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DOI: 10.46903/gjms/22.01.1499

ORIGINAL ARTICLE

OPEN READING FRAME 8 GENE SEQUENCE VARIATION OF CORONA VIRUS IN PATIENTS FROM ERBIL CITY/IRAQ

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ABSTRACT

Background: The rapid rate of mutation of corona virus, which is a significant characteristic, suggests that as it moves across various habitats, the viral genome undergoes new alterations. The objective of this study was to analyze SARS-COV2 ORF8 gene sequence variation among patients in Erbil city.

Materials & Methods: During the period of January to December 2022, ten SARS-CoV-2 positive patients (4 female and 6 male), who were detected during the pandemic at Erbil central Laboratory aged (20-70) years enrolled in this cross-sectional study. The single strand RNA extracted from patients and transformed to double stranded complementary DNA. The primers (forward and reveres)- were designed by Bioinformatics program, (Primer3 Plus). Polymerase chain reaction amplification for ORF8 gene was carried out. The products ran on gel electrophoresis to visualize the DNA products. Each species was bi-directionally sequenced to get sequence of DNA strand according to forward primer. The sequence editing was analyzed using BLAST NCBI to indicate the homology from closest species. Phylogenetic tree was constructed.

Results: In comparing to the wild type that stated by NCBI (Genbank Reference accession number: OP732758.1) sequencing of our samples showed mutations at different position in which codon number 35 nucleotide A changed to C which results in replacement of an amino acid Aspartic Acid by Alanine, in addition to that in codon 69 nucleotide T changed to C as a consequence amino acid Serine change to Proline..

Conclusions: Sequence variation in viral ORF8 gene is regarded as the primary hotspot for genetic recombination and mutation of spike protein of SARS-CoV-2. Monitoring frequent nucleotide substitutions in ORF8 gene could be extremely helpful in understanding how the virus evolved in the community. Formation of new clades in Erbil city may have impact on vaccination program or the infectivity of the virus.

KEY WORDS: SARS-CoV-2; ORF8; Spike protein; Mutation; Phylogenetic tree.

Cite as: Abdulla AK, AL-Barzinji RMG, Taher CA. Open reading frame 8 gene sequence variation of Corona virus in patients from Erbil city, Iraq. Gomal J Med Sci 2024 Jan-Mar;22(1):44-51. https://doi.org/1046903/gjms/22.01.1499

1-INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is rapidly spreading through countries, there has been considerable concern about whether the spread is due to mutations in the virus or other factors.[1] The rapid rate of mutation of this virus, which is a significant characteristic, suggests that as it moves across various habitats, the viral genome undergoes new alterations.[2-4]

SARS-CoV-2 belongs to the *Betacoronavirus* genus, which is divided into four lineages (A–D). Lineage B (subgenus *Sarbecovirus*) includes SARS-CoV-2 and SARS-CoV. Since SARS-CoV-2 is an RNA virus without a "proof reading" mechanism. Random mutations take place as it replicates.[5-7] Approximately 30,000 nucleotides make up the RNA genome of the SARS-CoV-2 virus. Upon entering the cell, the genomic RNA acts as a messenger RNA (mRNA) to create numerous proteins from open reading frames (ORFs) genes. About two-thirds of the genome is covered by two overlapping ORF genes, ORF1a and ORF1b.[7] Four structural proteins-the spike (S), envelope (E), membrane (M), and nucleocapsid (N)-as well as a number of 'accessory genes' that encode additional non-structural proteins including

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Date Submitted: 28-08-2023
Date Revised: 21-12-2023
Date Accepted: 23-01-2024

(ORF3a, ORF 6, ORF 7a, ORF 7b, ORF 8, and ORF 10), they are encoded by the remaining 3′ portion of the genome. Among accessory genes, ORF8 stands out because it exhibits a number of interesting characteristics that were discovered in SARS-CoV during the 2002–2003 SARS outbreak.[7] Furthermore researchers were able to distinguish three primary lineages, known as types I, II, and III, in phylogenetic analyses by comparing *Betacoronaviruses* lineage B.[7]

