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Comparison of L452R mutation variant diagnosis in SARS-COV-2 PCR positive samples with two different qPCR kits

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ABSTRACT

Objectives: Quantitative reverse transcription−polymerase chain reaction (qPCR) is used as the gold standard method to diagnose COVID-19 infection caused by SARS-CoV-2 which is the cause of the most important epidemic in world history. It was aimed to compare the results of two of the most commonly used commercial kits for the diagnosis of SARS-CoV-2 mutation in our laboratory during the pandemic.

Methods: Our study included 5000 SARS-CoV-2 PCR positive nasopharyngeal swab samples (2500 L452R mutation positive samples, 2500 L452R mutation negative samples). PCR positivity and negativity of the L452R mutation of the positive SARS-CoV-2 positive samples were identified with the Diagnovital® (DI-AGNO5plex NS SARS-CoV-2 Real Time PCR Kit [A1 Life Sciences Istanbul]) kit. The mentioned samples were also studied with a different commercial PCR kit, Bio-Speedy® (SARS-CoV-2 Emerging Plus Real Time PCR Kit [Bioeksen R&D Technologies Istanbul]).

Results: A total of 5000 samples included in the study were concluded as SARS-CoV-2 positive with both tests. One hundred and fifty of 2500 samples that were found positive for SARS-CoV-2 but negative for L452R mutations with the Diagnovital® kit were found positive with the Bio-Speedy® kit for SARS-CoV-2. The compatability between the two kits was found to be high (Kappa = 0.940). The mean Ct values of the samples found positive with the Diagnovital® kit and Bio-Speedy® kit were 24.15 ± 6.75 and 20.72 ± 7.17, respectively and the difference was statistically significant.

Conclusions: It was determined the two commercial kits included in the study were extremely compatible based on their analysis. Therefore both kits can be used safely for COVID-19 symptomatic patients.

Keywords: SARS-CoV-2, L452R mutation, COVID-19, qPCR, Laboratory diagnosis

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) is the most important infectious agent that caused more than 6 million deaths, embarked an unprecedented burden upon the national and international health systems/agencies and the global economy with the COVID-19 pandemic. Its diagnostic procedures are carried out with quantitative reverse transcription−polymerase chain reaction (qPCR) which is still the gold standard method for the task [1, 2]. While commercial qPCR tests used in daily practice

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in the diagnosis of SARS-CoV-2 virus detect some gene regions of the virus, they can not accuretely respond to the issues such as prolonged positivity, disease stage, reinfection and clinical condition of the patient [3]. Diagnostic tests with the qPCR method developed for the SARS-CoV-2 infection detection is one of the most important mechanisms that helps limiting the spread of the virus along with the measures to be taken by monitoring it [4]. SARS-CoV-2 qPCR kits basically targets "hemagglutinin-esterase" HE, "open reading frame 1" ORF1, "envelope glycoproteins spike" S, "RNA-dependent RNA polymerase" RdRp, "helicase" Hel, "nucleocapsid protein targets" N, "envelope" E, and "transmembrane" M [5]. "Cycle threshold" of the PCR test "Ct" represents the number of cycles in which the signal resulting from target gene amplification reaches the positivity threshold level. The Ct value is considered to be inversely proportional to the viral load in the sample in which the lower the Ct value, the higher the viral RNA copy number in the sample. There are publications supporting that the Ct value may be useful in following the clinical course and prognosis of COVID-19 patients [6,7]. It has been reported that the viral load is higher and the Ct value is low in the first 12 days depending on the disease severity in cases with strong symptoms requiring intensive care admission, poor prognosis or immunosuppression [7, 8]. Initially the kits supplied by the Ministry of Health of the Republic of Turkey and then the commercial kits approved by the Turkish Medicines and Medical Devices Agency were used in laboratories authorized by the Ministry of Health to diagnose SARS-CoV-2 in our country. Turkish Medicines and Medical Devices Agency has given pre-authorization to many diagnostic products during the COVID-19 pandemic, and the effectiveness of these kits has been assesed by the General Directorate of Public Health [9]. However, many new variants of SARS-CoV-2 have emerged during this time period, posing a public health concern. Increased transmission and reinfection risks, immune response evasion, decreased vaccine effectiveness and worsening of the clinical picture are threats that may adversely affect the course of the pandemic along with the variants emerging during the pandemic. Therefore, detecting SARS-CoV-2 variants and tracking their mutations is critical in the fight against the COVID-19. Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) variants are classified as "variants of concern (VOCs)" by World Health Organization (WHO) [10]. The B.1.1.7 variant has a higher contagiousness due to its unusually high mutation rate and poses a global threat [11]. The E484K mutation significantly reduces antibody neutralization [12]. It has been reported that E484K has a high effect for Oxford-AstraZeneca, Novavax and Gamaleya, a high to minimal effect for Moderna and Pfizer and a moderate effect for Sinovac and Sinopharm in evading post-vaccine neutralizing antibodies [13]. The L452R mutation in one of the VOCs (B.1.617) has significantly increased SARS-CoV-2 transmission rate in countries such as America and India where it was detected.

