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A screening strategy for identifying the dominant variant of SARS-COV-2 in the fifth peak of Kurdistan- Iran population using HRM and Probe-based RT-PCR assay

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A R T I C L E I N F O

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A B S T R A C T

By the emergence of SARS CoV-2 variants, many studies were developed to deal with it. The high transmissibility and mortality rate of some variants, in particular developing countries have caused the operation of simple diagnostic tests for genomic surveillance. In this study, we developed two assays of High Resolution Melting (HRM) and Probe-based RT-PCR as simple and inexpensive methods to identify the variants. We screened the mutations of del69–70, E484K, E484Q, D614G, L452R, and T478K in 100 cases from SARS-COV-2 positive patients in Kurdistan- Iran population. In general, the result of the two methods overlapped each other, nevertheless, we suggested HRM results be confirmed with a standard assay (Whole-Genome Sequencing). This work indicated that HRM as the rapid and inexpensive method could identify and categorize the variants of SARS CoV-2 and reduce the costs for carrying out sequencing.

1. Introduction

Since its discovery in late December 2019 in China, COVID-19 has become a huge public health problem. However, since there is no complete picture of COVID-19's genome, the most study has focused on genetic analysis. The spike (S), envelope (E), membrane (M), and nucleocapsid are the main protein encoded by several genes in the genome of SARS CoV-2 [1]. The two subtypes of S protein contain S1 and S2 have a high affinity to interact with the angiotensin-converting enzyme 2 (ACE2) of human cells [2]. The C-terminal domain (CTD), receptor-binding domain (RBD), and N-terminal domain (NTD) are three major domains of S1 protein monitored [3].

The lack of strong proofreading of RNA polymerase is considered the main cause of rising mutations frequencies in the COVID-19 genome. While there is no comparable distribution for known mutations, a number of studies have revealed that these mutations alter SARS-CoV-2 transmissibility and pathogenicity [4], through influence replication, the function of RNA polymerase[5]. The amino acid modification based on the Wuhan sequence (GenBank: MN908947.3) (Table 1) shows the mutations which are important and some of them cited for a specific variant. D614G as a common mutation identified in most variants of SARS CoV-2 is occurred in the nucleotide sequence number 23403, leading to the change of amino acid codon from aspartate to glycine codon[6]. B.1.617 variant with three sublines, including B.1.617.1 (Kappa), B.1.617.2 (Delta), and B.1.617.3 which are recognized according to three amino acid mutations, L452R, T478K, and E484Q [7]. Delta as a variant of concern (VOC) possesses L452R and T478K mutation, while the two other subtypes of B.1.617 cited with L452R and E484Q mutation [8]. The delta variation has been identified as a dominant variant with a global distribution [8]. The B.1.1.7 variety is

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Table 1
Amino acid modifications in comparison to the Wuhan sequence (GenBank: MN908947.3) in the B.1.617 sublineage and VOCs.

Lineage	Spike mutations
B.1.1.7 (Alfa)	**Δ69-70**, Δ144, N501Y, A570D, **D614G**, P681H, T716I, S982A, D1118H
B.1.351 (Beta)	D80A, D215G, Δ241-243, K417N, **E484K**, N501Y, **D614G**, A701V
P.1 (Gamma)	L18F, T20N, P26S, D138Y, R190S, K417T, **E484K**, N501Y, **D614G**, H655Y, T1027I, V1176F
B.1.617	**L452R**, **E484Q**, **D614G**, Δ681, Δ1072
B.1.617.1 (Kappa)	E154K, **L452R**, **E484Q**, **D614G**, P681R, Q1071H
B.1.617.2 (Delta)	T19R, G142GD, Δ156-157, R158G, **L452R**, **T478K**, **D614G**, P681R, D950N
B.1.617.3	T19R, Δ156-157, R158G, **L452R**, **E484Q**, **D614G**, P681R, D950N

another VOC that was first found in England in December 2020, with the rest of the world following suit in the months after [9]. A deletion at positions 69 and 70 linked with the B.1.1.7 mutation is thought to contribute to the transmissibility of SARS CoV-2 [10]. So, we decided to identify the possible major mutations of SARS CoV-2 variants highlighted in Table 1 and displayed in Fig. 1.

