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Novel Mutations Associated with *N*-Gene Target Failure in SARS-COV-2 Genome in Iran, Case Series

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ABSTRACT

Precision tracking and monitoring viral genome mutations are critical during a viral pandemic such as COVID-19. As molecular assays for diagnosing numerous infectious agents are being developed, RT-PCR is still deployed as the gold standard for detecting SARS-CoV-2. Despite its proofreading capability, SARS-CoV-2, like other RNA viruses, adopts several changes in its genome. If these mutations, especially deletions, occur in the target areas of primers and probes, they will hinder molecular detection methods from identifying the given gene. The authors describe the cases in which, despite the lack of the *N* gene detection, the *ORF1ab* gene was discovered with a relatively low cycle of threshold (Ct). Following sequencing, changes were discovered in the annealing region of the forward and reverse primers and probes used in the SARS-CoV-2 detection kit. Among the most significant mutations is a large deletion of 15 nucleotides in the *N* gene, which has never been seen in prior variants. This highlights the importance of persistent monitoring of hypervariable regions in the SARS-CoV-2 genome through sequencing and updating the molecular detection kits during the COVID-19 pandemic.

INTRODUCTION

The unprecedented pandemic of SARS-CoV-2, the etiological agent of COVID-19, has had a significant impact on public health worldwide. As of June 26, 2022, over 541 million confirmed cases of COVID-19, including more than 6.3 million deaths, have been documented globally [1].

Although the SARS-CoV-2 replication enzyme complex has the ability of mutation proofreading, it is estimated that the virus undergoes genetic variations with a rate of $1.13 \times 10{-}3$ mutations per site-year which equals one substitution in the genome every two weeks [2, 3].

Several variants of SARS-CoV-2 have been identified; some of them are called Variants of Concern (VOCs) owing to the alteration in phenotypic features such as antigenicity, transmissibility, and virulence as failure in molecular diagnostic tests [4-6]. Up to now, five VOCs have been reported, including 1) Alpha, B.1.1.7 lineage, first detected in the UK; 2) Beta, B.1.351 lineage, first detected in South Africa; 3) Gamma, P.1 lineage, first detected in Brazil; 4) Delta, B.1.617 lineage, first identified in India, and 5) Omicron, B.1.1.529 lineage, first documented in multiple countries [4, 5, 7, 8].

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There are some deletions and some substitutions in these variants; the most important ones include 69-70 Del, N501Y, E484K, D614G, P681H, and so on [4, 5, 7]. Among them, the mutations that occurred in the *S* surface glycoprotein gene of the virus have generated more concern among health scientists and governmental decision-makers [9]. Regarding high rates of mutation in the viral genome and millions of patients worldwide, the emergence of new variants in other locations is predictable.

Here, we reported six new cases with unique mutations in the *N* gene of the viral genome associated with *N*-gene target failure in the Real-Time PCR test.

CASE PRESENTATION

On February 8, 2021, a 54-year-old woman (case 1) and his 18-year-old son (case 2) were referred to a COVID-19 specialized clinic (Qazvin Province, Iran) due to fever, cough, sore throat, and headache. They had close contact with suspected COVID-19 patients with no history of recent traveling abroad.

Nasopharyngeal and oropharyngeal swabs were taken from both cases and subjected to a SARS-CoV-2 Real-Time reverse transcriptase-polymerase chain reaction (RT-PCR) test using a commercial kit targeting nucleocapsid (N) and *ORF-1ab* genes of the virus (PishtazTeb Zaman Diagnostics, Iran). Despite the low cycle of threshold (Ct) of the *ORF-1ab* gene (Ct 23), the *N* gene of SARS-CoV-2 was not detected in samples from both patients. Repeating RT-PCR tests twice revealed the same results. The samples were referred to COVID-19 National Reference Laboratory at Pasteur Institute of Iran (PII) for further investigation. The samples were tested with a different commercial RT-PCR kit targeting *N* and *ORF-1ab* genes of SARS-CoV-2 (Sansure Biotech, China). In contrast to the results from the first kit, amplification of both *N* and *ORF-1ab* genes was seen using the second assay. The Ct values for of *N* gene were 17 and 19 for case 1 and case 2, respectively. The Ct values of the *ORF-1ab* gene were 19 and 20 for case 1 and case 2, respectively. For all RT-PCR tests, internal control (*RNase P* gene) and positive and negative controls were included.

