hospitalised and non-hospitalised and Again, no significant difference was seen between the two subset of patients in terms of Ct-value and, indirectly, the viral load of their clinical sample. The results convey that IL-6 and D-Dimer was significantly high ($p=0.001$ and <0.001 respectively) in case of symptomatic patients. D-Dimer was significantly high ($p= <0.001$) in the patients who needed hospitalisation. IL-6 was significantly raised as well ($p=0.02$). Screening and confirmatory gene were found to have no significant relationship with IL-6 and D-Dimer, neither any correlation was observed with them.

Conclusion: Biomarkers such as Interleukin-6 and D-dimer can very well help in determining the severity and need for hospitalisation in a COVID-19 affected patient, but they have been found to have no relationship with cycle threshold value of RTPCR in our study.

Keywords: SARS COV-2; Ct value. IL-6; D-dimers.

1. INTRODUCTION

Novel Severe Acute Respiratory Syndrome Coronavirus -2 (SARS-COV-2) is identified as an agent for community acquired pneumonia and till now has affected 102,942,987 people and has claimed 349,263 lives, as recorded till third of February, 2021 [1].

After infection with SARS-COV-2, patient may develop disease ranging from being asymptomatic to severe respiratory tract infection [2]. According to Sudhir Bhandari et al, amongst 39 laboratory proven COVID-19 positive cases which were symptomatic, fever (79.47%), cough (74.35%), shortness of breath (36%) and sore throat (17.94%) were the most common presenting clinical manifestations [3].

Severity of the disease is determined by 1) sudden deterioration of the patient's condition around one to two weeks after onset; 2) much lower level of lymphocytes, especially natural killer (NK) cells in peripheral blood; 3) extremely high inflammatory parameters, including C reactive protein (CRP) and pro-inflammatory cytokines (IL-6, TNF α , IL-8, et al); 4) the majority of infiltrated immune cells in lung lesion are monocytes and macrophages, but minimal lymphocytes infiltration; 5) mimicry of vasculitis, hypercoagulability and multiple organs damage [4].

The main diagnostic test available to us is Reverse Transcription Polymerase Chain Reaction or RT-PCR. Real time RTPCR cycle threshold determines the number of amplification cycles required for target gene to exceed a threshold levels, and hence, is an indirect indicator of viral load [5].

IL-6 can be produced by almost all stromal cells and by immune system cells, such as B lymphocytes, T lymphocytes, macrophages, dendritic cells, monocytes, mast cells and many non-lymphocytes, such as fibroblast and endothelial cells [6].

IL-6 plays a crucial role in infectious diseases such as influenza where it has been shown how in a IL6^{-/-} mice influenza specific CD4⁺ T cell response is impaired. Loss of IL-6 results also in persistence of the influenza virus in the lung leading to extreme lung damage and death. This shows how IL-6 limits influenza-induced inflammation and protects against lung damage by promoting neutrophil survival in the lung [7].

Fibrinolytic system breaks down the fibrin mesh after the formation of clot. The D-dimer, which comprises two D fragments of the fibrin, is formed by the activation of the plasmin enzyme. This indicates the presence of a demolished

fibrin in the bloodstream. D-dimer represents the activation of coagulation and fibrinolysis systems. Technically, the amount of D-dimer level is measured using various commercial kits on the market, based on a monoclonal antibody. The D-dimer test is usually used in clinical practice to exclude a diagnosis of deep vein thrombosis (DVT) and pulmonary embolism (PE) and confirm the diagnosis of disseminated intravascular coagulation (DIC). The D-dimer levels rise almost in all patients with severe VTE. In physiological conditions such as pregnancy and pathological conditions such as cancer, inflammation, and surgery the elevated level of D-dimer can be seen frequently [8].

Our study intends to correlate the viral load (Ct value by RTPCR being an indirect indicator) with increase in laboratory markers such as IL-6 and D-Dimer in the patients of COVID-19, to ascertain the usefulness of such markers according to the disease severity.

2. AIMS AND OBJECTIVES

The study aims to correlate the Cycle threshold value obtained after chip-based RT-PCR with markers such as IL-6 and D-Dimers.

Objectives of this study are:

1. To analyse number of COVID-19 positive cases that were symptomatic.
2. To analyse symptomatic cases of COVID-19 that needed hospitalisation.
3. To study the Cycle threshold (Ct) value in such cases.
4. To correlate the findings of Ct value with IL-6 and D-Dimers.

3. MATERIALS AND METHODS

Study Design: A retrospective, observational study was carried out in a span of three months (August 2020 to October 2020).

