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Research paper

Development of a PCR-RFLP method for detection of D614G mutation in SARS-CoV-2

Seyed Ahmad Hashemi [a], Amirhosein Khoshi [b], Hamed Ghasemzadeh-moghaddam [b], Majid Ghafouri [a], Mohammadreza Taghavi [a], Hasan Namdar-Ahmadabad [b], Amir Azimian [b,*]

[a] Department of Infectious Diseases, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran
[b] Department of Pathobiology and Laboratory Sciences, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran

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ABSTRACT

In late 2019, an outbreak of respiratory disease named COVID-19 started in the world. To date, thousands of cases of infection are reported worldwide. Most researchers focused on epidemiology and clinical features of COVID-19, and a small part of studies was performed to evaluate the genetic characteristics of this virus. Regarding the high price and low availability of sequencing techniques in developing countries, here we describe a rapid and inexpensive method for the detection of D614G mutation in SARS-CoV-2. Using bioinformatics databases and software, we designed the PCR-RFLP method for D614G mutation detection. We evaluated 144 SARS-CoV-2 positive samples isolated in six months in Northeastern Iran. Our results showed that the prevalent type is S-D in our isolates, and a small number of isolated belongs to the S-G type. Of 144 samples, 127 (88.2%) samples have belonged to type S-D, and 13 (9%) samples typed S-G. The first S-G type was detected on 2020 June 10. We have little information about the prevalence of D614G mutation, and it seems that the reason is the lack of cheap and fast methods. We hope that this method will provide more information on the prevalence and epidemiology of D614G mutations worldwide.

1. Introduction

The novel *Coronaviridae* member named SARS-CoV-2 resulted in COVID-19 disease. From 2019 to date, this disease has spread in most parts of the world and had become a significant challenge for the World Health Organization. Many studies are running on clinical outcomes, epidemiology, and co-infections of this virus with other microorganisms. Some researchers evaluated and compared the whole genome sequence of SARS-CoV-2 isolated in various parts of the world and identified some mutations. Regarding the proteins encoded by mutant genes, they assumed that the mutations could affect the infectivity of this virus. The high-frequency mutations of the SARS-CoV-2 genome were seen in nsp6, RNA polymerase, helicase, membrane glycoprotein, RNA primase, nucleocapsid phosphoprotein, and spike protein genes (Yin, 2020). One of the most critical mutations is D614G in the spike protein gene. This mutation leads to a change of aspartate to glycine. Studies showed that S-G614 mutants are more infective than S-D614 strains due to the high transmission efficacy (Hu et al., 2020). The S protein of coronaviruses is the main factor of host and tissue tropism and also is a significant target of viral entry inhibitors, neutralizing antibodies, and vaccines (Du et al., 2009; Hoffmann et al., 2020). The S protein is cleaved to S1 and S2 subunits by host proteases. S1 acts for receptor binding and S2 for membrane fusion and entrance to host cells. Multiple proteases include transmembrane serine protease 2, cathepsin B/L, and furin, are critical for S protein cleavage and cell entrance (Hu et al., 2020). Recently researchers found a new serin protease, called elastase-2, cleavage site in S-G614 mutants (Bhattacharyya et al., 2020). It leads to an increase in enzymatic cleavage efficiency and enhances infectivity (Hu et al., 2020). From the beginning of the COVID-19 epidemic, many articles published on the evaluation of D614G mutation in SARS-CoV-2 (Bhattacharyya et al., 2020; Biswas and Majumder, 2020; Gong et al., 2020; Hu et al., 2020; Isabel et al., 2020; Korber et al., 2020a; Korber et al., 2020b; Maitra et al., 2020; Yin, 2020). Almost in all works, researchers worked on previously published SARS-CoV-2 genome sequences in data banks such as NCBI and GISAID database, or their sequences. Although whole-genome sequencing is a sensitive and precise method, but is expensive

* Corresponding author at: Department of Pathobiology and Laboratory Sciences, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran.
E-mail address: amir_azimian2003@yahoo.com (A. Azimian).

