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SARS-CoV-2 Omicron: Genotyping, Mutational Analysis, and Characterization of Subvariants in Iran

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ABSTRACT

Background: Several SARS-CoV-2 variants with distinct characteristics have emerged, with Omicron sub-variants such as BA.1 to BA.5 being predominant since late 2021. Distinguishing sub-variants using phylogenetic and molecular analyzes provides a valuable approach in the context of epidemiological research. .
Materials & Methods: Molecular epidemiology and sub-variants of SARS-CoV-2 omicron were investigated using 150 nasopharyngeal samples from COVID-19 patients in Tehran (Iran) from May 2022 to August 2023. Omicron lineages were differentiated using RT-PCR targeting Q493R, L452R, and Δ69-70 spike mutations. SARS-CoV-2 omicron sub-variants were determined by amplicon sequencing.
Findings: The mean age of the study participants was 44±7 years, comprising 38.6% males and 61.4% females, which may have an effect on transmission and susceptibility of different ages. Also, 117 (78%) samples were positive for one of the three lineages, while 33 (22%) was none of the lineages, which were referred to as conclusive and inconclusive results, respectively. 60.7% of the samples was the omicron lineage BA.4 or BA.5.
Conclusion: Considering the prevalence of BA.4 and BA.5 in the study population and their differences with the parental SARS-CoV-2 variant, the primary vaccine seems to be not effective against the current omicron sub-variants. These results underscore the importance of vaccination as a critical strategy to prevent the spread of these variants. The suggested primer sets could be an easy way to screen sample variants and lineages and are useful for screening and sequencing samples in countries with limited resources. Continuous monitoring of omicron sub-variants is recommended for preventing the resurgence of COVID-19.

Keywords: Severe acute respiratory coronavirus 2 (SARS-CoV-2), Omicron sub-variants, Genotyping, Molecular epidemiology

CITATION LINKS

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified as the causative agent of coronavirus disease 2019 (COVID-19), which is rapidly spreading worldwide [1-3]. Despite the large viral genome size, the mutation rate is high due to the low efficacy of the viral polymerase and high recombination, resulting in genomic diversity [4]. SARS-CoV-2 has been displaying multiple variants with different feature characteristics since 2019. In 2019 the SARS-CoV-2 outbreak began in Wuhan and later evolved into variants of concern, including alpha (B.1.1.7), beta (B.1.351), delta (B.1.617.2), and gamma (P.1) [5]. Surface proteins that are subjected to selection pressure by the immune system, such as neutralizing antibodies, are more likely to undergo antigenic drift than other viral proteins [6]. Genetic changes and mutations have led to the emergence of new variants with different characteristics [4]. The alpha variant containing the most common mutation (N501Y) has been reported to be the most prevalent variant in Canada [7, 8]. Meanwhile, the delta variant has been shown to be approximately 63 to 167% more transmissible than the alpha variant and 1.37 to 2.63 times faster than the alpha variant containing the L452R and P681H mutations in the spike [9]. The delta variant was first documented in India. In South Africa, the beta variant has been shown to be associated with higher hospitalization and mortality rates and potentially more transmissible than the alpha variant [10]. Omicron (B.1.1.529) was first identified in Botswana and South Africa in 2021. This variant has raised global concern due to its rapid spread and significant mutations in the spike protein. These mutations have led to increased binding affinity to ACE2 receptors, increased transmissibility, and evasion from neutralizing antibodies [11]. The omicron variant of SARS-CoV-2 is a rapidly spreading virus that has been of global concern since 2021 and remains the dominant strain of COVID-19 infection [12-13]. Omicron includes several sub-variants with different genetic characteristics, including BA.1 and BA.2, which emerged in some areas in early 2022 [14]; however, the BA.2.12.1 and BA.4/5 sub-variants became dominant in other countries, such as the United States and South Africa [15]. Between November 2021 and February 2022, there were two predominant circulating variants: delta and omicron 21K /BA.1 [16]. After that a recombinant variant of BA.1-BA.2, named "XE", emerged with increased transmissibility, which was indicated as a variant of concern. This sub-variant was potentially 10% and 43-76% more transmissible than BA.2 and BA.1 in 2022, respectively [17]. Another omicron sub-variant was XBB with different sub-lineages. One of them, called XBB.1, appeared in India in 2023 and led to more patient hospitalizations. Among amino acid mutations, Q493R, S373P, S375F, R408S, S447N, and N501Y were identified in XBB.1.16, increasing its binding affinity to ACE2. A study characterizing the properties of this sub-variant showed that these acquired mutations conferred a competitive advantage in both its binding affinity to ACE2 receptors and its ability to escape antibodies [18]. At the time of writing this article, a recent study reported L452R as one of the listed mutations occurring in BA.2 but not in BA.2.75 or XBB.1.16 sub-variant [19].

