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Original Article

Comparison of Circulating Variants during the Beginning, Middle and the End of the 4th Wave of COVID-19 in Tehran Province, Iran in 2021

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Abstract

Background: Whole viral genome sequencing with next generation sequencing (NGS) technique is useful tool for determining the diversity of variants and mutations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In this study we have attempted to characterize the mutations and circulating variants of the SARS-CoV-2 genome during the 4th wave of COVID-19 pandemic in Tehran, Iran in 2021.

Methods: We performed complete genome sequencing of 15 SARS-CoV-2 detected from 15 COVID-19 patients during the 4th wave of COVID-19 pandemic with NGS. Three groups of the patients at the beginning, middle and the end of the 4th wave were compared together.

Results: We detected alpha and delta variants during the 4th wave of the pandemic. The results illustrated a dominance of amino acid substitution D614G in spike, and the most frequent mutants were N-R203K, G204R, S235F, nsp12-P323L, nsp6-G106del, G107del and F108del.

Conclusion: The detection of the virus mutations is a useful procedure for identifying the virus behavior and its genetic evolution in order to improve the efficacy of the monitoring strategies and therapeutic measures.

Keywords: SARS-CoV-2; Genome sequencing; Mutation; Variant; COVID-19

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as a novel coronavirus has been emerged in Wuhan, China in Dec 2019 which is the causative agent of human coronavirus disease 2019 (COVID-19) (1). In Iran, the first cases of the COVID-19 were detected in 19 Feb 2020 from Qom city and after that whole genome sequencing of the SARS-CoV-2 circulating during the different waves of the pandemic were performed in Iran (2-4).

SARS-CoV-2 has categorized in *Coronaviridae* family, Betacoronavirus genus. The viral genome is positive-sense RNA with approximately 30 kb (5). Mutational rate in coronaviruses like the other RNA viruses is dramatically high, and genetic diversity influences by mutations (6). With perpetu-

ation of the COVID-19 pandemic, different lineages of the SARS-CoV-2 were appeared. WHO was determined and introduced different variants including variants of concern (VOC). VOCs harbor various mutations in the spike protein especially in receptor binding domain (RBD) and N-terminal region. They have high effect in virus transmission, pathogenesis and decreasing neutralization antibody efficacy (7, 8). The previously circulating VOCs variants include Alpha, Beta, Gamma and Delta. The Omicron as a currently dominant is the latest VOC has mainly replaced the other co-circulating SARS-CoV-2 variants (9). Monitoring of the SARS-CoV-2 mutations reveals different aspects of the viral features that allows tracking, controlling, optimal diagnosis and management of the COVID-19 outbreak (10). Genomic epidemiology along with whole-genome sequencing (WGS) as a powerful and excellent tool can be used to determine and analyze genetic diversity, significant mutations and circulating lineages of the SARS-CoV-2 (10, 11).

This study was designed for analysis of the genetic evolution and mutations in SARS-CoV-2 genome and detection of the SARS-CoV-2 variants during the 4th wave of the COVID-19 pandemic in Tehran with next generation sequencing (NGS).

Materials and Methods

The oropharyngeal swab (OPS) specimens were collected from 15 outpatient and hospitalized COVID-19 patients in the 4th wave of COVID-19 pandemic in Tehran from Apr 4, 2021 to Aug 8, 2021. The samples were taken to the laboratory in National Influenza Center (NIC), School of Public Health in Tehran University of Medical Sciences. These specimens were categorized into three groups including, the 1st group infected with SARS-CoV-2 in the beginning, the 2nd group in the middle and the 3rd group at the end of the 4th wave of the COVID-19 pandemic. The samples of the 1st, 2nd and 3rd groups were collected on April, June and June to August 2021, respectively.

Ribonucleic acid isolation and Real-Time PCR

After samples preparation, viral RNA was extracted from OPS specimens by using High Pure Viral Nucleic Acid kit (Roche, Germany) according to the manufacturer's instructions. The molecular diagnosis of the SARS-CoV-2 infection was confirmed by Real-Time PCR test. In order to virus detection, primers and probes for E and RdRp genes were used (12). The positive samples with Ct number<35 were selected for NGS process.