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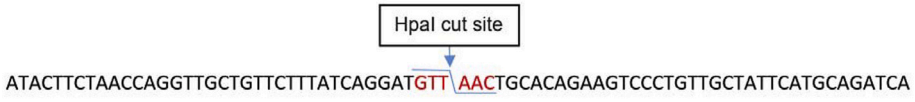

Fig. 1. The cut site of HpaI at the position 1845.

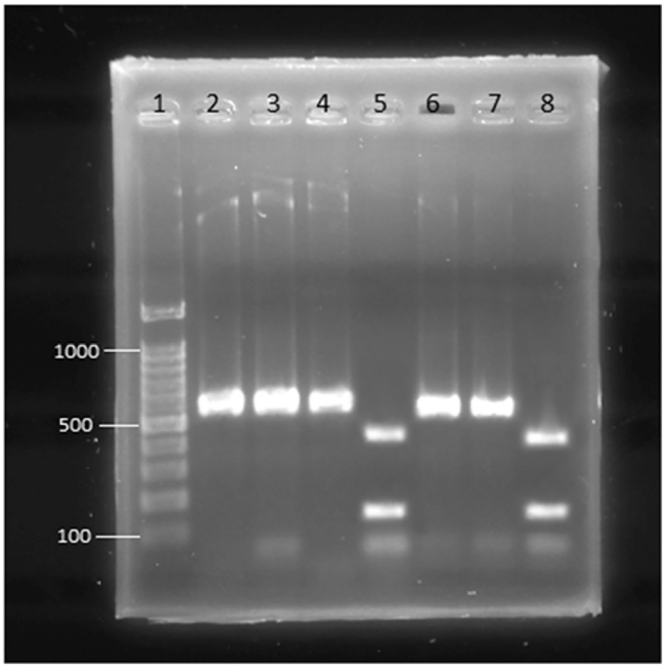

Fig. 2. Agarose gel electrophoresis of undigested and digested PCR products of S protein gene. Lane 1; 100 bp ladder, lane 2; 590 bp PCR product, lane 3, 4, 6 and 7; undigested PCR products, lane 5 and 8; digested PCR product to 433 and 157 bp pieces.

and puts researchers in a constraint on the number of samples. Given that D614G is the most important among all detected mutations, here we evaluated and optimized a fast and inexpensive method for detection of this mutation in SARS-CoV-2 in clinical samples.

2. Materials and methods

In the first step, we used the sequence of S protein of SARS-CoV-2, published in Gene bank with accession number MT252819.1, for appropriate restriction endonuclease selection and primer design. At position 1845 in the S-D type, the primary nucleotide is T that encode aspartate at position 614 of the amino acid chain. If the T to G mutation occurred at this position, then aspartate is replaced by glycine at position 614 of the amino acid chain, called S-G type.

At the next step, we evaluated the restriction endonucleases whose cleavage region covered the T-1845 at the S protein gene. Using the Gene runner software, *Hpa*I, target sequence GTTAAC, found as a suitable enzyme [Fig. 1]. Then we designed a primer pair that their product includes the cut site position. The forward sequence was 5- AATCTATCAGGCCGGTAGCAC -3, and the reverse was 5- CACCAATGGGTATGTCACACT −3. The PCR product size was 590 base pairs. If the T nucleotide is in position 1845, enzymatic digestion produces two pieces of 433 bp and 157 bp and, if the nucleotide G is in this position, digestion had no effect on PCR product, and the one 590 bp piece can be seen after agarose gel electrophoresis [Fig. 2]. Afterward, we selected a positive sample per day from patients admitted to the intensive care unit at intervals between 2020 March 5 and 2020 August 5, a total of 144 samples. The primary screening of positive samples was done using LightMix Modular SARS-CoV-2 probe and primers (TIB molbiol, Berlin, Germany)

Table 1
Components and concentrations in RT-PCR reaction.

