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Research article

Detection of SARS-CoV-2 spike protein D614G mutation by qPCR-HRM analysis

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ABSTRACT

Objectives: Monitoring the spread of the G614 in specific locations is critical as this variant is highly transmissible and can trigger the emergence of other mutations. Therefore, a rapid and accurate method that can reliably detect the D614G mutation will be beneficial. This study aims to analyze the potential use of the two-step Reverse Transcriptase *quantitative polymerase chain reaction - high resolution melting* analysis (RT-qPCR-HRM) to detect a specific mutation in the SARS-CoV-2 genome.

Methods: Six SARS-CoV-2 RNA samples were synthesized into cDNA and analyzed with the qPCR-HRM method in order to detect the D614G mutation in Spike protein of SARS-CoV-2. The primers are designed to target the specific Spike region containing the D614G mutation. The qPCR-HRM analysis was conducted simultaneously, and the identification of the SARS-CoV-2 variant was confirmed by conventional PCR and Sanger sequencing methods.

Results: The results showed that the melting temperature (T_m) of the D614 variant was 79.39 ± 0.03 °C, which was slightly lower than the T_m of the G614 variant (79.62 ± 0.015 °C). The results of the HRM analysis, visualized by the normalized melting curve and the difference curve were able to discriminate the D614 and G614 variant samples. All samples were identified as G614 variants by qPCR-HRM assay, which was subsequently confirmed by Sanger sequencing.

Conclusions: This study demonstrated a sensitive method that can identify the D614G mutation by a simple two-step RT-qPCR-HRM assay procedure analysis, which can be useful for active surveillance of the transmission of a specific mutation.

1. Introduction

Since it first appeared in Wuhan in late December 2019, the severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2), the cause of Coronavirus Disease 2019 (COVID-19), has spread worldwide and becomes the current major healthcare problem [1, 2, 3, 4, 5]. The viral mutation has occurred frequently since the first outbreak and has increased rapidly with increasing transmission [6]. One of the important mutations in the SARS-CoV-2 genome is the missense located in the Spike (S) protein, which led to the replacement of amino acid from Aspartic Acid (D) to Glutamic Acid (G), at residue 614 [6, 7]. This mutation is known as the D614G, which has been shown to affect the infectivity of SARS-CoV-2 *in vitro* and *in vivo* [8, 9, 10]. The G614 variant had higher infectivity and could become the dominant circulating variant and replace the original D614 variant [6, 9, 11]. An effective mutation surveillance study should be considered because of its high infectivity

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property, which causes a greater probability to reach out to the higher risk community [3]. This approach can be efficient in supporting rapid and cost-effective detection methods, instead of relying on the high-cost and time-consuming sequencing-based detection method [12].

Several approaches have been developed to rapidly detect the D614G mutation. Furthermore, it was shown that the engineered Cas12 guide RNA reliably differentiates between variant D614 and G614 [13]. The RT-qPCR assay was also specially developed to perform a D614G genotyping that could distinguish the two variants [14]. Another study illustrated that the Biosensing approach was also reliable in detecting the D614G mutation [15]. The PCR-RFLP method has also been described to detect the D614G mutation, but the data were ultimately misinterpreted [16, 17]. Another possible and reliable method for detecting SNP is real-time PCR and high-resolution melting curve analysis (HRM) [12]. In addition, it has been reported that the HRM analysis accurately distinguishes between GR and non-GR clades of SARS-CoV-2 [18].

The HRM analysis is widely used for many purposes, particularly for detecting mutations [19]. This approach is a post-PCR protocol and is considered a powerful method for SNP detection [12]. This can be performed by determining the differences in the melting temperature between the samples, whereby a single change in the base can affect the melting temperature of the PCR product [20]. This technique has made it possible to distinguish SNPs with high accuracy and low cost, which are widely used in many areas, such as human genetic screening for specific disease susceptibilities, genotyping of drug resistance, as well as intra-microbial species genotyping [12, 19, 21, 22, 23, 24, 25]. This suggests that SNP genotyping by RT-qPCR-HRM analysis can detect the D614G mutation in SARS-CoV-2.

This study developed a sensitive method for the detection of the D614G mutation of SARS-CoV-2. The differences between the D614 and G614 variants of SARS-CoV-2 were distinguish using the two-step RT-qPCR-HRM analysis method. Furthermore, this is the first study that describes the reliability of the qPCR-HRM assay for the detection of D614G mutations in SARS-CoV-2 and provides a model that can be developed for the detection of other mutations in SARS-CoV-2.