Library preparation and next generation sequencing

The RNA was extracted from selected OPS samples for NGS by High Pure Viral Nucleic Acid kit (Roche, Germany). First and second strand cDNA was synthesized by Thermo Scientific Maxima H Minus kit. Library preparation step was done by Nextera DNA Flex kit (Illumina, USA) according to the manufacturer's instructions. DNA tagmentation by Bead-Linked Transposomes (BLT) and adding adaptor sequences were performed. Then followed tagmented DNA amplification, clean-up steps were done. Subsequently, library constructed undergone probe hybridization using Respiratory Virus Oligo Panel kit (Illumina,USA). This step was followed by probe capture, enrichment, amplification and clean-up quantification. The constructed library concentration was quantified by Qubit (Thermo Fisher, USA). Finally, the pooled library loaded onto Illumina Next Seq 550 machine for sequencing.

Data analysis

SARS-CoV-2 complete genome sequencing on all of the 15 samples was done with high quality scores and the data acquired in FASTQ format were analyzed. The genome sequences aligned by BioEdit sequence alignment editor software and compared with reference strain (hCoV-19/Wuhan/WIV04/2019) in GISAID. For analyzing and determining the mutations in the SARS-CoV-2 genome, we utilized CoVsurver mutations App in GISAID (13).

Results

Demographic information and distribution of the SARS-CoV-2 variants in the COVID-19 patients

The patients were nine males (60%) and six females (40%) in range of 23-72 yr old. The sequences were submitted to the GISAID database and the accession numbers were recorded. At the beginning of the 4th wave, GRY clade (alpha variant) was circulating, but GK clade (delta variant) was also detected in the middle of the 4th wave. In the 3rd group, alpha and delta variants were detected. These results were shown in the Table 1.

Table 1: Accession numbers, variants, clades and date of collection samples were shown in three groups of the COVID-19 patients in the 4th wave in Tehran, Iran

No. Patients	*Status*	*Age*	*Sex*	*Date of Collection Sample*	*Accession Number*	*GISAID Clade*	*Variant*
1st Group							
1	Outpatient	72	M	April 2021	EPI_ISL_1993557	GRY	Alpha
2	Outpatient	45	M	April 2021	EPI_ISL_1993547	GRY	Alpha
3	Outpatient	-	M	April 2021	EPI_ISL_1993549	GRY	Alpha
4	Outpatient	-	M	April 2021	EPI_ISL_1993556	GRY	Alpha
5	Outpatient	62	M	April 2021	EPI_ISL_1993552	GRY	Alpha
2nd Group							
6	Hospitalized	49	M	June 2021	EPI_ISL_8754003	GRY	Alpha
7	Hospitalized	23	F	June 2021	EPI_ISL_14197129	GK	Delta
8	Hospitalized	43	F	June 2021	EPI_ISL_8754007	GRY	Alpha
9	Hospitalized	68	F	June 2021	EPI_ISL_8754026	GRY	Alpha
10	Hospitalized	64	M	July 2021	EPI_ISL_8754016	GK	Delta
3rd Group							
11	Hospitalized	51	F	July 2021	EPI_ISL_14158878	GRY	Alpha
12	Outpatient	24	F	August 2021	EPI_ISL_8753996	GRY	Alpha
13	Outpatient	-	F	August 2021	EPI_ISL_4803556	GRY	Alpha
14	Hospitalized	49	M	August 2021	EPI_ISL_ 8754025	GK	Delta
15	Hospitalized	72	M	July 2021	EPI_ISL_14197130	GK	Delta

F: Female M: Male

The mutations in structural proteins of SARS-CoV-2

In the 1st group, the highest frequency of changes was belonged to the spike protein, including H69del, V70del, Y144del, N501Y, A570D, D614G, P681H, T716I, S982A and D1118H (Table 2). Among several mutants in N protein D3L, R203K G204R and S235F were common in all of the patients. We have not found any mutations in *M* and *E* genes.

In the 2nd group, the analysis has identified some changes with higher frequency in the spike protein like N501Y, A570D, D614G, P681H, T716T, D950N, D1118H and nucleocapsid R203K and G204R in alpha variant. Also, N-P122L, S235F, R203M and G215C with lower frequency were detected. I82T and G79-stop were detected in M protein (Table 2). We could not detect any mutations in *E* gene in our isolates.

In the 3rd group, the range of variations in spike protein were wide. Y144del, N501Y, A570D,

D614G, P681H and D1118H were frequent. In N protein, R203K and G204R with high frequency were detected in alpha variants. In addition, D63G in two patients was detected and R203M, G215C and D377Y in patient-15 belonged to delta variant were seen. Moreover, in M protein I82T along with S197T have detected in delta variants (Table 2). However, *E* gene was without change.

