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Association of different biomarkers and CT value of RT-PCR with mortality in COVID-19 cases attended at tertiary care centre in India

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Abstract

Background: Severe Acute Respiratory Syndrome- Corona Virus-2 (SARS-CoV-2) infection becomes the pandemic of coronavirus disease 2019 (COVID-19) shows drastic changes in health care system.
Aims and objectives: 1) To assess different biomarkers in COVID-19 cases, 2) To correlate biomarkers with cycle threshold (CT) value of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and it's role for prognosis of severe cases.
Material and Methods: Consecutive patients with positive for SARS-CoV-2 by RT-PCR with CT value less than 36 were included. A CT values were found for envelope (E-gene) as a screening gene and RNA dependent RNA polymerase (RdRp) as a confirmatory gene were amplified during RT-PCR. The severity of illness was assessed using different biochemical parameters for C-reactive protein (CRP), serum ferritin, D-dimer, lactate dehydrogenase (LDH), serum urea, and creatinine.
Results: We enrolled 395 SARS-CoV-2 positive symptomatic patients. Among these, 322 (81.5%) were recovered and remaining succumbed 73 (18.5%). The mean age and distribution of gender were similar among death vs. recovered patients. We found that death seen in more severe cases 56/91 (61.5%), while recovery was good in mild-moderate [287/304 (94.4%)]. CRP was significantly higher among death than recovered cases ($p<0.05$). D-dimer, serum ferritin and LDH were also elevated among death alone and also in severe cases except serum ferritin.
Conclusions: We concluded that, mortality occurs more in patients with lower CT values (or early peak) than recovered cases ($p < 0.001$). The elevated level of CRP, serum ferritin, D-dimer and LDH including lower value of CT are associated with higher mortality in severe cases of COVID-19.
Keywords: SARS-CoV-2, RT-PCR, cycle thresholds (CT), biochemical markers, COVID-19, RdRp.

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1. Introduction

Towards the finish of December 2019, multiple cases of unusual pneumonia lead about by a corona virus disease 2019 (COVID-19) were first recognized in Wuhan, China [1]. The pathogenic organism was a β-corona virus homologous to bat corona viruses [2]. Later this virus was known as a "Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2). The first patient with COVID-19 was confirmed on January 30, 2020 and April 27, 2020 in India and Gorakhpur, Uttar Pradesh; respectively [3]. In view of its infectious nature, it causes quick spread and on the day of the end of our study, India was the second most common country in the incidence of SARS-CoV-2 positive

cases (10,758,619) globally. However, mortality rate was lower 154,428 (1.44%) in India than 2,246,806 (2.17%) worldwide [4]. The infected patients may remain asymptomatic or have mild conditions but approximately 13.8%-6.1% showed serious or critical life-threatening symptoms of flu-like illness or enteric or neurologic, hepatic diseases, potentially fatal pneumonia, and respiratory failure especially in elder subjects with co-morbidities [5,6]. These patients need necessary hospitalization and, in some cases, intensive care unit (ICU) admission. The identification of miserable biomarkers associated with higher mortality rate including risk stratification of the patients that needed hospitalization and ICU in the circumstance of insufficient resources and overburdened to our health care system.

Biochemical parameters can be useful in predicting negative outcomes in COVID-19 patients [7]. Several prognostic biomarkers of this disease predicting the severity have been validated till date [8,9]. However, in-hospital patient's clinical findings especially hematological, inflammatory markers, and biochemical parameters as well as viral load of SARS-CoV-2 are needed for the assessment of severity of COVID-19 infection. Biomarkers may be classified as a susceptibility or risk, diagnostic, tracking, prognostic and predictive for any disease including the classify patients that are more likely to have a certain outcome [10]. C-reactive protein (CRP) is a liver-produced protein; indicate the early indicator of infection and inflammation [11]. It has been identified as a key marker that changes dramatically in extreme COVID-19 patients [12]. D-dimer (coagulation factor) acts as a biomarker among severe COVID- 19 patients [13,14]. It were elevated among most deaths, may be caused by systemic microvascular thrombosis or coagulopathy, according to a recent autopsy [15]. Serum ferritin, lactate hydrogenase (LDH), serum urea and creatinine were also used as biomarkers of COVID-19 especially unexplained weakness or shortness of breath, inflammatory cell damage and signs of kidney or liver damage, respectively [8,16-17]. However, little is known about these biochemical markers in relation to COVID-19 progression and rate of mortality.