Following these two cases, from March 26 to April 16, 2021, four other samples, including a 59-year-old woman (case 3), a 52-year-old-man (case 4), a 60-year-old man (case 5), and a 30-year-old woman (case 6), from COVID-19 specialized clinic of Qazvin Province were sent to Iran's COVID-19 National Reference Lab for further analysis. These patients also went to the clinic with symptoms such as fever, fatigue, myalgia, sore throat, and occasionally diarrhea, vomiting, and cough. None of the cases have reported shortness of breath or been hospitalized, but case 5 had diabetes mellitus (DM) and non-accelerated nephrotoxic nephritis (NTN). The failure of the PishtazTeb Zaman Diagnostics kit to detect the *N* gene was a common feature of the subsequent samples with the first two samples. As with previous samples, the samples were tested and sequenced using the Sansure Biotech SARS-CoV-2 RT-PCR kit and Sanger Sequencing. The Ct values for the *N* gene were 12, 14, 16, and 18 for cases 1-4, respectively.

The inconsistent test results indicating *N* gene target failure led us to subject the samples to sequencing analysis of the *N* gene (Table 1) [10, 11]. RT-PCR products were sequenced bidirectionally using the Applied Biosystems 3500xl Genetic Analyzer. Raw sequencing data were trimmed, and the consensus sequences were constructed by the CLC Main Workbench 5.5 package (CLC bio, Denmark). The genetic variations were specified according to a reference sequence of SARS-CoV-2 (NC_045512). The sequencing results indicated several mutations in the *N* gene, including deletions and substitutions (Table 2).

The sequences were submitted to the GISAID database under the accession numbers EPI_ISL_5856454 to 5856467.

The SARS-CoV-2 genome's hypervariable regions can be analyzed using the Nextstrain online database. The *N* gene has several mutation positions, from the most to the least. By September 5, 2022, 14 codons had higher entropy, the uncertainty inherent in the possible nucleotides or codons at a given position, throughout the *N* gene (Table 3) (Fig. 1).

The Pasteur Institute of Iran's Ethical committee approved the study under the ethical code: IR.PII.REC.1399.073.

Table 1. The primers used for sequencing of SARS-CoV-2 *N* gene. The Sanger sequencing was performed bi-directionally using primers presented in the table.

Gene		Primer sequence	Primer binding sites, according to the Reference sequence (NC_045512)	PCR product length (bp)
N	9F	TTTGGTGGACCCTCAGATTC	28322	1239
	9R	CCATCTGCCTTGTGTGGTC	29561	
	10F	CAGACAAGGAACTGATTACAAA	29149	708
	10R	GAAGCTATTAAAATCACATGGG	29857	

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Table 2. The list of mutations in the SARS-CoV-2 *N* gene of the cases. The deletions and substitutions are associated with *N* gene target failure of RT-PCR assays.

No.	Nucleotide Changes	Position	AA Change	Case No.
1	Deletion	28274	Δ1M	3
2	GA>CT	28280-28281	D3L	3
3	T>A	28282	D3E	4
4	Deletion	28892-28906	Δ207-211 PARMA	1,2
5	C>T	28940	L223L	5,6
6	C>T	28854	S194L	5,6
7	Deletion	28881-28883	RG203KR	1,2
8	Deletion	28881-28884	Frameshift	3,4
9	Deletion	28888-28898	Frameshift	3,4
10	Deletion	28902-28904	MA210-211T	3,4
11	G>T	28907	G212C	4
12	C>T	28940	L223L	5,6
13	C>T	28977	S235F	3,4
14	C>T	29095	F274F	5,6
15	C>T	29250	P326L	1,2

Table 3. The list of variations presented in codon (amino acid) positions based on the entropy of given positions. The highest entropy score belongs to codons 413, 33, and 32, respectively.

No.	Codon	Protein	Nucleotide Position	Entropy
1	413	N	29510-29512	0.636
2	33	N	28370-28372	0.614
3	32	N	28367-28369	0.57
4	13	N	28310-28312	0.562
5	31	N	28364-28366	0.553
6	203	N	28880-28882	0.525
7	204	N	28883-28885	0.446
8	151	N	28724-28726	0.248
9	63	N	28460-28462	0.242
10	377	N	29402-29404	0.239
11	215	N	28916-28918	0.207
12	3	N	28280-28282	0.123
13	136	N	28679-28681	0.111
14	235	N	28976-28978	0.1

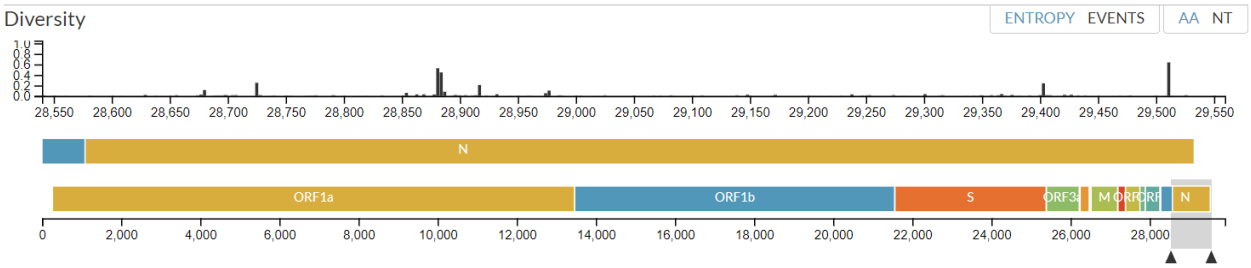

Fig. 1. Presentation of variations in the nucleotide sequence of SARS-CoV-2 *N* gene. The prevalence of variations is analyzed based on the entropy of given positions. The highest entropy score is assigned to nucleotide position 29510, which involves codon 413 of N protein [22].

