# Rapid, inexpensive methods for exploring SARS CoV-2 D614G mutation

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A B S T R A C T

A common mutation has occurred in the spike protein of severe acute respiratory syndrome coronavirus 2 (SARS CoV-2), known as D614G (A23403G). There are discrepancies in the impact of this mutation on the virus's infectivity. Also, the whole genome sequencings are expensive and time-consuming. This study aims to develop three fast economical assays for prompt identifications of the D614G mutation including Taqman probe-based real-time reverse transcriptase polymerase chain reaction (rRT PCR), an amplification refractory mutation system (ARMS) RT and restriction fragment length polymorphism (RFLP), in nasopharyngeal swab samples. Both rRT and ARMS data showed G614 mutants indicated by the presence of HEX probe and 176 bp, respectively. Additionally, the results of the RFLP data and DNA sequencings confirmed the prevalence of the G614 mutants. These methods will be important, in epidemiological, reinfections and zoonotic aspects, through detecting the G614 mutant in retro-perspective samples to track its origins and future re-emergence of D614 wild type.

## 1. Introduction

Since its first emergence in Wuhan, China in late December 2019, severe acute respiratory syndrome coronavirus 2 (SARS CoV-2) which leads to coronavirus disease 2019 (Covid-19), has been considered as a pandemic since March 2020 by World Health Organisation (WHO). The disease has caused approximately 107.8 million global infections and 2.37 million deaths by 12 February 2021 (COVID-19 Dashboard by the Centre for Systems Science and Engineering (CSSE) at Johns Hopkins University (JHU) https://coronavirus.jhu.edu/map.html). The genome of SARS CoV-2 contains several genes encoding structural proteins such as spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Ahmadpour et al., 2020). SARS CoV-2 interacts with angiotensin-converting enzyme 2 (ACE2) of human cells through its spike proteins, spike 1 (S1) and spike 2 (S2) (Walls et al., 2020), which are cleaved, by human type II transmembrane serine protease (TMPRSS2) and furin, to facilitate the viral envelope fusion with the targeted human cell membrane (Ahmadpour et al., 2020; Walls et al., 2020). It is worth mentioning that S1 protein has three main domains: C-terminal domain (CTD), receptor-binding domain (RBD) which binds to human ACE2, and N-terminal domain (NTD) (Ahmadpour et al., 2020).

However, SARS CoV-2 has proofread mechanisms for correcting its RNA replication errors, nevertheless, mutations occur in its genome leading to an increase in viral survival adaptations (Pachetti et al., 2020; Phan, 2020; Robson et al., 2020; Romano et al., 2020). One of the most common mutations occurred, since its emergence, has been recognized as D614G (A23403) at amino acid number 614 in the spike protein sequence (nucleotide sequence number 23403) of the reference Wuhan SARS CoV-2 genome, when nucleotide A in G**A**T encoding aspartate residue (D) altered to G creating G**G**T, a codon for glycine (Badua et al., 2020). The D614G mutation is located in the NTD of S1 that lies between RBD and S2 near the cleavage site (Bhattacharyya et al., 2020), where both S1 and S2 are cleaved by TMPRSS2 and furin (Ahmadpour et al., 2020; Walls et al., 2020). Therefore, this mutation may enhance the viral infectivity of SARS CoV-2 by increasing the attachment capability of the RBD to human ACE2 via decreasing interactions between S1 and S2 (Gupta et al., 2020).

Since the emergence of the SARS CoV-2 D614G mutation, tracing back to January 2020 from China to Europe (Xu et al., 2020), and it is currently highly prevalent in all continents of the world (Bhattacharyya

et al., 2020; Gómez-Carballa et al., 2020), there have been controversial studies on impacts of this single nucleotide variation (SNV), D614G, on the viral survival fitness (Isabel et al., 2020; Kim et al., 2020; Omotuyi et al., 2020; Zhang et al., 2020), immunogenicity and antigenic epitopes (Gupta et al., 2020; Hernández-Huerta et al., 2020; Islam et al., 2020; Kim et al., 2020; Koyama et al., 2020; Saha et al., 2020; To et al., 2020), antibody neutralizing sensitivity (Garcia-Beltran et al., 2020; Goldman et al., 2020; Hu et al., 2020; Klumpp-Thomas et al., 2020; Li et al., 2020; Mansbach et al., 2020; Plante et al., 2020), infectivity (Daniloski et al., 2020; Hu et al., 2020; Korber et al., 2020; Li et al., 2020), transmission (van Dorp et al., 2020) and fatality (Hernández-Huerta et al., 2020). The studies have mainly focused on bioinformatic simulation models, and sequence alignments comparing with other coronavirus genomes with the available SARS CoV-2 whole viral genome sequences. They predicted spike protein destabilizing effect of the D614G mutation, leading to rapid S1 detachment with S2 causing more ACE2 attachments. Few studies have conducted investigations of D614G mutation on in vitro viral pseudotype infected cells (Daniloski et al., 2020; Hou et al., 2020; Hu et al., 2020; Korber et al., 2020; Li et al., 2020; Wang et al., 2020), laboratory animal models (Plante et al., 2020), and infected populations, suggesting increases in infectivity, viral loads (Korber et al., 2020; Yurkovetskiy et al., 2020; L. Zhang et al., 2020), and fatality (Hernández-Huerta et al., 2020). Overall, data indicated the viral adaptability to human cells as a result of D614G mutation. However, few studies have considered this single mutation outside RBD as a consequence of random mutations (Dearlove et al., 2020; van Dorp et al., 2020; Grubaugh et al., 2020; Isabel et al., 2020). Thus, these discrepancies should be elucidated to see whether the mutation is due to viral fitness or a random process (Korber et al., 2020).