SARS-CoV-2 viral loads are detectable by reverse transcriptase polymerase chain reaction (RT-PCR) cycle threshold (CT) values. It represent the number of amplified cycle required the target gene like envelope (E) and RNA dependent RNA polymerase (RdRp) gene to exceed threshold level [18]. Several studies suggested that CT values can provide indirectly the copy number of viral load for the determination of likelihood transmission as well as severity and recovery rate of the SARS-CoV-2 infected cases [19,20].

COVID-19 prognosis with optimal value of biochemical parameters as well as CT values of RT-PCR on admission to predict mortality or recovered have not been well evaluated in our population.

1.1 Aims and Objectives

Accordingly, we undertook a prospective observational study with the aims of, (i) biochemical level assessment like liver function tests, CRP, D-dimer, serum ferritin, LDH and kidney function tests, and (ii) RT-PCR CT values among death and recovered cases, who attended at tertiary care centre, covered mainly the symptomatic patients of Northern (Poorvanchal area) India.

2. Material and Methods

This study was based on consecutive SARS-CoV-2 positive patients diagnosed by COVID-19 Viral Research Diagnostic Laboratory (VRDL) having Bio Safety Level-3 (BSL-3) standard molecular facility in the Department of Microbiology, Baba Raghav Das Medical College and associated Nehru Hospital, Gorakhpur in northern India during an 8-months period between June 2020 and January 2021. A total of 395 confirmed SARS-CoV-2 infected patients were included in this study.

The inclusion criteria of testing for SARS-CoV-2 are recommended by Indian Council of Medical Research under the Ministry of Health and Family Welfare, government of India used the selection of suspected COVID-19 with the following: (i) all subjects with at least one suggestive symptoms like fever, altered smell/taste or acute respiratory disease (cough and respiratory distress), (ii) all close contacts of RT-PCR positive cases for SARS-CoV-2, (iii) presence of clinical features with unexplainable by other diseases or suggested by computed tomography scan, (iv) people traveling back to the country from an international destination or where the infection was endemic in the previous 14 days before the onset of symptoms, and (v) other complaints, radiological and epidemiological features suggestive for atypical pneumonia and blood test results of patients were obtained from the patient's file. However, the exclusion criteria was (i) all the RT-PCR negative cases for SARS-CoV-2, (ii) patients or their attendants refuse to be enrolled in the study, (iii) presence of allergic bronchitis/pneumonia and (iv) SARS-CoV-2 positive case with congestive heart failure .

The demographic and clinical data were obtained by a hospital data management system and by an electronic online questionnaire assisted by telephonic interviews. Patients were quarantined either at home or hospitals depending on the severity of illness and followed-up either physically or telephonically.

2.1 Diagnosis of SARS-CoV-2 Virus

The naso- and oro-pharyngeal swab samples were obtained from the referral of the cases from districts of Gorakhpur, Deoria, Mahrajganj, and Kushinagar from the state of Uttar Pradesh as per standard protocol wearing personal protective equipment (PPE) kit [21]. The assay was composed of two steps; (1) RNA extraction from the patient specimen's samples with a column-based QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) under standard biosafety guidelines, (2) The RT-PCR for SARS-CoV-2 was performed with a single tube multiplex assay by QuantoStudioTM 5 Real-Time PCR system (Thermofisher, USA) as per instructions of the manufacturer. Briefly, the PCR condition included uracil-DNA glycosylases enzyme (mixed in COVISure RT master mix to prevent laboratory carryover contamination) incubation at 25°C for 2 minutes, which is an optional step followed by reverse transcription at 50°C for 15 minutes and activation at 95°C for 3 minutes. PCR amplification was performed denaturation for 40-45 cycles at 95°C for 10 seconds and annealing/extension at 60°C for 30 seconds.