Despite the annual outbreak of flu from November to late January in Iran, late February 2019 was the initial time COVID-19 was reported [11]. After that time, strict restrictions were imposed to prevent the SARS CoV-2. But, the following years, 2020 and 2021, with the emergence of new variants of SARS CoV-2, particularly, B.1.617.2 and B.1.1.7, a challenging period was experienced. This was more noticeable in the fifth wave of SARS CoV-2, starting the July to October 2021,where based on evidence, more infected patients suspected were to suffer from B.1.617.2 and B.1.1.7 variant. Therefore, because of a higher number of death as well as hospitalization, we came up to identify the dominated variants in the fifth wave- a critical time tackled with SARS CoV-2 in Kurdistan, Iran. We are the only PCR testing laboratory for SARS CoV-2 in Kurdistan Province, which is located in western Iran and shares a border with Iraq. We employed the two speedy and affordable approaches to report the significant SARS CoV-2 variations in Kurdistan because to restrictions on research capabilities, particularly whole-genome sequencing.

2. Material and method

2.1. Sample collection

100 cases were examined in this study. The only one patient infected with Delta variant passed away among the population of our study. 26 cases became under admission in hospital, and the 74 cases with minor symptoms were quarantined at home. Nasopharyngeal-Oropharyngeal swab samples in a 2 ml viral transport medium were sent to the Kurdistan molecular lab for testing by a SARS-CoV2 RT-PCR assay. At the fifth

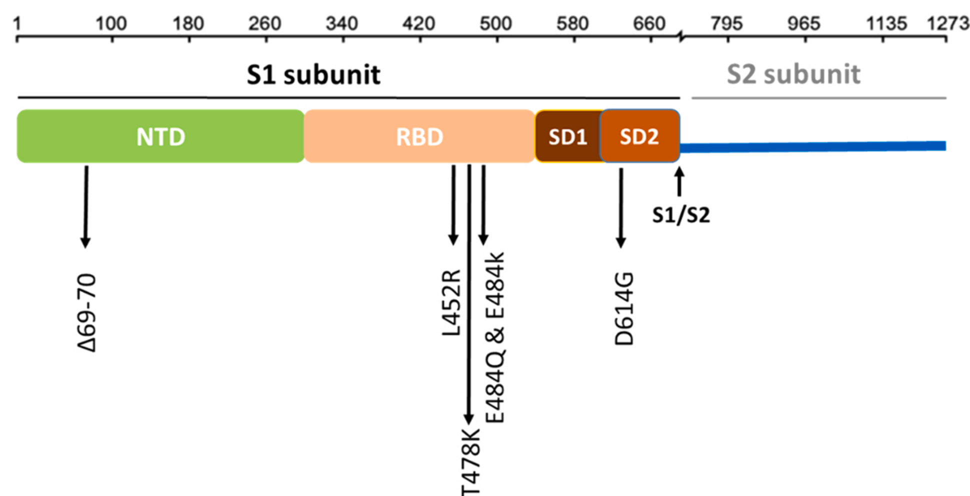

Fig. 1. Schematic of the mutations del69-70, E484K, E484Q, D614G, L452R, and T478K that were selected for dominant variant screening.b.

Table 2
Specific primer for HRM & Probe assay.