Reagent	Primary Concentration	Volume
2.5× buffer	2.5 X	8 µl
Enzyme solution	20 X	1 µl
Forward Primer	0.2 pm	1 µl
Reverse Primer	0.2 pm	1 µl
Dw	–	3 µl
Template RNA	10–40 ng	6 µl
Final volume	–	20 µl

Table 2
RT-PCR program.

Amplification step	Temperature	Time
cDNA synthesis	50 °c	30 min
Primary denaturation	95 °c	10 min
Denaturation	95 °c	30 s
Annealing	60 °c	30 s
Extension	72 °c	60 s
Go to repeat	40 cycles	
Final Extension	72 °c	5 min

and addbio one-step RT master mix (ADD BIO INC, Daejeon, Republic of Korea) and also novel coronavirus (2019-nCoV) nucleic acid diagnostic kit (Sansure, China). At the next step, we performed PCR on SARS-CoV-2 positive samples using the AddScript RT-PCR kit (Addbio, Korea) as a manufacture recommendation [Tables 1,2]. After PCR amplification, we performed RFLP based on manufacturing protocol. In 20 µl reactions; 2 µl buffer 10×, 0.4 µl enzyme solution, 4.6 µl PCR product, and 13 µl DW were mixed and incubated at 37 °c for 10 min. Finally, electrophoresis performed using 10 µl of digestion products on 1.5% agarose gel. We sent 10 S-D and 10 S-G related PCR product for sequencing and confirmation of our results. After the alignment of sequences of 590 bp PCR product, we saw the T in S-D isolates and G in S-G isolates [Fig. 3].

3. Results

We selected positive samples from ICU admitted patients in six months. Of 144 samples, 127 (88.2%) samples have belonged to type S-D, and 13 (9%) samples typed S-G. It should be noted that the 4 (2.8%) samples had mixed bands related to both S-D and S-G types. We repeated the test on these samples and got the same results. The first S-G type was detected on 2020 June 10. after that, the number of S-G strains increasingly raised to date [Fig. 4].

4. Discussion

Within six months, SARS-CoV-2 spread rapidly around the world. Many efforts are made to develop vaccines or monoclonal antibodies against this virus. The viral spike protein is one of the best target molecules for this purpose. This protein is usually stable; nonetheless, some researchers found mutations in this protein (Walls et al., 2020). The most crucial mutation is a missense mutation in amino acid 614. This mutation converts aspartate to glycine, which is more easily breaks by proteinases such as elastase. The D614G mutation was first identified in Germany (Phan, 2020). After that, Becerra-Flores et al. concluded that the S-G strains are the more pathogenic form of the virus and lead to a higher fatality rate in patients (Becerra-Flores and Cardozo, 2020).

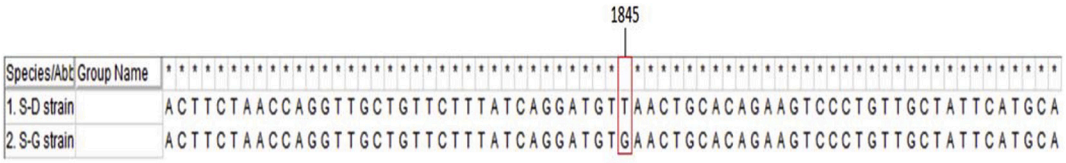

Fig. 3. Alignment of PCR product sequences. In the S-D strain detected with our method, nucleotide T were seen in the 1845 position and nucleotide G were seen at this position.