2.2 Assessment of severity of COVID-19 cases

The illness severity of COVID-19 patients was assessed as described previously—(i) "mild-moderate" means any criteria either- (a) only upper respiratory symptoms or although pneumonia was present, the patient did not require oxygen, (b) lung infection <50% involvement on either chest X-ray or high resolution computed tomography (HRCT), (c) lung imaging showed viral pneumonia, (d) blood oxygen saturation between 94-84%, (e) fever and cough or other respiratory symptoms and, (f) discharge after 10 days of symptoms onset. While "severe" means either- (a) required ventilator or needed oxygen or blood oxygen saturation < 84%, (b) lung infection > 50% involvement on either chest X-ray or HRCT, (c) shock and, (d) presence of acute respiratory distress syndrome (ARDS) or cardiac injury or multi-organ dysfunction [22]. Cases without symptoms and suspected cases with negative RT-PCR for SARS-CoV-2 were excluded from this study.

2.3 Blood sample collection for biochemical markers

The different biochemical assessment was done by using an aseptic technique and the machine used was fully automatic batch analyzer for serum or plasma preferred samples. These were the routine process among hospitalized patients recommended by clinicians, who were positive for the SARS-CoV-2. All the clinical and laboratory parameters like biochemical profile, especially CRP, serum ferritin, D-dimer, LDH, serum urea, and creatinine were measured. We have also collected biochemical profiles for liver function tests mainly alanine transaminase (ALT) or SGPT, aspartate amino transaminase (AST) or SGOT, and alkaline phosphates (ALP), and among all the patients.

2.4 Determination of the cycle thresholds (CT) values of RT-PCR

We have used a multiplex RT-PCR kit for the qualitative detection of different regions of RNA virus primer sequence by COVISure Genetix kit. The cycle thresholds (CT)-value was defined as the number of cycles required for the fluorescence signal to cross the threshold (i.e., exceed the background level). These CTs suggest the approximate measure of viral load. If CT values are ≥ 36 then the value is significant but again these values depend upon standard protocol provided by the manufactures as per the kit insertion [23]. The RNase P was used as an internal control; however, envelope (E-gene) as a screening gene and RNA dependent RNA polymerase (RdRp) as a confirmatory gene were amplified and tested for SARS-CoV-2 infection during RT-PCR.

2.5 Statistical analysis

Discrete data were represented as frequencies and percentages. Continuous data were represented as mean±standard deviation, especially age biochemical parameters and CT values of RT-PCR. Data were analyzed using by IBM SPSS 15. A 0.05 level of probability (p-value) was used as the criterion for statistical significance of studied data. Categorical data were analyzed by Chi-square test and continuous data were analyzed by independent sample's t-test. Dot blot graph was represented through GraphPad Prism software.

3. Results

3.1 Data collection and demographic characteristics

In this study, we enrolled 395 symptomatic patients, positive for SARS-CoV-2 by RT-PCR with CT value ≤ 36. Among these, 322 (81.5%) were recovered after the treatment. However, 73 (18.5%) were loss their life, and counted as a mortality rate (death) of our study. Male were higher than female among infected and hospitalized cases (275 [69.6%] vs.120 [30.4%], p=0.001).The mean age and distribution of gender of studied cases were similar among death vs. recovered patients (Table 1). We have also categorized the cases based on different age group and we found that death cases did not only belong to older age (61+ years) 22/96 (22.9%) but also the younger cases frequently died with age group 18-30 years and 31-40 years old as 16/70 (22.9%) and 15/76 (19.7%), respectively. If, we cover the cut of age 50 for high risk of mortality and we found no difference between more or less age among death vs. recovered (>50 years age: 27/155 (17.42% vs. 46/240 (19.17%), p=non-significant). All the cases were also divided among mild-moderate 304 (77.0%) and severe cases 91 (23.0%) and we have found that death cases had more severe illness than recovered cases (Table 1).