Name	Sequence 5 3	Position
E484K F	GGTAGCACACCTTGTAATG	22988–23006
E484K R	GTTGGAAACCATATGATTG	23039–23057
E484-Probe	**CY5**-TGTTGAAGGTTTTAATTGTTACTTTC-**BHQ1**	23008–23033
484k-Probe	**CY5.5**-TGTTAAAGGTTTTAATTGTTACTTTC-**BHQ2**	23008–23033
484Q-Probe	**CY5.5**-TGTTCAAGGTTTTAATTGTTACTTTC-**BHQ2**	23008–23033
D614G F	CAAATACTTCTAACCAGGTTGC	23367–23388
D614G R	CTGTAGAATAAACACGCCAAG	23457–23477
D614-Probe	**CY5**-TTCTTTATCAGGATGTTAACTGCACAG-**BHQ1**	23391–23417
614G-Probe	**CY5.5**-CTTTATCAGGGTGTTAACTGCACAGAA-**BHQ2**	23393–23419
L452R F	ATCTTGATTCTAAGGTTGGTG	22881–22901
L452R R	CGGCCTGATAGATTTCAG	22971–22988
L452-Probe	**CY5**-TTACCTGTATAGATTGTTTAGGAAG-**BHQ1**	22912–22936
452R-Probe	**CY5.5**-TAATTACCGGTATAGATTGTTTAGGA-**BHQ2**	22909–22934
Del69/70F	TCAACTCAGGACTTGTTCTTACC	21710–21732
Del69/70R	ATGGTAGGACAGGGTTATCAAAC	21796–21818
Del69/70 Probe	**CY5**-TGGTTCCATGCTATCTCTGGGACCA- **BHQ**	21752–21765×××××21772–21782
T478K F	TTTAGGAAGTCTAATCTCAAACC	22928–22950
T478K R	GAAACCATATGATTGTAAAGGA	23032–23053

Table 3
Characteristics of study population: The SPSS Software was used to determine the mean and standard deviation.

Characteristics		Count (Total=100)
Vaccine	YES (Dose)	10 (one dose)
	NO	90
Hospitalization	Inpatient	26
	Outpatient	74
Age	Mean ± SD	45.37 ± 15.99
Gender	Male	56
	Female	44

peak of the coronavirus in Kurdistan-Iran, July 2021 to October 2021, the mean positive tests of SARS-COV-2 were about 400. Among the positive samples with Ct values less than 25, 100 samples were randomly selected from residual samples (acceptable margin of error ± 10) [12]. The SPSS software was used to analysis the population characterization. The characteristics of the study population are provided in Table 3.

2.2. SARS-CoV-2 PCR assay

To detect SARS-CoV-2, nucleic acid was extracted using GB Viral DNA/RNA Extraction Kit (General Biologicals Corporation, Taiwan), and 5 μL of the eluate was added to a 15 μL of the reaction mixture of RT-PCR kit(Pishtaz, Iran). RT-PCR was performed using the QIAquant 96 5 plex (QIAGEN) with the following conditions: 50 °C for 20 min, 95 °C for 3 min, and 45 cycles of 94 °C for 10 s and 55 °C for 40 s

2.3. Detection of SARS CoV-2 mutations

2.3.1. Probe-based Real-Time Reverse Transcriptase PCR

SARS-CoV-2 positive samples were screened for del69-70, E484K, E484Q, D614G, and L452R mutations by five separate RT-PCR assays. These assays were carried out using the primer/probe sets, which were designed by Gene runner software from Wuhan strain (GenBank: MN908947.3) and shown in Table 2. The two channels were designed to detect the wild variant from the mutated one. CY5 channel was to detect the wild, and CY5.5 channel operated to detect the mutant of variant. In brief, 5.5 μL of RNA template was added to a 12 μL of the reaction mixture of Add-Probe RT-PCR Master Mix (Add bio, South Korea) and 2.5 μL of primer/probe set. RT-PCR was performed using the QIAquant 96 5 plex (QIAGEN) with the following conditions: 50 °C for 20 min, 95 °C for 10 min, and 45 cycles of 95 °C for 10 s and 55 °C for 40 s. Samples with the positive result for del69-70, E484K, E484Q, D614G, and L452R assays were considered screen-positive and collected for Sanger sequencing.

2.3.2. HRM analysis for determination of variants

SARS-CoV-2 positive samples were examined for D614G, L452R, and T478K mutations by High-resolution melting analysis. The same RNA extraction kit was used for Real-Time PCR Probe/HRM. Real-time PCR and subsequent analysis of melting curves were performed on 10 ng of extracted RNA templates 4 μL of EvaGreen-qPCR 5XMaster Mix (Solis BioDyne, South Korea) and 1 μL of SNP-specific primers (10 pmol/μL) on a Corbett RG-6000 machine (Australia). For this purpose, 40 cycles of two-step reactions, including step1: 5 s at 95∘C, step2: 30 s at the specific annealing temperature of primers; 58–62 ∘C were applied for the thermal cycler machine. HRM analysis setting was set to display the 0.1 difference of melting pattern of PCR product.