In 2022, the BA.4 and BA.5 variants spread to Europe and the United States, and BA.2.12.1, BA.4, BA.5, and BA.2 variants classified as variants of concern (VOC) were under investigation [20]. The spike proteins of BA.4 and BA.5 were shown to be most similar to those of BA.2. However, there were additional spike mutations such as 69–70 deletions, L452R, and revertant amino acid

at position Q493 in BA.4 and BA.5 compared to BA.2 [21]. Two BA.5 sub-variants, including BQ.1 and BQ.1.1, were found in October 2022, and several mutations were found in BQ.1 and BQ.1.1. Among these mutations, K444T, L452R, N460K, and F486V were found to be more dominant in the spike protein, although another mutation called R346T.6 may also be found in BQ.1.1. According to reports, symptoms related to BQ.1 and BQ.1.1 seem to be similar to those of other SARS-CoV-2 variants [22], and there is no evidence of the known Q493R mutation in BA.4/5 sub-variants and BQ.1 and BQ.1.1 sub-lineages [23].

Previous data have indicated that there is a common deletion (Δ69-70) in the NTD (N-terminal domain) region of all omicron variants, including BA.1, BA.1.1, BA.2, BA.3, and BA.4/5. However, an additional mutation (Q493R) occurs in the RBD of the spike protein of all omicron variants, except BA.4/5, where this mutation is reversed. Another important difference between BA.4/5 and BA.1/2 is the L452R mutation, which is present in BA.4/5 but not in BA.1/2. A study indicated that omicron sub-lineages BA.4 and BA.5 exhibited greater susceptibility to neutralizing antibodies induced by vaccination or prior infection compared to BA.1 and BA.2 sub-variants [24]. This led to another wave of omicron infections triggered by BA.4/5 in 2022 [24]. Notably, the hospitalization and mortality rates caused by BA.4 and BA.5 sub-variants were comparable to those of the first omicron wave. This may be due to the demographic pattern of the region, where the elderly are expected to be infected with BA.4 and BA.5 variants [20]. While many mutations may have deleterious or neutral effects on viruses, others make viruses more infectious, transmissible, and dominant [25]. Moreover, a study demonstrated that careful monitoring of lineages containing spike Δ69–70 deletions could be essential to distinguish different variants [26].

Objectives: The current study identified and determined omicron molecular sub-variants via genotyping of three differentiable mutations, Q493R, L452R, and Δ69-70, in patients with COVID-19 infection in Tehran, the capital of Iran. We planned to find a correlation between these mutations and the current omicron sub-variants.

Materials and Methods

Sample collection and preparation: From May 2022 to August 2023, nasopharyngeal specimens were collected from 150 COVID-19 positive patients referred to the COVID-19 center of Iran University of Medical Sciences and Keyvan Virology Laboratory in Tehran, Iran. In all samples a CT value of <45 considered as positive result for presenting of SARS-CoV-2. After extraction process, real-time polymerase chain reaction (RT-PCR) was used to detect SARS-CoV-2 in patients' nasopharyngeal swab samples using a commercial kit (Pishtaz Teb, Tehran, Iran). The Ethics Committee of Iran University of Medical Sciences reviewed and approved all aspects of this research (IR.IUMS.FMD.REC.1401.132).

Genotyping and molecular identification: Patients' nasopharyngeal samples were collected in viral transport medium (VTM). After confirming the presence of SARS-CoV-2 by commercial real-time PCR kit, the residual VTM samples were stored at -70 °C. Frozen VTM samples were subsequently thawed, and RNA was extracted manually using the AddPrep Viral Nucleic Acid Extraction Kit (AddBio, South Korea) according to the manufacturer's protocol. Extracted RNA samples were subjected to one-step reverse transcription real-time PCR (RT-qPCR) using a Rotor-gene Q thermocycler (Qiagen, Hilden, Germany) to determine the presence/absence of Q493R,

L452R, and Δ69-70 mutations in the S gene of SARS-CoV-2. Briefly, 5 µL of purified viral RNA was tested in a 20 µL reaction volume containing 10 µL of one-step Master Mix (Covitech, Zist Virayesh, Iran), 300 nM primers and probes for each target mutant (Table 1), and 4 µL of nuclease-free distilled water. The concentrations of primers and probes of Q493R and Δ69-70 mutations in the RT-qPCR mixture were 250 and 200 nM, respectively. The concentrations of primer and probe of L452R mutation were 400 and 250 nM, respectively. Real time PCR was performed at 55 ◦C for 10 min, followed by PCR amplification involving an initial denaturation step at 95 ◦C for 3 min and then 45 cycles of denaturation at 95 ◦C for 15 s and annealing/elongation at 59 ◦C for 30 s.