Table 2: Mutational frequency in the structural proteins (spike, nucleocapsid and membrane) of SARS-CoV-2 in alpha and delta variants during the 4th wave in Tehran, Iran

Gene	*Amino Acid Replacements and Deletions in Alpha Variant*	*Percentage*			*Gene*	*Amino Acid Replacements and Deletions in Delta Variant*	*Percentage*		
		1st Group	2nd Group	3rd Group			1st Group	2nd Group	3rd Group
S	H69del	100	40	40	*S*	T19R	-	40	40
S	V70del	100	40	40	*S*	T951	-	40	20
S	I100T	60	40	40	*S*	G142D	-	40	40
S	Y144del	100	40	60	*S*	E156G	-	40	40
S	N501Y	100	60	60	*S*	F157del	-	40	40
S	A570D	100	60	60	*S*	R158del	-	40	40
S	D614G	100	100	100	*S*	V213G	-	20	-
S	P681H	100	60	60	*S*	L452R	-	40	40
S	L699I	80	40	40	*S*	T478K	-	40	40
S	T716I	100	60	40	*S*	D614G	100	100	100
S	S982A	100	40	40	*S*	P681R	-	20	20
S	D1118H	100	60	60	*S*	I850L	-	20	40
N	D3L	100	-	40	*S*	D950N	-	60	40
N	D3E	-	-	20	*N*	D63G	-	40	40
N	R203K	100	60	60	*N*	R203M	-	20	20
N	G204R	100	60	60	*N*	G215C	-	20	20
N	S235F	100	20	40	*N*	D377Y	-	40	20
N	P122L	-	20	-	*M*	G79-Stop	-	20	-
					M	I82T	-	40	40
					M	S197T	-	-	20

Non-structural proteins (nsp1-nsp16)

In the 1st group, the alterations in non-structural proteins nsp2, nsp3, nsp5, nsp6 and nsp12 were observed. nsp3 had T183I, A890D and I1412T with 100% frequency in this group. Three deletions nsp6-S106del, G107del and F108del were detected in 80% of the patients. Meanwhile P323L in nsp12 has reported in all of the samples (Table 3).

In the 2nd group some changes have found in non-structural proteins like nsp2, nsp3, nsp4, nsp5, nsp6, nsp10, nsp12-16. In nsp3 several single mutations have observed in patient numbers 7,8 and 10. In nsp-6, three deletion mutants S106del, G107del and F108del seen in the 1st group were repeated again. In nsp12, P323L in three patients along with Q468H and G671S were observed. We have detected P77L, I42V, A394V and T112I respectively in nsp13, nsp14 and nsp15 that all were in patient number 7 with delta variant (Table 3).

In the 3rd group, among the non-structural proteins nsp3, nsp4, nsp6, nsp9, nsp12, nsp13, nsp14, nsp15 and nsp16, amino acid changes were seen. Specially nsp3 had many mutations. P323L in nsp12 in four patients (80%) and G671S in two

patients (40%) with delta variant were observed. P77L in nsp13 in two patients (40%) was detected. With progression of the 4th wave from middle to the end, we have faced many variations that mostly were in hospitalized patients. In the 2nd and the 3rd groups, we often have seen several common single mutations. Meanwhile, we did not detect any mutations in the nsp1of all groups (Table 3).

Table 3: Mutational frequency in the non-structural proteins of SARS-CoV-2 in the 4th wave in Tehran, Iran

Gene	*Amino Acid Replacements and Deletions*	*Percentage*			*Gene*	*Amino Acid Replacements and Deletions*	*Percentage*		
		1st Group	2nd Group	3rd Group			1st Group	2nd Group	3rd Group
nsp2	A205S	-	20	-	*nsp4*	T492I	-	20	20
nsp2	E457K	40	-	-	*nsp5*	G283S	40	-	-
nsp2	G517V	-	20	-	*nsp5*	P132H	-	20	-
nsp3	T6I	-	-	20	*nsp6*	T77A	-	20	40
nsp3	T48I	-	-	20	*nsp6*	S106del	80	60	40
nsp3	T183I	100	40	60	*nsp6*	G107del	80	60	40
nsp3	E130D	-	20	-	*nsp6*	F108del	80	60	40
nsp3	A488S	-	40	40	*nsp6*	Q208R	-	-	20
nsp3	G489S	-	20	-	*nsp9*	R39K	-	-	20
nsp3	T725I	-	-	20	*nsp10*	L138F	-	20	-
nsp3	A890D	100	20	60	*nsp12*	P323L	100	60	80
nsp3	A1006V	-	20	40	*nsp12*	Q468H	-	20	-
nsp3	A1105T	-	20	-	*nsp12*	G671S	-	40	40
nsp3	P1228L	-	20	40	*nsp13*	P77L	-	20	40
nsp3	I1412T	100	20	60	*nsp14*	I42V	-	20	-
nsp3	C1421F	-	-	20	*nsp14*	A394V	-	20	20
nsp3	P1469S	-	20	20	*nsp15*	T112I	-	20	-
nsp3	S1682F	-	20	-	*nsp15*	S147G	-	-	20
nsp3	A1775V	20	-	-	*nsp15*	F329L	-	-	20
nsp4	V242I	-	-	20	*nsp16*	K160R	-	20	40