On one hand, no adequate cohort clinical data have been obtainable for finding causality or even associations between this mutation and Covid-19 patients' severity. On the other hand, sufficient whole genome sequences might be available only in countries with highly developed genome services. However, whole genome sequencings are expensive and time-consuming. Moreover, there should have been plethora of SARS CoV-2 positive nasopharyngeal samples stored in developing countries without being sequenced due to lack of whole genome sequence facilities. Thus, retro-perspective studies are required to discover the origin of the virus imported into those countries.

Therefore, developing inexpensive and rapid methods for identifying SNVs, such as D614G, are essential for tracking this variant by epidemiologists, molecular virologists or immunologists collaborating with clinicians to compare Covid-19 patients with SARS CoV-2 D614 and G614 variants. A previous study mistakenly developed an RFLP method for identifying another mutation at residue 615 of the spike protein (Hashemi et al., 2020), but not D614G as commented by Niranji and Al-Jaf (Niranji and Al-Jaf, 2021). A study highlighted the necessity of SARS CoV-2 D614G mutant using biosensing and restriction enzyme methods including *Bts*CI endonuclease which can cleave the wild type D614 but do not cut G614 mutant (Zhang et al., 2021). Several SARS CoV-2 lineages have been circulating in the world (Cella et al., 2021). A recent research in Uganda has found an emergence of SARS CoV-2 lineage A23.1 variant, which contains the D614 wildtype residue, indicated circulating of this variant in the local regions (Bugembe et al., 2021).

Therefore, the purpose of this study is to develop three various methods such as Taqman probe-based rRT PCR, ARMS and RFLP to detect SARS CoV-2 D614G mutation in clinical nasal swab samples taken from Covid-19 patients. Furthermore, DNA sequencing was used for approving the validity of the methods.

## 2. Materials and methods

### 2.1. Sample collection and study area

Three (3) ml viral transport medium (VTM) containing nasopharyngeal swab samples were collected in Covid-19 clinically suspected individuals ($n = 67$) at Coronavirus Research and Identification Lab in the University of Garmian in Kalar town, Sulaymaniyah province, Kurdistan region of Iraq from June to October 2020. The VTM samples were preserved on ice or 4 °C or −85 °C until viral nucleic acids were extracted. Written consent forms were taken from the covid-19 suspected persons and the study was ethically approved by an ethical committee, which is adhered to WHO Guidelines on Ethical Issues in Public Health Surveillance and to the principles of the Declaration of Helsinki, at the department of biology, University of Garmian.

### 2.2. Viral RNA extraction

Sample processing was handled according to WHO standards under biological safety Level 2 using personal protection equipment (PPE) and biological safety cabinet (Labconco, Kansas City, MO, USA). Total viral RNA was extracted from the VTM preserved nasal swab samples using AddPrep Viral Nucleic Acid Extraction Kit (AddBio, Korea). According to the manufacturer's instructions, 200 μl of the VTM stored samples mixed with 350 μl lysis buffer and 3.5 μl β-mercaptoethanol (14.2 M), in a 1.5 ml microcentrifuge tube. After 10 min of incubating the lysed mixture, 150 μl of Isopropanol was added and this was followed by two successive washing steps, using 500 μl washing 1 and 2 solutions in a spin column, centrifuged for 13,000 rpm for 1 min. Finally, the bound RNA was eluted with 50 μl elution buffer and the purified RNA samples were kept at 4 °C for short-term storage or − 85 °C deep freeze for long period preservations.

