

Coronavirus Pandemic

Risk factors for prolonged viral RNA shedding in patients with COVID-19; a nested case-control study

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Abstract

Introduction: The coronavirus disease 2019 (COVID-19) pandemic continues to have a global impact. The behavior and viral course of severe acute respiratory syndrome coronavirus (SARS-CoV-2) remains unpredictable. We aimed to investigate the prediction factors associated with prolonged viral shedding in COVID-19 patients.

Methodology: This is a retrospective, nested, case-control study with 155 confirmed COVID-19 infected patients divided into two groups based on nucleic acid conversion time (NCT), a prolonged group (viral RNA shedding >14 days, n = 31) and a non-prolonged group (n = 124).

Results: The mean age of participants was 57.16 years, and 54.8% were male. Inpatient numbers were 67.7% across both groups. No statistically significant differences between the two groups were observed in terms of clinical manifestation, comorbidities, computer tomography, severity index, antiviral treatment, and vaccination. However, C-reactive protein and D-dimer levels were significantly higher in the prolonged group ($p = 0.01$; $p = 0.01$). Using conditional logistic regression analysis, D-dimer and bacterial co-infection were found to be independent factors associated with the prolonged NCT (OR: 1.001, 95% CI: 1.000-1.001, $p = 0.043$; OR: 12.479, 95% CI: 2.701-57.654, $p = 0.001$ respectively). We evaluated the diagnostic value of the conditional logistic regression model by using receiver operating characteristic curve analysis. The area under the curve was 0.7 (95% CI: 0.574-0.802; $p < 0.001$).

Conclusions: Our study design included controlling confounders. We showed a clear result associating predicting factors with prolonged NCT of SARS-CoV-2. D-dimer level and bacterial co-infection were considered as independent predictors of prolonged NCT.

Key words: COVID-19; prolonged; NCT.

J Infect Dev Ctries 2023; 17(5):610-616. doi:10.3855/jidc.17508

(Received 08 October 2022 – Accepted 10 January 2023)

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Introduction

Coronavirus disease 2019 (COVID-19) first appeared in China in 2019 and continues to affect the global population to this day [1]. The real time polymerase chain reaction (RT-PCR) test remains the most commonly used method of diagnosis of COVID-19 [2]. However, the viral course of severe acute respiratory syndrome coronavirus (SARS-CoV-2) continues to be unpredictable. Some recent studies have showed an association between severe outcomes and prolonged viral shedding for influenza and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) [3]. Additional studies have also showed an association between patients with COVID-19 infection who developed nosocomial pneumonia, and increased SARS-CoV-2 shedding days [4,5]. Due to this, identifying the risk factors impacting viral clearance duration is of significant importance.

Our study aimed to predict factors associated with prolonged nucleic acid conversion time (NCT) in

COVID-19 infection. We compared selected variables of patients with negative virus detection to those with persistent positive virus detection to evaluate the impact of different factors on the duration of SARS-CoV-2 viral shedding.

Methodology

Study design, clinical management, and data collection

This is a single-center, retrospective nested case-control study conducted in Izmir/Turkey. The study included 155 patients with confirmed COVID-19 infection between January 2020 and March 2022. Only patients who tested positive for SARS-CoV-2 RT-PCR (inpatient or outpatient) and underwent a second test performed at least 14 days after the first diagnostic RT-PCR were included in the study. Any patients < 18 years old were excluded. The participants were divided into two groups for evaluation based on the duration of SARS-CoV-2 RT-PCR positivity. In terms of viral RNA shedding, > 14 days was considered prolonged,

and 0-14 days was non-prolonged. In order to limit the effect of different SARS-CoV-2 variants on the duration of COVID-19 PCR positivity, a nested-case control study design was implemented. This meant that the controls were individually selected at the time when each case occurred. For each of the 31 patients found to have a prolonged PCR positivity, we randomly selected four matched controls who were active at the time of case diagnosis. Matching criteria included age, gender, and medical care (either inpatient or outpatient), for a total of 124 control patients.

Using G-Power 3.1.9.7 software, the calculation resulted in a sample size of 145, considering a power of 95% and a type 1 error rate of 5%. The effect size observed was 0.3.