The SARS-CoV-2 open reading frame 8 (ORF 8) gene is made up of 366 nucleotides, which are located in the viral genome at positions (27,894 to 28,259), these nucleotides encode for the 121 amino acid long ORF8 protein. Human isolates were discovered to have a distinct continuous ORF8 with 366 nucleotides and a predicated protein of 122 amino acids during the early stages of the SARS pandemic. However, the development and dissemination of SARS-CoV-2 strains with a 29-nucleotide deletion which produced two functional ORFs (ORF8a and ORF8b) that were projected to generate two small proteins, 8a with 39 amino acids and 8b with 84 amino acids, they were distinguished the middle and late phases of the SARS epidemics.[7-9] Within the endoplasmic reticulum lumen, the SARS-CoV-2 ORF8 protein interacts with a variety of host proteins, including numerous components thought to be involved in endoplasmic reticulum-associated degradation.[10] Open reading frame 8 is likely released rather than kept in the endoplasmic reticulum that it is why it is considered one of the key markers of SARS-CoV-2 infections.[11] SARS-CoV-2 utilizes its ORF8, accessary gene as unique mechanism in different situations. Exogenous overexpression of ORF8 in cells impairs IFN-I signaling.[12] Open reading frame 8 of SARS-CoV-2, but not ORF8 or ORF8a/ORF8b of SARS-CoV, has been demonstrated to down-regulate major histocompatibility-I (MHC-I) in cells.[13] According to previous findings, understanding the impact of ORF8 structure, function, and sequence variation on viral behavior, may be essential for comprehending how SARS-CoV-2 became a dangerous human disease.[14] Furthermore clinical evidence suggests that SARS-CoV-2 variants with ORF8 deletions have a greater transmission trend and a lesser illness rate, proposing that ORF8 is not required for viral genome replication and may contribute to pathogenesis process instead.[15] The objective of the study is to analyze SARS-CoV-2 ORF8 gene sequence variation among patients in Erbil city.

2. MATERIALS AND METHODS

2.1. Study design and sample collection:

Between January and December 2022, ten SARS-CoV-2 positive patients (4 female and 6 male)- who were detected at Erbil central Lab. Aged (20-70) years old were enrolled in this cross-sectional research. Nasopharyngeal swabs were taken from all patients for the molecular study.

2.2. Molecular tests:

2.2.1. RNA Extraction:

The kit's magnetic beads have certain polymeric groups of adsorbed nucleic acid (DNA/RNA) on their surfaces. Nucleic acids are released from cells or viruses into the samples under specific circumstances, such as hypersaline, and are specifically adsorbed by magnetic beads. The magnetic separator will separate the nucleic acids on the magnetic beads from the liquid phase when it is in operation. By washing with extraction reagent II, residual contaminants and inhibitors in the liquid phase are eliminated. Finally, to quickly and effectively separate the nucleic acids, the liquid phase conditions changed to escape the nucleic acids from the magnetic beads.[16]

2.2.2. Synthesis of complementary DNA (cDNA):

The single strand RNA extracted was transformed to double stranded complementary DNA through utilizing of AddScript cDNA synthesis Kit made in South Korea with a product code 22701. For cDNA processing in total volume of 20μl comprised 10 μl reaction buffer, 2.0μl of 10mM deoxynucleoside triphosphate (dNTP) mixture, 2.0 μl 10x oligo dT_{20} , 4μl virus RNA template, 1.0μl Add Script enzyme solution and 1μl of Nuclease-Free H_2O. The temperature profile was completed in thermocycler with temperature cycling protocol priming at 25℃ for 10 minutes, reverse transcription at 50℃ for 60 min, real time inactivation at 80℃ for 5 min. and held at 12℃ for one min.[17]