### 2.3. Detection of SARS CoV-2 RNA by rRT PCR

The RNA extracted samples were inspected for SARS CoV-2 using genesig® Real-Time PCR assay (PrimerdesignTM Ltd., UK). The assay has been originally designed to be one step rRT PCR for making both cDNA synthesis from viral RNA and amplification of the cDNA using primer-probes, specific for SARS CoV-2 RdRp, developed in a single-step reaction. According to the manufacturer's instructions, a mixture of 10 μl Oasig™ OneStep 2× RT-qPCR master mix and 2 μl primer-probe mix was prepared. The prepared RT qPCR mixture was mixed with either 8 μl RNA, negative or positive controls, amplified using CFX Connect Real-Time PCR Detection System (Bio-Rad, Germany) at the following amplification programs: reverse transcription at 55 °C for 10 min, initial denaturation at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 60 s.

### 2.4. Detection of SARS CoV-2 D614G mutations

SARS CoV-2 RNA samples, identified by Gensig rRT PCR kit, with Cq values <30, were selected for detection of the D614G mutation by using Taqman probe-based rRT PCR, ARMS, and RFLP.

#### 2.4.1. Taqman probe-based rRT PCR

Forward and reverse primers (D614G IN F and D614G IN R), designed to cover both sides of the D614G mutation (A23403G), with a product size of 169 bp, from the Wuhan strain (GenBank: MN908947.3), were carefully inspected using NCBI online database for checking melting temperatures, GC contents, lengths, product sizes and locations of the primers. Taqman probes including D614 D-FAM and G614 G-HEX (Macrogen, South Korea) were designed to be specific for each wildtype Adenine (A) and mutant Guanine (G), respectively, as shown in Table 1 and Fig. 1B. The lyophilized primers and probes were reconstituted with RNase/DNase Free Water to 100 μM which was further diluted to 10 μM. Ten (10) μl of Addprobe rRT PCR master mix (Addbio, South Korea) was mixed with 0.5 μl (10 μM) of each primer (D614G IN F and D614G IN R) and probes (D614 D-FAM and G614 G-HEX) followed by adding 8 μl of RNA, giving 20 μl total volume with 250 nM final concentrations of primer probes. The rRT PCR reactions were performed as follow: reverse transcription at 50 °C for 20 min, initial denaturation at 95 °C for 10

**Table 1**
Primers and probes for rRT PCR, ARMS and RFLP.

| Primers | Sequence | TM | GC % |
|---|---|---|---|
| D614G In F | GAGATTCTTGACATTACACCATG | 59 | 39 |
| D614G In R | CTGTAGAATAAACACGCCAAG | 59 | 43 |
| D614 D-FAM | FAM- TTCTTTATCAGG**A**TGTTAACTGCACAG-BHQ1 | 64 | 37 |
| G614 G-HEX | HEX- TTCTTTATCAGG**G**TGTTAACTGCACAG-BHQ2 | 65 | 41 |
| D614G Out F | AACAATTTGGCAGAGACATTG | 60 | 38 |
| D614G Out R | CTATTAAACAGCCTGCACGT | 60 | 45 |
| D614 ARMS mis A F | CAGGTTGCTGTTCTTTATCAaG**A** | 57 | 39 |
| G614 ARMS mis G R | AGGGACTTCTGTGCAGTTAAtA**C** | 58 | 43 |

min, followed by 50 cycles of denaturation at 95 °C for 10 s, and annealing at 61 °C for 60 s using CFX Connect Real-Time PCR Detection System (Bio-Rad, Germany).

### 2.4.2. Amplification-refractory mutation system PCR (ARMS-PCR)

Four primers were designed and used in a single tube multiplex reaction including D614G outer and ARMS specific primers as shown in Table 1 and Fig. 1C. Outer primers, D614G Out F and D614G Out R that amplify a PCR product size of 266 bp in which both A and G variants were located. D614G ARMS specific primers were designed as follows: D614 ARMS **A** F amplifies the wildtype **A** with the D614 Out R creating a 134 bp PCR product size. G614 ARMS **G** R amplifies the mutant **G** with the D614 Out F primer creating a 176 bp PCR product (Table 1 and Fig. 1C). ARMS primers were designed in a manner that the wild type **A** nucleotide is located at the 3'end of the forward primer while the mutant **G** nucleotide is located at the 3'end of the reverse primer. To reduce non-specific amplification, a nucleotide mismatch was introduced, just two nucleotides before the 3'end of each ARMS primer. The ARMS PCR reaction mixtures were as follows: 0.5 μl of ARMS primers (10 μM) added to 10 μl of Addscript RT PCR master mix (Addbio) and 8 μl RNA. The PCR program was set using conventional Lightcycler (Eppendorf, Germany) as follow: reverse transcription at 50 °C for 20 min, initial denaturation at 98 °C for 10 min, followed by 36 cycles of (denaturation at 95 °C for 15 s, annealing at 61 °C for 45 s, and extension 72 °C for 30 s) and then a final extension at 72 °C for 5 min.