2.4. Sanger sequencing

After analyzing the HRM and Primer-Probe the PCR products were sent to the Pishgam Company, Iran for sequencing. We opted to utilize the existing primer for the sequencing-forward primer of L452R and the

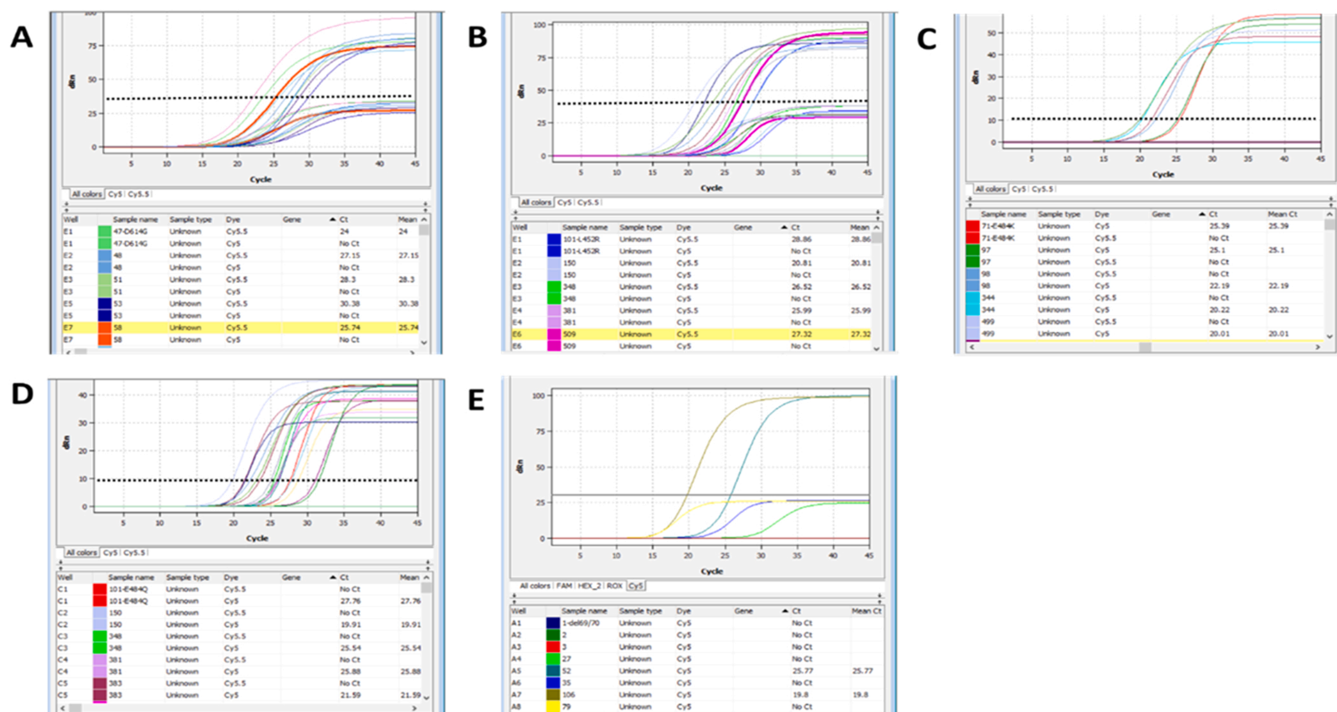

Fig. 2. Probe-based Real-Time PCR analysis. A) Amplification curves D614G using D614G primers and probes. D614 was detected in the CY5 channel and G614 was detected in the CY5.5 channel. B) Amplification curves L452R using L452R primers and probes. L452 detected in CY5 channel and 452R detected in CY5.5 channel. C) Amplification curves E484K using E484K primers and probes. E484 was detected in the CY5 channel and 484 K was detected in the CY5.5 channel. D) Amplification curves E484Q using E484Q primers and probes. E484 was detected in the CY5 channel and 484Q was detected in the CY5.5 channel. E) Amplification curve Del69/70 was detected in the CY5 channel.