2.2.3. Primers design:

Oligonucleotide primers for ORF8 gene were synthesized chemically by joining nucleotides together. The main property of primers is that they must correspond to sequences on the template molecule (must be complementary to template strand). The primers (forward and reveres) was designed by bioinformatics program, Primer3 Plus, on website https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi. This web included blank space for nucleotide of desire gene. The target gene was ORF8 which was only related to SARS-Cov-2 gene. Our target gene was selected from National Center for Biotechnology Information (NCBI) GenBank (https://www.ncbi.nlm.nih.gov/nucleotide) by accession number (NC_045512.2, Severe Acute Respiratory Syndrome Coronavirus 2) so applied it to Primer3Plus program to pick primers. After adding sequences of gene (ORF8) clicked on picked primers several forward and revere primers were available, two primes of forward and revere have chased that can amplified 850 nucleotides as shown in (table 1). The primers were designed in Micro-gene Co. of South Korea.

Table (1) Primer's information and their properties

Primer Name	Sequence	Length	Tm	GC%
COVID/ ORF8-F	ACGTGCCAGATCAGTTTCACCT	22bp	59 Cº	50
COVID/ ORF8-R	GGGAATTTAAGGTCTTCCTTGCC	23bp	60 Cº	50

2.2.4. Polymerase Chain Reaction (PCR) Amplification of Partial Polyproteins

Coat Gene of virus:

PCR amplification for ORF8 gene was done in 50 µl of reaction mixture containing; master mix 25 µl, forward primer 3 µl, reverse primer 3 µl, DNase free water 14 µl and cDNA template 5 µl. The thermal cycling parameters of our PCR experiments were as following: step one was an initial denaturation at 95 ºC for 5 min, step two followed by 35 cycles of a denaturation at 95 ºC for 35 second, a primer annealing at 60 ºC for 40 sec, an extension at 72 ºC for 1 min and final step an extra extension at 72 ºC for 10 min, Bioresearch PTC-200 Gradient thermocycler was used for processing our samples.[18]

2.2.5. Gel preparation and visualization of DNA fragments:

An intercalating dye of Ethidium Bromide was added to 1.5% of melted agarose gel in 1X Tris-Borate-EDTA (TBE) buffer, followed by running for 30 minutes within the electric field of electrophoresis in 80 volt electric field, the locations of the bands were determined through examining of the gel under UV transilluminator.

2.2.6. Sequencing of DNA:

The PCR products were purified through utilizing of FavorPrep™ PCR Clean-UP Kit manufactured by Korea.[19] DNA sequencing was performed to determine the nucleotide sequence viral cap protein region. All PCR products were applied to ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Microgene Center in Korea.[20] Each species was bi-directionally sequenced to get sequence of DNA strand according to forward primer.

2.2.7. Data analysis:

The chromatograms were converted to FASTA format using Finch TV chromatogram viewer software. The DNA sequences in application binary interface (ABI) file were manually edited through using BioEdit V.7.0.5. Results of sequence editing were analyzed using BLAST (Basic local alignment search tool) NCBI to indicate the homology from closest species. Phylogenetic tree was constructed using maximum likelihood method, calculation using Bootstrap with 1000 times of repetition in molecular evolutionary genetic analysis 11 (MEGA 11) software program.[21]

3. RESULTS:

The PCR product was electrophoresed and visualized by 1.5% Agarose gel as shown in Figure 1.

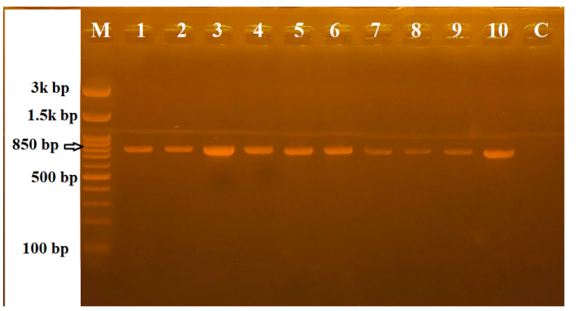

Figure 1: PCR amplification of partial viral spike gene from 10 patients' samples in which the first lane is DNA marker (3k bp-100bp) and the lane number 1 to lane number 10 are the PCR products from patients samples, with band size of 850 bp. the last lane (C) is negative control without any band.