Demographic and laboratory data, comorbidities, clinical manifestation, antiviral treatments received, and computer tomography (CT) results of participants were extracted from the electronic health records of the hospital.

The upper limits of all biochemical test parameters were based on the reference limits determined by the Biochemistry Laboratory. CT results were classified according to the 'CT severity index'; mild score 1 (< 5%), moderate score 2 (5–25%) and score 3 (26–50%), severe score 4 (51–75%) and score 5 (> 75%) [6].

Other notable cross-referenced outcomes included; vaccination status, bacterial co-infection, and oxygen demand of participant. Bacterial co-infection was defined as the patient having a clinically or microbiologically proven secondary bacterial infection. We also defined those who had received a full dose vaccination at least four weeks prior to time of infection, as being vaccinated participants [7]. We considered asymptomatic patients as those who had applied for PCR at their own request, and were subsequently found to be positive.

All patient follow-ups were carried out in strict accordance with the recommendations of the Turkish Ministry of Health, COVID-19 Guidelines [7]. This study was approved by the institutional review board and the research and ethics committee at Bozyaka Education and Research Hospital (2022/75).

SARS-CoV-2 detection test

All patients in our study received a positive RT-PCR test result in specimens collected via nasopharyngeal swabs. The SphaeraMag® DNA/RNA Isolation Kit (Procomcure Biotech, Thalgau, Austria) was used for isolating nucleic acids from nasopharyngeal swabs with automated nucleic acid extraction systems. Following that, a PhoenixDx®

SARS-CoV-2 Detection Kit was used for RT-PCR (Procomcure Biotech/Thalgau, Austria). This kit detects the SARS-CoV-2 qualitatively by targeting open reading frame 1ab, and nucleocapsid protein genes. We used the Roche COBAS Light Cyclor® 480 system to determine SARS-CoV-2 (Roche Diagnostics, Rotkreuz, Switzerland). A positive test was defined by the typical S-shaped amplification curves of FAM and ROX fluorescence channels, as well as Ct values below 40.

Statistical analysis

According to the Kolmogorov-Smirnov test, continuous variables were reported as mean with standard deviation (SD) or median with interquartile range (IQR). The categorical variables were distributed using the number (n) and percentage (percent) distributions. The Mann-Whitney U test was used as a non-parametric test, and the Chi square test and Fisher's exact test for categorical data, to compare the prolonged (> 14 days) and non-prolonged (\leq 14 days) viral shedding groups. Statistical significance was defined as $p < 0.05$. Multivariate analyses were used to evaluate independent risk factors for extended viral shedding by including statistically significant variables in univariate studies. Since the groups were matched, conditional logistic regression models were used to calculate adjusted odds ratios (aOR) and 95% confidence intervals (CI) [8]. The model fit was evaluated using Hosmer-Lemeshow goodness of fit statistics. The logistic regression model's prediction was assessed using a receiver operating characteristic (ROC) curve analysis. 2017 IBM SPSS statistics (version 26.0) was used for statistical analysis.

Results

Patient characteristics

We determined that 31 patients met our inclusion criteria in the prolonged group, matching them with 4 controls per case for a total of 124 controls in the non-prolonged group. The demographic and clinical characteristics of COVID-19 patients at the time of their first SARS-CoV-2 positivity have been summarized in Table 1. The mean age was 57.16 years in the prolonged and non-prolonged group and 54.8% of the patients were male. We observed that 67.7% of the participants were inpatient, and 32.3% were outpatients in both groups.

On examination of the patients at admission, fatigue and cough presented as the most common symptoms, while 33 (21.2%) of the patients were completely asymptomatic. We also observed that approximately

70% of the patients had one or more chronic diseases; with hypertension and diabetes mellitus being the most prevalent.