2. Methods

2.1. Sample collection and primer design

Six RNA samples from specimens confirmed positive for SARS-CoV-2 were used. These samples are part of the sample collection from the COVID-19 Diagnostic Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia. Furthermore, two samples were used as reference strains, one of which was identified as variant D614 (Sample ID: 3.09) and the other as variant G614 (Sample ID: 3.13). Four samples with unknown variance were used as research samples (Sample ID: 2.14, 3.08, 3.17, and 3.24). The research protocol has been approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia (EC: KE/FK/0511/EC/2020).

A primer pair was designed to target the specific region containing the D614G mutation (Table 1). Furthermore, the primers amplified the target region from nucleotide position 23.286 to 23.434 of the Wuhan-Hu-1 reference sequence (NC_045512.2). The primers were used for qPCR-HRM assay as well as for PCR and sequencing procedures.

Table 1. The primers for qPCR-HRM assay.

Primer Name	Type	Primer Sequence	Primer Length	Primer GC Content (%)	Amplicon Size (bp)
F3.CoV-2-S	Forward	CTG TCC GTG ATC CAC AGA CAC T	22	55	172
R8.CoV-2-S	Reverse	GTA GGA GTA AGT TGA TCT GCA TG	23	42	

2.2. cDNA synthesis

The RNA was converted to the first strand of cDNA using the *Reverse Transcription Kit II (ExcelRT™ series, Smobio)*, according to the manufacturer's instructions. The RNA was incubated with the primer mixture (50 μM oligo $(dT)_{20}$ and 100 μM random hexamers) in a total volume of 10 μl at 70 °C for 5 min, followed by incubation in ice for at least 1 min. The first reaction volume of cDNA synthesis of 20 μl was completed by adding 5x RT Buffer, RTase, and DEPC-treated water, followed by a subsequent incubation at 20, 50, and 85 °C for 10, 60, and 5 min, respectively.

2.3. qPCR and HRM

qPCR was performed using *THUNDERBIRD™ SYBR™ qPCR Mix (Toyobo)*, according to the manufacturer's instructions. The cDNA concentration was equalized to 100 ng/μl. A total of 20 μl qPCR reactions, each containing 0.2 μM forward and reverse primer, cDNA template, qPCR mix, and nuclease-free water, were incubated according to the qPCR amplification steps: 95 °C 1 min; 45 cycles of 95 °C 5 s and 60 °C 30 s.

The qPCR product was used for post-PCR analysis with HRM. The qPCR product was incubated at 95 °C for 1 min and 60 °C for 1 min, followed by melting curve steps from 65 to 95 °C with the increment temperature 0.1 °C/s. The melting curve-RFU data from 78.3 to 81.5 °C were analyzed using the *PyHRM* code of *Python* (ver. 3.8.3) in order to generate the normalized melting curve and the difference curve of the HRM profile [26].

2.4. PCR and sequencing

PCR was performed using *Gotaq ® Green Mastermix (Promega)*. A total of 25 μl PCR reactions, comprising 0.2 μM of each forward and reverse primer, cDNA template, PCR mix, and nuclease-free water, was incubated following the PCR amplification steps: 95 °C 3 min; 35 cycles of 95 °C 30 s, 60 °C 45 s, and 72 °C 45 s; and final extension 72 °C 2 min.

The amplicon was sequenced using the Sanger method. All sequencing procedures using *BigDye® Terminator v3.1 cycle sequencing kit chemistry (Applied Biosystems)* were performed by DNA Sequencing Services (Genetika Science, Indonesia; 1st Base, Singapore).

3. Results

3.1. qPCR-HRM analysis of the D614 and G614 variant specimens

The preliminary HRM analysis of the two control specimens identified as the D614 variant (Sample ID: 3.09) and the G614 variant was performed (Sample ID: 3.13) in triplicate, resulting in a different melting temperature between the two groups (Table 2). Furthermore, the G614 variant showed a slightly higher melting temperature (79.62 °C) compared to the D614 variant (79.39 °C). Low standard deviation values (0.015–0.03) between the two variants allowed differentiation in a high-resolution difference, as shown in the normalized melting and difference curve of HRM profile (Figure 1).

Table 2. The melting temperature (T_m) of D614 and G614 variant amplicon.