It has also been reported that this mutation has the effect of avoiding neutralizing antibodies formed after vaccination [14]. The L452R mutation, which can be found in many other lineages, including B.1.1.7, should be tested regardless of lineage [13]. It has been difficult to provide quality assurance in diagnostic tests and laboratories from time to time with the increase in the number of samples studied with the spread of SARS-CoV-2 variants and use of a difficult method such as qPCR in the pandemic laboratories with inexperienced personnel in molecular methods [15]. There have been frequent changes in qPCR kit versions used in SARS-CoV-2 diagnostic laboratories and it has not been possible to obtain objective data about the performance of qPCR kits and eliminate uncertainities due to the vast amount of samples. Several researches were performed worldwide to compare different qPCR kits for SARS-CoV-2 identification [16-18].

It has been demonstrated that diagnostic kits give similar results in terms of accuracy and sensitivity even though different gene regions have been investigated in the studies. The aim of this study is to compare and independently evaluate the results of two of the most frequently used commercial kits in our laboratory for detecting L452R mutation in delta variants during the pandemic.

METHODS

Ethics committee approval of Yıldırım Beyazit University Yenimahalle Training and Research Hospital (Decision Number: 2022-47) was obtained for the study.

Specimen Collection

Our study was carried out at Ankara Provincial Health Directorate Public Health Molecular Diagnosis Laboratory, which is one of the pandemic laboratories with the highest sample working capacity in our country. Randomly selected 5000 nasopharyngeal swab samples (2500 SARS-CoV-2 L452 mutation positive, 2500 SARS-CoV-2 positive L452 mutation negative) were determined Sars-CoV-2 positive with Diagnovital® (DIAGNO5plex NS SARS-CoV-2 Real Time PCR Kit) kit and analyzed with a different commercial PCR kit, Bio-Speedy® (SARS-CoV-2 Emerging Plus Real Time PCR Kit) in the study.

The test outfits did not require any extra RNA extraction phase during the qPCR process since the samples were transfered directly into Viral Nucleic Acid Buffer (vNAT) with nucleic acid extraction. Extracting RNA of swap samples with vNAT solution required only the the vortex.

Molecular Assays

SARS-CoV-2 specific 'Orf1ab' and 'N' genes, mutation genes of Spike (S) E484K, Nucleocapsid (N) D3L and Spike (S) L452R are investigated with Diagnovital® kit. The sample-induced inhibition control and the kit reagent control include an internal control containing the RNase P gene. 7.5 µl reaction mixture and 2.5 µl sample were combined and reverse transcription (RT) was carried out at 52°C for 5 minutes, denaturation at 95°C for 20 seconds followed by 40 cycles of 1 second at 95°C and 60°C as suggested by the kit manufacturer. Bio-Speedy® kit, which we used as a comparison kit, is a multiplex-based quantitative real-time PCR kit using labeled oligonucleotides specific to target gene regions for SARS-CoV-2 found in nasopharyngeal swabs, oropharyngeal swabs, nasal swabs, nasopharyngeal aspirates, saliva and bronchoalveolar lavage samples. The Bio-Speedy® kit allows complementary DNA (cDNA) synthesis and qPCR reaction to be performed in the same tube. SARS-CoV-2 specific 'Orf1ab' and 'N' genes, mutations of Spike (S) E484K, Nucleocapsid (N) D3L, Spike (S) L452R along with Human RNase-P mRNA genes were targeted with this kit. Sample-induced inhibition control and kit reagent control were performed thanks to the RNaseP mRNA gene which was used as internal control.

