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Association between ABO blood groups genotype variation and sever infection of COVID-19 in Iraqi patients

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***Abstract*---**Coronavirus disease (COVID-19) is a pandemic caused by new beta-coronavirus SARS-CoV-2. Recently, several studies provided evidences to confirm that factors such as blood group type and the genetic variations play a significant role in severe infection. The current study focused on ABO blood group type and genetic suitability. 100 (male & female) sample were collected. The number of infected males was higher than female number (60% male), while the females were (40 %), the highest number of patients within age group 25-35 cases in male. while female showed the highest percentage infection in the age group 15-25. The blood group A among patients with was significantly higher 34 %. While the lowest proportion of blood group AB. We applied Sanger sequencing method to identify the genetic variation distribution patterns of single nucleotide polymorphisms regions of the *ABO* genetic locus rs657152 at the 9q34.2c. Initially, genomic DNA was extracted successfully for all cases followed amplification *ABO* gene locus, further alignment of sequences samples against a Homo sapiens in the NCBI showed two mutants type SNPs for mostly A/C and C/C associated sever infection corresponding the sequence the control A/A. To conclude, that blood group A scored the high for highest percentage while the blood group O is the lowest. Our results successfully supporting the evidence of association between ABO type genetic differs and determined specific mutants SNPs patients and SARS-CoV-2/COVID-19 and its provide the first step of population genome-wide analysis in Iraqi population, further analysis need to be done to confirm our result with larger sample and longer time.

***Keywords*---**COVID-19, ABO gene, Coronavirus 2 (SARS-CoV-2), DNA, COVID-19 suitability, ABO genetic locus rs657152.

Introduction

The COVID-19 is a pandemic leading to public health crises and caused over million people death in worldwide (Vannabouathong et al., 2020). There is a considerable variation human response towards SARS-CoV-2 infection from asymptomatic to severe respiratory infection which needs intensive care. Several factors have been found increasing the ability to infected and develop COVID-19 virus infection such as age, gender, blood group type and the human genetic variations (Rashedi et al., 2020, Sharma et al., 2020).

Previous study found that an important relationship between ABO blood group and the development of severe acute respiratory syndrome coronavirus (SARS-CoV) infection in a group of medical staff who treated patients with SARS patient and who were not wearing any personal protective kit (Cheng et al., 2005). Several researchers proved that the ABO blood group might be play significant role in the pathogenesis of SARS-CoV-2 infection (Li et al., 2020, Silva-Filho et al., 2020).

Clinically, bacterial, parasitic, and viral infections blood types have been linked to ABO group Cheng (Cheng et al., 2005, Kim et al., 2021). Numerous studied focused on the relation between blood group and associations with infectious diseases and blood group antigens play a significant role in infection over many mechanisms. The International society of blood transfusion(ISBT) classified the four blood group according to four antigens—A; B; A, B; and A1 based on the specificity of the antibodies. Biochemical and molecular genetic studies have clarified the molecular basis of the blood group ABO histo- logical system. (Kominato et al., 2020, Hakomori, 1999). According to (Ellinghaus et al., 2020) SARS-CoV-2 SARS-CoV-2 association with blood antigen grouping

Other researchers found that the molecular genetic origin of the ABO system is varying and there are several of weak phenotypes have been found to be associated to single nucleotide polymorphisms in the coding exons and splicing sites and hybrid formation between common alleles (Daniels, 2013, ISBT, 2022). However, mutant SNPs associated with ABO blood group for which no variant apparently exists in the coding exons have been reported (Olsson and Chester, 2001, Iwasaki et al., 2000). A study conducted by Kominato in 2020 to confirm the molecular mechanism that for the regulator of ABO gene expression in a specific cell type manner. This mechanism exists during normal cell differentiation and important to understand how ABO gene transcription is regulated. (Kominato et al., 2020, Kim et al., 2022).

Recently, Further investigations found that (Goel et al., 2021) reported that individuals with ABO blood group have antigens are oligosaccharides expressed on red cells and other tissues particularly endothelium. The researchers suggested hypotheses to clarify the variances in SARS-CoV-2 infection by ABO type. For instance, anti-A and or anti-B antibodies (e.g. present in group O

persons) may bind to matching antigens on the viral envelope and involve to viral neutralization. Consequently, preventing target cell infection. The SARS-CoV-2 virus and SARS-CoV spike (S) proteins could be bound by anti-A is agglutinins which existing in group O and group B individuals, which may prevent interactions between virus and angiotensin-converting-enzyme-2-receptor and that can cause preventing entry into lung epithelial cells.