Phylogenetic analysis: RT-qPCR was conducted on 33 inconclusive samples (based on initial clinical diagnosis), and the samples were re-evaluated to gain a clearer understanding of the variant using conventional PCR, followed by Sanger sequencing. For this purpose, conventional PCR was performed on some selected specimens in 25 µL of amplification reaction mixture. After confirming the amplicon size on a 1.5% (w/v) electrophoresis gel, one-way sequencing was performed using a reverse primer designed for the mutant omicron gene. Phylogenetic analysis was performed using the neighbor joining method (CLC Genomics Workbench Version 20) with a bootstrap value of 1000 to estimate the consistency of clusters (Figure 2).

Statistical analysis: All descriptive and analytic statistics were analyzed by SPSS statistical software Version 22 (SPSS Inc. Chicago, IL, USA). Demographic data were evaluated by descriptive statistics.

Findings

Demographic and clinical data: The

Table 1) Oligonucleotide primers and probes used for detection of Q493R, L452R, and Δ69-70 mutation in the S gene of SARS-CoV-2.

Mutation	Primer Sequences (5′→3′)	Amplicon size (bp)	Probe Sequences (5′→3′)	Ref.
Q493R	Fwd:CCTTGTAATGGTGTTGAAGGTTTT Rev: CTGGTGCATGTAGAAGTTCAAAAG	130	FAM-TTTACGATCATATAGTTTCGACCC-BHQ1	[16]
L452R	Fwd: GGTTGGTGGTAATTATAATTCCGG Rev: CCTTCAACACCATTACAACGTT	123	FAM-TCTCTCAAAAGGTTTGAGATTAGACTTCC-BHQ	[16]
Δ69-70	Fwd: TCAACTCAGGACTTGTTCTTACCT Rev: TGGTAGGACAGGGTTATCAAAC	107	FAM - TGGTTCCATGCTATCTCTGGGACCA -BHQ1	[26]
Sequencing	Fwd: GAAGTCAGCCAAATCGCTCC Rev: GGATCACGGACAGCATCAGT	521	-	

mean age of the patients was 44±7 years, 58 (38.6%) were male, and 92 (61.4%) were female. It seems that in different area the mean age for susceptibility is different. However, the mean age likely is important for contribution to community transmission of COVID-19. The clinical characteristics and vaccination status of the participants are shown in Table 2. None of the included patients had any underlying diseases. The most visible symptom was fever (66%).

Variant analysis: Positive controls for each PCR were collected from clinical specimens, and Sanger sequencing was used to validate variants and lineages. Table 3 reveals the reporting pattern of the results. In this study, 117 (78%) samples tested by recommended triple-target real-time PCR were positive for one of the three lineages, while 33 (22%) belonged to none of the mentioned lineages. Table 4 presents a summary of the sample tests and variants. Out of all samples tested, 60.7% were omicron variants BA.5 or BA.4. The frequency of omicron variants BA.1 and BA.3 was found to be 14 and 3.3%, respectively. Finally, 3% of the samples were identified as BA.2 sub-variant.

There was no significant correlation between symptoms and sub-variants (p> 0.15). Sanger sequencing and then neighbor-joining method were used to analyze samples that belonged to none of the mentioned sub-lineages. Most of the samples in the phylogenetic tree were linked to the BA.4 lineage. Sanger sequencing data indicated that 14% of inconclusive results were linked to the BA.4 sub-variant (Figure 1). This means Inconclusive samples belong none of the mentioned lineages and their sublineages.

Although, for 68% of patients the status of vaccination was not available, considering to the vaccination status, there was no significant difference among various type of vaccination and their symptoms.

