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Retrospective review of re-positive qPCR tests for SARS-CoV: do they indicate presence of reinfection?

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Abstract. – OBJECTIVE: In 2019, the Coronavirus Disease 2019 (COVID-19) pandemic broke out, caused by the coronavirus called Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Reinfections can be observed with various respiratory viruses, including human coronaviruses. Moreover, they may result from weak or waning initial immune response, reinfection with another genotype/subtype, or the rapid antigenic changes in the virus. The aim of this study was to investigate the likelihood of reinfection in COVID-19 patients that had a positive qPCR test result at least 60 days after a negative test result in patients that were confirmed with COVID-19 on qPCR.

MATERIALS AND METHODS: The quantitative polymerase chain reaction (qPCR) results of a total of 105,000 samples that had been obtained between April 1, 2020, and February 1, 2021, in two separate authorized laboratories were retrospectively analyzed. 22 samples from 11 patients included in the study, qPCR tests were repeated for each sample using the Rotorgene Q PCR system with Diagnovital SARS-CoV-2 (RTA Labs, Turkey) Real-Time PCR kits. Positive samples were screened for B.1.1.7 and E484K mutations using the qPCR method on the Rotorgene Q PCR system with Bio-Speedy SARS-CoV-2 Variant Plus kits (Bioeksen Technology, Turkey).

RESULTS: The 105,000 individuals comprised 55,614 men and 49,386 women. In the qPCR test, 14,511 (13.82%) individuals were found to be positive for SARS-CoV-2. Of these, 11 (0.076%) patients were included in the study based on the inclusion criteria. Accordingly, the risk of reinfection was calculated as 0.076% (95% confidence interval [CI]: 0.056%-0.096%) and the incidence was 1.04 per 10,000 population (95% CI: 0.62-1.38 per 10,000). No patient was admitted to the intensive care unit or died during both episodes. Moreover, no B.1.1.7 or E484K mutation was detected in any patient.

CONCLUSIONS: The high frequency of COVID-19 infection poses serious risks for the development of new variants and the currently used vaccines are likely to lose their efficacy against new variants. To reduce these risks and to be successful in the fight against the pandemic, we suggest compliance with personal protective measures as well as rapid and widespread application of vaccination not only in developed countries but also in the whole world and the modification of currently used vaccines in such a way to fight against newly emerged variants.

Key Words:
COVID-19, Reinfection, Variants of COVID-19.

Introduction

Coronaviruses belong to the Coronaviridae family of the Nidovirales order. These viruses are non-segmented positive-stranded RNA viruses and are roughly spherical, enveloped particles approximately of 120 nm in diameter, and typically have petal shape projections (spike proteins) on their surface[1]. Coronaviruses cause infections in a wide range of hosts, including mammals, birds, and humans. Prior to 2002, some coronaviruses were known to cause non-fatal common cold in humans, particularly during the winter months. In 2002, however, severe acute respiratory syndrome (SARS), which was caused by a new coronavirus and had a fatal course contrary to what was known, was defined. This virus was named SARS-CoV, with reference to the disease it caused, and the SARS-CoV epidemic ended shortly after it started. Additionally, in 2012, a coronavirus infection named Middle Eastern Respiratory Syndrome (MERS), which had a mortality of over 30%, emerged in Jeddah, Saudi Arabia[1,2]. In 2019, the Coronavirus Disease 2019 (COVID-19) pandemic broke out, caused by the coronavirus called Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). It is transmitted among people *via* respiratory droplets and contact routes; the SARS-CoV-2 has severely disrupted the global healthcare system due to its rapid spread and

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high numbers of cases and death, and it has also brought socioeconomic activities to a standstill[3,4]. As of 27 February 2022, over 433 million confirmed cases of COVID-19 and over 5.9 million deaths have been reported globally[5].

Reinfections can be observed with various respiratory viruses, including human coronaviruses. Moreover, they may result from weak or waning initial immune response, reinfection with another genotype/subtype, or the rapid antigenic changes in the virus[6].

Literature indicates that although specific antibodies were detected in survivors up to 24 and 34 months after the SARS-CoV-1 and MERS-CoV outbreaks, respectively. No information could be obtained regarding the protection of antibodies against reinfection due to the low probability of a second exposure to these viruses[7,8]. In patients with endemic coronaviruses, immunity has been shown to be transient, lasting from a few months to several years. In addition, reinfection has been reported after experimental and natural infection[9].