These results propose a molecular mechanism by which *ABO* polymorphism impacts susceptibility to SARS-CoV-1 infection and severity (Guillon et al., 2008). Recently (Goel et al., 2021) found also there is a variation response between different ABO blood group type. And they concluded that SARS-CoV-2 virus and SARS-CoV spike (S) proteins could be bound by anti-A iso-agglutinins (e.g. present in group O and group B individuals), which may block interactions between virus and angiotensin-converting-enzyme-2-receptor, thus avoiding entrance into lung epithelial cells

It has been identified that a 3p21.31 gene cluster of the ABO blood-group system as a genetic susceptibility locus in patients with COVID-19. One study, in particular, found that ABO polymorphism was associated with susceptibility to infection with SARS-CoV-1 (Chen et al., 2022). In a study which set out to determine analyzed single (SNP) sites from 35 patients with severe COVID-19 disease defined as respiratory failure genome-wide association studies (GWAS) (2012) found that Significant associations were observed with SNPs on chromosome 3p21.31 and on 9q34.2 involve sever infection (Ellinghaus et al., 2020). The current study aims to find out the reason behind COVID-19critical diversity between humans Najaf population. tried to explain the reason behind the ability of some people to be infected with COVID-19more than others. This variant could be belonging to the ABO blood group genetic factor. Detection of the single allele difference of gene *ABO* associate with severe infection (mutant) and compare it with the normal sequence in Iraqi patients.

Methods

Samples Collection

Sinces1st November 2021 to 1st February 2022 100 (male & female) sample were collected (20 control and 80 sample are infected with COVID -19) from Al-Amal Specialized Hospital corona virus centers in Najaf. Oral questionnaire was used. Followed statistical analysis data was analyzed using Excel and GraphPad Prism Version 5.00 for window normally distributed data. Patients confirmed with positive result using RT-PCR by using nasopharyngeal swab for SARS-2. The median age was ranging between (16) years to (90) year. vf(4) ml of fresh venous blood samples was collected from COVID-19 infected patients by sterile syringes

Molecular assays methods

DNA extraction; Total DNA was extracted from whole blood (2ml collected in EDTA tube) by using gSYNCTM biotecnology kit (Tiwan) according to the manufacturer's protocol. PCR were used for detection *ABO* gene in the blood of COVID -19 patients by the following procedure

Gene Amplification *ABO* blood group

PCR was used for detection *ABO* gene blood group genomic DNA COVID -19 blood. Firstly, novel forward and reverse primers were generated.
ABO- F ACAAGGGCAAGCCTGAAAG
ABO– R ATGGTTGTCAGGGTCAGGA

Amplification reaction kit. (Master mix10 µL**,** forward and revers primer 2µL of each one, 2-4 µL) the final volume was completed with nuclease free water till volume 20 µL and distributed in the PCR tubes & run according to the Thermal cycling conditions in addition, (Initial denaturation 95°C 5 min, denaturation 95°C 30 sec, annealing 58 °C, extension 72°C).

Analytical agarose gel electrophoresis To separate and detect different DNA fragments agarose gels were prepared and submerged in 0.5 × TBE buffer. PCR DNA fragments sequencing Extract PCR products. Subsequently, nucleic acid solution was diluted in 20 µL ddH2O to a concentration of 100 ng/µL. PCR fragment products sequencing was carried out by the company 'by Macrogen, Korea using Sanger sequencing method.

Results and discussion

The results obtained from the preliminary analysis shows that number of infected males was higher than female number (60cases- 60% male), while the female were (40 cases - 40 % male) (Figure 1) this result showed the agreement with statistical analysis of the WHO rescores and (Jin et al., 2020). Also, this finding is showing clear similarity with Iraqi researchers in 2021 published by (Suad G. J. Al kufi et al., 2021).

COVID-19 and Blood Group

The proportion of blood group A among patients with COVID-19 was significantly higher 34 % (Table 1) (Figure-2). These findings are consistent with similar risk patterns of ABO blood groups for other coronavirus infection found in previous studies (Zhao et al., 2020, Li et al., 2020). While the proportion of blood group O and B are approximately equal percentage patients with COVID-19, being 23% in the former vs 22 % in the later. A number of studies have found that recognized a higher proportion of group A, and a lower proportion of group O, among COVID-19 patients, as compared to healthy controls (Goel et al., 2021, Cheng et al., 2005, Leaf et al., 2020). These studies involved patients with SARS-CoV-2 pneumonia from mild to severe risky ill which needing mechanical ventilation or intensive care (Zeng et al., 2020).

In the other hand lowest percentage is group AB. These findings inconsistent with other coronavirus infection found in previous study (Zhao et al., 2020, Goel et al., 2021). Meanwhile, the proportion of blood group O in patients with COVID-19 was significantly lower than other blood groups in both genders. According to Goel, Bloch and his colleagues in 2021 recent studies the variation percentage incidence of COVID-19 related with ABO groups depend on population and our study support this opinion.

Since 1900 the ABO blood group system, discovered by Karl Landsteiner and reported an essential classification ABO blood group system to ensure the safety of blood transfusion. Later on the International Society of Blood Transfusion ISBT defines four antigens—A; B; A, B; and A1—based on the specificity of the antibodies to identify the ABO phenotypes or subgroups. The system is constituted of two carbohydrate antigens, A and B, and their antibodies (Kominato et al., 2020, Hakomori, 1999, Yamamoto, 2001). Previous studies, have been proved that people with different blood group types infection have a significant ability variation responses against different infectious diseases such as bacterial, parasitic, and viral infections (Cheng et al., 2005, Kim et al., 2021).

