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Genetic analysis of SARS-CoV-2 spike gene using next generation sequencing from COVID-19 patients in Erbil/Iraq

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Abstract

SARS-CoV-2 has been identified by the WHO as a new virus causing mild to severe respiratory illnesses that belong to the Coronavirus family. The virus underwent rapid and continuous changes in the genetic material, especially the S gene, during COVID-19 pandemic and generated a number of new variants announced by WHO in late 2020. Mutations in the S gene have greatly affected virus pathogenesis as the spike protein is responsible for many critical processes. Delta and Omicron variants were studied extensively due to increased mortality and morbidity rates associated with their pandemic waves. This study aimed to analyse the S gene through NGS in an attempt to identify and characterize the circulating variants among the infected population in Erbil/Iraq. Nasopharyngeal and throat swab samples were collected from hospitalized and non-hospitalized patients with COVID-19 symptoms in Erbil City/Iraq from the 1st of November 2021 to the 28th of February 2022. Following confirmation of SARS-CoV-2 infection by RT-PCR, 15 samples were selected and sent to Intergen Lab (Ankara/Turkey) for NGS and analysis. Following analysis and alignment of the received sequences with the Wuhan-Hu-1 strain (wild-type), Delta variant was identified in 13 samples, and Omicron in two. On the whole, different mutation classes have been observed including nonsynonymous, synonymous, non-frameshift deletions and a non-frameshift insertion. The Delta-specific set of mutations, L452R, T478K and P681R, was detected in all Delta isolates. Both Omicron variants appeared to have 35 mutations. D614G variation was conserved in both variants.

Keywords: COVID-19, Delta, SARS-CoV-2, NGS, Omicron, RT-PCR, S gene, VOCs.

1. Introduction

Coronavirus disease 2019 (COVID-19) caused a global health crisis in Wuhan-China late in the year 2019 [1]. The disease was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which belongs to the Coronaviridae family and constitutes viruses causing acute respiratory illnesses [2].

Virus spike protein situated on the surface of the virus and encoded by the S gene facilitates viral attachment to cell angiotensin-converting enzyme 2 (ACE2) receptors [3]. Variation in number, class, and position of nucleotides in the S gene generated different variants of concern (VOCs) announced by WHO in late 2020 [4]. VOCs like Delta (B.1.617.2) and Omicron (B.1.1.529) have received global attention and studied extensively as they showed significantly high replication rates, infectivity and transmission, unusual activation of the immune system and escape from neutralizing antibodies, decreased vaccine effectiveness and probably increased morbidity and mortality rates compared to infection caused by the wild type (Wuhan-Hu-1) and other variants [5-7].

Infection with Delta variant was first identified in India in late 2020 and caused an increase in COVID-19 morbidity and mortality worldwide and remained the dominant variant until early December 2021 accounting for 81% of all epidemic strains worldwide [8]. Later, in late December 2021, Omicron was identified for the first time in Botswana and South Africa and was swiftly disseminated throughout South Africa and the rest of the world. Since then, Omicron has dominated and remained the VOC causing SARS-CoV-2 infection worldwide [9]. In February 2022, the Ministry of Health in the Kurdistan Region has announced laboratory-confirmed cases of Delta variant in the third wave and Omicron variant in January 2022, as the fourth wave of COVID-19 infection in Erbil/Iraq [10].

As a result of the instability of the virus genome (similar to other RNA viruses), periodic sequencing of the virus genome, especially the S gene, is necessary to understand disease pathogenesis [11]. One of the recently applied methods of sequencing in this field is Next Generation sequencing (NGS). With little time, effort, or mistake, the NGS method made it possible to sequence a full genome. It made it also possible to detect novel or rare variants with better discovery potentials [12]. This study aimed to analyse the S gene through NGS in an attempt to identify and

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characterize the circulating variants among the infected population in Erbil/Iraq during the study period in an attempt to keep pace with global efforts to study and understand the virus and the associated clinical picture.

2. Materials and methods

2.1. Subjects

This study included 104 COVID-19 patients who visited Erbil Public Health Laboratory or admitted to the three COVID-19-specific hospitals; Al Emarati, West Erbil Emergency and Lalav Hospitals in Erbil City/Iraq, from the period of the 1st of November 2021 to the 28th of February 2022. Samples were collected from patients who agreed to participate in the study.

2.2. Sample collection

Nasal and throat swab specimens were collected from each patient using specific collection swab in virus transport medium (VTM). The collected samples were sealed in plastic bags (usually one plastic bag per sample) and transported to the laboratory for detection of SARS-CoV-2.