Table 1: Correlation of different demographic parameters with severity of COVID-19 cases

	Distribution of total patients (N=395)		P-value*
	Death(n =73)	**Recovered (n = 322)**	**Death vs. Recovered cases (p*-value)**
Gender distribution			
Male	49 (17.8%)	226 (82.2%)	
Female	24 (20.0%)	96 (80.0%)	0.196
Severity of cases			
Mild-moderate	17 (23.3%)	287 (89.13%)	
Severe	56 (76.7%)	35 (10.9%)	**< 0.001**
Age in years (Mean±S.D.**)	47.5±15.86	47.3±17.07	0.222
Age distribution			
18-30	16 (22.9%)	54 (77.0%)	
31-40	15 (19.7%)	61 (80.3%)	
41-50	15 (16.0%)	79 (84.0%)	0.162
51-60	5(8.5%)	54 (91.5%)	
>60	22 (22.9%)	74 (77.0%)	

Abbreviation**:** *p value: Fisher exact test; **S.D.: Standard deviation

3.2 Biochemical profile

The different biochemical parameters across death and recovered cases were represented as mean and standard deviation among normally distributed data. CRP was significantly higher among death than recovered cases, however, major liver function tests like SGOT, SGPT, and ALP were comparable among them (Table 2). D-dimer, an indicator of unknown cause of symptoms was significantly higher and serum ferritin was also elevated with trend significance value among death than recovered (Table 2). LDH was also elevated among death cases (467.9±265.69 vs. 414.9±193.96, p<0.001). The rest of the other biochemical tests like serum urea and creatinine were similar among death and recovered (Table 2).

Table 2: Correlation of different biochemical parameters and RT-PCR CT values among COVID-19 cases (both recovered and death)

			Total No. of cases (N = 395)	Death (n = 73)	Recovered (n=322)	*p value (Death Vs. Recovered)
	Unit	Normal value	Mean±S.D	Mean±S.D	Mean±S.D	
AST (SGOT)	IU/L	0-40	64.7±27.4	64.19±54.57	65.15±27.51	0.788
ALT (SGPT)	IU/L	0-45	63.3±49.8	54.57±43.53	63.2±48.94	0.166
ALP	IU/L	0-270	162.6±112.9	150.93±119.35	164.97±116.6	0.356
CRP	mg/L	> 10	48.0±30.5	55.43±23.71	46.73±30.79	**0.024**
D-dimer	mcg/ml	< 0.4	3.7±5.2	5.98±10.41	3.35±3.41	**< 0.001**
Serum Ferritin	ng/ml	20-250: M 10-120: F	755.5±604.2	876.36±569.78	733.13±583.69	**0.058**
LDH	U/L	290-2000	424.78±209.7	467.91±265.69	414.9±193.96	**< 0.001**
Serum Urea	mg/dl	8-24:M 6-21:F	43.2±17.1	41.64±16.33	43.03±17.33	0.532
Serum Creatinine	mg/dL	0.5-1.5	2.36±1.39	2.36±1.39	2.13±1.147	0.149

Abbreviations: p value: Independent sample two tailed t- test, AST: Aspartate transaminase, ALT: alanine transaminase, ALP: alkaline phosphates, CRP: C-reactive protein, LDH: lactate hydrogenase,

3.3 Association between CT values of RT-PCR and mortality (death cases)

When focusing on the issue of viral load, SARS-CoV-2 RT-PCR recommended genes especially RdRp, and envelope (E) gene was assessed in our study and found mean viral load was 28.48±5.90 and 28.06±5.63, respectively. We found that death cases showed significantly lower values (or early peak) of CT than recovered patients (RdRp: 21.45±5.90 vs. 30.07±5.13 and E-gene: 20.87±3.82 vs. 29.69±4.61, p<0.001) in our North Indian population (Figure 1).