Table 2) Clinical symptoms and background medical conditions of the participants

Variables		Number (%)
Headache		14 (9)
Myalgia		46 (30)
Fever		66 (44)
Cough		36 (24)
Sore throat or rhinorrhea		50 (33)
Vaccination status	BIBP (Sinopharm)	30 (20)
	ChAdOx1 (Oxford/ AstraZeneca)	32 (21.3)
	Other vaccines	5 (3.3)
	Not vaccinated	15 (10)
	Non available	68 (45.3)

Table 3) Pattern of RT-qPCR results obtained from different Omicron subvariants

Controls	Q493R	L452R	Δ69-70
BA.1, BA.3	<30	NEG	<30
BA.2	<30	NEG	NEG
XBB.1.16	NEG	NEG	<30
BA.4 or BA.5 (BQ.1 and BQ.1.1.)	NEG	<30	<30
Mean Ct value± SD	20±8.6	26±1.6	19±8.1
<30: Ct value less than 30			

Table 4) A summary of evaluated samples and variants

Variant	Detected Number (%)		
BA.1, BA.3	21 (14)		
BA.2 (XBB.1.16)	5 (3.3)		
BA.4 or BA.5 (BQ.1 and BQ.1.1.)	91 (60.7)		
Total detected	117 (78)		
Inconclusive	33 (22)	**Sanger sequencing**	
		BA.4	14
		Inconclusive*	5
		Total	19

* Inconclusive samples belong none of the mentioned lineages and their sublineages (BA.1, BA.2 (XBB.1.16), BA.3, BA.4, BA.5 (BQ.1 and BQ.1.1.)

Omicron variant/mutation	Δ69-70	Q493R	L452R
BA.1	POS	POS	NEG
BA.2	POS	NEG	NEG
XBB.1.16	POS	NEG	NEG
BA.4/5	POS	NEG	POS
BQ.1 and BQ.1.1.)	POS	NEG	POS

SP: Spike Promoter
NTD: N-terminal domain
RBD: SARS-CoV-2 receptor binding domain
RBM: SARS-CoV- 2 Spike receptor binding motif
S2: S2 subunit inside the s protein, a prefusion conformation
TM: Transmembrane domain
CT: C-terminal

Figure 1) Schematic of the experimental process to investigate the relation between different mutations and Omicron subvariants.

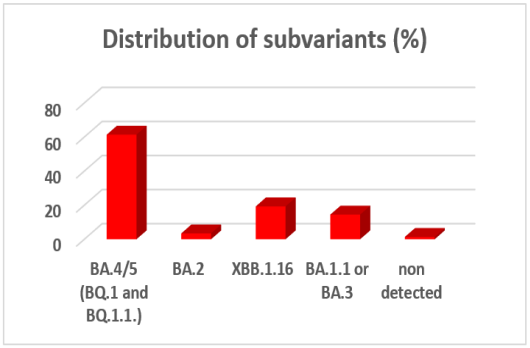

Figure 2) Distribution of the Omicron and its subvariants. BA.4/5 including BQ.1 and BQ.1.1. sublineages (63%), BA.1.1 (14%) and BA.2 including XBB.1.16 sublineage entered Tehran in May 2022 to August 2023.

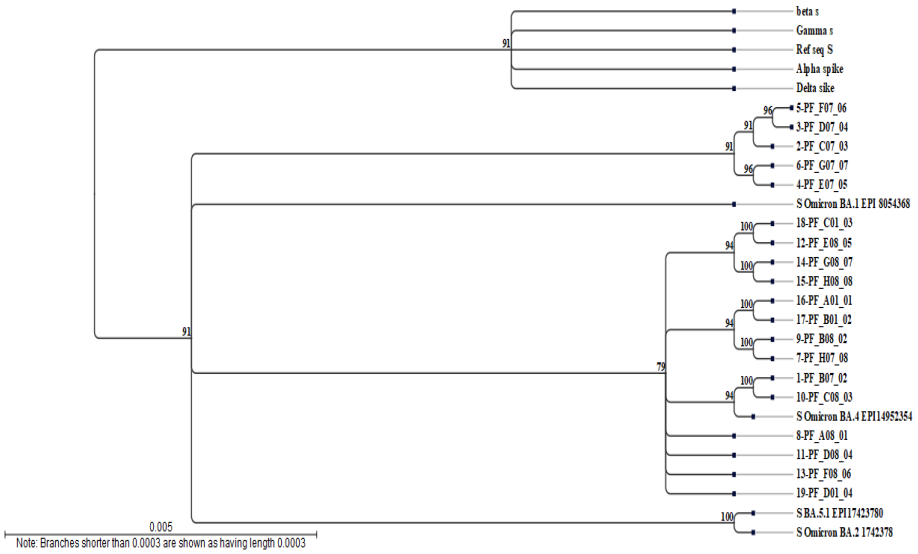

Figure 3) Cladogram phylogenetic tree for evaluated samples with Sanger sequencing. The tree was based on Neighbor joining method and 1000 bootstrap with 70% cut off.

