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Association of RT-qPCR Ct Values and Disease Severity among COVID-19 Patients Visiting a Tertiary Care Hospital in Nepal

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Abstract

COVID-19 pandemic due to SARS-CoV-2 has been one of the major global health issues of this aeon. The aim of this study was to evaluate the association of SARS-CoV-2 cycle threshold (Ct) values with multiple factors among COVID-19 patients visiting a tertiary care hospital in Sudurpashchim province of Nepal. A retrospective analysis was performed on the data of randomly selected COVID-19 cases among the total RT-qPCR tested patients from March 2020 to April 2022. The Ct values at the time of patient admission and their clinical outcomes (discharge or death) were compared. Among the COVID-19 patients, survivor group had significantly higher initial Ct value compared to non-survivors [median Ct values 23.21 and 24.39 (P < 0.0001)]. Selected haematological parameters; white blood cells (P<001), neutrophils (P<001), and monocytes (P<0.0001), and all the biochemical parameters were significantly different between these two groups (p < 0.005). Furthermore, significantly increased CRP (61.54±63.00, P<0.0017), D-dimer levels (0.8979± 1.480, P<0.0001), creatinine (0.7931±0.2551, P<0.0001), monocytes (0.6782±0.7981, P<0.0001), and random blood sugar (152.4±34.32, P<0.0001) were observed among non-survivors indicating as cause of disease severity in COVID-19. The findings of this study imply that the Ct value, CRP and D-dimer levels could be a crucial marker for the early detection of severe COVID-19 patients or those at higher risk of developing severe disease. This will eventually help to identify cases requiring immediate and critical medical care and reduce mortality.

Keywords: SARS-CoV-2, COVID-19, RT-qPCR, Cycle Threshold-Ct, Disease Severity, Severity Marker, Nepal

INTRODUCTION

Human Coronavirus was first confirmed in 1960, 23 years after its description in birds. More than 80% infections caused by this virus are zoonotic in nature. Taxonomically, Coronavirus belongs to the family coronaviridae and subfamily beta coronavirus.[1] Coronavirus is an enveloped single-stranded RNA virus with a genome length of approximately 27-30 kb.[1] Different coronaviruses evolved with time and at least seven of these viruses have been linked to human infections including the most recent Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) which caused the COVID-19 pandemic.[2-4] SARS-CoV-2 infected more than 769 million people with 6,955,141 deaths globally as of 12 August 2023.[1,2]

SARS-CoV-2 infection in humans is believed to have originated from wild animals although the intermediate host is not known yet.[3] This speculation was based on: 1- it was first reported from a seafood market in Wuhan, China, and 2-coronaviruses spilled over from bat, pivot cat and other wild animals to humans showed similar nucleotide sequence as SARS-COV-2 suggesting the evidence of zoonotic transmission.[4]

Nepal, a landlocked country, borders with India and China. Due to an open border between India and Nepal, there have been massive cross-border movement of people for various social and economic activities. By the time China controlled the first wave of COVID-19 pandemic, it was rapidly growing in India.[2] Despite the countrywide lockdown to contain virus transmission in Nepal and India, the existing porous borders allowed an uncontrollable infusion of people across borders that led to a surge in cases and subsequent bigger clusters in Nepal. Therefore, the number of COVID-19 suspected/confirmed cases and people under quarantine outnumbered in the bordering areas of Nepal compared to other parts. Diagnostic facilities in Nepal had been upgraded and expanded but were not adequate to meet the increased demand indicating the underreporting of the actual community transmission during the early pandemic phase. Subsequently, the situation became worse with the evolution of newer variants of SARS-CoV-2 that caused different waves of pandemic similar to the global trends. Since the first report of COVID-19 on January 13, 2020,[4-6] a total of 1,003,424 cases have been confirmed by RT-PCR with 12,031 confirmed deaths as of September 13, 2023.[1-7] Simultaneously, SARS-CoV-2 RNA was detected by qPCR in 60% (50/84) in tested waste, river water and hospital centers in Nepal conducted by Tandukar *et al.*[8] In Sudurpaschchim province (eight districts), the first case of COVID-19 was gulf returnee confirmed on March 27, 2020, at Seti Provincial Hospital, Dhangadhi, a total of 6,70,765 COVID-19 cases

were detected by RT-qPCR and antigen of 637 reported deaths as 30/09/2023 according to Sudurpaschchim Province of Health Directorate, Nepal.[9]