Sample size: 799

Target Population: All symptomatic patients who tested positive in the Laboratory for COVID-19 by chip-based RT-PCR.

3.1 Exclusion Criteria

1. All patients for whom the test was requested pre-procedure.

2. Patients with incomplete Sample Referral Forms (where clear history was not indicated.)

3.2 Laboratory Proceedings

3.2.1 Chip-based real time reverse transcription polymerase chain reaction

Specimen type- Nasopharyngeal swab

Initial processing: Sample was collected using Trueprep® AUTO Transport Medium for Swab Specimen Pack by Molbio Diagnostics Pvt. Ltd. Keeping universal precautions under consideration.

Samples were pretreated using Lysis buffer.

Extraction and purification of nucleic acids was done using Trueprep® AUTO Universal Cartridge Based Sample Prep Kit and Trueprep® AUTO Universal Cartridge Based Sample Prep Device.

3.2.2 Nucleic acid amplification

Nucleic acid amplification was performed using Truelab Quattro Real Time micro PCR Analyzer.

Principle: Real Time Reverse Transcription Polymerase Chain Reaction (RT PCR) based on Taqman chemistry Initially, Screening gene (E gene) was analysed using Truenat™ Beta CoV and the samples positive for E gene were confirmed by RdRp gene using Truenat™ SARS CoV-2 kit with RNase P as internal control.

Later, with E gene as screening and Orf1a gene as confirmatory, samples were analysed using Chip-based Real Time Duplex PCR Test for COVID-19 with RNase P as internal control.

Result interpretation was done as per kit insert [6] Ct value was taken as ≤ 32 .

Sensitivity of the test: 100%

Specificity of the test: 100%

3.3 IL-6 Detection

Sample type: EDTA-plasma

IL-6 was determined using Roche e411 machine and Elecsys IL-6 kit by Cobas.

Principle: Electrochemiluminiscence Assay (ECLIA)

Performance of the test as per kit insert:

- Sensitivity of the test: 84 % (95 % CI: 60 % to 97 %)
- Specificity of the test: 63 % (95 % CI: 44 % to 80 %)
- Limit of Detection: 12 ng/mL

3.4 D-Dimer Detection

Sample type: Citrate-plasma

D-dimer were run in Abbott Architect machine using Quantia D-dimer kit.

Principle: Quantitative analysis by Chemiluminiscence Assay

Performance of the test as per kit insert:

- Sensitivity of the test: 100 %
- Specificity of the test: 100 %
- Limit of Detection: 12 ng/mL

4. RESULTS

Total number of subjects enrolled were 799, with mean age of the subjects being 46.80± 17.55 years. In the study, males were found to be affected by COVID-19 more than females with

ratio of male to female being 1.65:1. 498 (62.3%) of males presented with COVID-19 while it was observed in 301 (37.6%) females.

Out of 799 subjects, 289 (36.2%) were symptomatic and out of 289 subjects, 140 (17.5% of total subjects) required hospitalisation (Table 1).

4.1 Value of Cycle threshold (Ct-value)

Cycle threshold values of both screening as well as confirmatory genes were determined separately in the cases of symptomatic and asymptomatic cases as given in Table 2.

As seen in Table 2, there was no significant difference between the Ct values in cases of symptomatic and asymptomatic patients.

Symptomatic patients were subcategorised under hospitalised and non-hospitalised, results for which are tabulated below (Table 3).

Again, no significant difference was seen between the two subset of patients in terms of Ct-value and, indirectly, the viral load of their clinical sample.

Table 1. Table depicting number of patients that were symptomatic and number of patients who needed hospitalisation

Total Subjects= 799		
	Symptomatic (289/36.2%)	Asymptomatic (510/63.8%)
Require Hospitalisation	140/17.5%	32/4%
Do not Require Hospitalisation	149/18.6%	478/59.8%

Table 2. Mean Ct-value of symptomatic and asymptomatic cases and their significance

Mean Ct Values In Symptomatic and Asymptomatic Cases			
	Symptomatic (289/36.2%)	Asymptomatic (510/63.8%)	p Value (SIGNIFICANT-≤0.05)
Screening/E Gene	22.23±6.46	23.04±6.07	0.123
Confirmatory/ RdRp or ORF1 Gene	21.77±6.37	22.69±6.00	0.122

Table 3. Mean Ct-value of hospitalised and non-hospitalised cases and their significance

Mean Ct Values in Hospitalised and Non- Hospitalised Patients			
	Hospitalised (140/17.5%)	Non-hospitalised (659/82.5%)	p Value (Significant-≤0.05)
Screening/E Gene	21.99±6.71	22.38±6.32	0.44
Confirmatory/ RdRp or ORF1 Gene	21.72±.59	21.80±6.26	0.48

We also compared value of Interleukin-6 in symptomatic and asymptomatic cases, and following are the results (Table 4). Out of 289 symptomatic patients, 95(32.9%) had got tested for rise in IL-6. In case of 510 asymptomatic cases, only 56 (10.9%) had IL-6 tested.