The primers yielded a band size of 850 bp, but from 850 bp only 366 bp were complete target ORF8 gene which picked for sequencing. Sequenced data were checked for quality using BioEdit V.7.0.5 software. Homology, insertions, deletions, stop codons, and frameshifts were tested through utilizing NCBI- BLAST, the website was compared our query sequence with other biological sequences to find out similarity and nucleotide variation with other targets. BankIt, a WWW-based submission tool with wizards to guide the submission process was used. The GenBank database was intended for new sequence data that was determined and annotated by the submitter. All sequences were uploaded to GenBank as shown in (Table 3). The GenBank gave accession numbers for all ten submitted samples separately started from OP978658 to OP978667 and as shown in Table2.

Sequence analysis of ORF8 gene revealed nucleotide variation at different positions as shown in (table 3). with probable changes in their protein product. In codon number 35 nucleotide A changed to C which consequently may led to changing of Aspartic Acid to Alanine in addition to that in codon 69 nucleotide T changed to C which may led to replacing of Serine by Proline also in codon number 100 nucleotide T changed to G with probable change of Valine to

Table 2: GenBank accession no. of SARS-CoV-2 showing different accession numbers for all 10 submitted samples each with different nucleotide variation at different positions

No. of Samples	**Name of the virus**	**Accession No.**
1	Severe acute respiratory syndrome coronavirus 2	OP978658
2	Severe acute respiratory syndrome coronavirus 2	OP978659
3	Severe acute respiratory syndrome coronavirus 2	OP978660
4	Severe acute respiratory syndrome coronavirus 2	OP978661
5	Severe acute respiratory syndrome coronavirus 2	OP978662
6	Severe acute respiratory syndrome coronavirus 2	OP978663
7	Severe acute respiratory syndrome coronavirus 2	OP978664
8	Severe acute respiratory syndrome coronavirus 2	OP978665
9	Severe acute respiratory syndrome coronavirus 2	OP978666
10	Severe acute respiratory syndrome coronavirus 2	OP978667

Table 3: ORF8 nucleotides variation at different positions and probable changes at codon number of amino acids of each sample.

Sample	ORF8 position sequences	Variant position	Nucleotide changed	Amino acid changed	Codon number changed	Genbank Reference accession number
OP978658	27868-28233	27971 28018 28107 28154 28166	A-->C A-->G A-->G T-->G T-->G	Aspartic Acid->Alanine (D-A) Silent silent Silent Valine->Glycine (V-G)	35 50 80 96 100	OP732758.1
OP978659	27868-28233	27971 28018 28107 28154 28166	A-->C A-->G A-->G T-->G T-->G	Aspartic Acid->Alanine (D-A) Silent silent Silent Valine->Glycine (V-G)	35 50 80 96 100	OP732758.1
OP978660	27868-28233	27971 28018 28107 28154 28166	A-->C A-->G A-->G T-->G T-->G	Aspartic Acid->Alanine (D-A) Silent silent Silent Valine->Glycine (V-G)	35 50 80 96 100	OP732758.1
OP978661	27868-28233	28107	A-->G	silent	80	OP732758.1
OP978662	27868-28233	28107	A-->G	silent	80	OP732758.1
OP978663	27868-28233	27971 28018 28072 28107 28154 28166	A-->C A-->G T-->C A-->G T-->G T-->G	Aspartic Acid->Alanine (D-A) Silent Serine-> Proline (S-P) silent Silent Valine->Glycine (V-G)	35 50 69 80 96 100	OP732758.1
OP978664	27868-28233	27971 28018 28107	A-->C A-->G A-->G	Aspartic Acid->Alanine (D-A) Silent Silent	35 50 80	OP732758.1
OP978665	27868-28233	27971 28018 28107	A-->C A-->G A-->G	Aspartic Acid->Alanine (D-A) Silent Silent	35 50 80	OP732758.1
OP978666	27868-28233	28107	A-->G	silent	80	OP732758.1
OP978667	27868-28233	27971 28018 28072 28107	A-->C A-->G T-->C A-->G	AsparticAcid->Alanine (D-A) Silent Serine-> Proline (S-P) silent	35 50 69 80	OP732758.1

Glycine. These alterations occurred in ORF8 gene at position (27868-28233), when compared to GenBank reference gene with accession number OP732758.1. As shown in Figure 2.

