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Original Article

SARS-CoV-2 clearance in term of Cycle Threshold (Ct) during first two waves of COVID-19 in Pakistan: a phenomenon of delayed viral clearance post-corticosteroid treatment

Eliminação do SARS-CoV-2 em termos de limite de ciclo (Ct) durante as duas primeiras ondas de COVID-19 no Paquistão: um fenômeno de eliminação viral retardada pós-tratamento com corticosteroides

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Abstract

SARS-CoV-2 is recently emerged virus, which caused millions of deaths, all over the world. To tackle COVID-19 pandemic, there is an utmost need for in-depth analysis of viral replication. We aimed to examine viral load in SARS-CoV-2 patients during first two waves of COVID-19 in Pakistan. 225,615 suspected subjects from 75 different regions of Pakistan were selected in the study. SARS-CoV-2 RNAs were detected *via* real time PCR. During first wave (period of June-July, 2020) of COVID-19 the prevalence of SARS-CoV-2 was 20.38%. However, during second wave (period of November-December, 2020) of COVID-19, the rate of prevalence was 9.41%. During first wave of COVID-19 96.31% of participants remained PCR positive for 14 to 21 days, 3.39% of subjects showed positive results for 22 to 35 days, while delayed Ct values were observed among 0.26% of participants for 36 to 49 days. However, during second wave of COVID-19 89.31% of the subjects exhibited symptoms and showed real-time PCR positive results for 14 to 21 days, 9.42% showed positive results for 22 to 35 days, while significantly delayed Ct value results were observed among 1.026% of participants for 36 to 63 days (3.95 times higher than first wave). In contrast to first wave of COVID-19, the factors that were different in second wave were neither viral (different strains) nor host (same population). But treatment factors changed significantly. As during second wave besides azithromycin, corticosteroid dexamethasone consumption was increased consequently causing delayed Ct value negativity. This suggests that corticosteroid treatment might be linked with delayed Ct value or viral clearance. This study is crucial for re-considering effective therapeutic options against COVID-19.

Keywords: SARS-CoV-2, COVID-19, Ct value delay, Pakistan, corticosteroid.

Resumo

O SARS-CoV-2 é um vírus que surgiu recentemente e causou milhões de mortes em todo o mundo. Para enfrentar a pandemia de COVID-19, necessita-se de uma análise aprofundada da replicação viral. Nosso objetivo foi examinar a carga viral em pacientes com SARS-CoV-2 durante as duas primeiras ondas de COVID-19 no Paquistão. Foram selecionados para o estudo 225.615 indivíduos suspeitos de 75 regiões diferentes do Paquistão. Os RNAs do SARS-CoV-2 foram detectados por PCR em tempo real. Durante a primeira onda de COVID-19 (período de junho a julho de 2020), a prevalência de SARS-CoV-2 foi de 20,38%. No entanto, durante a segunda onda de COVID-19 (período de novembro a dezembro de 2020), a taxa de prevalência foi de 9,41%. Durante a primeira onda de COVID-19, 96,31% dos participantes permaneceram PCR positivos por 14-21 dias, 3,39% dos indivíduos apresentaram resultados positivos por 22-35 dias, enquanto valores tardios de Ct foram observados em 0,26% dos participantes por 36-49 dias. No entanto, durante a segunda onda de COVID-19, 89,31% dos indivíduos tiveram sintomas e resultados positivos de PCR em tempo real por 14-21 dias, 9,42% apresentaram resultados positivos por 22-35 dias, enquanto resultados de valores de Ct significativamente atrasados foram observados em 1,026% dos participantes durante 36-63 dias (3,95 vezes superior à primeira onda). Em contraste com a primeira onda de COVID-19, os fatores que

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foram diferentes na segunda onda não foram nem virais (estirpes diferentes), nem hospedeiros (mesma população). No entanto, os fatores de tratamento mudaram significativamente. Assim como durante a segunda onda, além da azitromicina, o consumo de corticosteroide dexametasona aumentou, causando, consequentemente, negatividade tardia do valor de Ct. Isso sugere que o tratamento com corticosteroides pode estar associado ao atraso no valor de Ct ou na depuração viral. Este estudo é crucial para reconsiderar opções terapêuticas eficazes contra a COVID-19.

Palavras-chave: SARS-CoV-2, COVID-19, atraso no valor Ct, Paquistão, corticosteroide.