Table 4
The sensitivity & specificity of HRM & Probe assay in comparison with Sanger assay.

Methods	Sensitivity	Specificity	ROC Area	95%Conf.Interval
HRM	93.68%	100%	0.9684	(0.9148–0.9937) 95%CI
Primer-Probe	98.95%	100%	0.9947	(0.946–0.9997) 95%CI

reverse primer of D614G since the mutations addressed were in close proximity to each other. Finally, a 598 PCR product containing L452R, E484K/Q, T478K, and D614G were synthesized, and then sequencing was done. Control positive for Delta variant and UK variant used which screened by Primer-probe, then proved by sequencing.

3. Results

3.1. Primer Probe based-Real Time PCR

Primer Probe based-Real Time PCR method was able to identify 99 cases of our samples into two groups, 94 cases infected with the Delta variant, and like HRM analysis, the same 5 cases were determined UK variant (Fig. 2). 94 cases were detected in the CY5.5 channel for the

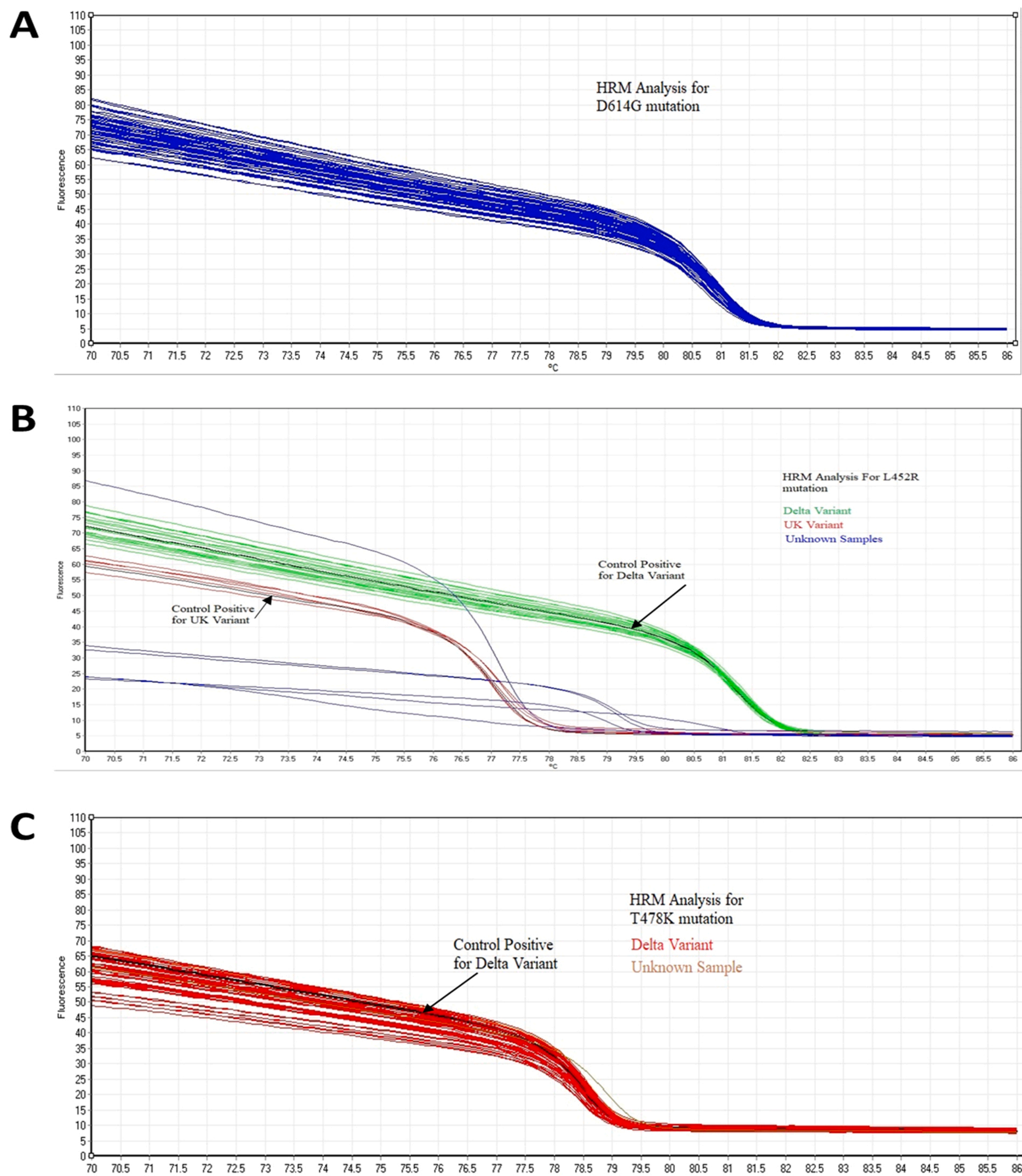

Fig. 3. A) HRM analysis for D614G mutation: all samples had the same patterns B) HRM analysis for L452R mutation, UK variant plus 6 samples with different patterns compared to L452R and UK