Alignment and detection of variant amino acid:

Among 10 submitted sequences of SARS-CoV-2 showing entirely three diverse variation of amino acids according to alignment using MEGA program version 11. There were probable changes to new amino acids in different position in which codon number 35 nucleotide A changed to C this may lead to replacement of Aspartic Acid by Alanine (D-A) identified by red color change that represents (D) to yellow color that represents (A) as shown in Figure 2, in codon 69 nucleotide T changed to C consequently may led to change Serine to Proline (S-P) which identified by green color change (S) to turquoise color which represents (P). Furthermore in codon number 100 nucleotide T changed to G this led to changing of Valine to Glycine (V-G) which identified by changing yellow color (V) to purple color (G) as shown in Figure 2.

Phylogenetic inferences:

MEGA 11 program of phylogenetic analysis with more than 50% automatic program resampling (Bootstrap) based on ORF8 complete gene of SARS-Cov-2 revealed grouping of 5 examined different mutations on expected lines. From sequence divergence similarity data, the phylogeny constructed, which revealed that mutations among 10 sample sequences belonging to respective genera were close to each other. The none mutant samples of the virus were grouped in one cluster with high similarity to the GenBank reference sequences, in addition to that phylogenetic analysis showed genetic distance between them were 0.00-0.01 percentage according to GenBank sequences. Based on the present similarity between patients samples in ORF8 genes, 5 groups were designated as 5 clades; Clade I, Clade II, Clade III, Clade IV, Clade V. Clade I, included the none mutant samples of the virus that grouped in one cluster with high similarity to the GenBank reference sequences. Clade II, composed of mutant samples that have alteration of (Aspartic acid to Alanine). Clade III, included samples that had alteration of (Aspartic acid to Alanine, and Serine to Proline). Clade IV comprised the samples that had alteration of (Aspartic acid to Alanine, Serine to Proline and Valine to Glycine). Clade V includes those samples that had shifting of (Aspartic acid to Alanine and Valine to Glycine). As shown in Figure 3.

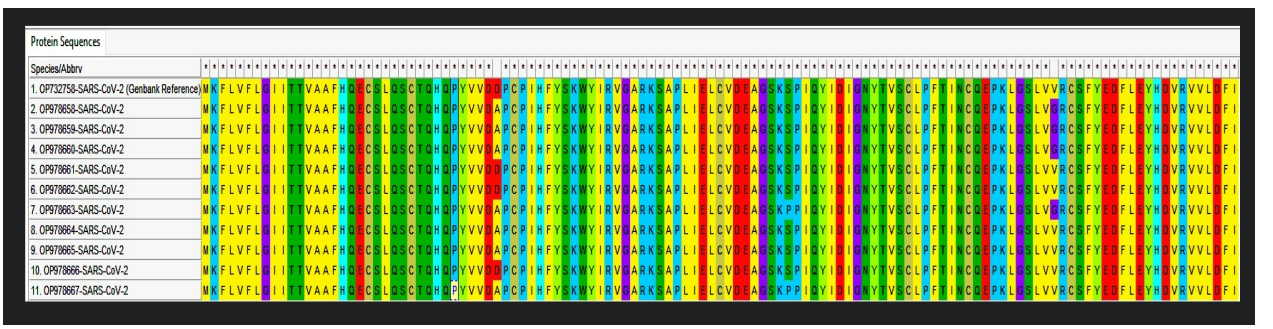

Figure 2: Multiple protein sequence alignment analysis of ORF8 gene among ten SARS-CoV-2 with one gene bank as reference compression