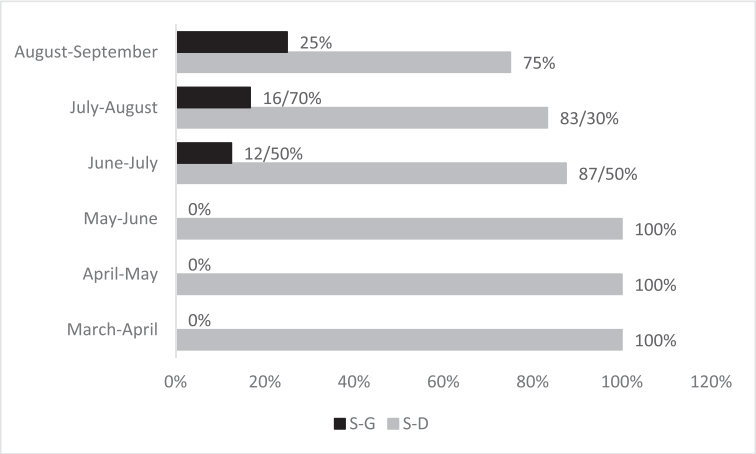

Fig. 4. Monthly prevalence of S-D and S-G types.

D614G mutants began expanding in Europe and rapidly became dominant species (Bhattacharyya et al., 2020). To date, reports around the world indicate an increase in the prevalence of D614G mutation. In previous reports, researchers evaluated the whole genome sequences published in databases such as GISAID, and in some papers, researchers worked on their sequences (Biswas and Majumder, 2020; Gong et al., 2020; Korber et al., 2020b; Maitra et al., 2020; Yin, 2020). The whole-genome sequencing is an expensive method and cannot be done for a large number of samples. Regarding this and also the importance of D614G in comparison to other mutations, here we evaluated the rapid and inexpensive PCR-RFLP method for the detection of this mutation. This method is cheap and rapid compared to sequencing methods, and it can do in many molecular laboratories with common facilities around the world. We evaluated the 144 samples of ICU admitted patients, in six months in North Khorasan, Iran. The results of this method were the same in different samples. Most of our strains (88.2%) have belonged to the S-D type, and a little part (9%) has belonged to the S-G type. Interestingly 4.8% of samples had a mix of both types. On the contrary, Bhattacharyya et al. reported that the dominant type of SARS-CoV-2 in Europe and China is the S-G (Bhattacharyya et al., 2020), while in our samples, the current type was S-D. Gong et al. reported the D614G mutation in Taiwanese patients and patients who had a history of travel to Europe, Turkey, and Iran (Gong et al., 2020). This study is vital for us because of the lack of data about D614G mutation in Iran. They reported SARS-CoV-2 clade S-G strains in patients returned from Iran. All of the evaluated samples in their report have been isolated in the first three months of 2020, while our first D614G mutant isolated in June. It should be noted that we evaluated the samples in North-Eastern Iran and not all parts of the country. In another study, Eden et al. reported the viral genome sequences to include D614G mutation in the travelers who returned from Iran (Eden et al., 2020). Generally, there is little information about the prevalence of mutations in Iran and worldwide, and it seems that the reason is the lack of cheap and fast methods. We hope that this method will provide more information on the prevalence and epidemiology of D614G mutations worldwide.

5. Conclusion

Our results showed that the designed method had consistent and reproducible results consistent with the protein S gene sequence of the studied strains. Using this method, we also found that the G614 mutant is increasingly raised during the time. We need to test a higher number of positive samples to evaluate the prevalence of G614 mutation in this region and also its relationship with the transmission rate and severity of the disease.

Contributors' statement

Seyed Ahmad Hashemi; found acquisition and supervised data collection.

Amirhosein Khoshi; collected laboratory data, carried out the RFLP test.

Hamed Ghasemzadeh-moghaddam; carried out RT-PCR tests.

Majid Ghafouri and Mohammadreza Taghavi; supervised data collection and reviewed the manuscript.

Hasan Namdar-Ahmadabad: carried out RT-PCR tests.

Amir Azimian; conceptualized and designed the study, carried out the RT-PCR and RFLP tests, drafted the initial and final revision of the manuscript.

All authors approved the final revised manuscript as submitted and agree to be accountable for all aspects of the work.

Declaration of Competing Interest

The authors have no conflicts of intrests.

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References

Becerra-Flores, M., Cardozo, T., 2020. SARS-CoV-2 viral spike G614 mutation exhibits a higher case fatality rate. Int. J. Clin. Pract. 74 (8), e13525.