Accessory proteins (NS3, NS7a, b and NS8)

In the 1st group, S83L in NS7a at 60% of the patients and in NS8 protein Q27-stop, R52I, K68-stop and Y73C with more frequency were reported. In NS3 only W131C in two patients was detected (Table 4).

In the 2nd group, NS3, NS7a, NS7b had less changes. However, NS8 had more changes which R52I has observed in 60% of the patients. S83L in NS7a in two patients with alpha variant was detected (Table 4).

In the 3rd group, NS3, NS7a, NS7b and NS8 had various changes. V82A and T120I in NS7a were detected and patient number 15 in NS7a had consecutive deletion mutations in amino acids numbers 49-79 in protein sequence LADNKFALTCFSTQFAFACPDGVKHVYQLRA. A single mutation T40I in NS7b in two patients and in NS8 stop codons at K68 (K68-stop), R52I and Y73C were detected (Table 4).

Table 4: Mutational frequency in the accessory proteins of SARS-CoV-2 in the 4th wave in Tehran, Iran

Gene	*Amino Acid Replacements and Deletions*	*Percentage*		
		1st Group	2nd Group	3rd Group
ORF3	K16T	-	20	40
ORF3	S26L	-	40	20
ORF3	G100C	-	-	20
ORF3	W131L	-	-	20
ORF3	W131C	40	-	-
ORF7a	T39I	-	-	20
ORF7a	S83L	60	40	20
ORF7a	V82A	-	-	40
ORF7a	T120I	-	20	40
ORF7a	K79-R79del	-	-	20
ORF7b	T40I	-	20	40
ORF8	Q27-stop	80	40	40
ORF8	R52I	60	60	60
ORF8	S67F	-	-	20
ORF8	K68-stop	60	20	40
ORF8	Y73C	100	40	60
ORF8	E110stop	-	-	20
ORF8	D119del	-	-	20
ORF8	F120del	-	-	20

Discussion

In order to clarify the genetic variations in the SARS-CoV-2 genome, numerous studies have been published up to now which virus genomic changes have shown. We reported complete genome sequences of SARS-CoV-2 in the 15 COVID-19 patients in three groups from the beginning until the end of the 4th wave.

Our findings showed that in the 1st group alpha variant was dominant and, in the 2nd, and 3rd groups alpha and delta variants were observed. For mutational analysis, in the 1st group, changes of the spike protein were in agreement with indicator mutations in alpha variant. In the 2nd and 3rd groups, changes in the spike gradually enhanced when circulating variants have shifted from alpha towards delta variant in some cases. Yavarian, et al. confirmed dominance of alpha and delta variants in the 4th wave of the COVID-19 in Iran (3).

Many changes in the alpha variant occurred in spike protein, especially in RBD which impacted on enhancement of the infectivity rate and changing in the sensitivity to neutralizing antibodies. P681H resists to antiviral function of IFNβ on the lungs cells (14). In our study P681H with high prevalence in all of the alpha variants was observed.

Among the nine accumulated mutations in the S protein of delta variant, some key mutants T478K and L452R have contributed in increasing the virus affinity to the human receptor and limited recognition by immune system. P681R is located in the effect site of furin enzyme that involved in facilitating viral fusion and increasing cell to cell infection (15). P681R mutant enhances S2 subunit cleavage in pseudoviruses. The pseudoviruses harboring D614G/ P681R had S2 subunit cleavage higher D614G alone (16). In our data, P681R in two patients with delta variant in the 2nd and 3rd groups were detected.

The involvement of the spike protein in viral pathogenesis and its interaction with host emphasizes on its important roles in virus lifecycle. D614G is considered as the hotspot mutant in RBD domain identified globally in all variants (since June 2020). D614G led to increasing the virus transmission and viral infectivity (17). Yavarian, et al. investigated the 54 Iranian full genome SARS-CoV-2

during five waves of COVID-19 pandemic and in the 4th wave that started on April until June 2021, amino acid substitution D614G was dominant mutant. They reported that after the 1st wave S-D614G was detected in all of the Iranian sequences (3). Our results were in line with their study and all of the virus isolates that collected in the same time harbored D614G.