Despite the rapid development of different platforms for COVID-19 diagnosis, real-time quantitative polymerase chain reaction (RT-qPCR) has been the gold standard method because of reasonably high sensitivity and specificity.[7,10] Apart from the SARS-CoV-2 diagnostic purpose, the potential utility of RT-qPCR Ct values in COVID-19 severity identification/ prediction has not been studied adequately in Nepal, although there are reports coming from other parts of the world.[7,10,11] This is very important during the pandemic specially in the health care resource limited countries like Nepal to enable rationale and timely use of intensive facilities for severe patients.[6-8] Viral dynamics and Ct values could predict the disease severity in COVID-19 when combined together with other factors such as symptoms, underlying health conditions (including comorbidities), routine laboratory parameters (haematological, immunological and biochemical profiles), chest x-ray, and chest CT scan, etc. Ct values can be influenced by several factors such as specimen collection technique, specimen type, sampling time, viral kinetics, transport and storage conditions, nucleic acid extraction, viral load, primer designing and real-time PCR efficiency and platforms used.[7,11] Nevertheless, Ct value could still be a simple yet useful marker for disease severity in COVID-19. Therefore, the main objective of this study was to evaluate the association of Ct values with multiple factors among COVID-19 patients visiting a tertiary care hospital in Sudurpashchim province of Nepal and explore its potential utility in severity prediction.

METHODS

Study site and its justification

The site, Seti Provinical Hospital (SPH) in Kailali districts was selected because it is the major tertiary-care public hospital in Sudurpashchim province of Nepal. Moreover, this hospital was one of the first to establish COVID-19 PCR laboratory in the Sudurpashchim province. The SPH catchment population area includes one of the major entry and exit portals of Nepalese people visiting India for different purposes including job, treatment and tourism.

Study design

This was a retrospective study conducted on randomly selected COVID-19 patients who were admitted and suspected COVID-19 patients at SPH. Purposive sampling was performed to select cases confirmed with RT-qPCR Ct values and multiple parameters related to COVID-19 disease severity. From March 2020 to April 2022, a total of 127,196 suspected COVID-19 patients were tested by RT-qPCR at SPH and 23,966 of them were confirmed as COVID-19 cases (Ct < 40). Of these confirmed COVID-19 cases, 313 positive cases with a history of discharge and 131 death cases were randomly selected from the SPH database. The main criteria for this selection were based on the availability of complete laboratory parameters obtained within one-week of onset of infection to allow the inclusion of eligible cases.

Specimen collection and RNA extraction

Nasopharyngeal (NP) and oropharyngeal (OP) swab samples were collected from admitted and suspected patients at SPH. The collected swabs were placed in viral transport medium and immediately transported to the COVID-19 laboratory for further analysis maintaining cold chain as per the WHO guidelines.[12] Manual and automated, viral RNA extractions were performed following the manufacturer's instructions (General Biologicals, Hsinchu, Taiwan[13] and Nanjing Zhongkebio Medical Technology, Nanjing, China).[14]