variant. We could not analyze these 6 samples by HRM analysis C) HRM analysis for T478K mutation. In this run, we faced with sample which its HRM patterns differed from other samples. This sample was the only sample that was not detectable in Primer- Probe assay.

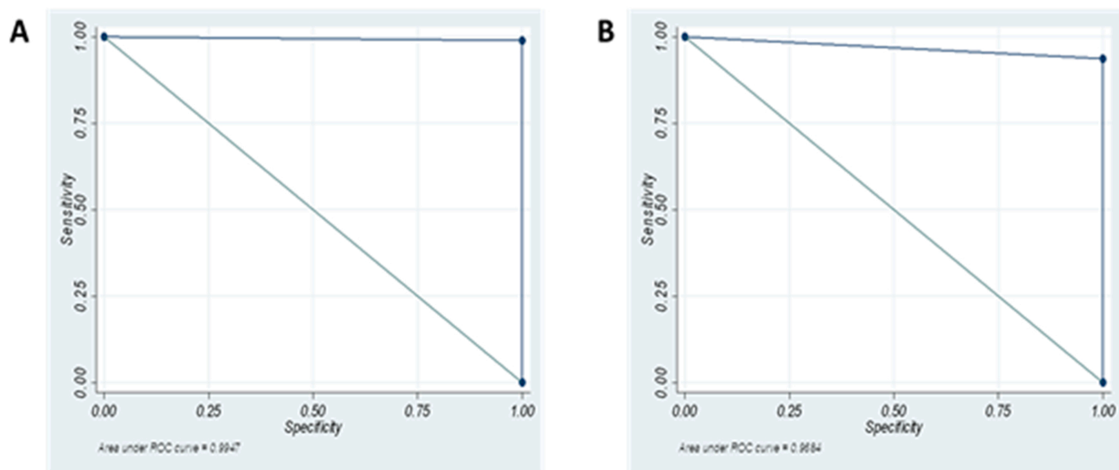

Fig. 4. Roc curve of HRM and Probe assay. A) Roc curve of HRM technique. B) Roc curve of Primer-Probe assay. The Sensitivity and specificity of both methods compared to the sequencing results by STATA SE12 Software. This comparison revealed that both methods are reliable and applicable in detecting SARS CoV-2 variants.

mutant type of D614G and L452R mutations (Fig. 2, A&B). In the CY5 channel, the wild type of E484Q and E484K were amplified in the 94 samples (Fig. 2, C&D). The 5 cases were detected for the Del69/70 (Fig. 2E). Sequencing results overlapped with the results of the Primer-Probe assay, 94 samples had the D614G and L452R mutations with 5 samples encompassing Del69/70 (Fig. 4, Table 4). The only one sample which was not detected, sequencing results confirmed it as Delta variant along with V483F mutation (Fig. 5).