SARS-CoV-2 Variant	Sample ID	Tm (°C)	
		Mean	SD
D614	3.09	79.39	0.03
G614	3.13	79.62	0.015

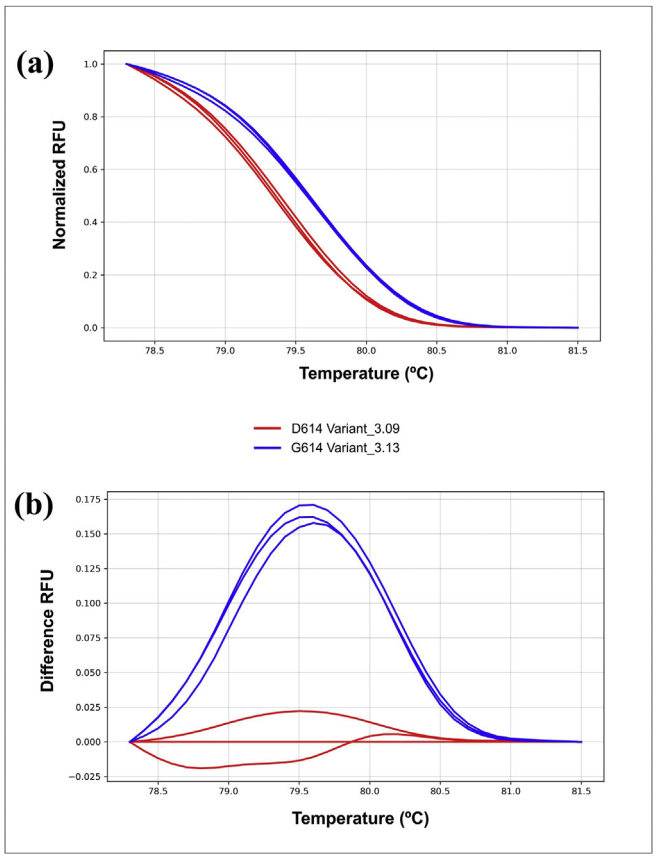

Figure 1. HRM profiles of D614 variant (Sample ID: 3.09) and G614 variant (Sample ID: 3.13). HRM profiles showed that D614 and G614 variants clustered distinctively, both by the visualization of the normalized melting curve (a) and difference curve (b).

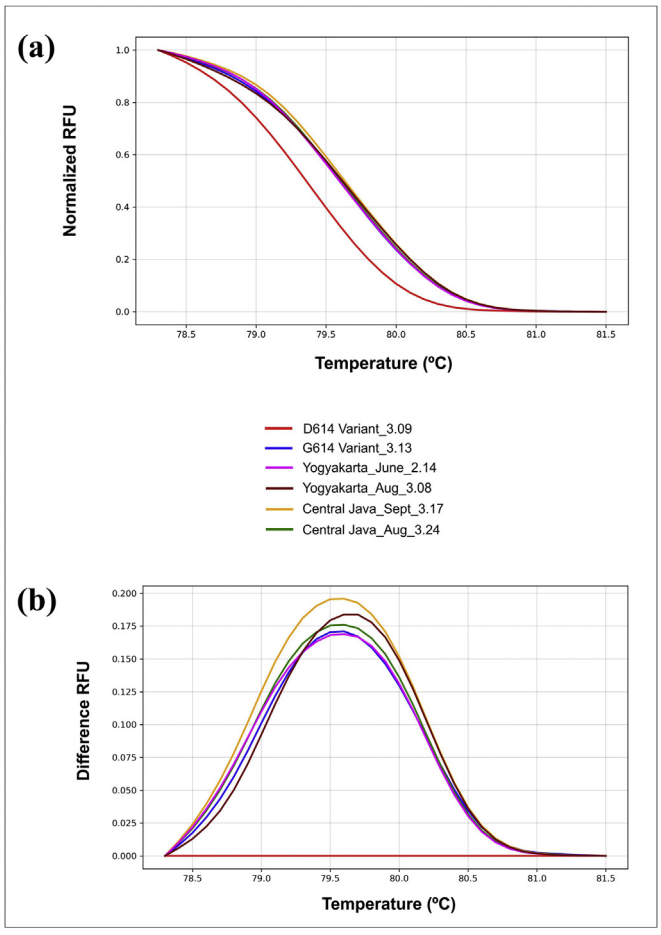

Figure 2. HRM profiles of unidentified-variant samples. HRM profiles showed that all four unidentified-variant samples (Sample ID: 2.14, 3.08, 3.17, 3.24) were identified as the G614 variant, as they clustered together with our G614 variant reference strain (Sample ID: 3.13), clearly indicated in both the normalized melting curve (a) and the difference curve (b).