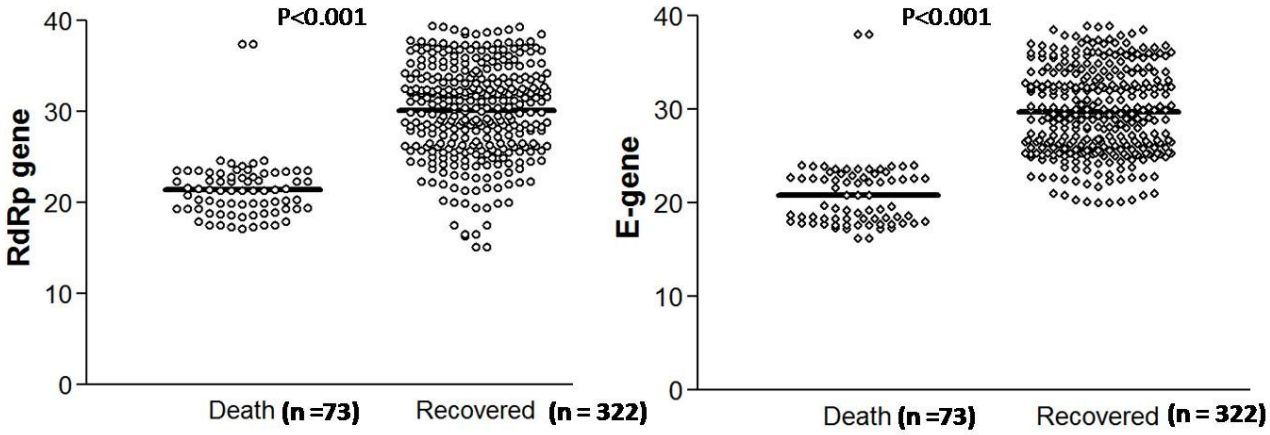

Figure 1: Comparison of CT values of RT-PCR SARS-CoV-2 assay among death versus recovered COVID-19 cases; Horizontal straight lines indicating the mean value of data, (A) RdRp (RNA dependent RNA polymerase) gene, (B) E-gene (envelope gene).

3.4 Association of biochemical and CT values with mortality (death) based on patients severity

The univariate analysis was done among the death and recovered cases based on mild-moderate and severe groups. All biochemical markers were comparable except ALP (233.26±198.73 vs. 170.23±120.66, p = 0.046) among death versus recovered especially in mild-moderate cases (Table 3). If we stratified the severe cases, we found that CRP levels were higher among death than recovered (57.63±24.82 vs. 34.68±34.66, p<0.001) but similar among the mild-moderate group. D-dimer was showed trend elevated level among death than recovered; however serum ferritin was comparable among them (Table 3).We have also found that LDH was higher while serum urea was lower among death than recovered cases (Table 3).

We have stratified the data, among mild-moderate and severe patients for analysis of RT-PCR CT values of RdRp, and envelope (E) gene of SARS-CoV-2 (Figure 2). We found that CT values was lower among death than recovered cases in both the mild-moderate (RdRp: 23.59±5.47 vs. 30.75±4.69 and E-gene: 21.89±6.52 vs. 30.14±4.49, p<0.001) and severe conditions (RdRp: 20.84±2.21 vs. 24.44±5.17 and E-gene: 20.55±2.51 vs. 26.08±4.01, p < 0.001) in our study. Our data also showed lower CT values among severe cases than mild-moderate cases.

Table 3: Biochemical parameters and RT-PCR CT values on the basis of different level of severity among COVID-19 cases

Bio-chemical parameters	Mild-Moderate cases (n = 304)			Severe Cases (n = 91)		
	Death (n = 17)	Recovered (n = 287)	p –value	Death (n = 56)	Recovered (n = 35)	p –value
AST (SGOT)	62.91±28.95	64.33±27.7	0.837	64.59±27.20	71.87±25.19	0.204
ALT (SGPT)	51.12±35.06	64.67±51.11	0.282	55.63±46.03	51.17±21.73	0.593
ALP	233.26±198.73	170.23±120.66	**0.046**	125.94±66.3	121.84±61.29	0.769
CRP	48.21±18.4	48.2±30.02	0.998	57.63±24.82	34.68±34.66	**< 0.001**
D-dimer	4.58±2.62	3.42±3.52	0.182	6.40±11.89	2.79±2.25	**0.078**
Serum Ferritin	672.84±399.81	702.82±558.87	0.828	938.14±601.50	981.74±719.64	0.756
LDH	657.13±286.26	435.99±204.32	0.133	501.54±252.28	377.20±172.42	**0.012**
Serum Urea	46.31±11.42	42.36±17.09	0.349	40.22±17.39	48.52±18.55	**0.034**
Serum Creatinine	2.54±0.62	2.17±1.18	0.202	2.3±1.56	1.82±0.76	0.092