3.2. HRM analysis

89 samples of our cases were detected by HRM analysis as Delta Variant, and 5 cases were classified UK variants (Fig. 3). Those samples with negative results for E484K, E484Q, and positive results for D614G, L452R were screened by HRM analysis plus T478K mutation specified for the Delta variant. The same melting curves patterns were detected in all cases for the D614G (Fig. 3, A). The 89 cases for L452R had the same patterns (Fig. 3, B). The 5 samples had detected by the Primer-Probe UK variant had the same melting curves that differed from 89 cases containing D614G, L452R, and T478K mutations (Fig. 3, B). The 6 samples had different HRM patterns (Fig. 3, B). The same 89 cases had similar patterns for T478K (Fig. 3, C). We had one sample with melting curves that differed from those of the Delta and UK versions. Unlike the Prime-Probe test, which failed to identify one sample, this sample was discovered using the HRM approach, and the sequencing confirmed it as a Delta variation, as indicated above. (Fig. 3C).

4. Discussion

Since the spread of SARS-CoV-2 was commenced, numerous studies were struggled to scrutinize the complex pathophysiology of SARS-CoV-2-induced disease. Hopefully, produced vaccines have effectively combated it, particularly by inspiration of genome-based studies. As the new variants of SARS-CoV-2 are constantly created with different distribution as well as different symptoms, occasionally with fast transmissibility and acute indication, rapid and accurate methods are required to categorize the existed variants and likely follow the new variants. However, although whole genome sequencing is regarded the gold standard for detecting novel variations, it is costly and not accessible in some developing countries. Hence, we came up to determine the dominated variants of SARS-CoV-2 in the fifth wave- July to October 2021 in Kurdistan via the two rapid and inexpensive methods, HRM analysis and Probe-based real-time PCR. According to evidence, Delta variants and UK variants were more with a high spread in Iran in the fifth wave. But, in our study, we tried to report the distribution of the exact variants in Kurdistan province. Due to the limitation on equipment and fund, we decided to utilize the HRM analysis and Probe assay to determine the variants. Nevertheless, sequencing the part of the genome of SARS-CoV-2, which the mutations examined in our study situated, was carried out to prove the results of two methods used.

Primer-Probe assay as the sensitive method was run for the initial screen of samples in our study, followed by HRM analysis. The results of Primer-Probe assay showed it could be considered a reliable method to detect the mutations. However, it is an expensive method in comparison with HRM analysis. Therefore, we decided to operate HRM analysis, because it is less costly [13]. Ultimately, the result of the two methods had the high overlapped each other as well as the results of sequencing (Fig. 4, Table 4), because the high level of sensitivity and specificity were provided (Table 4). Based on the results of the Primer-Probe and HRM, the two variants of SARS CoV-2 were detected, Delta variant and the UK variant. Utterly, 94 cases were evaluated with HRM analysis and 99 samples with Primer-Probe assay. Despite the fact that HRM was only able to identify 94 samples, the findings showed that it had the ability to categorize known mutations. Unfortunately, we were unable to analyze six instances due to confusing melting curves and HRM tests (Fig. 3, B). Despite this, we were able to identify these instances as Delta variations after sequencing. However, HRM technique detected 94 cases compared to the Primer-Probe assay, the one sample that had a different HRM pattern (Fig. 3, C) showed that HRM could be more effective because this sample was not detected by Primer-Probe assay. Hence, we concluded that HRM as a rapid, inexpensive method is applicable to identifying and categorizing the variants with relatively high sensitivity and specificity (Fig. 4, Table 4). Our study outcomes suggested that HRM analysis can be applied in other regions of the world to quickly identify SARS CoV-2 variants (Fig. 5).

In conclusion, our study succeeded in introducing HRM analysis as a sensitive approach to detect the SARS CoV-2 variant. However, further procedures may need to confirm the results of HRM analysis.

Statement

This project was approved by the research ethics committee of the Kurdistan University of Medical Science.