RT at 52°C for 3 minutes and holding at 95°C for 10 seconds, followed by 1 second at 95°C, 12 seconds at 60°C, 5 cycles of reproduction at 85°C, 1 second, at 60°C, 35 cycles of denaturation, annealing, and extension, duplication and reading, respectively, were carried out for 1 second in compliance with the manufacturer's specifications. Bio-Rad CFX96 TouchTM device was used in our study. The test was applied once for each sample and Ct values were recorded.

Analysis of the Results

The interpretation of test results was carried out following the manufacturer's instructions for all two kits. For each sample, samples with a Ct value of 36 and below (Ct ≤ 36) in the internal control (Rnase P) in the HEX channel from the fluorescent reading channels when the reaction was completed were included in accordance with the manufacturer's recommendations in the Diagnovital® kit. The samples seen as sigmoidal curve and Ct ≤ 38 in both FAM and Cy5.5 channels are determined as positive for SARS-CoV-2, but a variant containing L452R mutation (Delta, Epsilon, Kappa) is positive while Alpha variant (B.1.1.7) and variants containing E484K (Beta, Gamma, Zeta, Eta, Theta, Iota) were considered negative when the reaction is completed according to manufacturer recommendations. Samples with a sigmoidal curve and Ct ≤ 38 only in the FAM channel were SARS-CoV-2 positive, but Alpha variant (B.1.1.7), variants carrying E484K (Beta, Gamma, Zeta, Eta, Theta, Iota) and variants carrying L452R (Delta, Epsilon, Kappa) were considered negative. Samples with non-sigmoidal curves were excluded from the study.

For each sample, in accordance with the manufacturer recommendations, samples with a Ct value of 32 and below (Ct ≤ 32) in the internal control (Human mRNA) in the HEX channel, one of the fluorescent reading channels, were included in the study when the reaction was completed with the Bio-Speedy® kit. In accordance with the manufacturer recommendations, when there is a sigmoidal curve in both FAM and Cy5.5 channels and Ct ≤ 33, the Ct differences between Cy5.5 and FAM and Cy5 are calculated and if [Ct_Cy5.5 – Ct_FAM] < 4 and [Ct_Cy5.5 – Ct_Cy5] < 0 then the result is found to be positive for SARS-CoV-2 and one of the variants containing L452R mutation (Delta, Epsilon, Kappa) was positive while Alpha variant (B.1.1.7) and variants containing E484K (Beta, Gamma, Zeta, Eta, Theta), iota) were consid-

ered negative. Samples with a sigmoidal curve and Ct ≤ 33 only in the FAM channel was SARS-CoV-2 positive, but Alpha variant (B.1.1.7), variants carrying E484K (Beta, Gamma, Zeta, Eta, Theta, Iota) and variants carrying L452R (Delta, Epsilon, Kappa) were considered negative. Samples with non-sigmoidal curves were excluded from the study.

Statistical Analysis

The results of both tests were recorded in SPSS Statistics (IBM, version 22) and evaluated with Kappa analysis and t test in dependent groups.

RESULTS

A total of 5000 samples included in the study were concluded as SARS-CoV-2 positive with both tests. One hundred and fifty of 2500 samples that were found positive for SARS-CoV-2 but negative for mutations with the Diagnovital® kit were found positive with the Bio-Speedy® kit for SARS-CoV-2 and one of the variants containing the L452R mutation (delta, epsilon, kappa). The compatability between the two kits was found to be high (Kappa = 0.940, p = 0.5) in Kappa analysis. The mean Ct values of the samples with positive delta mutation (n = 2650) with Bio-Speedy® kit were 20.72 ± 7.17, while the mean Ct values of samples with positive delta mutation with Diagnovital® (n = 2500) were 24.15 ± 6.75. The difference between the two groups was statistically significant ($p < 0.05$). Table 1 shows the positivity and negativity rates of both tests. The Ct value of 51.01% of the samples in the Bio-Speedy® kit is below 20. Table 2 shows the Ct value ranges of both kits.