SARS-CoV-2 detection by RT-qPCR

Viral detection was performed by using the RT-qPCR kit (Uni-Medica, Shenzhen, China)[15] according to the manufacturer's instructions. Briefly, RT-qPCR were performed in a 40 µl reaction mixture containing 18.5 µl reaction buffer, 1.5 µl enzyme mix and 20 µl of RNA template. The reaction was executed in a thermo-cycler (D-Lab, Beijing, China) with the following conditions: reverse transcription at 55°C for 10 min, Taq activation with pre-denaturation at 95°C for 3 min, denaturation at 95°C for 10 s, followed by 45 cycles of amplification with a final annealing, extension

and fluorescence acquisition at 60°C for 1 min. For interpretation analysis, samples with a Ct value < 40 of all Orf1ab/N/E genes were considered as positive and Ct value > 40 of Orf1ab/N/E genes as negative for SARS-CoV-2.[15]

Data collection

Information on RT-qPCR-positive patients' demographic details including age, gender, Ct value, hospitalization status, health status of patients, laboratory test results (haematological, immunological and biochemical markers), and chest x-ray of COVID-19 patients and clinical outcomes were retrieved from the electronic medical records and database of respective hospital departments. According to Shah *et al*,[16] Ct values were categorically grouped into high (Ct 31–40), moderate (21–30), and low (11–20). Among COVID-19 patients (survivors and non-survivors), Ct values were compared based on clinical outcomes (severe, moderate, and mild disease). The following criteria used to classify as mild, moderate and severe cases if COVID-19 patients had: (i) mild clinical symptoms without evidence of breathlessness or hypoxia (normal saturation), dyspnea or abnormal chest imaging, ii) fever, cough and evidence of lower respiratory disease during clinical assessment or imaging, including oxygen saturation (SpO_2) <94% (range 90–94%) on room air, respiratory rate ≥24 per

minute and iii) clinical signs of pneumonia plus one of the following; respiratory rate >30 breaths/min, severe respiratory distress with oxygen saturation (SpO_2) < 90% at room air, requirement for mechanical ventilation, and/or admission to intensive care unit (ICU).[17,18]

Data analysis

RT-qPCR Ct values and all available clinical and laboratory parameters were retrieved/transcribed into the Excel spread sheet followed by data cleaning and verification. Ct values of confirmed COVID-19 cases were further analysed to ascertain the correlation of Ct with clinical manifestations, haematological and biochemical markers, chest x-ray findings and COVID-19 severity levels. GraphPad Prism 9.4 software was used for data analysis. For continuous variables, Student's t-test and Mann-Whitney U test were used for parametric and non-parametric data, respectively. Chi-square was used for categorical variables. A P-value < 0.05 was considered statistically significant.

Ethics

Ethical approval was obtained from the Institutional Review Committee - Institute of Science and Technology (IRC-IoST), Tribhuvan University, Kathmandu, Nepal (IRCIOST-23-0090). We obtained completely de-identified COVID-19
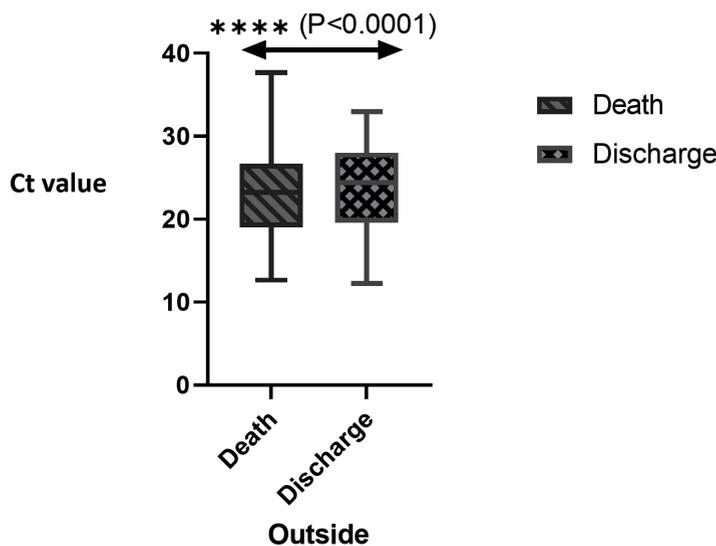

Figure 1. Cycle threshold (Ct) values and clinical outcomes of COVID-19 patients, Nepal

data of patients. As this was a retrospective analysis conducted in the de-identified data, informed consent was not required.