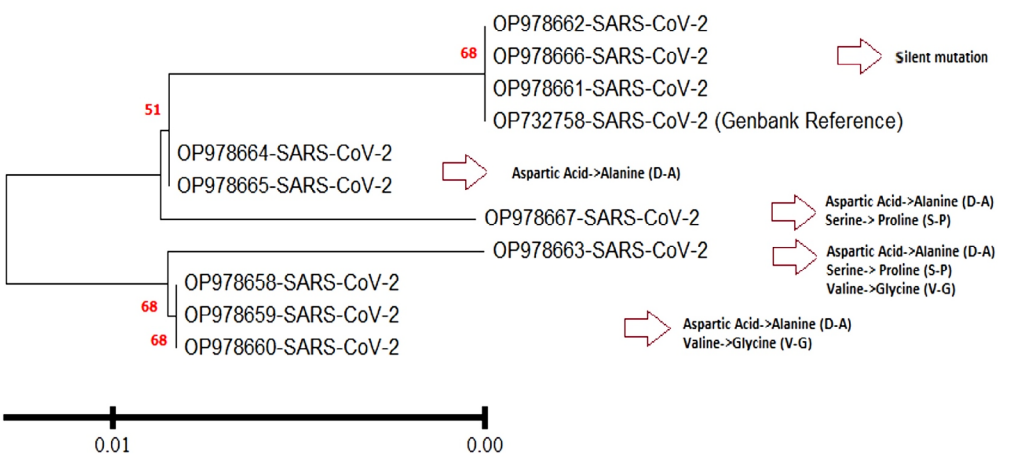

Figure 3: Employing maximum Likelihood with bootstrap with red numbers of Mega 11 program, show phylogenetic positioning of each mutant of 10 samples with similar GenBank sequences of ORF8 complete gene of SARS-Cov-2.

4. DISCUSSION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), have a specific connection to the human SARS-CoV, shares many similarities with each other.[22] SARS-CoV-2 and Beta-CoV of bats were found to have the highest observed similarity, suggesting that the virus likely originated from bats and transmitted to humans via an intermediate host.[23] At least six primary viral open-reading frames (ORFs) that encode the spike protein, envelop, nucleocapsid, and viral membrane are found in the genome, which is composed of positive single-strand RNA molecules. Recently, it was discovered that the structural and non-structural sites of the SARS-CoV-2 viral RNA are commonly acquired mutations and subjecting to various genetic reassortments that made the virus able to mutate and to create new variants.[24-26] Interestingly, the viral ORF8 gene is regarded as the primary hotspot for genetic recombination and mutation.[27] Although the viral ORF8 gene's functional significance is still up for debate, monitoring frequent nucleotide deletions and substitutions in this region could be extremely helpful in understanding how viruses evolve through intermediate hosts and how humans develop adaptive immune systems.[28]