2.3. RNA extraction and RT-PCR

Nucleic acid extraction from nasopharyngeal and throat swab samples was performed using a specific kit (Zybio/China). Following RNA extraction, detection of SARS-CoV-2 was done using SARS-CoV-2 RT-PCR nucleic acid detection kit (Zybio/China) that can specifically detect the target genes (ORF 1ab and N genes) of the virus. All the positive samples were subsequently placed at -70˚C until use.

2.4. Next Generation Sequencing of the S gene

Next Generation Sequencing (NGS) of the spike gene was totally done by the Intergen Genetic Diagnosis andResearch Center in Ankara/Turkey as this technique was not available in Erbil/Iraq. It is well-known that this technique is cost-effective, as a result, only 15 RNA extracts from mild and severe patients were selected to be sent for molecular sequencing and analysis of the S gene. Following receiving the RNA samples in the Intergen lab, they were tested for verification and RNA integrity. Using standard protocol of Ipsogen RT kit (Qiagen/Germany), RNA extracts were reverse-transcribed to obtain the cDNA. The prepared cDNA samples were stored at -20°C until PCR processing. Primer Designer V.2.0 (Scientific& Educational Software) was used to create primers targeting a specific location. The amplification process was then performed and the obtained PCR product was checked up using a 2% agarose gel electrophoresis for indexing and tagmentation of each individual product. Later, obtained PCR products were purified using NucleoFast® 96 PCR clean-up kit (MACHEREY-NAGEL GmbH/Germany) and quantified using Nanodrop 1000 micro-volume spectrophotometer (Thermo Inc./USA). Later, NGS and analysis were carried out in which samples were first prepared for NGS using Nextera XT sample preparation kit (Illumina Inc./USA), then the sequencing process was carried out using MiSeq (Illumina Inc/ USA) according to protocols fixed in the kit. Finally, obtained sequences were aligned to the Wuhan type SARS-CoV-2 genome (Wuhan-Hu-1) on NC_045512.2 with the Burrow -Wheer aligner.

3. Results

Details about the 15 nasopharyngeal and throat swab specimens from mild and severe COVID-19 patients subjected to RNA extraction, NGS and analysis of the S gene are shown in Table 1. As the samples were collected from the 1st of November 2021 to the 28th of February 2022, it seemed that it was the period of transition from SARS-CoV-2 Delta (B.1.617.2) variant to the Omicron (B.1.1.529) variant. Delta variants were identified from 13 samples and 2 were Omicron.

Following the S gene sequence alignment of the analyzed samples with the Wuhan-Hu-1 strain (wild-type) SARS-CoV-2, various mutations were identified, some of which were repeated among the same variant, some were unique and others were conserved in all VOCs (Supplementary Table 1). The total nucleotide number of the S gene was found to be 3816 and 3813 for each of Delta and Omicron variants. Delta variants showed interesting differences in number (10 to 14 mutations), position and

Table 1. Variants of concern identified following NGS and analysis of SARS-CoV-2 S gene from COVID-19 patients.

Sample	VOCs	Patient Group	Survival
1	Delta (B.1.617.2)	Mild	Survived
2	Delta (B.1.617.2)	Mild	Survived
3	Delta (B.1.617.2)	Mild	Survived
4	Delta (B.1.617.2)	Mild	Survived
5	Delta (B.1.617.2)	Mild	Survived
6	Delta (B.1.617.2)	Mild	Survived
7	Delta (B.1.617.2)	Mild	Survived
8	Delta (B.1.617.2)	Mild	Survived
9	Delta (B.1.617.2)	Severe	Survived
10	Delta (B.1.617.2)	Severe	Died
11	Delta (B.1.617.2)	Severe	Died
12	Delta (B.1.617.2)	Severe	Died
13	Delta (B.1.617.2)	Severe	Died
14	Omicron (B.1.1.529)	Severe	Died
15	Omicron (B.1.1.529)	Severe	Died

type of mutations, except two isolates (isolates no. 10 and 11) which were totally identical as they were isolated from two members of the same family both of which have been passed away following admission to ICU. On the other hand, the 35 mutations in both Omicron variants were found to be totally the same.

On the whole, Different mutation classes have been observed including nonsynonymous single nucleotide variations (SNV) that constituted the most abundant type of mutation, multiple synonymous SNVs, 4 non-frameshift deletions one in Delta variants (156_158del) and 3 in Omicron (68-70del, 142-145del and 211-212del), and a single non-frameshift insertion (ins214 EPE) in Omicron.

Samples were selected from two clinically different patient groups, mild and severe, in an attempt to find out a specific mutation related to severity and/or mortality, but such relation has not been fixed.