RESULTS

Ct values and clinical outcomes

Among 127,196 RT-qPCR-positive cases, 444 cases (131 deaths and 313 survivors) were randomly selected for the analysis. More than half (66.56%, 300/444) were male patients. The median age was 45 years [IQR (63-29)] and the median Ct value was 24.01 (IQR, 19.35-27.750). Comparison of the initial Ct value with clinical outcomes (discharge or death) showed that the survivors had a significantly higher Ct than non-survivors [median Ct (IQR), 23.39 (19.55-28) and 23.21 (19.08-26.68)] (p < 0.0001; Figure 1). The frequency and average Ct values were also analysed according to disease severity; mild (33.55%, median (IQR): 23.16 (18.15-27.70); moderate (25.90%, 24.88 (19.8-28.23) and Severe (40.76%, 24.01 (20.38-27.65) by among the 444 patients included (p> 0.05). Of the death

cases, majority had severe pneumonia followed by cardio-respiratory failure, acute respiratory distress syndrome and hypertension (data not shown) which could be the cause of death in COVID-19 patients.

Table 1 provides detailed information on patient demographics and descriptive statistics. Among death group (n =131), majority of patients had severe conditions (103/131, 78.62%) followed by moderate conditions (26/131, 19.84%) and mild conditions (2/131, 1.52%) based on Ct value and clinical outcomes as per the classification criteria.[13-15] In contract, among survivors, most patients had mild (147/313, 46.96%), moderate (89/313, 28.43%) followed by severe conditions (77/313, 24.60%). Male patients had higher frequency of severity (124-68.89%) than females (56-31.11%). Moreover, among the elderly patients above 60 years, majority had severe conditions (68-37.78%) and moderate condition (31-29.96%) compared to other age groups. Similarly, age group 18-25 predominantly had mild condition (51-34.23%) (Table 1).

Table 1. Demographic characteristics and severity levels of COVID-19 patients, Nepal

Variable	Category	Mild (n =149)	Moderate (n = 115)	Severe (n = 180)	Chi-square	P value
Clinical outcome	Survival	147 (46.96%)	89 (28.43)	77 (24.60%)	χ^2 =125.93	P < 0.00001*
	Death	2 (1.52%)	26 (19.84%)	103 (78.62%)		
Gender	Male	104 (69.80%)	76 (66.09%)	124 (68.89%)	χ^2 =0.43	P = 0.802
	Female	45 (30.30%)	39 (33.91%)	56 (31.11%)		
Age group	18-25	51 (34.23%)	22 (19.13%)	18 (10%)	χ^2 =55.43 24.66±13.63	P < 0.00001*
	26-33	28 (18.79%)	16 (13.91%)	20 (11.11%)		
	34-42	22 (14.77%)	13 (11.30%)	16 (8.89%)		
	43-50	17 (11.41%)	16 (13.91%)	24 (13.33%)		
	51-60	7 (6.09%)	17 (9.44%)	34 (18.89%)		
	>61	24 (16.11%)	31 (29.96%)	68 (37.78%)		

* P < 0.05 was considered statistically significant.

Biochemical parameters of COVID-19 cases

The biochemical parameters i.e. CRP (P<0.0017), D-dimer (0.0017), creatinine (P<0.0001) and potassium (P<0.0001) showed a statistically significant difference (P<0.05) in COVID-19 cases between survival and death groups (Table 2). Interestingly, CRP (< 5 mg/L, 35.15-61.54, dimer (< 0.5mg /dl, 0.895-1.475), alanine aminotransferase (5-42 IU/L, 67.82-72.94), and aspartate aminotransferase (5-37 IU/L, 40.05-48.13) were found to be significantly higher than the normal reference range in comparison of survival with death group.