DISCUSSION

Measures taken to reduce the spread of COVID-19 depend on rapid and accurate identification of the disease in SARS-CoV-2-infected individuals with the most sensitive and specific method available. qPCR is still a fast and accurate method for SARS-CoV-2 virus diagnosis in the world [19]. Although many diagnostic tests became available at the laboratories with rapid approval, many studies support that the kits are sensitive and reliable [20]. In this study, two locally manufactured and frequently used commercial kits that perform mutation analysis in samples deterimed to be positive for SARS-CoV-2 were compared and the compatability between them was found to be extremely high. False negative results are the main issue for qPCR in the early stages of infection, Incorrect application of reagents and incomplete extraction in qPCR cause false negative results [21]. Incorrect result qPCR problem caused issues during the delta variant intensive period. In addition, qPCR requires trained medical personnel, special tools and technical labor [22]. This sample constitutes only 3% of our group. The mean Ct values of the samples found positive with the Diagnovital® kit and Bio-Speedy® kit were 24.15 ± 6.75 and 20.72 ± 7.17 respectively and the difference was statistically significant ($p < 0.05$). The high compatability between the two kits with the positivity rates and average Ct values determined in this study shows that both kits can be used in the diagnosis of SARS-CoV-2 variants.

Van Kasteren *et al*. [20] also tested kits specific for common betacoronavirus and/or only SARS-CoV-2 virus and targeting very different gene regions at different dilutions with 13 samples with different viral

Table 1. Comparison of positivity and negativity of both kits

Method	Bio-Speedy® SARS-CoV-2 Emerging Plus Real Time PCR Kiti			
	Results	Positive	Negative	Total
Diagnovital® DIAGNO5plex NS SARS-CoV-2 Real Time PCR Kiti	Positive	2500	0	2500
	Negative	150	2350	2500
Total		2650	2350	5000

Table 2. Ct value ranges of mutation-positive samples with Diagnovital® DIAGNO5plex NS SARS-CoV-2 Real Time PCR Kit and Bio-Speedy® SARS-CoV-2 Emerging Plus Real Time PCR Kit

		Method			
		Diagnovital®		Bio-Speedy®	
		n	%	n	%
Ct value	< 20	410	16.4	1352	51.01
	20.00-24.99	1048	41.92	798	30.11
	25-29.99	945	37.8	400	15.09
	30-32.99	77	3.08	100	3.77
	33-37.99	20	0.8	0	0

Ct = Cycle threshold

loads and stated that all kits can be used in routine diagnosis in symptomatic patients. WHO recommends first performing qPCR testing targeting two different gene regions of the virus, or specific to the common gene region of the betacoronavirus family, and then confirming the test result with partial or whole genome sequencing specific to the SARS-CoV-2 virus in periods or places where the virus prevalence is low. On the other hand, an qPCR test targeting a single gene region specific to SARS-CoV-2 virus is sufficient in regions where SARS-CoV-2 is common [23].

Studies show that the performance of the qPCR test can be affected by many factors such as patient's viral load, disease stage, sample source (upper or lower respiratory tract), sample collection technique, sample handling conditions [24]. In the mutation analysis of the Bio-Speedy® kit, the Ct value differences between Cy5.5, FAM and Cy5 were calculated in accordance with manufacturer recommendations, and one of the variants containing the L452R mutation was evaluated as positive and the variants containing B.1.1.7 and E484K as negative. It has also been observed in our study that this proces complicates the work of doctors during the test evaluation phase and that it creates an extra burden on the result evaluation stress in a laboratory with a high daily case rate.

The Diagnovital® and Bio-Speedy® PCR kits were compared in another study which showed that both of the methods produce on par results for the negative and positive clinical specimens. However, it has been reported that the amplification graph of the Diagnovital® PCR kit was a more distinctive curve than the one produced by the Bio-Speedy® PCR kit. It was stated that the use of diverse kits targeting different genes in samples of unknown virus presence based on COVID-19 clinical symptoms could provide a more definitive SARS-CoV-2 diagnosis [25].

CONCLUSION

Different nucleic acid amplification tests were developed and used during the COVID-19 Pandemic. It has been understood that it is extremely important to determine the kits with low sensitivity and specificity so the health professionals can ward off false positive patients while they avert false negative patients from further spreading the infection and becoming infectious in the pandemic. In this study, there was no significant difference between the Diagnovital® kit and the Bio-Speedy® kit even though the test specificity was similar between the two kits. It is thought that both kits can be used safely in the diagnosis of COVID-19 infection and SARS-CoV-2 variants in patients with symptoms.

Authors' Contribution

Study Conception: BGG; Study Design: BGG, GGA; Supervision: GGA; Funding: BGG, GGA; Materials: BGG, GGA; Data Collection and/or Processing: BGG, GGA; Statistical Analysis and/or Data Interpretation: BGG, GGA; Literature Review: BGG, GGA; Manuscript Preparation: BGG and Critical Review: BGG, GGA.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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