Discussion

Since the emergence of the SARS-CoV-2 omicron variant in 2021, several sub-variants have emerged and circulated worldwide, including BA.1, BA.2, BA.1.1, BA.3, and BA.4/5 [27]. The spike protein has undergone multiple mutations in the RBD region, which has different amino acids in each sub-variant. Some of these mutations are present in both omicron and earlier variants like delta, and some are distinct to each omicron sub-variant [28]. Neutralizing antibodies are unable to recognize the spike structure of these new sub-variants, which could negatively impact the efficacy of COVID-19 monoclonal vaccines and treatments. However, in this study, the antibody status of the patients was not investigated, performing such a study might help find a better correlation between each omicron sub-variant and the immune status of patients. The spike protein of each omicron sub-variant contains commonly occurring mutations in its RBD, including G339D, S477N, T478K, and E484A. In a study, the Q493R mutation was present in both lineages BA.1 and BA.3 [29]. Our data also showed that the Q493R mutation was present in BA.1 and BA.3 but not in BA.2 and BA.4/5. In contrast, L452R was only present in BA.4/5, which is in agreement with a previous study [30]. Although the reversion of L452 to L452R in BA.4/5 has been linked to infection severity due to increased cleavage of the spike protein [31], no significant difference in patient symptoms was found between the BA.4/5 sub-variant and other omicron sub-variants. Other research has indicated that the 69-70 deletion is present in BA.3 and BA.4/5 variants as well [32, 33]. Using TaqMan PCR assay, a study by Hirotsu et al. (2022) revealed that all 127 patients in whom the BA.1/BA.1.1 variant was confirmed by whole genome sequencing (WGS) were positive for the Δ69-70 deletion, whereas all 44 patients with the BA.2 lineage tested negative [29]. This deletion at positions 69–70 of the S protein (Δ69-70) of the omicron variant causes false-negative results in the S-assay using the widely-used PCR method. This feature, also known as S-gene target failure (SGTF), could be used to detect the omicron variant by molecular assays [34]. The specific primer for Δ69-70 revealed that all sub-variants contained this deletion. However, further research is needed to identify inconclusive results with only positive Δ69-70.

In this study, the correlation between different mutations and omicron sub-variants in Iran was investigated from May 2022 to August 2023 (Figure 1).

The findings revealed that the majority of the tested samples were of the omicron variant lineages BA.5 or BA.4 (60.7%). Furthermore, the BA.4 lineage accounted for the majority of the samples in the phylogenetic tree. Sanger sequencing and then neighbor joining method were performed on inconclusive samples (22%), resulting in the identification of BA.4 in 14% of cases (Figure 2). Given that at the time of testing the samples, sub-lineages BQ.1 and BQ.1.1 were identified in several geographic areas in the world, with the detection of the L452R mutation, we believe that the majority of BA.4 and BA.5 samples might be associated with the BQ.1 or BQ.1.1 sub-lineage. The cladogram of SARS-CoV-2 was inferred based on the nucleotide sequence of the spike gene. The evolutionary relationship of SARS-CoV-2 was constructed using the neighbor-joining method by comparing the nucleotide sequences of the spike gene of SARS-CoV-2 isolates from patients in this study with nucleotide sequences of known assemblages retrieved from GenBank. In Figure 3, bootstrap values obtained from 1000 replicates are indicated as percentages on the branches; only bootstrap values > 70% are shown. Evolutionary analyses were

conducted in MEGA X.

Although the omicron variant has been reported to be more infectious and contagious than the previous variants, research suggests that mutations in this variant have contributed to its higher infectivity, its evasion from the immune system, and reduced vaccine efficacy [35]. One of the limitations of this study was the limited sample size. Also, the vaccination status of the patients was not examined, which may have an effect the severity of COVID-19 and the manifestation of symptoms caused by each sub-variant, and this was another limitation of the current study. To precisely interpret the data and understand the correlation between each sub-variant and the vaccination status of the population, it is necessary to study more samples in each COVID-19 wave. The identification of mutations that are clinically significant is crucial in planning public health policies and vaccination programs. To maintain immunity and protect the population against COVID-19 infection, an additional booster vaccine is recommended. Considering the efficacy of the previous dose of vaccine designed against previous sub-variants, the use of a third booster dose based on the new omicron sub-variant is crucial [36].