DISCUSSION

Since January 2020, a new coronavirus named the SARS-CoV-2 pandemic has affected people worldwide. Respiratory disease caused by SARS-CoV-2 is from mild to highly severe and life-threatening. WHO highly recommended the detection, isolation, and treatment of confirmed patients [12].

Some mutations of VOCs might affect the performance of PCR detection kits. The well-known 69-70 del, for instance, could disrupt the detection quality of PCR SARS-CoV-2 detection kits which use an *S* gene target, named *S*-gene target failure (SGTF) [13]. Although detection kits that target different viral genes might be more reliable and could reduce the risk of false negative

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results, the concern of missing new variants still exists. We experienced this situation where one SARS-CoV-2 detection kit missed an *N* gene, but another could detect it. The only reason is that the target sequence on the *N* gene of SARS-CoV-2 is mutated; therefore, primers and probes cannot attach and find gene targets.

The deletion in the *N* gene of cases 1 and 2 (PARMA207-211 del) detected in Qazvin Province, Iran, was not observed in other VOCs. PARMA207-211 del is on the *N* gene in the viral RNA genome, which translates nucleocapsid (N) protein. N protein of SARS-CoV-2 is highly immunogenic and is abundantly expressed during viral infection [14]. As its name implies, N protein is responsible for making a nucleocapsid around the single-stranded positive-sense RNA genome of SARS-CoV-2. N protein also is a vital antigen not only for inducing antibody response but also for T-cell cytotoxic activity [15]. The PARMA207-211 del in the *N* gene of SARS-CoV-2 is probably related to immune response. More research is required to see whether these mutations and deletions affect an immune escape, neutralization reduction, and anti-N serological assay failure. However, its effect on molecular detection assay is not ignorable. If primers and probes target the mutated region by chance, it fails virus detection in molecular assays explaining the reason behind overlooking some cases with the common symptoms of COVID-19.

On the other hand, in molecular diagnosis, the WHO recommends a minimum of two genes to reduce the risk of false results [16]. In other words, mutations and deletions lead to either impeding primer annealing or dropping the probe out. The dropout assay is utilized to detect VOCs and differentiate between VOCs [17]. Therefore, if only the *N* gene is not detected by SARS-CoV-2 commercial detection molecular kits, following up with the patient and sequencing the samples are highly recommended. Some studies in other countries reported the primer dropout of SARS-CoV-2's *N* gene [18, 19]. In a study, the results of the Allplex assay, a commercial multiplex RT-PCR, showed *N*-gene dropouts or delayed Ct value in the Alpha variant compared with other assays that target RDRP and *S* gene [20].

The PishtazTeb Zaman Diagnostics SARS-CoV-2 Real-Time PCR detection kit uses China the CDC primers and probes that anneal to positions 28881 to 28902 by the forward primers, 28958 to 28979 for reverse primers, and 28934 to 28953 for probe of *N* gene, all located in the hypervariable region. Our sequencing demonstrated that the mutations had occurred in the annealing regions of either primers or probes used in the PishtazTeb Zaman Diagnostics kit. However, primers and probes developed from other institutions such as the Centers for Disease Control (US CDC), the University of Hong Kong (HKU), the National Institute of Infectious Diseases of Japan (NIID), and the National Institute of Health of Thailand (NIH), are located outside his range [21]. The set of primers and probes used in Sansure Biotech SARS-CoV-2 Real-Time PCR detection kit might also target regions in the *N* gene that do not overlap with the hypervariable region. The 69-70 Del, in association with N501Y mutation, led to disruption of *S* gene detection (SGTF). It could be concluded that deletions and mutations in the *N* gene that cause target failure might be introduced as a potential *N*-gene target failure (NGTF).

In general, it is noteworthy that mutations and variants of SARS-CoV-2 must be constantly monitored by health researchers working in diagnostic laboratories and medical companies manufacturing SARS-Co-V-2 molecular detection kits, that if the number of mutations in the region annealing to the primers and probes are increasing, immediately report it and redesign and produce a new kit according to these mutations. This is crucial to avoid false negative results that might lead to the spread of the SARS-CoV-2 pandemic.

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Author contributions; A.M. wrote the manuscript. Z.F., A.H.N., and P.Y-S., carried out the experiments. M.H. and A.E. contributed to the sample collection. M.T., A.M., and M.S.V. analyzed the data. M.S.V. designed and supervised the project. A.M. and M.S.V. contributed to the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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