At the same time, D-Dimer was determined for the subjects and results are given in Table 5.

The results convey that IL-6 and D-Dimer was significantly high ($p=0.001$ and <0.001 respectively) in case of symptomatic patients.

When the same analysis was done in hospitalised and non-hospitalised patients, following were the results.

The above results denote that D-Dimer was significantly high ($p= <0.001$) in the patients who needed hospitalisation. IL-6 was significantly raised as well ($p=0.02$).

When the values of IL-6 and D-Dimer were correlated with Ct-values of screening and confirmatory gene, following were the results.

Table 4. Median value of Interleukin-6 in symptomatic and asymptomatic cases and their significance

IL-6	Symptomatic (95/32.9%)	Asymptomatic (56/10.9%)	p Value (Significant- ≤ 0.05)
Median	18.71	6.86	0.001
Inter-quartile Range	4.96-61.62	1.06-20.61	

Table 5. Median value of D-Dimer in symptomatic and asymptomatic cases and their significance

D-Dimer	Symptomatic (152/52.6%)	Asymptomatic (28/5.9%)	P value (significant- ≤ 0.05)
Median	371.89	262	<0.001
Inter-quartile Range	174-895.27	111-595.39	

Table 6. Median value of IL-6 in hospitalised and non-hospitalised cases and their significance

IL-6	Hospitalised (140/17.5%)	Non-hospitalised (659/82.5%)	P value (significant- ≤ 0.05)
Median	18.11	10.52	0.02
Inter-quartile Range	5.00-68.59	2.85-31.45	

Table 7. Median value of D-Dimer in hospitalised and non-hospitalised cases and their significance

D-Dimer	Hospitalised (140/17.5%)	Non-hospitalised (659/82.5%)	p Value (Significant- ≤ 0.05)
Median	519.11	251.60	<0.001
Inter-quartile Range	184.50-1033.61	120.25-489.11	

Table 8. Table showing significance of IL-6 and D-Dimer with respect to Screening (E) gene

Screening gene (E gene)	IL-6	D-Dimer
p VALUE (Significant- ≤ 0.05)	0.18	0.09
Correlation coefficient (r)	0.10	0.09

Table 9. Table showing significance of IL-6 and D-Dimer with respect to confirmatory (RdRp/ORF1) gene

Confirmatory gene (RdRp/ORF1 gene)	IL-6	D-Dimer
p Value (Significant- ≤ 0.05)	0.19	0.19
Correlation coefficient (r)	0.10	0.07

Screening and confirmatory gene were found to have no significant relationship with IL-6 and D-Dimer, neither any correlation was observed with them.

5. DISCUSSION

We included 799 patients in our study who were COVID-19 positive by chip-based RT-PCR. Mean age of the patients was 46.80 ± 17.55 years, which is supported by F. Zheng et al in their study that states median age as 45 years [7]

In our study, 62.3% males were affected by COVID-19 while positivity rate was less in females with 37.6%. Similar observation was made by Kaijin Xu et al who noted that Male sex is a factor for delayed viral shedding [8]. This may be due to following potential reasons: 1. Males express more ACE2 receptors in their lung and heart as compared to females [9]. 2. TLR-7 helps control the innate immunity is expressed more due to an extra X chromosome in females [10] 3. Estrogen is believed to build a strong immune response and inhibits viral replication in females, leading to lower mortality and morbidity in them [11].

Arons MM et al in their study found 56% of patients who were tested for COVID-19 as asymptomatic [12]. In our study as well, 63.8% of patients who were tested positive were asymptomatic. Data from Mizumoto K. et al differ with ours with their asymptomatic cases being 17.9% [13]. The major reason behind that could be the need for test only with the presence of symptoms.

When we compared average Cycle threshold values for screening and confirmatory genes in both symptomatic and asymptomatic cases, mean value for E gene was 22.23 ± 6.46 in symptomatic cases and for asymptomatic cases was 23.04 ± 6.07 ($p=0.123$) in case of symptomatic cases. However, for RdRp/ORF1 gene, mean Ct value was 21.77 ± 6.37 in symptomatic cases and 22.69 ± 6.00 in asymptomatic cases ($p=0.122$). We can observe from the above findings that there was no significant difference between the cycle threshold value in symptomatic and asymptomatic cases. This observation is backed by another study by Jae-Sun Uhm which claims that there were no significant difference in Ct-value of E, N and RdRp gene of symptomatic and asymptomatic patients [14]. Another study by Barnaby E Young, MB BChir et al also quoted that they did

found no relationship between illness severity and duration of viral shedding or PCR Ct values [15]. Another indian study by Shah et al it was concluded no difference in the initial cycle threshold (a surrogate marker of viral load) or time to swab negativity of patients with severe vs. those with mild disease [16].