Haematological parameters of COVID-19 cases

Non-survivors (death group) had significantly lower count of WBC (p<0.0001), neutrophil (p<0.0001), and significantly higher levels of monocytes (p<0.0001), haemoglobin (p<0.0001), platelets (p=0.0022), and random blood sugar(P<0.0001) (p < 0.005), while there was no significant difference in lymphocytes (p=0.2489), and platelets counts (p=0.0022) with (p > 0.0.05) (Table 3). Remarkably, random blood sugar level was found significantly higher than the normal reference range (80-120 mg/dl) in both death (152.4±34.32) and survivor groups (128±39.22) with statistically significant difference (p < 0.0001).

DISCUSSION

In the current study, high SARS-CoV-2 viral load (Ct:11-20) was observed more frequently

Table 2. Biochemical parameters and clinical outcomes COVID-19 patients, Nepal (n=444)

Parameters	Reference range	Survived (n=313)	Death (n=131)	P-value[b]
CRP (mg/L)	<5	35.15±31.53	61.54±63.00	< 0.0017
D-dimer (mg/L)	<0.5	1.475±2.018	0.8979±1.480	< 0.0001
HbA1c (%)	3.8-5.8	10.86±10.14	8.376±3.813	0.7248
Urea (mg/dL)	15-45	27.96±14.78	25.46±11.01	0.0541
Creatinine (mg/dL)	0.6-1.5	0.6986±0.4656	0.7931±0.2551	<0.0001
Albumin (mg/dl)	3.2-5.5	3.077±0.4180	3.199±0.7377	0.0104
Sodium (Na$^+$) (mmo/l)	135-150	138±6.468	137.5±3.427	0.4333
Potassium (K$^+$) (mmo/l)	3.5-5.3	4.667±4.415	3.884±0.5583	< 0.0001
ALT (U/L)	5-42	67.82±23.11	72.94±47.19	0.4937
AST (U/L)	5-37	48.13±29.99	40.05±21.44	0.0389
ALP (U/L)	45-132	84.30±18.72	86.28±12.86	0.1631

[a]Mean ±standard deviation; [b]Mann-Whitney U test; P < 0.05 was considered statistically significant.
CRP: C-Reactive Protein, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, ALP: Alkaline Phosphatase

Table 3. Haematological parameters and clinical outcomes of COVID-19 patients, Nepal (n=444)

Parameters	Reference range	Survival[a] (n=313)	Death[a] (n=131)	P value[b]
WBC (10×3/ul)	4.5-11	10.47±3.711	8.604±3.492	<0.0001**
NEU (10×3/ul)	1.8-7.7	8.85±1.940	7.0±2.167	<0.0001**
LYM (10×3/ul)	1.50-3.0	1.221±0.7660	1.267±0.8525	0.2489
MON (10×3/ul)	0.3-0.5	0.4981±0.1600	0.6782±0.7981	<0.0001**
Hb (g/dl)	11.5-15.5	12.59±1.607	13.21±1.553	<0.0001**
PLT (10×3/ul)	150-400	179.2±67.69	197.9±55.27	0.0022
RBS (mg/dl)	80-120	128.4±39.22	152.4±34.32	<0.0001**