In some cases of SARS-CoV-2, the antibody titers were found to wane, which strengthens the assumption that inadequate immune development and consequent reinfections are possible[10]. On the other hand, occurrence of reinfection is the most critical issue regarding the course of the COVID-19 pandemic, success of the vaccine, and the development of appropriate vaccination policies. Although some studies[11,12] have reported that neutralizing antibodies develop rapidly after infection, some other studies[13,14] have found that antibody titers begin to decrease 1-2 months after acute infection. As with other viruses, changes may occur in the genetic material of SARS-CoV-2 over time. However, most of these changes have little or no impact on the properties of the virus, as with other viruses. Moreover, these changes can sometimes have an impact on the spread rate and severity of the disease as well as on diagnostic tools, vaccines, and therapeutic drugs. For this reason, the World Health Organization (WHO), together with numerous individuals, institutions, and organizations, has been monitoring and evaluating the genetic changes of SARS-CoV-2 since January 2020. In late 2020, after the emergence of variants that pose an increased risk to global public health, various classifications were developed for these variants with the aim of promoting global monitoring and research. Accordingly, variants to be monitored were grouped into three classes: (i) Variants of Interest (VOI), (ii) Variants of Concern (VOC), and (iii) Variants of High Consequence (VOHC)[15,16]. In February 2021, the Public Health England (PHE) published a case of the B.1.1.7 variant with spike E484K mutation, which raised a concern that the variants with E484K mutation might be associated with reduced efficacy of the vaccines developed to date[17]. It has been reported that in some patients that were discharged after a negative reverse transcription quantitative polymerase chain reaction (RT-qPCR) test result, the qPCR test was positive in later tests. The studies[18] suggested that this phenomenon was due to prolonged viral shedding around the limit of detection in these individuals. Some other studies[19] proposed that reinfection might have occurred in such patients. Accordingly, it appears that the reason for re-positive test results after negative qPCR test results in some individuals remains a major concern.

The aim of this study was to investigate the likelihood of reinfection in COVID-19 patients that had a positive qPCR test result at least 60 days after a negative test result in patients that were confirmed with COVID-19 on qPCR.

Materials and Methods

The qPCR results of a total of 105,000 samples that had been obtained between April 1, 2020 and February 1, 2021 in two separate authorized laboratories at Diyarbakır and Şanlıurfa provinces in Turkey were retrospectively analyzed. Demographic characteristics such as age and gender were not taken into account in order to eliminate the risk of not detecting possible reinfection cases.

Selection of Patients Suspected with Reinfection

The cases to be included in the study were selected based on the following criteria:

1. A first-time diagnosis of COVID-19 infection confirmed by qPCR test (cycle threshold [Ct]<35);
2. Having two consecutive negative qPCR test results and signs of clinical improvement;
3. A second positive qPCR test result for SARS CoV-2 infection obtained at least 60 days after the last positive test result (Ct<35).

The only exclusion criterium was: a second positive SARS CoV-2 qPCR test result obtained within 60 days of the last positive test result.

Assessment of Reinfection Risk and Incidence Rate

Reinfection risk was determined as the ratio of the number of cases diagnosed with reinfection according to the established criteria to the number of all laboratory-confirmed SARS-CoV-2 cases. The incidence of reinfection was calculated by dividing the number of cases diagnosed with reinfection by the total number of samples evaluated.

Laboratory Workup

The qPCR tests of the combined nasopharyngeal-oropharyngeal swab samples transferred to both authorized laboratories were performed using Rotorgene Q (Qiagen, Germany) and Light Cycler 96 (Roche Diagnostics, Germany) PCR systems with the test kits distributed free of charge by the Turkish Public Health Institution in line with the manufacturers' recommendations. The presence of SARS CoV-2 RNA was evaluated by the reverse transcriptase-polymerase chain reaction analysis by using the Bio-Speedy COVID-19 RT-qPCR kit (Bioeksen Technology, Turkey) and in all cases the same type of PCR tests used. Samples with positive results were stored at -80°C. After thawing 22 samples from 11 patients included in the study, qPCR tests were repeated for each sample using the Rotorgene Q PCR system with Diagnovital SARS-CoV-2 (RTA Labs, Turkey) Real-Time PCR kits in line with the manufacturer's recommendations. Positive samples were screened for B.1.1.7 and E484K mutations using the qPCR method on the Rotorgene Q PCR system with Bio-Speedy SARS-CoV-2 Variant Plus kits (Bioeksen Technology, Turkey) in accordance with the manufacturer's recommendations. This study was carried out in Dicle University Medical Faculty Medical Microbiology Laboratory.

Statistical Analysis

Patients were analyzed according to age, gender, and symptoms at the time of diagnosis. Statistical analysis was performed with Jamovi Project (version 1.1.9) and JASP Team (version 0.11.1) softwares. A p-value <0.05 was deemed as statistically significant. In the intergroup analysis of continuous variables, normality analyses were performed using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Analyses between the two measurements were performed using the t-test. We also investigated the correlation of Ct values with Pearson correlation analysis test. Data were expressed as mean±SD (standard deviation).