3.2. qPCR-HRM analysis of unidentified-variant specimens

A total of two specimens from Central Java (Sample ID: 3.24 and 3.17) and two from Yogyakarta (Sample ID: 2.14 and 3.08), collected from June to September 2020, were analyzed by the same protocol as described in the previous section. Furthermore, specimens 3.09 and 3.13 were used as reference strains of the D614 and G614 variants, respectively. The HRM analysis showed that all four unidentified-variant specimens were identified as G614 variants, as they clustered with the G614 variant reference strain (Sample ID: 3.13) (Figure 2).

3.3. Sequence read of HRM-Analyzed samples

All four samples that have been detected as the G614 variant by HRM analysis were sequenced using the Sanger Method. The sequencing results showed and confirmed the HRM analysis that all samples were identified as G614 variant by the presence of missense mutation A to G at position 23403 (NCBI Reference Sequence Wuhan-Hu-1/NC_045512.2 = 23403A > G) which causes changes in the amino acid, Aspartic Acid (D) into Glycine (G) at the Spike residue 614 (D614G) (Figure 3).

4. Discussion

This study described the qPCR-HRM analysis method as a sensitive method that can reliably detect D614G mutation in the S gene of SARS-CoV-2. A primer pair was designed (F3.CoV-2.S and R8.CoV-2.S), which amplified 172 bp-length regions containing the hotspot mutation of D614G within the *S* gene. Furthermore, HRM analysis of reference samples, the D614 variant (Sample ID = 3.09) and the G614 variant (Sample ID = 3.13) showed different melting temperatures (T_m). The G614 variant was slightly higher (79.62 ± 0.015 °C) than the D614 variant (79.39 ± 0.03 °C) (Table 2). The G614 variant carried the missense mutation 23403A > G (Reference Sequence = NC_04512.2), which allowed the higher melting temperature, as Guanine (G) base needs 3 hydrogen bonds to form a base pair, whereas Adenine (A) base needs only 2 hydrogen bonds [27]. This created two distinct plots, which can differentiate the D614 and G614 variants in a high-resolution normalized melting and difference curve (Figure 1).

To validate this method, four additional samples were examined by qPCR-HRM analysis after cDNA synthesis. All samples were identified as G614 variants (Figure 2) as they were all clustered with the G614 variant reference sample and this was confirmed by the sequencing results (Figure 3). Furthermore, the qPCR-HRM method was proposed and is capable of detecting the D614G mutation by reliably distinguishing the D614 and G614 variants. A similar study examining the main clades of SARS-CoV-2 also proved that HRM analysis can effectively differentiate between variants of SARS-CoV-2 [18]. The other study described that HRM analysis also can rapidly differentiate Influenza A virus subtypes [28]. Therefore, this study suggests that a qPCR-HRM method is a powerful tool for high-throughput screening and epidemiological study of circulating SARS-CoV-2 variants in a specific location, as this technique is fast, efficient, and cost-effective, compared with the time-consuming and high-cost sequencing-based method.

Surveillance for the D614G mutation is important because the variant that contains this mutation, or the G614 variant, is more transmissible