Clinical outcome

In the inpatient group, 17 (34%) patients were supported by mechanical ventilation, 14 (28%) required noninvasive ventilation, and 19 (38%) required nasal cannula oxygen. In terms of receiving antiviral treatments, 38 (24.5%) of the participants were given favipiravir and 11 (7%) hydroxychloroquine or lopinavir/ritonavir. A steroid therapy was also administered in 48 cases (30.9%). Due to our study time frame incorporating the beginning stage of the pandemic, only 16 (10.3%) patients had received full vaccination status prior to infection. A thorough review of medical records also revealed 17 (10.9%) bacterial co-infections (9 bacterial pneumonia, 6 bloodstream, 1 urinary tract, 1 catheter-related bloodstream infection).

Risk factors for prolonged viral shedding

This study detected a median of 7 days (IQR: 7-8 days) of viral RNA shedding from disease onset in patients. No statistically significant differences were observed in terms of comorbidities, CT severity index, antiviral treatment, or vaccination status, between the prolonged and non-prolonged groups. However, the values in the prolonged group were higher than those in non-prolonged group. Participants in the prolonged viral RNA shedding group also had significantly higher CRP and D-dimer ($p = 0.01$; $p = 0.01$), as did the percentage of patients with bacterial co-infection and mechanical ventilation. ($p = 0.03$; $p < 0.001$ respectively) (Table 1). In the univariate logistic regression analysis, we found crude ORs for bacterial co-infection (OR: 7.959, 95% CI: 2.725-23.245), mechanical ventilation (OR: 3.325, 95% CI: 1.150-9.611), CRP (OR: 1.003, 95% CI: 0.998-1.008), and D-dimer (OR:1.001, 95% CI:1.000-1.001) (Table 1).

Table 1. Clinical characteristics, laboratory and radiographic findings of COVID-19 patients on admission.

	Viral shedding > 14 days (n = 31)	Viral shedding ≤ 14 days (n = 124)	p value
Comorbidity			
Hypertension	7 (22.6)	28 (22.6)	1.00
Diabetes mellitus	7 (22.6)	27 (21.8)	0.92
Cardiovascular disease	6 (19.4)	10 (8.1)	0.07
Chronic kidney disease	1 (3.2)	3 (2.4)	0.59
Cerebral infarction	1 (3.2)	1 (0.8)	0.36
Chronic lung disease	3 (9.7)	9 (7.3)	0.44
Cancer	2 (6.5)	4 (3.2)	0.35
Clinical manifestation			
Fever	5 (16.1)	24 (19.4)	0.68
Cough	8 (25.8)	31 (25)	0.93
Dyspnea	12 (38.7)	37 (29.8)	0.34
Fatigue	10 (32.3)	36 (29.0)	0.43
Headache	3 (9.7)	4 (3.2)	0.14
Myalgia	3 (9.7)	21 (16.9)	0.24
Bacterial co-infection	10 (32.3)	7 (5.6)	< 0.001
COVID-19 vaccination	6 (19.4)	10 (8.1)	0.07
Hospitalized treatment			
Mechanical ventilation	7 (22.6)	10 (8.1)	0.03
Favipiravir	5 (16.1)	33 (26.6)	0.26
Laboratory results			
WBC, 10 ⁹ /mL	7100 (4950-9400)	6650 (4900-8700)	0.39
Lymphocytes, 10 ⁹ /mL	1500 (930-2000)	1500 (1000-1852)	0.58
CRP, mg/L	26 (9.8-114.5)	9.9 (2-69)	0.01
D-dimer, mg/mL	490 (287.5-1875)	313 (177.8-585.5)	0.01
Ferritin, ng/mL	182 (69.8-430.3)	121 (45-308)	0.15
Urea, mg/dl	38 (24-64.8)	33 (24-44.8)	0.43
Creatinine, mg/dl	0.8 (0.5-1)	0.9 (0.7-1)	0.49
Chest CT			
Normal	13 (41.9)	63 (50.8)	
Mild	5 (16.1)	20 (16.1)	
Moderate	4 (12.9)	18 (14.5)	0.62
Severe	9(29.1)	23 (18.6)	

Data are presented median (IQR) and n (%). p values were calculated by Mann-Whitney U test, Chi-square test, or Fisher's exact test, as appropriate. WBC: white blood cell; CRP: C-reactive protein; CT: computer tomography.