### 2.4.3. RFLP method for D614G variant

This method was developed using NEB cutter (https://nc2.neb.com/NEBcutter2/index.php). Interestingly, *Bts*CI restrictions site (5'-GG**A**TGNN-3') was found in the wild type D614 (G**A**T) but not in G614 mutant (G**G**T). Thus, this enzyme was used to distinguish between D614 from G614 as the former nucleic acid sequence is cleaved but the latter remains uncut (Fig. 2A). Therefore, PCR products, amplified by D614G Out primers, were incubated with BtsCI restriction endonuclease at 50 °C for 30 min in BtsCI buffer and inactivated at 80 °C for 20 min as recommended by the manufacturer (New England Biolabs, Ipswich, MA, USA). Furthermore, to confirm the action of the enzyme, a positive control was exploited by amplifying a human cytochrome *b* gene using universal primers (Kocher et al., 1989) since its DNA sequence contains *Bts*CI restriction site (5'…NNCATCC…3') using the NEB cutter as shown in Fig. 2B.

### 2.4.4. DNA sequencings

Three (3) PCR products, amplified by conventional RT PCR using D614G Out primers, were randomly sent for Sanger sequencings (Macrogen Co., Seoul, KR), to confirm D614G mutants and the sequences were submitted to NCBI using Bankit (Benson et al., 2015).

## 3. Results

The current study has developed three methods including probe-based rRT PCR, ARMS and RFLP. For the first time, using fast, cost-effective methods, this study has identified G614 mutants in 67 naso-pharyngeal samples in Iraq.

### 3.1. Probe-based rRT PCR

In the primer-probe rRT PCR method, two primers (D614G IN forward and reverse) with two probes (D-FAM and G-HEX) were designed for detecting D614 and G614 variants of SARS CoV-2, respectively. The probes and primers worked in 250 nM final concentrations at 61 °C as shown in Fig. 3.

### 3.2. Arms PCR

The single plex reactions (Panel A) performed for D614 AF and Out R primers showed no PCR products (Fig. 4-A-1) that indicate wild type D614 variant is lacking. The second single plex reaction performed using G614 GR and Out F primers, a PCR product of approximately 176 bp indicates G614 mutant (Fig. 4-A-2). The third single plex reaction shows PCR products (266 bp) using Out F and Out R primers confirmed that the outer primers worked (Fig. 4-A-3). Multiplex ARMS PCR, using all primers in a single tube (Panel B), shows two PCR products with sizes of 176 bp and 266 bp, which indicated G614 mutant and outer PCR products, respectively. The results of ARMS PCR have corresponded with the rRT PCR data.

### 3.3. RFLP

Incubations of PCR products, amplified by D614G Out primers, with *Bts*CI enzyme, produced no cleavages indicated that only G614 mutants of SARS CoV-2 are prevalent in the region. The enzyme validity was checked by incubations with human cytochrome *b* PCR products amplified by universal primers as described in Section 2.4.3. The results showed that the BtsCI enzyme has cleaved the PCR products of the human cytochrome *b* (Fig. 5).

### 3.4. DNA squences

The DNA sequence results showed only G614 variants as Genbank accession numbers (MW405786, MW405787 and MW405788) were released in NCBI online database.

## 4. Discussions

In the current study, we developed three rapid and inexpensive techniques that can be used to discriminate between D614 and G614 variants. The methods included rRT PCR, ARMS and RFLP. Data obtained by these methods revealed that only G614 mutants are prevalent in the studied region. The results of all of the three methods are corresponding with each other and the DNA sequencing data. We believe our methods are advantageous over SARS CoV-2 whole genome sequences, particularly in developing countries for tracking the D614G mutation and its association with severity, infectivity, and fatality which have been controversial in various studies. It is worth remembering that the origin of the virus has not been found. However, WHO has been searching for the viral emergence in Wuhan suggested that the virus may have arisen from frozen wildlife or SARS CoV-2 related viruses of animals circulated outside China (Wacharapluesadee et al., 2021). The first SARS CoV-2 cases from Wuhan China were considered as an original wild type D614. Thus, the future re-emergence of the D614 is not unlikely. Therefore, reasonable diagnostic techniques are required for discovering future D614 outbreaks or tracking previous retro-perspective samples preserved in developing countries. For example in

## A) A part of amino acid sequences of D614

RVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNGLTGTGVLTESNKKFLPFQQFG
RDIADTTDAVRDPQTLEILDITPCSFGGVSVITPGTNTSNQVAVLYQDVNCTEVPVAI
HADQLTPTWRVYSTGSNVFQTRAGCLIGAEHVNNSYECDIPIGAGICASYQTQTNSPR

Wild type: G<u>A</u>T is codon for <u>D</u>614 (Aspartate)= A23403
Mutant: G<u>G</u>T is codon for <u>G</u>614 (Glycine)= G23403