Abbreviations: *p value: Independent sample two tailed t- test

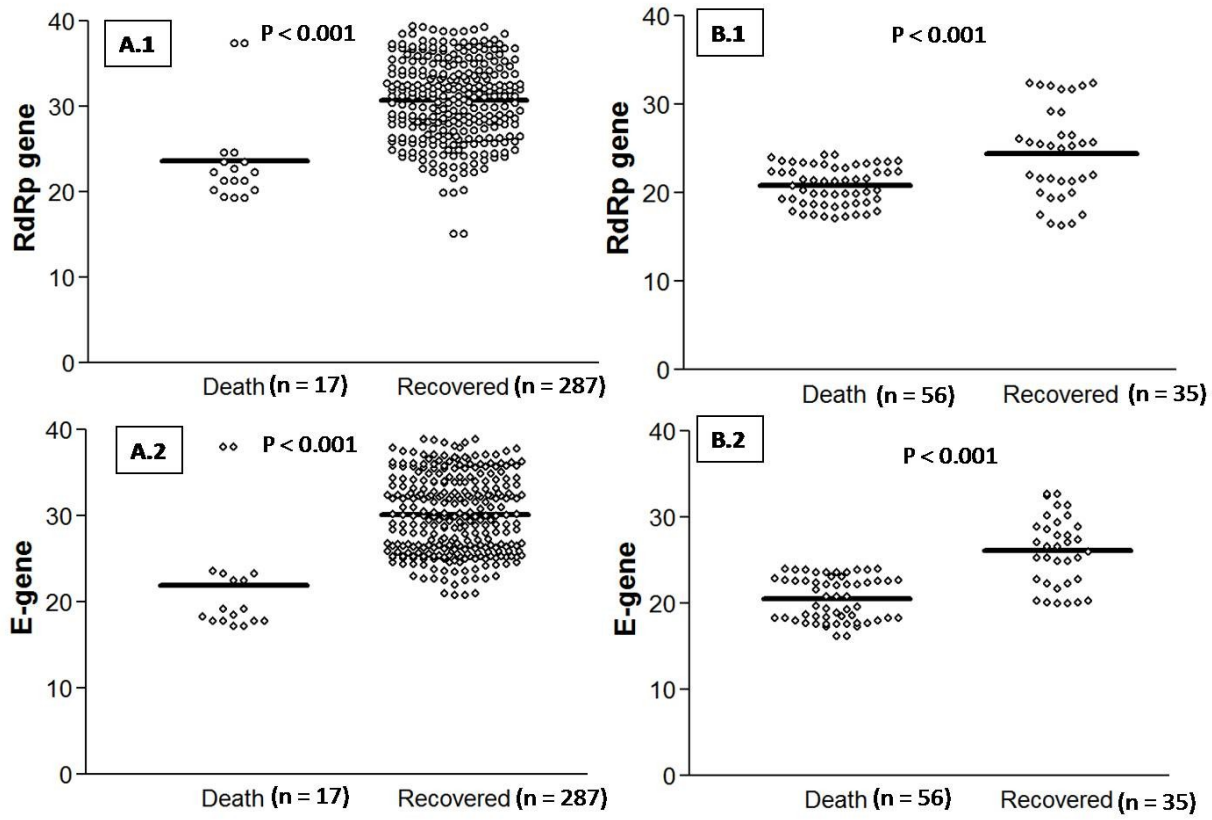

Figure 2: Comparison of CT values of RT-PCR SARS-CoV-2 based on severity of COVID-19 illness among death versus recovered cases; Horizontal straight lines indicating the mean value of data, (A.1) and (A.2) are indicating the difference in mild-moderate, while (B.1) and (B.2) indicating the difference in severe cases

4. Discussion

The present prospectively collected data revealed that, (a) The mean age and distribution of gender were comparable among death and recovered cases, (b) Death cases did not only belong to older age (>50 yrs) but also the younger cases frequently died, (c) CRP, D-dimer, serum ferritin and LDH were elevated among death than recovered cases, (d) however; CRP, D-dimer and LDH was only higher among death cases with severe symptoms, and (e) we have also found that death cases showed significantly lower values (or early peak) of CT than recovered patients not only alone but also in severe cases in our North Indian population.