According to the multivariate conditional logistic regression analysis, bacterial co-infection (adjusted OR (aOR): 12.479, 95% CI: 2.701-57.654, $p = 0.001$) and D-dimer (aOR: 1.001, 95% CI:1.000-1.001, $p = 0.043$) were found to be independent risk factors for prolonged NCT. The p value was 0.191 for Hosmer-Lemeshow goodness of fit test.

We evaluated the diagnostic value of our conditional logistic regression model by using ROC curve analysis. Area under the curve (AUC) of the conditional logistic regression model was 0.7 (95% CI: 0.574-0.802; $p < 0.001$), which was significantly higher than CRP (AUC = 0.659, 95% CI: 0.562-0.755; $p = 0.001$) and D-dimer (AUC = 0.672, 95% CI: 0.572-0.773; $p = 0.001$) (Figure 1).

Discussion

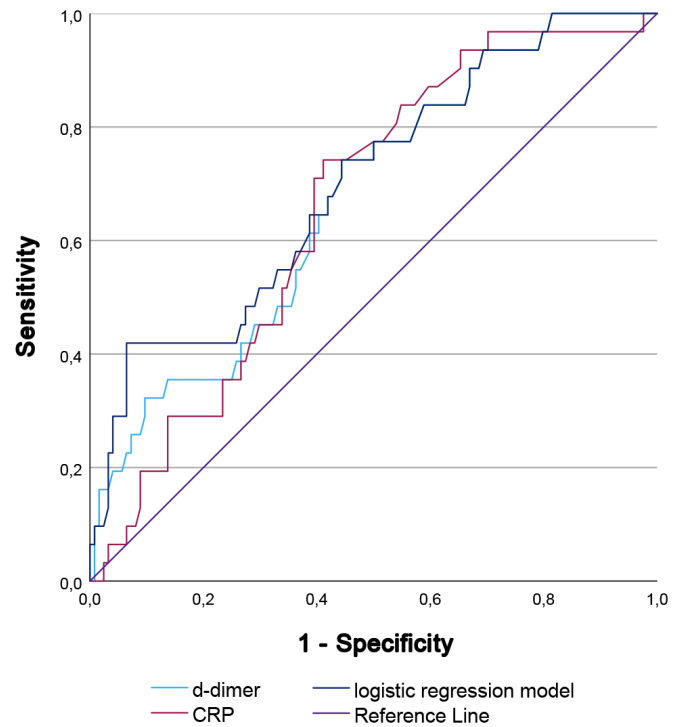
COVID-19 continues to have a huge impact on the world; however, information regarding the importance of prolonged NCT remains limited. Many observers remain unconvinced in terms of a relationship between prolonged viral shedding and clinical outcomes. However, several recent studies have outlined a relationship between prolonged NCT, secondary pulmonary infection, and mortality [4].

It is also generally accepted that immunosuppressed patients infected with SARS-CoV-2 often experience prolonged virus shedding. This may be a critical component of the prolonged NCT story, because in some studies it has been hypothesized that immunocompromised hosts with prolonged NCT, may play a pivotal role in the emergence of new viral variants [9,10].

Herein, we have taken this one step further by outlining specific risk factors of prolonged NCT in terms of various perspectives in the patient group, including both symptomatic and asymptomatic patients.

It is generally considered that older age is associated with severe outcomes for COVID-19 infection [11]. A recent study, including symptomatic patients, also showed an association between age and viral RNA persistence, but couldn't establish a relationship between RNA persistence and gender, or

Figure 1. ROC curve analysis of D-dimer, CRP and multivariate conditional logistic regression model in predicting the prolonged viral shedding.



co-morbidities [12]. A study from Ding Shi *et al.*, using 99 hospitalized patients, however, did suggest that male gender was associated with prolonged NCT [13]. We could not find any relationship between clinical manifestations or comorbidities and prolonged NCT. This may be related to the range of health status of our patients. All these studies were conducted using only symptomatic patients. Our study, however, also contained asymptomatic participants. In similar studies that included both asymptomatic and symptomatic patients no statistically significant differences based on these variables were observed [14]. Due to this we considered that gender, age, and chronic disease may play a specifically more important role in the severe group for prolonged NCT.