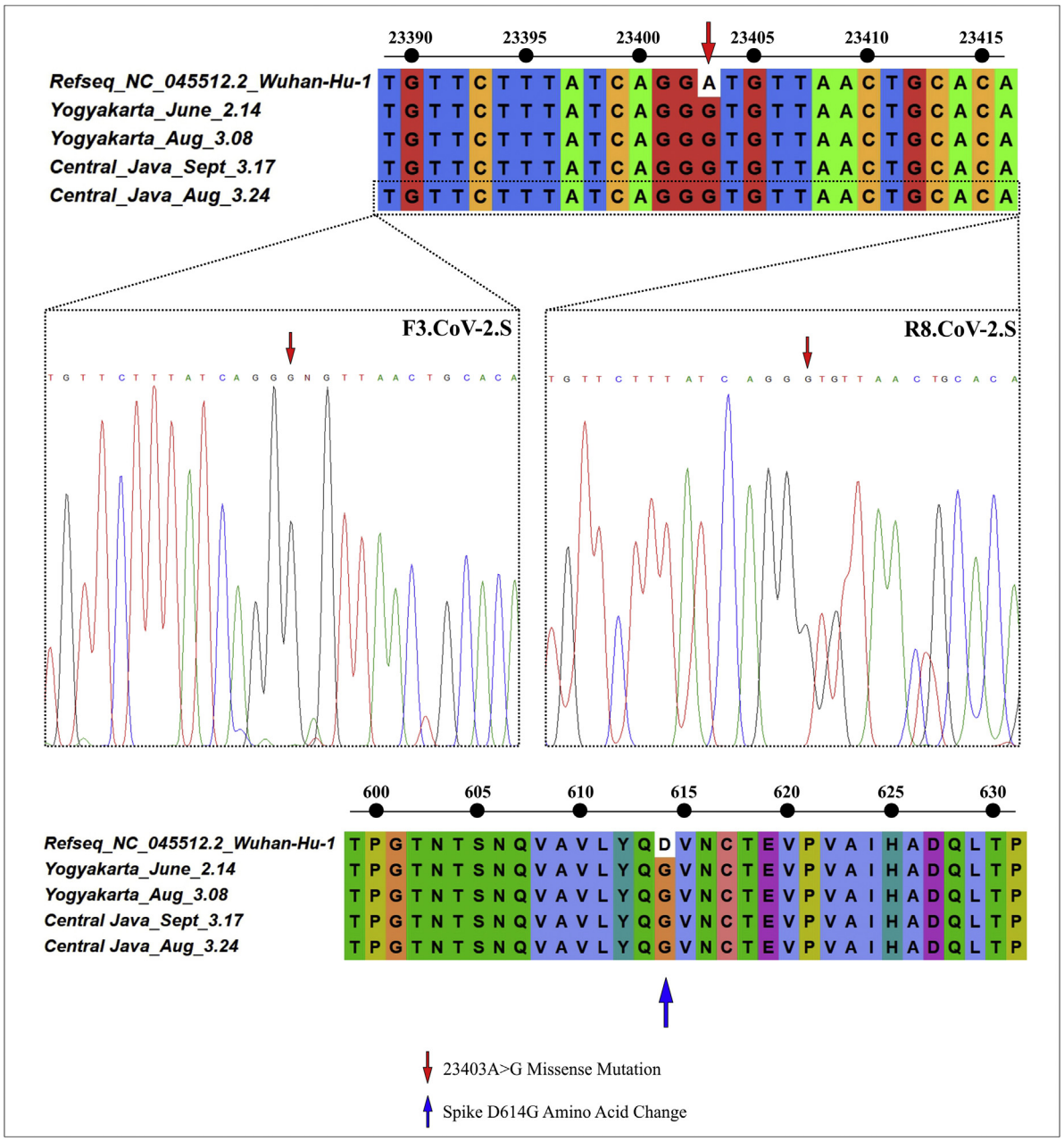

Figure 3. Sequence reads of HRM-analyzed samples. All four unidentified-variant specimens (Sample ID: 2.14, 3.08, 3.17, 3.24) which have been identified as the G614 variant by the qPCR-HRM analysis were confirmed as the G614 variant by Sanger sequencing.

and has become the dominant variant of SARS-CoV-2 that has been circulated around the world, including Indonesia [5, 6]. Compare with other countries like Costa Rika, which was dominated by the G614 variant by 98.9%, the prevalence of the G614 variant detected in Indonesia was only 65% in September 2020 [5, 29]. In addition, all variants of concerns (VOCs) of SARS-CoV-2, which are considered to be more transmissible, pathogenic, and immune-resistant, were generated from the G614 variant lineage [30, 31, 32, 33]. Therefore, the qPCR-HRM method might help to rapidly and efficiently monitor the spread of this G614 variant in a specific area. For further study, this qPCR-HRM method can be improved by designing primers for specific mutations of certain SARS-CoV-2 variants, including the VOCs, thereby highlighting the importance of the qPCR-HRM method for SARS-CoV-2 variants surveillance.

However, this qPCR-HRM assay has several limitations. Any further mutation in the target area can affect the melting temperature and lead to misinterpretations. Surveillance of the mutations by DNA sequencing should be performed regularly to determine whether there are other mutations besides D614G, which might affect the HRM results. The development of the one-step RT-qPCR method for HRM analysis can improve efficiency and time, as well as reduce the risk of cross-contamination.

In conclusion, this study suggests that the D614G mutation of SARS-CoV-2 Spike protein can be detected by the qPCR-HRM assay, which will help to rapidly trace the circulating SARS-CoV-2 variants in certain locations.

Declarations

Author contribution statement

Faris Muhammad Gazali: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Matin Nuhamunada: Conceived and designed the experiments; Wrote the paper.

Rahma Nabilla: Performed the experiments; Analyzed and interpreted the data.

Endah Supriyati, Edwin Widyanto Daniwijaya: Performed the experiments; Wrote the paper.

Mohamad Saifudin Hakim, Eggi Arguni, Titik Nuryastuti, Sofia Mubarika Haryana: Conceived and designed the experiments; Wrote the paper.

Tri Wibawa: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nastiti Wijayanti: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

The data will be published in the GISAID and/or NCBI after the manuscript is published.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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