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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 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...
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.

### Table 1

| 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, D138V, R190S, K417T, E484K, N501V, D614G, H655Y, T1027I, V1176F |
| B.1.617       | L452R, E484Q, D614G, Δ681, Δ1072                                                |
| B.1.617.1     | E154K, L452R, E484Q, D614G, P681R, Q1071H                                    |
| B.1.617.2     | T19R, G142GD, Δ156-157, R158G, L452R, T478K, D614G, P681R, D950N             |
| (Delta)       |                                                                                |
| B.1.617.3     | T19R, Δ156-157, R158G, L452R, T478K, D614G, P681R, D950N                     |

### Table 2

| Name            | Sequence 5’3’ | Position   |
|-----------------|---------------|------------|
| E484K F         | GGTAGCACACCTTGTAATG | 22988-23006 |
| E484K R         | GTGGAAACCATATAGTGG | 23039-23057 |
| E484-Probe      | CYS-TGGTGAAGGTTTATAATGTGATCTTT-ChlQ | 23008-23033 |
| 484Q-Probe      | CYS-5TTTAAGGTTTATAATGTGATCTTT-ChlQ2 | 23008-23033 |
| D614G F         | CAAATCTTCTAACCAGGTTGC | 23367-23388 |
| D614G R         | CTGTAGAATAAACACGCCAAG | 23457-23477 |
| L452R F         | ATCTTGATTCTAAGGTTGGTG | 22881-22901 |
| L452R R         | CGGCCGATAGATTTTCC | 22971-22988 |
| L452-Probe      | CYS-5TTTAAGGTTTATAATGTGATCTTT-ChlQ2 | 23393-23419 |
| 452R-Probe      | CYS-5TTTAAGGTTTATAATGTGATCTTT-ChlQ | 23393-23419 |
| De69/70F        | ATCTTGATTCTAAGGTTGGTG | 23367-23388 |
| De69/70R        | CTGTAGAATAAACACGCCAAG | 23457-23477 |
| De69-70F Probe  | CYS-TGGTGAAGGTTTATAATGTGATCTTT-ChlQ | 23008-23033 |
| T478K F         | TTAGGAATCATAATCTTACC | 22928-22950 |
| T478K R         | GAAACCATATGATTGTAAGGA | 23032-23053 |
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.

### 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     |
| Male            | 56                |
| Female          | 44                |

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 10 min, and 45 cycles of 95 °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. CYS 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 reverse primer of L452 for sequencing.
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

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

Table 4

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

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.
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 novel variants and likely follow the new mutations. 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
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. Darirush 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.

References

[1] Chatterjee, S., Understanding the nature of variations in structural sequences coding for coronavirus spike, envelope, membrane and nucleocapsid proteins of SARS-CoV-2. Envelope, Membrane and Nucleocapsid Proteins of SARS-CoV-2 (March 28, 2020), 2020.

[2] Smith, M. and J.C. Smith, Repurposing therapeutics for COVID-19: Supercomputer-based docking to the SARS-CoV-2 viral spike protein and viral spike protein-human ACE2 interface. 2020.

[3] Ahmadpour, D., Ahmadpour, P., Rostaing, L., 2020. Impact of circulating SARS-CoV-2 mutant G614 on the COVID-19 pandemic. Iran. J. Kidney Dis. 14 (5), 331–334.

[4] Yin, W., Mao, C., Luari, X., Shen, D.D., Shen, Q., Su, H., Wang, X., Zhou, F., Zhao, W., Gao, M., Chang, S., Xie, Y.C., Tian, G., Jiang, H.W., Tao, S.C., Shen, J., Jiang, Y., Jiang, H., Xu, Y., Zhang, S., Zhang, Y., Xu, H.E., 2020. Structural basis for inhibition of the RNA-dependent RNA polymerase from SARS-CoV-2 by remdesivir. Science 368 (6498), 1499–1504.

[5] Farooqi, T., Malik, J.A., Mulla, A.H., Al Hagbani, T., Almansour, K., Ubaid, M.A., Alghamdi, S., Anwar, S., 2021. An overview of SARS-COV-2 epidemiology, mutant variants, vaccines, and management strategies. J. Infecti. Public Health 14 (10), 1299–1312.

[6] Badua, C.L.D., Baldo, K.A.T., Medina, P.M.B., 2021. Genomic and proteomic mutation landscapes of SARS-CoV-2. J. Med. Virol. 93 (3), 1702–1721.

[7] Wilhelm, A., Toptan, T., Pallas, C., Wolf, T., Goetsch, U., Gottschalk, R., Vehreschild, M., Ciesek, S., Widera, M., 2021. Antibody-mediated neutralization of authentic SARS-CoV-2 B.1.617 variants harboring L452R and T478K/E484Q. Viruses 13 (9), 1693.

[8] Kannan, S.R., Spratt, A.N., Cohen, A.R., Naqvi, S.H., Chand, H.S., Quinn, T.P., Lorson, C.L., Byrareddy, S.N., Singh, K., 2021. Evolutionary analysis of the Delta and Delta Plus variants of the SARS-CoV-2 viruses. J. Autoimmunity 124, 102715.
[9] Grint, D.J., Wing, K., Houlihan, C., Gibbs, H.P., Evans, S., Williamson, E., McDonald, H.I., Bhaskaran, K., Evans, D., Walker, A.J., Hickman, G., Nightingale, E., Schultz, A., Renton, C.T., Bates, C., Cockburn, J., Curtis, H.J., Morton, C.E., Bacon, S., Davy, S., Wong, A., Mehrkar, A., Tomlinson, L., Douglas, I. J., Mathur, R., MacKeana, B., Inglesby, P., Croker, R., Parry, J., Hester, F., Harper, S., DeVito, N.J., Hulme, W., Tazare, J., Smeeth, L., Goldacre, B., Eggo, R. M., 2021. Severity of SARS-CoV-2 alpha variant (B.1.1.7) in England. Clin. Infect. Dis. ciab754-.

[10] Peterson, S.W., Liddell, R., Daigle, J., Wonitowy, Q., Nagasawa, A., Mulvey, M.R., Mangat, C.S., 2021. RT-qPCR detection of SARS-CoV-2 mutations S 69–70 del, S N501Y and N D3L associated with variants of concern in Canadian wastewater samples. Sci. Total Environ., 151283

[11] Ghafari, M., et al., Ongoing outbreak of COVID-19 in Iran: challenges and signs of concern with under-reporting of prevalence and deaths. medRxiv, 2020.

[12] Conroy R., Sample size Arough guide. Retrieved from http://www.beaumontethics.ie/docs/application/sample size calculation pdf. 2015.

[13] Aoki, A., Adachi, H., Mori, Y., Ito, M., Satoh, K., Okuda, K., Sakakibara, T., Okamoto, Y., Jinno, H., 2021. A rapid screening assay for L452R and T478K spike mutations in SARS-CoV-2 Delta variant using high-resolution melting analysis. J. Toxicol. Sci. 46 (10), 471–476.