Several studies are reported as SARS-CoV-2 is highly contagious for elderly individuals and shows critical illness [24,25]. However, in our study mortality rate was quite similar in all symptomatic hospitalized cases among all the age groups. A similar study also in agreement that aging populations are at an even higher risk than the younger age [26]. Our findings suggest that elders are not only associated with high predictive mortality situation, but also the younger cases needed more intensive care without the fact of their age.

The recognition of biomarkers for COVID-19 progression risk stratification, as well as their molecular characterization, is essential for optimizing treatment and identifying therapeutic options [27]. Among inflammatory biomarkers especially CRP is very important predictor for condition causing inflammation [28]. The level of CRP increases 20 to 50 mg/L on average among patients with COVID-19 [29,30]. In our study, CRP was significantly higher among death than recovered cases; however, other liver function tests like SGOT, SGPT, and ALP were comparable among them.

When plasmin cleaves fibrin to break down the clots, one of the fragments formed known as D-dimer. Assessment of plasma D-dimer is commonly used as part of a diagnostic algorithm to rule out thrombosis. Any pathologic or non-pathologic process that increases fibrin development or breakdown raises the level of D-dimer [31]. According to previous research, D-dimer levels are frequently higher in community-acquired pneumonia (CAP) and chronic obstructive pulmonary disease (COPD), and may be used as a prognostic biomarker [32]. D-dimer was elevated among study group of death than recovered cases as well as in stratified group of severe in our findings. The elevated level of D-dimer, has been linked to an increased

risk of in-hospital death in COVID-19 patients in many studies [33].

Lactate dehydrogenase (LDH) enzyme activity increases after tissue breakdown [34].Since LDH (isozyme 3) is present in lung tissue, patients with severe COVID-19 may expect to release more LDH into the bloodstream due to severe type of interstitial pneumonia that often progresses to acute respiratory distress syndrome. In our study, LDH was also elevated among death than recovered cases including severe conditions. In previous findings, Middle East Respiratory Syndrome (MERS) which was similar to SARS-CoV-2 infection also showed elevated LDH levels [35,36].

Lower RT-PCR CT values of SARS-CoV-2 infections are generally correlates with higher viral loads, although these CT values not are directly proportional to log viral load because of the linear dynamic range and presence of potential inhibitory factors [37]. We found that death cases showed significantly lower values (or early peak) of CT than recovered patients**.** A study conducted in China on 308 hospitalized patients also finds the similar results [38]. We also found that CT values was lower than recovered cases among both the mild-moderate and severe conditions. The results of this study indicate that lower (early peak) CT values are potentially associated with worse outcomes in COVID-19 especially in severe patients.

5. Conclusions

We concluded that, the continuous research must be required to understand the progression of the SARS-CoV-2 infection, its pathophysiology and for the progression of disease by using different biomarkers as well as close monitoring of CT value of RT-PCR. Therefore, the search for biochemical or other suggestive laboratory parameters are extremely necessary in this scenario to evaluate and establish early clinical diagnosis of SARS-CoV-2 infection. These parameters included D-dimer, LDH, CRP and serum ferritin, ALT, AST, ALP could be helpful to monitor the severity and progression of the disease as compared with RT-PCR, because it's costly, required lot of high end medical equipments as well as technically sophisticated test (gold standard) [39,40]. The presence of elevated level of biochemical biomarkers especially CRP, D-dimer, serum ferritin and LDH as well as lower SARS-CoV-2 CT values have been associated with the higher chance of progression to the severe disease and increased mortality rate. Thorough, special attention towards the patients with these above findings should be considered, and may be helpful to reduce the death in COVID-19 cases.

Ethics approval and consent to participate:

This study was already approved by institutional ethical committee (register IHEC/BRDMCGKP/06/09-2020) and written consent form was also obtained from all the cases/participants or patient attendants at the time of sample collection.

Data Availability

All data was original, non-duplicate included in the manuscript were produced/analyzed from this tertiary care COVID-19 treatment facility.

Conflicts of Interest

The results used for this study was only used for our area of research and country. All authors declare that there is no conflict of interest.

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Author's Contributions

All authors contributed to the study hypothesis and design. Data collection and laboratory investigations were performed by AKS, SK, SP, IPA and VG. The first draft of the manuscript was written by AKS, SK and SP and reviewed by all authors and commented on every versions of the manuscript. All authors read and approved the final manuscript before final submission.

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