Table 2 presents the frequency and specificity of amino acid variations in Delta and Omicron variants. It was found that D614G and T478K were the most frequent variations and were present in all the 15 SARS-CoV-2 isolates. D614G was the first mutation that appeared after the Wuhan-Hu-1 strain and conserved in all previously identified variants from Alpha variant (B.1.1.7) to the Omicron, but T478K was first identified in Delta variant and conserved in Omicron variant as well.

Variations like T19R, G142E, L452R, P681R, D950N and 156-158del were detected only in Delta isolates. L452R, T478K and P681R were the basis of identification of Delta variants as they were identified by the WHO as signature mutations of Delta variant. It is worth highlighting a number of variations observed among Delta variants that were isolate-specific; never repeated in two or more isolates, naming D80Y, L176F, G181V, L229L, D294D, T302T, T345T, F543F, Q613H, N657N, N658H, A688V, A771S, A783T, H1101Y, V1104L, E1195A and V1264L. Details about amino acid variation types and frequencies in the spike of Delta variants are presented in Figure 1.

On the other hand, both identified Omicron variants had unusual number of new amino acid changes in the spike protein including A67V, G339D, R346K, S371P, S371F, S373P, S375F, K417N, N440K, G446S, S477N, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F, D1146D, 214inseEPR, 68-7-del, 142-145del and 211-212del.

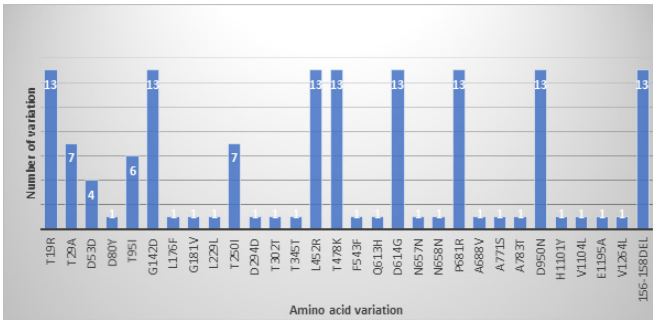

Fig. 1. A summary of amino acid variation types and frequencies in the spike of Delta variants identified in this Study.

4. Discussion

COVID-19 pandemic began in Wuhan/China in late 2019 and triggered a global health crisis [1]. The infection was caused by an RNA virus, SARS-CoV-2, that caused mild to severe respiratory tract infections [13]. The polymerases of the vast majority of RNA viruses lack 3′ exonuclease proofreading activity and hence are error-prone and undergo continuous mutation that could impact disease pathogenesis [14]. However, nothing proved regarding SARS-CoV-2 virus to undergo genetic reassortment or antigenic shift. The latter process is a unique feature of influenza A virus that possesses a segmented RNA genome. Reassortment occurs when two or more viruses infect a single host cell, they can package each other's genome segments into a newly produced virion, thereby producing a hybrid progeny. If the newly acquired RNA segment is that part of RNA genome that codes for hemagglutinin (HA) receptor, a new virus subtype will be more likely to emerge. Changes in amino acid sequences of HA may result in increased virus potential to bind to the sialic acid receptor on the respiratory tract of a new host range. Examples were N5H1; an avian influenza A virus subtype originated from Hong-Kong in 1997, and H1N1; a swine influenza A virus originated from Mexico and Southern United States in 2009, that caused pandemic infections in human [15].

SARS-CoV-2 infects human cells by the interaction of the spike protein with the ACE2 protein receptor. It was found that the mutation rate in the S gene is 4-5 times higher than that of other genomic locations [6,7]. Increased rates of hospitalization and death seen among the studied COVID-19 patients infected with Delta variant compared to Omicron in our study. The same observation was reported by previous studies [16,17] and greatly attributed to the S gene mutation pattern in Delta variant [18]. In this study, all the Delta variants have been identified with the unique set of mutations; L452R, T478K, and P681R, that have been identified by the WHO as signature mutations of Delta variant [4]. L452R is located in the RBD and was detected by the previous studies and was shown to restructure the genome and lead to increasing interaction with the ACE-2 receptor. It has also been shown to aid virus replication and transmissibility [19,20]. Saberiyan et al. (2022) reported that this mutation aids the virus to escape from immune cells such as CD4, allowing virus survival and activity in the body [21]. The second mutation with a significant effect on disease pathogenicity was P681R when proline is replaced by arginine at position 681. This change has been reported to promote furin-mediated cleavage of the spike protein and, in turn, better fusion of the virus particle with the host cell. Additionally, this mutation has also been linked to a partial decrease in neutralizing antibodies [22-24]. On the other hand, both Omicron variants had an unusual number of changes namely; non-synonymous mutations included A67V, T95I, G339D, R346K, S371P, S371F, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F, a single synonymous mutation D1146D, along with 68_70, 142_145, and 211_212 deletions and a single 214 EPE insertion. Interestingly, all the above changes were reported by previous studies [25,26]. Fifteen of the recorded mutations in this study were located in the RBD of Omicron spike protein, including S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, N501Y, and Y505H. The occur-

Table 2. Frequency and specificity of amino acid variations in Delta (B.1.617.2) and Omicron (B.1.1.529) variants.