1. Introduction

The ongoing pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused more than 153 million cases of Coronavirus disease-19 (COVID-19), resulting into 3.2 million deaths worldwide, worst affected areas include United States and India (Worldometer, 2021). The virus preferentially infects respiratory tract cells, but may also cause severe damage to other organs such as conjunctiva, pharynx, brain, lungs, liver, kidneys, heart and several others (Zou et al., 2020; Hui et al., 2020; Puelles et al., 2020; Drosten et al., 2003). The virus constitutes of envelop (E), matrix core protein (M), nucleocapsid (N), and surface spike glycoprotein (S). The virus penetrates the host Alveolar type 2 progenitor (AT2) cells expressing angiotensin-converting enzyme 2 (ACE2) through which spike surface glycoprotein interact with receptor binding domain. The virus hijacks cellular metabolic pathways such as G-protein-coupled receptors (GPCRs) linked pathways, c-Jun N-terminal Kinase (JNK), Janus Tyrosine Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) signaling pathways to favor enhanced viral transmission (Singh et al., 2020).

In Pakistan, the first case of COVID-19 was reported from Karachi on February 26, 2020, while afterwards, SARS-CoV-2 rapidly sprout nationwide causing infection in millions. During first wave of SARS-CoV-2 it has been intimated by the prediction analysis of Medical Research Council Centre for Global Infectious Disease, Imperial College London that the expected number of deaths due to COVID-19 might exceed 80,000 per day, however through admirable management skills of National Command Operation Center (NCOC) of Pakistan and implementation of standard operating procedures via efficient smart lock down policies, the first wave of COVID-19 was controlled. These efforts were recognized worldwide and appreciated by WHO and several other countries. And there were <10 deaths per day at that time and 1st wave recorded down with aforementioned efforts (Mawngat, 2020).

The second wave of COVID-19 emerged due to poor compliance, political gathering, festivals etc. which resulted in rise of numbers of COVID-19 patients. In second wave based upon new global guidelines, the inflammatory damage caused by SARS-CoV-2 significantly caused critical COVID-19. In such patients, steroids and azithromycin showed promising results in term of morbidity and mortality. Of note in-appropriate usage of azithromycin for SARS-CoV-2-related disease could also worsen Extensively Drug Resistant (XDR) typhoid in Pakistan, and may not be prescribed during early phase of SARS-CoV-2 infection (Saeed et al., 2021a). Since, it has been reported that usage of corticosteroids in patients with early SARS and MERS infection were linked with delayed viral clearance with no clear benefits in term of length of hospitalization, survival or mechanical ventilation. And usage of corticosteroids for management during initial phase of SARS-CoV-2 infection is not recommended (Dequin et al., 2020). However among critically ill SARS-CoV-2 infected ones, corticosteroids treatment showed clear survival benefit (Spagnuolo et al, 2020). Accordingly, World Health Organization recommends systemic corticosteroids treatment for critically SARS-CoV-2 infected patients (Angus et al., 2020). There is a dearth of research on SARS-CoV-2 viral clearance after steroid treatment. The aim of this study was to estimate SARS-CoV-2 viral load (cycle thresh hold (Ct) values) and consequent viral clearance during initial two waves of COVID-19 in Pakistan.

2. Material and Methods

To compare the viral clearance in term of cycle threshold (Ct) values of real-time PCR during first two wave of COVID-19 epidemics in Pakistan, 225,615 COVID-19 suspected subjects were selected across 75 different regions of Pakistan including federally-administered Islamabad Capital Territory, Punjab, Khyber Pakhtunkhwa, Sindh, Balochistan and Azad Kashmir. About 225,615 suspected SARS-CoV-2 infected patients were included in the study during first two COVID-19 epidemics across the nation between June-July 2020, and November-December 2020.

After pre-test counseling *via* trained professional counselors, history of SARS-CoV-2 suspected patients and comprehensive medical examination was conducted. Ethical approval was obtained from all study participants. The institutional review board of Islamabad Diagnostic Center approved the study. After obtaining patients formal consent, the specimens were sent for molecular analysis at IDC specialized center for COVID-19, G-8 Markez Islamabad, Pakistan. The study investigators had professional background, full understanding and knowledge of specific contents of the protocol and all indicators through training related to SARS-CoV-2 diagnostics. The accuracy and integrity of COVID-19 testing was verified using gold standard means. The National Institute of Health (NIH), Islamabad, Pakistan Quality Control Laboratory approved IDC laboratory to ensure the standardization of experimental operating procedures.