## B) rRT PCR

                              D614G In F

23281 tgatgctgtc cgtgatccac agacactt**ga gattcttgac attacaccat g**ttcttttgg

23341 tggtgtcagt gttataacac caggaacaaa tacttctaac caggttgctg ttctttatca

23401 gg**A**tgttaac tgcacagaag tccctgttgc tattcatgca gatcaactta ctccta**cttg**
      <u>G</u>
            D614G In R

23461 **gcgtgtttat tctacag**gtt ctaatgtttt tcaaacacgt gcaggctgtt taataggggc

D614 FAM: FAM- TTCTTTATCAGG<u>A</u>TGTTAACTGCACAG- BHQ1
G614 HEX: HEX- TTCTTTATCAGG<u>G</u>TGTTAACTGCACAG- BHQ2

## C) ARMS-PCR

                              D614G Out F

23221 tgagtctaac aaaaagtttc tgcctttcca ac**aatttggc agagacattg** ctgacactac

23281 tgatgctgtc cgtgatccac agacacttga gattcttgac attacaccat gttcttttgg

                             D614 ARMS mis A F

23341 tggtgtcagt gttataacac caggaacaaa tacttctaac **caggttgctg ttctttatca**

23401 **ggA** <u>tgttaac tgcacagaag tccctgttgc tattcatgca gatcaactta ctcctacttg</u>
     <u>G</u>  G614 ARMS mis G R
                         D614G Out R

23461 gcgtgtttat tctacaggtt ctaatgtttt tcaaac**acgt gcaggctgtt taatag**gggc

**Fig. 1.** Locations of primers and probes in a specific region around D614G (<u>A</u>23403<u>G</u>) mutation in SARS CoV-2 isolate Wuhan-Hu-1, complete genome. GenBank: MN908947.3.
A part of amino acid sequences showing amino acid D614 which is mutated to G614 (A). Locations of rRT PCR Primers and probes (B). Locations of ARMS PCR primers (C).
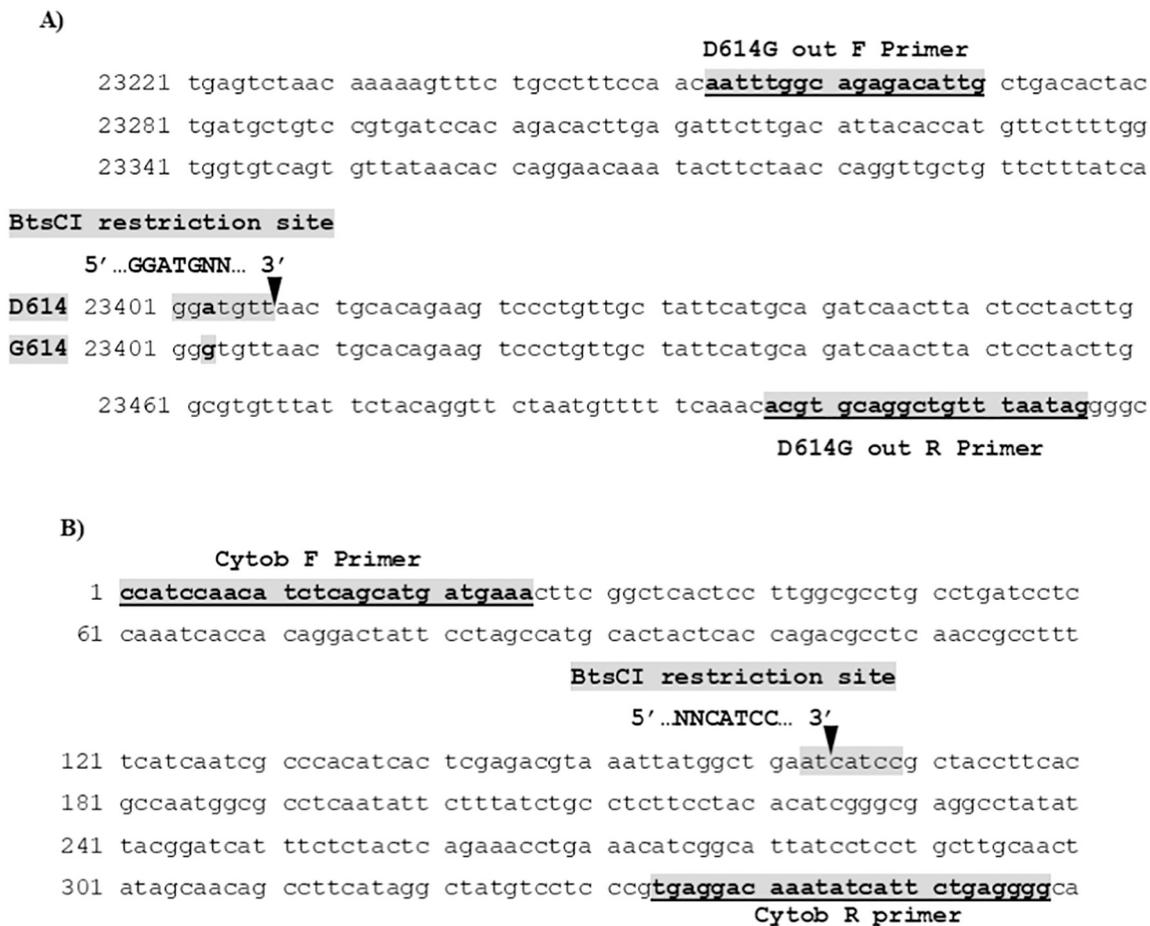
A)

```
                                              D614G out F Primer
    23221  tgagtctaac  aaaaagtttc  tgcctttcca  acaatttggc agagacattg  ctgacactac
    23281  tgatgctgtc  cgtgatccac  agacacttga  gattcttgac  attacaccat  gttcttttgg
    23341  tggtgtcagt  gttataacac  caggaacaaa  tacttctaac  caggttgctg  ttctttatca
```