Spike amino acid variation	Variation frequency	Delta (B.1.617.2)	Omicron (B.1.1.529)
T19R	13	+	-
T29A	7	±	-
D53D	4	±	-
A67V	2	-	+
D80Y	1	±	-
T95I	8	±	+
G142D	13	+	-
L176F	1	±	-
G181V	1	±	-
214EPE	2	-	+
L229L	1	±	-
T250I	7	±	-
D294D	1	±	-
T302T	1	±	-
G339D	2	-	+
T345T	1	±	-
R346K	2	-	+
S371P	2	-	+
S371F	2	-	+
S373P	2	-	+
S375F	2	-	+
K417N	2	-	+
N440K	2	-	+
G446S	2	-	+
L452R	13	+	-
S477N	2	-	+
T478K	15	+	+
E484A	2	-	+
Q493R	2	-	+
G496S	2	-	+
Q498R	2	-	+
N501Y	2	-	+
Y505H	2	-	+
F543F	1	±	-
T547K	2	-	+
Q613H	1	±	-
D614G	15	+	+
H655Y	2	-	+
N657N	1	±	-
N658H	1	±	-
N679K	2	-	+
P681R	13	+	-
P681H	2	-	+
A688V	1	±	-
N764K	2	-	+
A771S	1	±	-
A783T	1	±	-
D796Y	2	-	+
N856K	2	-	+
D950N	13	+	-
Q954H	2	-	+
N969K	2	-	+

L981F	2	-	+
H1101Y	1	±	-
V1104L	1	±	-
D1146D	2	-	+
E1195A	1	±	-
V1264L	1	±	-
68-70del	2	-	+
142-145del	2	-	+
156-158del	13	+	-
211-212del	2	-	+

rence of these mutations was also reported by Harvey et al. (2021], Li et al. (2021) and Mohapatra et al. (2022) and has been proven to potentially influence ACE2 receptor binding and antibody binding [27-29]. This is in addition to the persistence of a set of previously discovered mutations such as K417N, N440K, G446S, T478K, and E484A that have been linked to immunological escapes and evasion from neutralizing antibodies [30]. How the Omicron variant generated such a large number of mutations, notably on the spike protein, is still a mystery. Less selection pressure was placed on the virus, as this variant was first isolated from immunocompromised patients in South Africa, and the prolonged duration of infection could be possible reasons for such a huge evolution [9,10]. The most frequent variations that were present in all the 15 SARS-CoV-2 isolates in this study were D614G and T478K. Both mutations were also reported by previous literatures to be found in both Delta and Omicron variants [31]. The asparagine-to-glycine substitution at amino acid position 614 (D614G) was one of the most critical mutations that has become so dominant in all VOCs and VOIs globally, including both Delta and Omicron variants [32]. Furthermore, Studies have revealed that the D614G mutation dramatically boosts the virus particles' ability to replicate in the respiratory system, thus, it has been potentially linked to increased transmission rates [33-35]. It is noteworthy that T19R, G142D, L452R, T478K, D614G, P681R, D950N mutations and a deletion in the nucleotide position 156 and 157 were only detected in Delta isolates and disappeared in Omicron isolates. These results were in accordance with other studies that reported that above 8 mutations were found in almost all delta variants [36].

In conclusion, The NGS and analysis of the S gene identified two SARS-CoV-2 VOCs in Erbil City/Iraq during the study period; Delta and Omicron. Different mutation classes have been observed in which the non-synonymous mutation exhibited the most abundant type. Delta variants exhibited different pictures of mutations in the S gene (but all shared Delta-specific L452R, T478K and P681R mutation set) indicating the rapidly mutated nature of the virus. The two Omicron variants exhibited 35 mutations of different classes; some of which were conserved mutations but most were totally new. Mutations in the S gene of all isolates were located in critical regions that probably enhanced virus pathogenicity and negatively affected the clinical picture of COVID-19.

Conflict of interests
The authors have no conflicts with any step of the article preparation.

Consent for publications
The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
All procedures performed in this study were in accordance with the institutional and/or national research committee's ethical standards and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent
Informed consent was obtained from all individual participants included in the study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

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Authors' contributions
Asmaa Ameen Ghareeb: Sample collection and laboratory procedures and manuscript writing, Sazan Moffaq Abdulaziz: Research design, supervision, manuscript writing and editing.

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