After RNA extraction through Auto pure 32 Zybio China, from suspected COVID-19 samples, using standard Primerdesign, SARS-CoV-2 RNA positivity was detected *via* real-time polymerase chain reaction (Roche, USA). The assay included positive control template and RNA internal extraction control (Saeed et al., 2021b). World

health organization recommended USFDA approved triple target genes design (including SARS-CoV-2 RNA-dependent RNA polymerase gene, Sarbecovirus E gene, SARS-CoV-2 N gene) was used along with Seegene kit (#RP10244Y Allplex™ 2019-nCoV Assay, Seegene South Korea) with detection limit of (100) copies/ml. The positive samples having exponential growth curve and Ct value < 40 were considered COVID-19 positive. Discordant results were repeated or excluded. From each sample's nucleic acid, 5 µl of RNA template was used along with 15 µl of the One-step real-time PCR Master-mix. The conditions for thermocycling were; 30 min at 48 °C for reverse transcription, 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C (Saeed et al., 2021c). The Seegene kit was stored at -20 °C freezer, however extracted RNA was stored at -70 °C deep freezer. All of the test procedures were applied in accordance with standardized manufacturing protocols.

3. Results

To examine the impact changes of second COVID-19 wave (Nov-Dec, 2020) on delayed negative cycle threshold (Ct) values and associated course of illness or outcomes, in comparison to first wave of SARS-CoV-2 infections (June-July, 2020) in general population of Pakistan, we selected 225,615 SARS-CoV-2 suspected patients across the country. A total of 48,723 and 176,892 suspected SARS-CoV-2 infected patients were selected during the period of 1st June to 31st July 2020, and 1st November to 31st December 2020, respectively. Among these patients, SARS-CoV-2 RNA positive cases *via* real time PCR were selected for examining correlation between Ct values and disease progression.

The prevalence of SARS-CoV-2 during period of June-July, 2020 and November-December, 2020 among general population of Pakistan was 20.38% and 9.41%, respectively. Among selected subjects during first and second COVID-19 waves 66.92% (June-July, 2020) and 65.7% (Nov-Dec, 2020) were males, 33.07% (June-July, 2020) and 34.32% (Nov-Dec, 2020) were females, and 4% (June-July, 2020) and 12.7% (Nov-Dec, 2020) were children. During second wave of COVID-19 attack, the rate of infection among children was increased to approximately three times than first wave.

During first peak wave of COVID-19 attack among Pakistani population (during June-July) COVID-19 infection detected *via* real-time PCR with considerably Ct values persisted for approximately 2 to 3 weeks (14 to 21 days) among 96.31% of participants. And afterwards became real-time PCR negative. While 3.39% of participants showed COVID-19 symptoms and real-time PCR positive results for 3 to 5 weeks (approximately 22 to 35 days). Delayed Ct positive values were observed only among 0.26% of the participants for 5 to 7 weeks (approximately 36 to 49 days) as shown in Figure 1.

However, during November-December, 2020 among real-time PCR confirmed positive patients, Ct value or viral loads and disease severity persisted for 2 to 3 weeks (approximately 14 to 21 days) among 89.31% of the participants. 9.42% of the participants exhibited symptoms and real-time PCR positive results and Ct values for 3 to 5 weeks (approximately 22 to 35 days). This demonstrated that during the second wave the phenomenon of delayed Ct value between 22 to 35 days increased by three times as compared to the first peak wave of COVID-19. Significantly Delayed Ct positive values were observed among 1.026% of participants for approximately 5 to 9 weeks (36 to 63 days), indicating that the phenomenon of delayed Ct values for more than 5 weeks (> 36 days) increased by 3.95 times during second COVID-19 wave as shown in Figure 1.

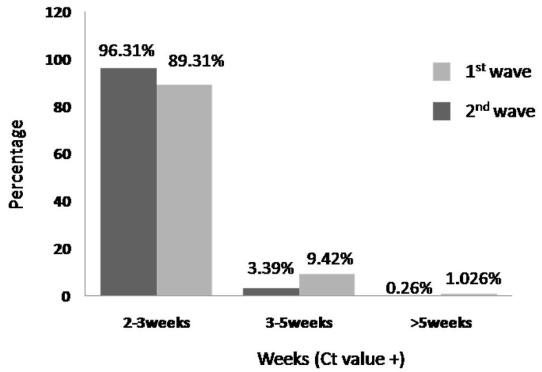

Figure 1. Comparison of Ct value positivity with respect to time among patients infected during first and second waves of COVID-19.