BtsCI restriction site

         5'...GGATGNN... 3'
                        ▼
```
D614  23401  ggatgttaac  tgcacagaag  tccctgttgc  tattcatgca  gatcaactta  ctcctacttg

G614  23401  gggtgttaac  tgcacagaag  tccctgttgc  tattcatgca  gatcaactta  ctcctacttg

      23461  gcgtgtttat  tctacaggtt  ctaatgtttt  tcaaacacgt gcaggctgtt taataggggc
```
                                          D614G out R Primer

B)

         Cytob F Primer
```
    1  ccatccaaca tctcagcatg atgaaacttc  ggctcactcc  ttggcgcctg  cctgatcctc
   61  caaatcacca  caggactatt  cctagccatg  cactactcac  cagacgcctc  aaccgccttt
```

BtsCI restriction site

             5'...NNCATCC... 3'
                          ▼
```
  121  tcatcaatcg  cccacatcac  tcgagacgta  aattatggct gaatcatccg ctaccttcac
  181  gccaatggcg  cctcaatatt  ctttatctgc  ctcttcctac  acatcgggcg  aggcctatat
  241  tacggatcat  ttctctactc  agaaacctga  aacatcggca  ttatcctcct  gcttgcaact
  301  atagcaacag  ccttcatagg  ctatgtcctc  ccgtgaggac aaatatcatt ctgaggggca
```
                                        Cytob R primer

**Fig. 2.** DNA sequencings of both SARS CoV-2 D614G and human cytochrome *b* used for RFLP: Panel (A) Nucleic acid sequences of SARS CoV-2 shows restriction site (GGATGNN) in the wild type D614 which is cleaved by *Bts*CI enzyme while G614 mutant sequence is not cut by the enzyme. Both D614G Out F and R primers are also located. Panel B) Nucleic acid sequences of human cytochrome *b* gene shows restriction site (5'…NNCATCC…3') which is cleaved by BtsCI enzyme. Both universal F and R primers are also located.
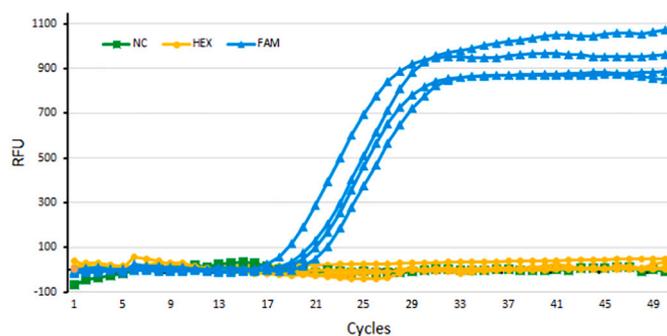


**Fig. 3.** Amplification curves using AddProbe rRT PCR master mix (AddBio) using D614G IN primers and probes including D614 D-FAM and G614 G-HEX probes. Only HEX (blue) showed amplification curves indicating the G614 mutant, but FAM (yellow) did not form amplification curves indicating that wild type D614 is absent. NC (green) is a negative control.

Iraq, a whole genome sequencing has been performed for only one sample concluding for discovering the G614 mutation that may help to understand the spread of the virus (Al-Rashedi et al., 2021). Therefore, retro-perspective studies for hunting both D614 and G614 are an area of interest in developing countries.