Nucleocapsid protein involves in RNA packaging and promotes virus assembly (5). The frequency of R203K and G204R mutants have associated with COVID-19 severity, increasing virulence and speed of transmission (18). We observed D3L, R203K, G204R, and S235F during the starting of the 4th wave. In the 2nd and 3rd groups, frequency of R203K/G204R were decreased relatively, and other mutations relevant to delta variant in N protein have occurred like D63G, R203M and D377Y.

Membrane as an O-linked glycoprotein participates in the assembly of the virion and it is possible to be involved in the SARS-CoV-2 pathogenesis, viral propagation and immune escape (5). I82T as a fit mutant has a possible role in glucose uptake process during viral replication additionally and suggested that it is necessary to be involved in continuous sequence surveillance (19). We observed I82T in all of the patients with delta variant.

ORF1ab is the largest genomic region with vitally important roles in the virus life cycle and host-virus interaction. It encodes non-structural proteins nsp1-nsp16 (replication and transcription complex). ORF1ab seems to have contribution in virus evolution process and have multiple single nucleotide polymorphisms (SNPs) in its different genes. In our results many mutations in ORF1ab region were detected. Herein we scrutinized the variations in the non-structural proteins.

nsp3 is a large protein, also known as papain-like protease which participates in polyprotein processing and viral replication. In agreement with the other studies, our data showed a similar pattern about the distribution of these mutations. In Pakistan, nsp3-A488S and P1228L were reported in the 4th wave in the delta variant (8). We detected similar mutations in four and three patients respectively in delta variant.

nsp6 stimulates the formation of ER-derived autophagosomes and contributes in the organization of the replication-transcription complexes (RTCs) (20). We observed nsp6_S106del, G107del and F108del with amino acid sequence SGF in alpha variant. nsp6-T77A in three patients with delta variant was detected. Fooladinezhad et al. analyzed 6.5 million protein sequences of nsp3, nsp4, and nsp6 of SARS-CoV-2 from Jan 2020 to Jan 2022 in the different geographical zones. Their results highlighted the increasing frequency of nsp3-A488S, P1228L, P1469S and nsp6-T77A and nsp4-T492I mutants. On the other hand, they mentioned a significant association and co-occurrence between nsp3, nsp4, and nsp6 mutations (21).

nsp12, also known as RNA dependent RNA polymerase enzyme synthesizes new strands of RNA and has important role in viral replication (22). P323L in nsp12 is a common mutant that was reported in many countries among all variants. It is located in the interface domain (residues A250-R365) of the nsp12 protein and perhaps has a severe effect on the function of RdRp (23). Our results showed that both of the outpatients and inpatients (taking remdesivir) had P323L. The extensive use of antiviral drugs like remdesivir against SARS-CoV-2 can cause the emergence of the scape mutations in nsp12, and global monitoring is necessary to prevention of the drug resistance during COVID-19 pandemic in patients cured with this drug.

A study assessed the low frequency remdesivir escape mutants worldwide and suggested that SARS-CoV-2 RdRp can be a permanent target for antiviral drug development (24). nsp12-G671S is fixed mutant detected in 97.8% of delta variant isolates and in agreement with the same study, we detected nsp12-G671S in all of the delta variants. G671S mutant can biochemically raise the stability of the nsp12 (22, 25).

Among the accessory proteins of SARS-CoV-2, NS7a has an antagonized role to the IFN-I response (26). Nguyen et.al reported 14 sequential deletions (F63-Q76) in NS7a protein, and another record had one deletion at L77- position in protein sequence (27). In our analysis, one patient in the

3rd group has shown 30 consecutive deletions of NS7a protein in the same position. Previous studies proposed that some structural and accessory coding genes like *E, M, NS6*, *NS7a, NS7b* and *NS10* were more stable. These genes were conserved in the South Asian countries and as a consequence these genes can be appropriate target for designing of vaccine and drug (27, 28).
In our study we faced with two important limitations including sample size and inaccessible clinical findings of the patients. Thus, we could not discuss about the relation between symptoms and disease severity with detected SARS-CoV-2 mutations and variants.

Conclusion

Our findings demonstrated the circulation of alpha and delta variants in the 4th wave in agreement with the other studies worldwide. Whole genome analysis illustrated the top and rare mutations in different genes in some hospitalized patients that deserve further investigations in-vitro. All of these arguments lead to the conclusion that SARS-CoV-2 is changing speedily, and continuous molecular epidemiology studies can help to track the dangerous mutations and determining their effects on the disease's severity, viral fitness and evolutionary trends.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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