Of note, among COVID-19 patients during first wave, majority of patients empirically consumed hydroxychloroquine and ciprofloxacin. While during the second wave majority of the patients consumed corticosteroid medications (Table 1). It can be speculated that possibly corticosteroid dexamethasone consumption caused delay of Ct value during second wave of COVID-19.

4. Discussion

RNA viruses are rampantly mutating and proliferating and it is speculated that soon these viruses might emerge as highly dangerous entities in the world (Saeed et al., 2021a, b, c). Identification of disease progression patterns is necessary to reduce global burden of disease. It has been reported that viral load and progression to severe disease are correlated (Rao et al., 2020). Also, the mortality among the Patients with and without cancer can be predicted through the viral load among patients hospitalized with COVID-19 (Westblade et al., 2020).

Among hospitalized patients several studies reported association between Ct value and severity of disease (Liu et al., 2020a, b, c, Yu et al., 2020; Zheng et al., 2020; Schwierzeck et al., 2021). Among COVID-19 patients Lower Ct values were correlated with higher lactate dehydrogenase levels, neutrophil counts and high-sensitivity troponin-1 levels; and with lower lymphocyte, T-cell counts, and serum albumin levels (Liu et al., 2020a, Huang et al., 2020; Yuan et al., 2020; Azzi et al., 2020).

Clinical knowledge of SARS-CoV-2 and COVID-19 disease progression is expanding at higher rates. However clinical implications associated with severe aliments or prognoses

Table 1. SARS-CoV-2 positive patients and clinical assessment during first two waves of COVID-19 in Pakistan.

COVID-19 waves	Samples	Ct value	RT-PCR	Hydroxychloroquine	Ciprofloxacin	Azithromycin	Corticosteroid Treatment	Dyspnea	Fever	Cough
First wave (June-July)	Male (66.92%)	(15-39)	+	+	+	-	-	+	+	+
	Female (33.07%)	(11-37)	+	+	+	-	-	+	+	+
	Children (4%)	(25-38)	+	+	+	-	-	-	+	+
Second wave (November-December)	Male (65.7%)	(12-38)	+	-	-	+	+	+	+	+
	Female (34.32%)	(11-39)	+	-	-	+	+	+	+	+
	Children (12.7%)	(15-35)	+	-	-	+	+	-	+	+

such as mortality are needed to be further explored. The multivariate logistic regression analyses from viral titers of real-time PCR SARS-CoV-2 positive patient samples revealed that for single unit increase of Ct value, the infectivity (defines as growth in cell culture) decreased by 32%. In other words, lower Ct values were linked with increased probability of viral culture (Bullard et al., 2020).

A study conducted in 12 patients showed negative correlation of Ct value with C-reactive protein levels. Among several patients lower Ct values were significantly correlated with higher levels of creatinine kinase myocardial band (Liu et al., 2020b; Huang et al., 2020). The SARS-CoV-2 epidemics analysis in Pakistan revealed delayed cycle threshold (Ct) values in patients infected during second wave of SARS-CoV-2 infections during November and December 2020.

The prevalence rate of COVID-19 during first wave of COVID-19 in Pakistan was 2.17% higher than the second wave hit during November and December (Data from the current study). However, intriguingly, frequent delayed cycle threshold values were observed among COVID-19 patients infected during second wave of SARS-CoV-2 attack. During the second wave of COVID-19, SARS-CoV-2 variants were not prevalent in Pakistan. We asked that among both COVID-19 waves of attack why Ct values varied significantly?

Although people carefully practiced standardized SOPs for prevention against SARS-CoV-2, yet mode of treatment among COVID-19 patients was relatively different. Majority of the people consumed steroids for treatment during second wave of COVID-19. Furthermore, it was observed that usage of corticosteroids significantly delayed Ct values among participants. It can be inferred that frequent usage of corticosteroid might enhance SARS-CoV-2 replication. The preliminary findings of current study can further be confirmed through investigating distinct intracellular molecular signaling and in-depth biochemical pathways affecting SARS-CoV-2 replication (Saeed et al., 2023a, b, c, 2024; Saeed and Piracha, 2023; Piracha and Saeed, 2023, Piracha et al., 2024; Piracha et al., 2023).

5. Conclusion

In conclusion, our study demonstrated that during second wave of COVID-19, the delayed SARS-CoV-2 clearance was associated with early use of corticosteroids. Current study is important for medical research councils at national and international forums and provides useful information for government strategic organizations for re-considering effective therapeutic options against COVID-19.

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