Our finding is close to results found by Habeeb NJ *et al.,* who found similar band size for PCR amplification of ORF8 region of COVID-19 samples in the majority of their samples.[29] Sequencing of our patients samples were compared to reference sample from GenBank, the results were showing mutations at nucleotide level, to document that all 10 samples were submitted to GenBank as shown in (Table 3). The GenBank gave accession numbers for all ten submitted samples separately started from OP978658 to OP978667 as shown in Table 3, that could answer why the virus behaves differently in different countries. Further analyzing the impact of mutations at gene level to their protein product using MEGA11 revealed that among the 10 submitted sequences of SARS-CoV-2 ORF8 gene, appeared that three diverse variations of amino acids occurred according to alignment using MEGA program version of 11 (Figure 3). In comparing to the wild type which stated by NCBI (Genbank Reference accession number: OP732758.1), there were changes to new amino acids in different positions. These findings may be attributed to the fact that SARS-CoV-2 genes and more specifically ORF8 gene sequence mutation, is a hotspot for the majority of viral nucleic base substitution and deletion.[29] Mutation of ORF8 gene over time may provide the virus a greater chance to evade the immune system, as evidenced by the global increase in ORF8 gene mutations in SARS-CoV-2 variants, which were evidently connected with altering the host immunological response to viral infection, in addition to that, down regulating MHC-I and lowering T cell cytotoxicity against virus-infected cells are two ways that ORF8 gene may aid immune evasion[15,30], as a result, ORF8 gene acts as a major coordinator of the virus-host hybrid network's activities toward the creation of new virions.[31] New strains shown to have a big impact on the world's vaccination programs, based on this fact we believed that the mutation happened in our samples might have an impact on the vaccination program, since new clades formed.[30] In the current study genetic variations occurred within the region 27868-28233 of ORF8, which was near to results found by Mohammad S, *et al.,* who noticed the variations of ORF8 gene within the same region 27848:28229.[30] In a proteomic study, that was done by Shah M *et al*., they found amino acids substitutions of spike protein of SARS-CoV-2 there was replacement of Valine by Lysine, Proline by Alanine and insertion of glycine.[32] Since angiotensin-converting enzyme 2's (ACE's) Aspartic acid amino acid and Lysine amino acid of the viral spike have a powerful interaction as consequence, the result showed by Shah M. *et. al*., amino acid substitution facilitate stronger viral protein interaction with ACE2 which further promote high infectivity.[32] In addition to that the neutralization of SARS-CoV-2 by anti-SARS-CoV-2 monoclonal antibodies has also been hindered by the transition of Proline-Alanine.[32]

Tracking viral evolution and the emergence of pathogenic variations is one advantage of studying viral nucleotide substitutions.[30,33] The SARS-CoV-2 genome underwent an early phase of evolution during human-to-human transmission and separated into at least three significant phylogenetic groups globally. One of them was characterized by the appearance of single point mutations at genomic locations 28077 and 28144 of the ORF8 gene, which changed the amino acid Valine to Leucine and the amino acid Leucine to Serine in the ORF8 protein, respectively[4,33-35], these changes were within the same region of our target genome (ORF8, 27868-28233) .

Further analyzing the impact of nucleotide variation in our samples with probable change in their amino acid product, we did phylogenetic analysis with more than 50% automatic program resampling (Bootstrap) based on ORF8 complete gene of SARS-Cov-2. It revealed that the 5 groups designated as 5 Clades; Clade I, Clade II, Clade III, Clade IV, Clade V. According to data from other investigators, the S1 subunit change D614G was detected considerably more frequently than other S variation locations, and it is the hallmark of a large SARS-CoV-2 subclade (clade G). SARS-CoV-2 variants with G614 in the S protein have been the main form circulating globally since March 2020, replacing the initial D614 variants.[36] Patients infected with the G614 variant had higher viral loads than those infected with the D614 variant.[36] Our gene target also located in S1 subunit of spike protein of SARS-CoV-2.

5. CONCLUSION

Sequence variation in viral ORF8 is regarded as the primary hotspot for genetic recombination and mutation of spike protein of SARS-CoV-2. Monitoring frequent nucleotide substitutions in ORF8 gene could be extremely helpful in understanding how the virus evolved in the community. Formation of new clades in Erbil city may have impact on vaccination program or the infectivity of the virus. The modification of the spike protein may be a potential method employed by SARS-CoV-2 to increase viral transmission by evading the inhibitory effects of ORF8 that reflects viral ORF8 is crucial for immune surveillance.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.
GRANT SUPPORT AND FINANCIAL DISCLOSURE
None declared.

AUTHORS' CONTRIBUTION

The following authors have made substantial contributions to the manuscript as under:

Conception or Design:	AKA
Acquisition, Analysis or Interpretation of Data:	AKA, RMGAB, CAT
Manuscript Writing & Approval:	AKA, RMGAB, CAT

All the authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.