Bhattacharyya, C., Das, C., Ghosh, A., Singh, A.K., Mukherjee, S., Majumder, P.P., Basu, A., Biswas, N.K., 2020. Global spread of SARS-CoV-2 subtype with spike protein mutation D614G is shaped by human genomic variations that regulate expression of TMPRSS2 and MX1 genes. bioRxiv. https://doi.org/10.1101/2020.05.04.075911.

Biswas, N.K., Majumder, P.P., 2020. Analysis of RNA sequences of 3636 SARS-CoV-2 collected from 55 countries reveals selective sweep of one virus type. Indian J. Med. Res. 151 (5), 450–458.

Du, L., He, Y., Zhou, Y., Liu, S., Zheng, B.-J., Jiang, S., 2009. The spike protein of SARS-CoV—a target for vaccine and therapeutic development. Nat. Rev. Microbiol. 7, 226–236.

Eden, J.-S., Rockett, R., Carter, I., Rahman, H., De Ligt, J., Hadfield, J., Storey, M., Ren, X., Tulloch, R., Basile, K., 2020. An emergent clade of SARS-CoV-2 linked to returned travellers from Iran. Virus Evolution 6, veaa027.

Gong, Y.-N., Tsao, K.-C., Hsiao, M.-J., Huang, C.-G., Huang, P.-N., Huang, P.-W., Lee, K.-M., Liu, Y.-C., Yang, S.-L., Kuo, R.-L., 2020. SARS-CoV-2 genomic surveillance in Taiwan revealed novel ORF8-deletion mutant and clade possibly associated with infections in Middle East. Emerg. Microb. Infect. 1–37.

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T.S., Herrler, G., Wu, N.-H., Nitsche, A., 2020. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. Cell. 181 (2), 271–280.

Hu, J., He, C.L., Gao, Q., Zhang, G.J., Cao, X.X., Long, Q.X., Deng, H.J., Huang, L.Y., Chen, J., Wang, K., 2020. The D614G mutation of SARS-CoV-2 spike protein enhances viral infectivity. bioRxiv. https://doi.org/10.1101/2020.06.20.161323.

Isabel, S., Grana-Miraglia, L., Gutierrez, J.M., Bundalovic-Torma, C., Groves, H.E., Isabel, M.R., Eshaghi, A., Patel, S.N., Gubbay, J.B., Poutanen, T., 2020. Evolutionary and structural analyses of SARS-CoV-2 D614G spike protein mutation now documented worldwide. bioRxiv. https://doi.org/10.1101/2020.06.08.140459.

Korber, B., Fischer, W., Gnanakaran, S.G., Yoon, H., Theiler, J., Abfalterer, W., Foley, B., Giorgi, E.E., Bhattacharya, T., Parker, M.D., 2020a. Spike mutation pipeline reveals the emergence of a more transmissible form of SARS-CoV-2. bioRxiv. https://doi.org/10.1101/2020.04.29.069054.

Korber, B., Fischer, W.M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., Hengartner, N., Giorgi, E.E., Bhattacharya, T., Foley, B., 2020b. Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell. 182 (4), 812–827.e19.

Maitra, A., Sarkar, M.C., Raheja, H., Biswas, N.K., Chakraborti, S., Singh, A.K., Ghosh, S., Sarkar, S., Patra, S., Mondal, R.K., 2020. Mutations in SARS-CoV-2 viral RNA identified in eastern India: possible implications for the ongoing outbreak in India and impact on viral structure and host susceptibility. J. Biosci. 45.

Phan, T., 2020. Genetic diversity and evolution of SARS-CoV-2. Infect. Genet. Evol. 81, 104260.

Walls, A.C., Park, Y.-J., Tortorici, M.A., Wall, A., McGuire, A.T., Veesler, D., 2020. Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. Cell. 181 (2), 281–292.e6.

Yin, C., 2020. Genotyping coronavirus SARS-CoV-2: methods and implications. Genomics. 112 (5), 3588–3596.