Some studies have shown that the CT score is positively correlated with severity. It is also generally considered that CT score can predict short-term outcomes specifically [15]. In our study we did not

Table 2. Multivariate conditional logistic regression analysis for prolonged viral shedding.

	B	SE	Wald	OR (95% CI)	p value
CRP, mg/L	-0.007	0.005	2.118	0.993 (0.984-1.004)	0.146
D-dimer, mg/mL	0.001	0.001	4.111	1.001 (1.00-1.001)	0.043
Bacterial co-infection	2.524	0.781	10.449	12.479 (2.701-57.654)	0.001
Mechanical ventilation	0.042	0.874	0.002	1.043 (0.188-5.780)	0.941

Nagelkerke R²: 0.198; Hosmer and Lemeshow test: $p = 0.191$; ⁻² log likelihood: 134.357; CRP: C-reactive protein; OR: odds ratio; CI: confidence interval; SE: standard error.

observe any association between the CT severity index and prolonged NCT. In terms of influenza virus infection, prolonged viral shedding has been associated with a delay in antiviral treatment [16]. However, in the case of COVID-19 infection, this has, thus far, not been the case. In our study, we also found that antiviral treatment and vaccination did not affect viral shedding, although our study included patients across two years, and vaccination status and patient treatment ratios may not be robust enough for serious consideration. More research is needed to effectively evaluate this aspect.

Serum CRP, D-dimer, and ferritin levels are strongly associated with outcomes due to diffuse inflammatory activation and diffuse coagulopathy [17]. Some previous studies have shown that there is no correlation between the COVID 19 severity and viral shedding [18,19]. However, the alteration of pro-inflammatory markers on admission has been more frequent with prolonged NCT [20,21]. Interestingly, no previous independent association has been observed after carefully accounting for confounding factors. We were able to show that the level of D-dimer on admission was, in fact, an independent factor for prolonged NCT. We also found that CRP and D-dimer values were higher in the prolonged group.

A previous retrospective study reported that 8% of patients experienced bacterial/fungal co-infection during the COVID-19 infection period. While we observed no fungal co-infection in our study, our ratio for bacterial co-infection was almost the same [22]. To date, there is only one study available in the literature that shows bacterial co-infection as an independent risk factor for NCT [23]. Our study, however, supports this data by ruling out the confounders.

COVID-19 is a new disease with many aspects that are still not completely understood. Additional studies on long-term effects of prolonged viral shedding are required to gain a solid picture of all the factors involved. When considering the studies of immunosuppressed patients with prolonged NCT, it has been suggested that the prolongation of the virus elimination period may lead to the formation of new mutations. Based on this, there should be focus on the prevention of prolonged NCT going forward. Our investigation into the risk factors is a small but vital step.

Our study has several limitations; firstly, the retrospective data collected from medical records may contain imperfections regarding the original recording of the information (chronic disease, symptoms, etc.). Secondly, as mentioned earlier, the ratio of vaccinated people or patients receiving treatment may not be

appropriate for evaluation. Finally, this study is a single-center study. Therefore, our findings might be subject to bias of the local population. The real strength of our study, however, is that the groups were matched according to age, gender and treatment (inpatient or outpatient) to control confounders; contrary to previous studies on the same subject. We know that confounders can influence outcomes and controlling them is vital for a more realistic clinical picture. Thus, we have been able to show a clear result for prediction factors associated with prolonged NCT of SARS-CoV-2.

Conclusions

D-dimer level and bacterial co-infection can be seen as independent factors in predicting prolonged shedding. The duration of viral shedding may hold vital importance due to a suspected link to concomitant outcomes or the emergence of new COVID-19 infection mutations. Any risk factors for prolonged viral shedding must be determined. While larger, more robust studies are needed, this study does provide a very useful reference in this area.

Authors' contributions

Nadir Y. conceived of the presented idea and wrote the manuscript. Kiran P. verified the analytical methods and provided critical feedback.

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Conflict of interests: No conflict of interests is declared.