Results

The 105,000 individuals comprised 55,614 men and 49,386 women. In the qPCR test, 14,511 (13.82%) individuals were found to be positive for SARS-CoV-2. Of these, 11 (0.076%) patients were included in the study based on the inclusion criteria. All the 11 patients were not vaccinated against SARS-CoV-2. Accordingly, the risk of reinfection was calculated as 0.076% (95% confidence interval [CI]: 0.056%-0.096%) and the incidence was 1.04 per 10,000 population (95% CI: 0.62-1.38 per 10,000).

The 11 patients comprised 7 men and 4 women with a median age of 37 (range, 9-55) years. Mean time to the second episode was 104 (range, 63-186) days. In the qPCR test, the Ct value was below 35 in all samples. Since the data were normally distributed according to Kolmogorov-Smirnov and Shapiro-Wilk tests, paired t-test was used for comparison. Result: p=0. Through the analysis of Ct values, the second Ct value was 27.30±2.89; which was higher than that of the first value (22.16±2.67). There was no correlation in the Pearson analysis test and the difference was not statistically significant (p=0.371, r=0.299) (Table I).

Pearson analysis shows that there is not a significant positive correlation between Ct values (r=0.299, p=0.371).

All the 11 patients included in the study were admitted to the hospital with the complaint of respiratory tract infection in both episodes. Of these, only two patients received inpatient care in the first episode, while no patient was hospitalized during the second episode. No patient was admitted to the intensive care unit or died during both episodes. Moreover, no B.1.1.7 or E484K mutation was detected in any patient.

Discussion

To et al[12] reported the first case of COVID-19 reinfection in the world who was an asymptomatic patient and was found to be positive for SARS-CoV-2 on PCR test during mandatory airport screening in August 2020, 142 days after the first COVID-19 infection. The authors performed Whole-genome sequencing (WGS) and reported that the virus in the second episode came from a different lineage, and thus, the patient was defined as a case of reinfection. Duggan et al[20] reported on a patient that had previously recovered from COVID-19 infection and was diagnosed with re-

Table I. Demographic and clinical characteristics.

Patient	Gender	Age (years)	Date of first episode	Ct value	Date of second episode	Ct value	Time between two episodes (days)	Difference between two Ct values
#1	Female	37	29.06.2020	**24.03**	30.08.2020	**28.90**	**63**	**4.87**
#2	Male	31	12.07.2020	**18.26**	28.09.2020	**28.06**	**78**	**9.80**
#3	Female	38	21.07.2020	**19.08**	03.02.2021	**26.79**	**186**	**7.71**
#4	Male	41	29.07.2020	**18.16**	27.11.2020	**26.14**	**121**	**7.98**
#5	Male	23	29.07.2020	**23.54**	08.12.2020	**30.60**	**132**	**7.06**
#6	Male	55	30.07.2020	**22.04**	26.11.2020	**26.25**	**119**	**4.21**
#7	Male	31	31.07.2020	**22.11**	15.10.2020	**24.10**	**76**	**1.99**
#8	Male	28	06.08.2020	**24.52**	18.11.2020	**22.19**	**104**	**-2.33**
#9	Female	53	27.08.2020	**22.12**	23.12.2020	**26.41**	**87**	**4.29**
#10	Female	9	03.10.2020	**26.37**	21.12.2020	**32.70**	**79**	**6.33**
#11	Male	42	05.10.2020	**23.62**	12.01.2021	**28.26**	**99**	**4.64**

infection based on new respiratory, radiographic, and laboratory findings and a positive RT-PCR result. Additionally, two more probable reinfection cases were reported from USA, both of whom had a history of domestic contact and developed a second episode of COVID-19 with more severe symptoms approximately two months after the first confirmed episode of COVID-19. In both patients, WGS detected several potential variations between the two viruses over some parts of the viral genome and the authors described these patients as cases of reinfection[21,22]. Xiao et al[23] conducted a clinical study with 70 COVID-19 patients among whom 15 (21.43%) patients had a second positive qPCR test result after two consecutive negative results. The authors reported that these 15 patients were not evaluated as cases of reinfection due to false positives of the qPCR test and prolonged nucleic acid shedding. The authors also noted that the qPCR test may not always provide accurate results since its performance can be affected by numerous factors including patient's viral load, disease stage, source of the sample (upper or lower respiratory tract), sample collection technique, sample transport conditions, and the kits used in the test[24]. Based on these findings, it can be asserted that the negative qPCR test results obtained during the recovery period after the first episode may not always be accurate and thus the presence of reinfection is questionable. Some researchers argue that there may be cases of SARS-COV-2 reinfection due to the decrease in antibody titers caused by the immune response to endemic coronaviruses over time and, in some cases, due to relatively high and stable antibody titers. The researchers support this view with the decrease in antibody titers in the early stages following infection in some cases of COVID-19[9,10].