To differentiate between different omicron sub-lineages, three useful mutations associated with the omicron variant were selected in this study. If the mutation is caused by a small deletion in the spike gene, the PCR assay may not be able to detect the omicron mutation. However, these deletions have caused BA.4, BA.5 (BQ.1 and BQ.1.1.), and BA.3 to be closely related and have comparable properties. Studies have shown that neutralizing antibodies against BA.1 are not sufficient to protect people from BA.4 and BA.5, but they definitely confer protection against symptomatic infection. There is no doubt that new SARS-CoV-2 strains will emerge and circulate among people in the future, but by predicting future mutant strains through computer research, specific primers could be developed for these mutations in the form of unique tests. Multiplex PCR could quickly detect new strains and also provide a solution to reduce the symptoms of unpredictable new strains. Identification of co-circulating lineages in the Iranian population, which have co-occurred in other countries, could be achieved using these mutants, which are useful for epidemiological studies. Although only three specific mutations related to BA.1, 2 (XBB.1), 3, 4, and BA.5 (BQ.1 and BQ.1.1.) were investigated, it would definitely be useful if more mutations related to other omicron sub-variants were investigated, such as K444T and F486V for BQ.1 and BQ.1.1 as well as F486P for XBB.1, which were not investigated in this study. The results underscore the necessity of robust genomic surveillance to track the evolution of SARS-CoV-2 variants. Using molecular assays to detect specific mutations such as Q493R and L452R could help identify and differentiate variants in clinical settings. Identifying co-circulating variants in Iran and their relation to global strains could inform targeted public health interventions and vaccination campaigns. Implementing multiplex PCR assays could enhance the speed and accuracy of variant detection, allowing for timely responses to new outbreaks.

The use of computational models to predict future mutations appears to be vital for proactive public health measures. Developing specific primers for anticipated mutations could streamline testing and improve the management of potential future waves of COVID-19. The ongoing evolution of the SARS-CoV-2 virus, particularly the emergence of the omicron variant and its

various sub-variants, presents a significant challenge for public health. Finally, our detailed study highlights the complex interplay between viral mutations, immune evasion, and vaccination efficacy, particularly focusing on the omicron sub-variants BA.2, BA.4, and BA.5 and their sub-lineages.

Conclusion

As of the time of writing this article (from May 2022 to August 2023), the high prevalence of the BA.4/5 sub-variant (60.7%) among all study samples and co-circulating variants highlights the importance of monitoring sub-variants in the population to control future waves of COVID-19. As the virus continues to evolve, genome sequencing of positive specimens is a useful method to detect various virus variants; thus, by identifying different mutants related to each sub-variants and constructing a phylogenic tree, the screening of omicron variants and lineages could be performed even in resource-limited conditions in certain countries. Therefore, molecular epidemiology with sequencing and screening panels could help prioritize the samples that should be sequenced first, allowing for focus on the most important cases in each sub-variant of concern and continued monitoring by global and national health authorities. This will help inform public health policies and vaccination strategies to mitigate the impact of future variants. In conclusion, the findings provide valuable insights into the dynamics of SARS-CoV-2 variants in Iran and the world. Continued research and vigilance are essential to adapt and respond effectively to the challenges posed by these evolving viral strains.

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Ethical permissions: The research was directed ethically according to the World Medical Association Declaration of Helsinki. The study subjects gave their written informed consent, and the study was approved by the Medical Ethics Committee of Iran University of Medical Sciences [IR.IUMS.FMD.REC.1401.132].

Conflicts of Interests: There is no conflict of interest in this study.

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Authors' contributions: L. Mousavizadeh conceptualized and designed the study and wrote the manuscript. M. Roozbehani contributed to performing all experiments and edited the manuscript. H. Keyvani reviewed the manuscript. A.Tabibzadeh analyzed experiments. F. Bokharaei Salim provided samples.

Data availability statement: All data generated or analyzed during the current study are included in this article. Further inquiries could be directed to the corresponding author.

Consent to participate: All samples were collected from 150 COVID-19 positive patients referred to the COVID-19 center of Iran University of Medical Sciences and Keyvan Virology Laboratory in Tehran. There was no need to take consent paper from patients.

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