Previous in vitro and simulation studies have found that G614 mutation is considered as more infectious variant than the D614 wildtype (Daniloski et al., 2020; Hou et al., 2020; Hu et al., 2020; Korber et al.,

2020; Li et al., 2020; Wang et al., 2020). Studies showed that D614G mutation is associated with antigenic epitopes (Gupta et al., 2020; Hernández-Huerta et al., 2020; Islam et al., 2020; Kim et al., 2020; Koyama et al., 2020; Saha et al., 2020; To et al., 2020), antibody neutralizing sensitivity (Goldman et al., 2020; Hu et al., 2020; Klumpp-Thomas et al., 2020; Li et al., 2020; Mansbach et al., 2020), infectivity (Daniloski et al., 2020; van Dorp et al., 2020; Hu et al., 2020; Korber et al., 2020; Li et al., 2020), transmission (van Dorp et al., 2020) and fatality (Hernández-Huerta et al., 2020). Plante et al., 2020 have suggested that SARS CoV-2 G614 variant has roles in increasing upper respiratory viral loads, transmissions and survival fitness of the virus (Plante et al., 2020). To explore whether this SNV was as a result of either founder effects or viral fitness, caused by random mutation in the viral genome, larger clinical data could be linked with this SNV by comparing SARS CoV-2 disease severity with each D614 or G614 subtypes.

The global vaccines against SARS CoV-2 have been designed using the wildtype D614 virus. One of the most problematic features, which play roles in succeeding vaccines and monoclonal antibodies, is viral SNVs that make the virus resist neutralizing antibodies produced by the vaccines or immune-therapeutics. Therefore, some vaccines or designed antibodies may not be effective against all SARS CoV-2 variants. Thus, identifications of SARS CoV-2 SNVs may help researchers to examine vaccination programs and immunological drugs (Fernández, 2020a, 2020b), before vaccine or drug trial steps.

There have been controversies around the impacts of D614G mutations on vaccine developments. For instance, in a study that used both in
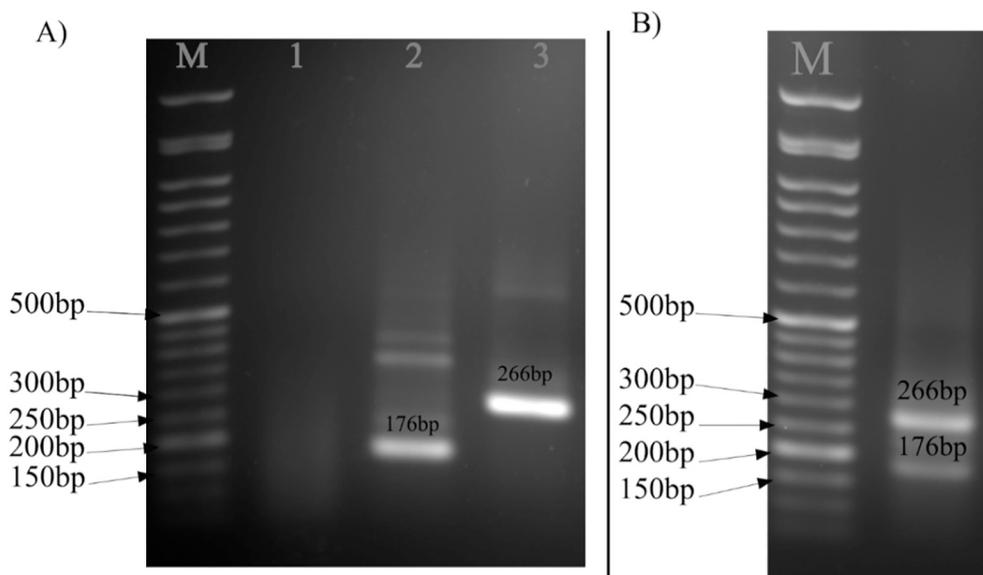
**Fig. 4.** PCR products on 1.5% agarose gel electrophoresis: Addscript RT PCR master mix (AddBio) using ARMS PCR primers. Panel A: PCR products using primers separately. Well 1: PCR products using D614 AF and Out R primes. Well 2: PCR products using G614 GR and Out F primers generating 176 bp. Well 3: PCR products using Out F and Out R primers creating 266 bp. Panel B: an example of a PCR Product using all primers D614 AF, G614 GR, Out F and Out R in a single tube reaction that created 176 bp (for G614 mutant) and 266 bp (for outer primers).
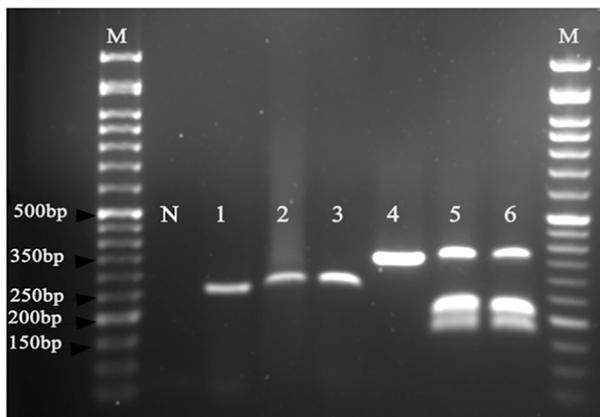