[a]Mean ±standard deviation; [b]Mann-Whitney U test; P < 0.05 was considered statistically significant.
WBC: White Blood Cell, NEU: Neutrophils, LYM: Lymphocytes, MON: Monocytes, HG: Haemoglobin, PLT: Platelet, RBS: Random Blood Sugar

in admitted patients with the consequence of death, which is comparable to the previous reports.[15,16,19,20] The reason behind the high viral load could be due to disease severity associated with other endemic, pandemic as well as seasonal respiratory viruses. However, others respiratory viruses, laboratory diagnosis was not performed among the randomly selected COVID-19 patients to draw a conclusion.[21-25] Pre-pandemic avian influenza and seasonal influenza cases with high viral loads were associated with more severe form of disease and poorer treatment outcomes similar to SARS-CoV-2 infection. The correlation of viral load with clinical outcome and disease severity has been investigated with different endemic respiratory viruses, but most of the results remained inconclusive.[21-25] The association of viral load with clinical consequence and severity of COVID-19 were assessed by employing haematological, biochemical and immunological markers (Table 2 and Table 3).[22] Additionally, numerous common laboratory parameters that likely cause severity in COVID-19 infection may play an crucial role during the disease prognosis and forecast for treatment decisions.[23-26] Most of the previous studies revealed to be association of disease severity with common parameters include C-reactive protein (CRP), D-dimer, creatinine, potassium, alanine aminotransferase (ALT), alkaline phosphatase (ALP), white blood cell, neutrophil, monocytes and lymphocytes.[23-26] Assessment of initial Ct value with clinical consequences exhibited that the survivors had significantly higher levels than non-survivors ($P<0.0001$; Figure 1). Regarding the Ct values of COVID-19 cases with different clinical outcomes (mild, moderate and severe), current study did not show any significant difference (Figure 2). Ct values could be indirectly associated with the majority of final clinical decisions when combined with routine laboratory profiles, radiological findings and physician's clinical observation (i.e. clinical signs/scores and disease progression). Therefore, Ct value could be a useful tool for physicians in the management of COVID-19 patients and to make clinical decisions.[27]

We found, more than half of the patients had severe pneumonia (B/L pneumonia in the lower zone) followed by cardiorespiratory failure, (ARDS) and hypertension among the death group. Similarly, chest x-ray finding in COVID-19 patients was able to detect severe pneumonia as in the previous study where the presence of severe symptoms significantly associated with abnormal chest x-ray findings. Previous findings by Yang *et al.*[28] and Rousan *et al.*,[29] suggesting that the combination of clinical symptoms and radiological findings with Ct value could be helpful as a tool in the diagnosis, follow up of COVID-19 pneumonia and drawing clinical conclusions for prompt treatment of COVID-19 cases in resource-limited countries like Nepal.

Similarly, haematological parameters (WBC, neutrophils, and monocytes) were significantly different between survivor and non-survivor COVID-19 cases which is comparable to previous studies.[25,30,31] On the other hand, random blood sugar level was found significantly higher than normal reference range in COVID-19 patients (Table 2). Other studies found that WBC, monocytes, and random blood sugar increased during the viral infection while the neutrophil and lymphocytes counts decreased.[27,29] In contrast,
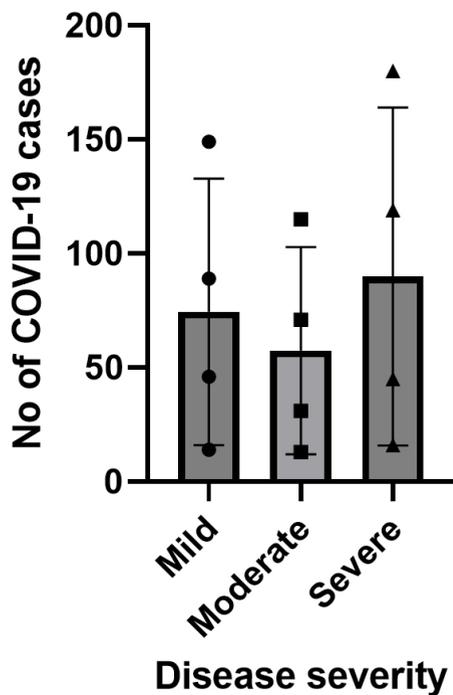

Figure 2. Cycle threshold (Ct) values and levels of severity in COVID-19 patients, Nepal

a previous study on COVID-19 patients showed an elevated AST, and low leukocyte, neutrophil, lymphocyte, eosinophil, and monocyte counts.[32] According to Cheng-Fu *et al.*,[32] decreased CBC was found in immuno-compromised conditions or other inflammatory responses during COVID-19 disease progression. Of note, Wang *et al.*[33] showed that these values were within the normal range in initial stage of COVID-19 disease and subsequently elevated during disease progression with a decreased lymphocyte count.