Author statement

Mohammad Moradzad: Led the project, performed the experiment, and wrote the paper. Shohreh Fakhari: sDesigned the experiment and

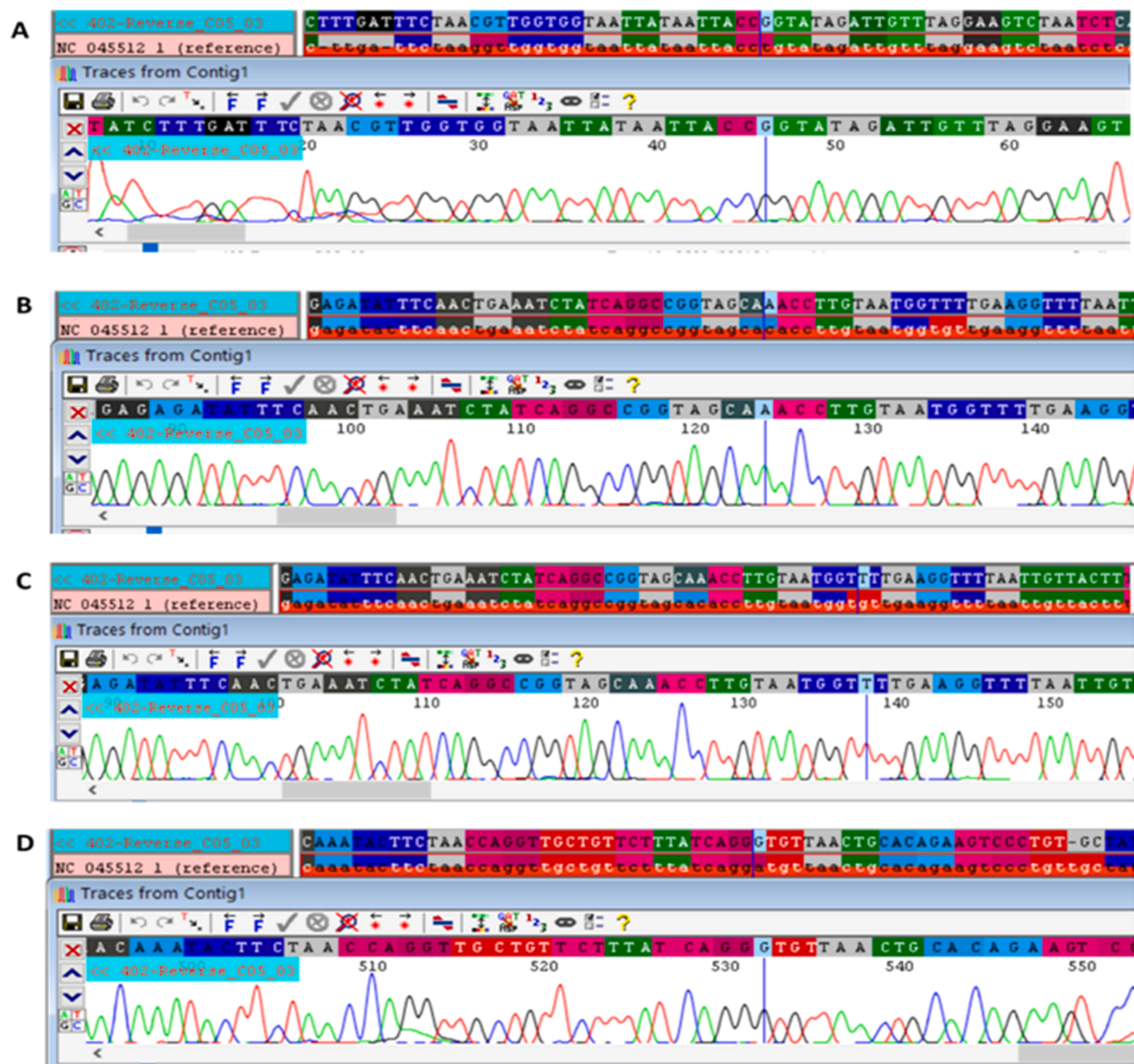

Fig. 5. The presence of A) L452R mutation, B) T478K mutation, C) V483F mutation and D) D614G mutation in one sample that was not detected by RT-PCR. The data of Sanger sequencing was analyzed by CodonCode Aligner software.

wrote the paper. Hasan Soltani: Contribution in experimental procedures and edited the paper. Hamid Salehi: Contribution in experimental procedures. Khaled Rahmani: Analysed the results of this paper. Dariush Khateri: Contribution in experimental procedures. Diman Az: Contribution in experimental procedures. Mohammad Ziad Rahimi: Contribution in experimental procedures.

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Conflict of interests and disclosure

The authors declare that they have no competing financial into interest Ethics.

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