**Fig. 5.** PCR products on 1.5% agarose gel electrophoresis using Addscript RT PCR Master mix (AddBio) and ARMS PCR primers. M = DNA marker 50 bp. N = negative control. Lane 1 = undigested PCR product amplified by D614G Out primers. Lanes 2 and 3 = PCR products amplified by D614G Out primers and incubated with BtsCI enzyme at 50 °C. Lane 4 = undigested human cytochrome *b* PCR products amplified by universal primers. Lanes 5 and 6 = the human cytochrome *b* PCR products were cleaved by the BtsCI enzyme.

vitro experiments and structural modelling, G614 has been shown to have no effects on vaccines (McAuley et al., 2020). However, it has been revealed that SARS CoV-2 mutations impact the viral spike antigenic epitopes which are normally recognized and neutralized by antibodies produced by B- lymphocytes (Fernández, 2020b).

A limitation of this study was the absence of the D614 wild type subtype in the area where SARS CoV-2 has been spreading. However, in our study, three assays, as Taqman probes, ARMS, and RFLP all validated each other, in addition to the DNA sequencing data. For instance, if a D614 strain is available, the D614 probe in the rRT-PCR assay may cross-react with that the G614 probe to give a positive result for a D614 strain. To address this concern, the RFLP method can validate both rRT PCR and ARMS-PCR methods before they can be widely deployed.

Another point of caution, the D614 vs G614 strains are usually encoded by GAT vs GGT. However, D can also be encoded by GAC and G can also be encoded by GGA, GGC, and GGG. While such mutations are highly unlikely in the context of retrospective studies, for screening the mutants, it is possible that this could lead to misleading results. To

overcome this, some samples could be sent for sequencing by using Out primers used in this study. Additionally, the researchers should be reminded that our methods are actually testing for the A23403G nucleotide mutation, which mostly encodes the D614G amino acid mutation.

Co-segregations of SNVs are also essential for future understanding of the roles of viral pathogenesis and host immune responses against the virus. For instance, identifications of common co-occurred mutations in SARS CoV-2 spike proteins at a particular population would overcome problems of the viral antibody resistance and host cell binding capacity of the virus. By 12 February 2021, the D614G (A23403G) is the most prevalent single nucleotide variations (SNVs) in the world that is about 439,559 sequences (95.5%). Other common mutations in the spike (S) protein, including A222V (C22227T), L81S (C21614T), and S477N (G22992A), are co-occurred with D614G in 100,401 sequences (21.8%), 45,943 sequences (10%) and 23,426 sequences (5.1%), respectively (Covid19 CG data: https://covidcg.org/?tab=group). Moreover, a UK variant, N501Y, has been emerged since October 2020 and currently spreading in the country (Leung et al., 2021) and has become a public threat in more than 30 countries (WHO, 2020). Therefore, future work should concentrate on these four mutations to see whether their co-occurrences with D614G are associated with disease severity, infectivity, transmissions, and antibody sensitivity.

The virus may have 'spilled over' from animals to humans led to the pandemic and the animal reservoirs could play roles in the emergence of the disease (Parolin et al., 2021). Moreover, mutations play roles in zoonotic transmissions from animals to humans and vice versa. A recent study has shown that mammals such as mink harbour SARS CoV-2 mutations, which make the virus resistant to neutralizing antibodies from recovered patients (Oude Munnink et al., 2020). However, minks been have naturally infected with both D614 and G614 variants of SARS CoV-2, no evidence shows the impact of G614 mutations in zoonotic outbreaks in this species of mammals. Nonetheless, this suggests that mink and other mammals could have been reservoirs for both D614 and G614 variants that their outbreaks might occur in the future. Further studies using large numbers of samples are necessary to discover this effect. Besides, re-infections with SARS CoV-2 are also rare in the world (Goldman et al., 2020; To et al., 2020) and it is also essential to study the roles of mutations including G614 variants in re-infections. Rapid and cost-effective methods are also essential to study the roles of mutations in zoonosis, animal reservoirs and re-infections. We have recently developed methods for detecting N501Y mutation (Al-Jaf and Niranji, 2021), which can be used with the methods of the current study to

explore SARS CoV-2 reinfections (Niranji and Al-Jaf, 2021).

## 5. Conclusions

Up to our best knowledge, this is the first assay developed specifically for exploring SARS CoV-2 D614G mutation using specific primers and probes for detecting D614G mutant by using real-time, conventional PCR and restriction endonuclease. This will help developing countries to conduct further research on this mutation concerning the origin of the virus using previously preserved samples and re-infections that occurred between the pandemic waves. Future research should focus on other common co-segregated mutations that occurred SARS CoV-2 genome.

## Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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