Our findings on biochemical parameters in COVID-19 cases demonstrated an increase in CRP, creatinine, ALT, and ALP (Table 3) and there was a significant increase in death group compared to survivor group. An inflammatory disorder that changes fibrin levels is linked with elevated D-dimer levels.[34] This is similar to a report by Tang *et al.*[35] which showed a considerable increase in D-dimers, CRP and liver enzymes in non-survivors. Mardani *et al.*[36] detected increased levels of biochemical parameters i.e. ALT, AST, CRP, bilirubin and albumin so as to forecast COVID-19 progression. Furthermore, study findings also suggest that Ct value, clinical outcome, haematological and biochemical parameters may be useful to predict the progression of disease in the early phase and thereby help forecasting the clinical management of COVID-19 patients. Our finding is in accordance with a previous report by Yang *et al.*[28] which showed a significant increase in the levels of D-dimer, CRP, and creatinine in severe COVID-19 patients compared to non-severe. These findings would be of potential utility to guide treatment, diagnosis and evaluate prognosis. Moreover, CRP and D-dimer can also be considered as an early marker for inflammatory reactions elevated in COVID-19 disease.[35-39]

In summary, SARS-CoV-2 viral dynamics demonstrated by RT-qPCR Ct values correlated to disease severity in COVID-19 patients at a tertiary care hospital, and this association was influenced by multiple factors including haematological, biochemical, chest x-ray and clinical findings. Our study findings suggest that Ct values could be used as an important tool for the identification of potential severe patients at the early stage COVID-19 thereby supporting in initial diagnosis, and clinical management when combined with haematological, biochemical, radiological and clinical findings.

Thus, viral and human genetics, genomics, and immunology research studies will be necessary to further understand those affecting factors. Further longitudinal studies with larger sample size will also be needed to understand the actual mechanism of clinical illness, COVID-19 severity and their consequences by employing RT-qPCR Ct values.

CONCLUSION

The results of this study imply that the Ct value could be a crucial tool as an early detection marker of severe COVID-19 disease or in identifying those at higher risk of disease severity. The Ct value also helps to identify cases requiring immediate and critical medical care and can be utilized for initial diagnosis, and prediction of severity and treatment options in COVID-19 when combined with clinical symptoms, haematological, biochemical, and chest x-ray findings. Nevertheless, these findings might be useful for policymakers or planners in improving the strategies to diagnose and treat on time to prevent further transmission of SARS-CoV-2 to healthy populations, as well as in preparedness plans.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

JJ, OPJ, SBK, GRA, YS, RS, SK, PRP, SR, SRS, DKP and HRJ conceptualized and designed the

study. JJ, YS, HRJ, RPO, CRJ, KP, SPD, RSD, LRB, GRA, KSK and BDP performed methodology. JJ, SPD, YS, KP, RS, GRA and BDP supervised the study. JJ, OPJ, YS, RPO, SK, SR, PRP, KSK and BDP wrote original draft. JJ, OPJ, SBK, GRA, CRJ, KP, SPD, YS, HRJ, RS, LRB, RPO, RSD, GRA, SBS, SK, SR, PRP, SRS, DKP, KSK and BDP wrote and edited the manuscript. All authors have read and approved the final manuscript for publication.

FUNDING

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This study was apporved by Institutional Review Committee – Institute of Science and Technology (IRC-IoST), Tribhuvan University, Kathmandu, Nepal (Ref IRCIOST-23-0090).

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