Again, when the data for Ct-value in hospitalised patients was analysed against those patients who did not require hospitalisation, in both the genes we could not find significance, neither we observed any significance of Ct-value with inflammatory markers IL-6 and D-dimer. Damion Jacot. et al, also in their study also approve of this where they found that patients with disease progression from mild to severe disease did not have significant high viral load, in fact, patients in ICU were found to have lower viral load in some cases explaining that inflammatory response dominated over viral replication in their case [17]. Their study also states that COVID-19 severity is not just determined by amount of viral replication but also by unregulated inflammatory responses by the host.

A study provides us with logical reasons behind the insignificance of the Ct-value, since it's value is dependent upon various variables like a) quality of the sample because of the collection, transport and storage prior to test, b) site of collection, c) assay's gene target, d) extraction platform, e) PCR amplification chemistry [18]. Some studies also state that Ct-value can vary upto 5 cycles if same sample is processed by different assays [19].

One interesting study, however, gives a very useful insights on the autopsies in case of COVID-19 cases. They conclude that individual Ct value did not correlate with the organ damage. The Ct values for the non-autopsy cases were correlated with the duration of disease and hospitalization, respectively, which did not reveal statistical significance [20].

When we correlated the values of IL-6 and D-dimer in symptomatic and asymptomatic patients, then IL-6 and D-dimer were found to be raised significantly ($p=0.001$ and <0.001 respectively). Even when their values were compared among the patients who required hospitalisation and non-hospitalised patients, the significant difference is clear ($p= 0.02$ and <0.001). This was also observed in the study done by Tobias Harold MD et al. In their study, they found that risk of respiratory failure was 22

times more in the patients with IL-6 value of ≥ 80 pg/mL, as compared to the patients with lower or non-detectable IL-6 levels [21]. This leads us to believe about IL-6 that it is still not concluded whether it is merely a biomarker or a pathogenetic element, but it must be used as a parameter to assess the severity.

Another study reasons the raise in IL-6 levels in symptomatic cases or cases that need hospitalisation by postulating that elevated IL-6 drives immune dysregulation and respiratory failure rapidly as it contributes to lymphopenia, impaired lymphocyte cytotoxicity, and endothelial activation, leading to "Cytokine storm" [22].

A study by Oliver J McElaveny et al also found that plasma levels of IL-6 in the cases requiring hospital admission were significantly high ($p=0.0001$) [23], leading us to believe that increased IL-6 points towards impending pulmonary damage.

When we analysed D-dimer as a predictor of severity in our study, we found that not only was significantly high in the symptomatic patients, it also helped in determining if the patient needed hospitalisation.

Elevated D-Dimer is a biomarker of abnormal coagulation function, and has proven to be a factor in disease progression [24-25]

This finding is supported by Litao Zhang et al who observed that D-Dimer levels above $2\mu\text{g/mL}$ had a higher incidence of mortality as compared to the patients with lower D-Dimer levels [26-27]. D-dimer originates from the formation and lysis of cross-linked fibrin and reflects activation of coagulation and fibrinolysis.

In a study by Yong Gao et al, When IL-6 and D-Dimer were jointly predicted, the ROC curve integral of severe COVID-19 was 0.840 ($P < .01$) as good predictors of severe COVID-19 under the ROC curve, and the combined detection effect was better [28].

Keeping above findings under consideration, we can safely imply that IL-6 and D-Dimer have potential to predict the severity of the course of COVID-19 with great efficiency.

Our study, however, had certain limitations. Since it was done in a stand-alone laboratory, we could not follow-up on the patient outcome. Also, we could not stratify under "patients requiring

ICU admission" and "patient not requiring ICU admission". Moreover, other known markers such as Interleukin-8, Tumor Necrosis Factor were not determined in our study.

6. CONCLUSION

In conclusion, our study did not find any correlation between low Ct-value (indirectly, high viral load) in the sample and severity or need for hospitalisation. Neither Ct value found any correlation with increase in IL-6 or D-Dimer. But, we can safely conclude that IL-6 and D-Dimer must be used as predictors of severity when risk stratification is done.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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