Although new reinfection cases diagnosed by WGS are increasing day by day, the accuracy of this test remains controversial[25]. For this reason, there is need to establish an agreeable and applicable definition and criteria for the diagnosis of reinfection. The European Center for Disease Prevention and Control (ECDC) published a report that indicated that the use of WGS for the diagnosis of reinfection is restricted due to its limited availability[26]. Yahav et al[6] developed a set of recommendations for the definition of reinfection cases, to which they added a clinical and epidemiological definition as well as a precise definition similar to the definition proposed in ECDC recommendations (Table II). In the present study, the recommendations of ECDC and Yahav et al[6] were taken as the basis for determining the criteria used to be used for identifying possible reinfection cases and our findings were similar to the findings of few similar studies in the literature[27,28].

Abu-Raddad et al[27] reviewed 133,266 laboratory-confirmed cases and evaluated 243 cases that had a second positive qPCR test result more than 45 days after the first SARS-CoV-2 episode. Of these, 54 (0.043%) cases were diagnosed with reinfection and the incidence was 1.09 per 10,000 population. Similarly, in our study, the reinfection rate was 0.043% and the incidence was 1.04 per 10,000 population. In a study conducted in Italy, Flacco et al[28] detected reinfection in 24 (0.33%) out of 7,173 COVID-19 patients. This rate is higher than the rate found in our study, which could be due to the inclusion of qPCR-positive asymptomatic patients in our study. In a similar way to our study, two cohort studies[29,30] also reported the reinfection rate as below 1%. Some case studies[12,21,22] have shown that the second episode of COVID-19 is often as-

Table II. Yahav's recommendations for the definition of reinfection.

1. Confirmation of the diagnosis: **a.** Confirmation of initial infection (reanalysis of the initial sample and Ct <35), **b.** Confirmation of virus in subsequent infection (by PCR or cell culture), **c.** Confirmation of infection with two different phylogenetic strains by genome sequencing **d.** Documentation ofat least one and ideally two negative RT-PCR test results on two different samples collected between the first and second episodes
2. Clinical definition: Recurrence of clinical symptoms consistent with COVID-19 confirmed by a positive PCR test (Ct < 35) 90 days after the onset of primary infection, supported by a history of close contact with infected individuals or an outbreak
3. Epidemiologic definition: Any positive RT-PCR test result obtained within 90 days after the first episode regardless of symptoms

ymptomatic or milder than the first episode. Similarly, in our study, no patient had a more severe second episode compared to the first episode and all the cases had either similar or milder findings in the second episode. Moreover, in a study by Abu-Raddad et al[27], only 1 out of 54 patients was hospitalized and the patient had milder symptoms in the second episode than in the first episode. On the contrary, Flacco et al[28] reported that 4 out of 24 patients that were suspected with reinfection received inpatient treatment during the second episode and one of these patients, aged 77 years old, died in the hospital. However, Hansen et al[29] and Vitale et al[30] found no data on this subject in their studies. Due to these contradictions, we could not reach a definite judgment regarding the severity of COVID-19 reinfection. On 26 November 2021, WHO designated the SARS-CoV-2 variant B.1.1.529, named the Omicron variant, as a VOC[31]. This variant contains a large number of mutations associated with the evasion of innate immunity and thus is likely to result in reinfections. In a confirmatory manner, in a study conducted after the emergence of the Omicron variant, Juliet et al[32] reported that 35,670 suspected reinfections were detected among the 2,796,982 subjects with laboratory-confirmed SARS-CoV-2. In the second episode, Ct elevation was found to be statistically significant. Viral genome copies in upper respiratory tract swabs are informative and differences between variants may be detected in virus load variations[33]. In addition, we consider that this result may be due to the rapid development of the secondary immune response in case of reinfection.

Sequencing methods used for SARS-CoV-2 genomes could not be performed due to the lack of laboratory facilities employed for the samples obtained in both episodes, and this was considered a limiting factor for the research.

Conclusions

The high frequency of COVID-19 infection poses serious risks for the development of new variants and the currently used vaccines are likely to lose their efficacy against new variants. To reduce these risks and to be successful in the fight against the pandemic, we suggest compliance with personal protective measures as well as rapid and widespread application of vaccination not only in developed countries but also in the whole world and the modification of currently used vaccines in such a way to fight against newly emerged variants. Future studies using larger sample sizes may shed more light on the situation.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

The study was approved by Dicle University Medical School Noninterventional Clinical Research Ethics Committee (Approval No: 2021/291).

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Informed Consent

All subjects gave written informed consent prior to participation.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contribution

All authors contributed, read, and approved the final version of the manuscript.

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