Different researchers tried to find the reasonable explanations which related to the type ABO group and COVID-19 different infection behaviors in different blood group type. Firstly, the interaction of viral outer protein surface (spike) that specific viral protein structure will lead to differences in susceptibility. For instance, anti-A and or anti-B antibodies (e.g. present in group O people) could bind to the same antigens on the viral envelope and involve to the virus neutralization, so inhibiting target cell infection. The SARS-CoV-2 virus and SARS-CoV spike (S) proteins may be bound by anti-A iso-agglutinins (e.g. present in group O and group B individuals), which may stop connections between virus and angiotensin-converting-enzyme-2-receptor, thus inhibiting entrance into lung epithelial cells. Secondly suggestion unique virus gene and patients encoded construction. In This study, further gene susceptibility analyses have been done to confirm the study hypothesis.

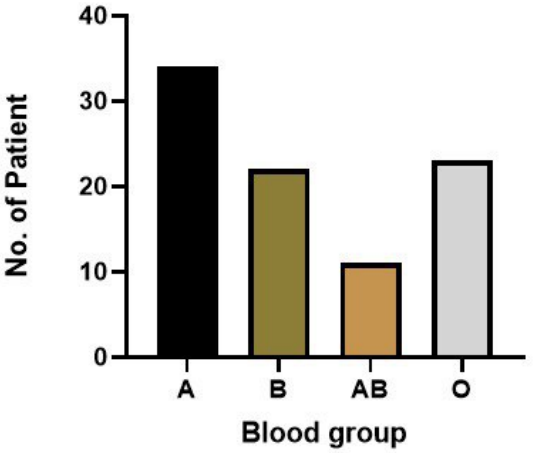

Figure 1: - Diagram illustrated the distribution number of patients infected with COVID-19 according with ABO blood group. A blood group is the highest numbers and the lowest is the AB blood group and blood group B and O are equal percentage approxitmetly.

COVID-19 severity analysis

The current study cases of COVID-19 divided to different categories depending on the infection severity. The below table 1 presented the number of patients have severe infection 65 cases, people with moderate infection degree are 15 while the

number of the mild infection patients are 7 cases. In the other hand, number of non-infected cases are 13. The 65 infected patients in our study 26 they do not have chronic disease, while 25 are having different chronic diseases 8 of them have diabetes type 1, 4 of them have diabetes type 2, 2 have Kidney frailer and 25 have other chronic disease mostly hyper tension as can see in table 2.

Table 1 Different cases categories divided according to COVID -19 severities

Degree of infection	No.
Sever	65
Moderate	15
Mild	7
Cases not infected	13
Total	100 cases

Table 2 Distribution of severe COVID-19 cases divided according chronic disease.

Chronic disease	No
Diabetes type 1	8
Diabetes type 2	4
Kidney frailer	2
Other Chronic disease	25
No infected with chronic diseases	26

Genetic Association of COVID-19

The initial results from above confirm that COVID-19 associations with type of blood group. The higher incidence of blood group A in this study on ABO blood group infected with SARS-CoV-2 they have also severe disease that match with other recent studies. This finding supports previous research into this main area which links COVID-19 high incidence of Najaf patients' blood group A. (Goel et al., 2021) In order to prove the ABO blood group and COVID-19 infection predisposition. Polymerase chain reactions were achieved. Firstly, novel oligonucleotides were generated forward and revers. PCR reaction fragments products were submitted for further DNA sequence analysis.

The PCR fragments products DNA sequencing

The amplification reactions of *ABO* gene were submitted. It is apparent from the figure 3A and B that all samples were successful with correct gene amplification 465 pb size. Nine sever patients were selected as high risk group patients. In addition, these patients have diabetes type one or two and have kidney failure.

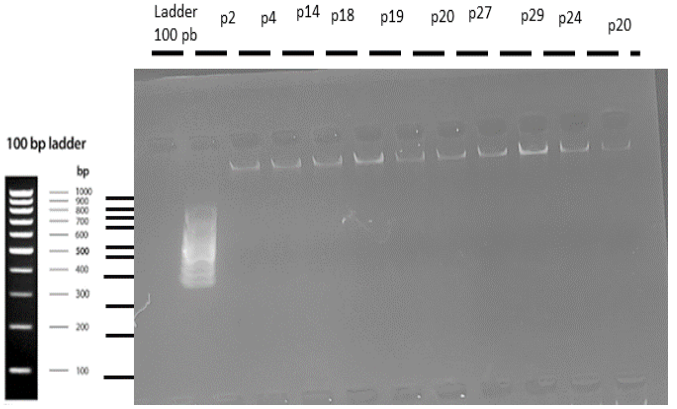

Figure 2: - Isolated genomic DNA from control samples who infected and people with COVID-19 from P2 to P20 DNA extraction patient. (Agarose gel 8% ladder 100pb) at 5 v/cm² for 45 min.

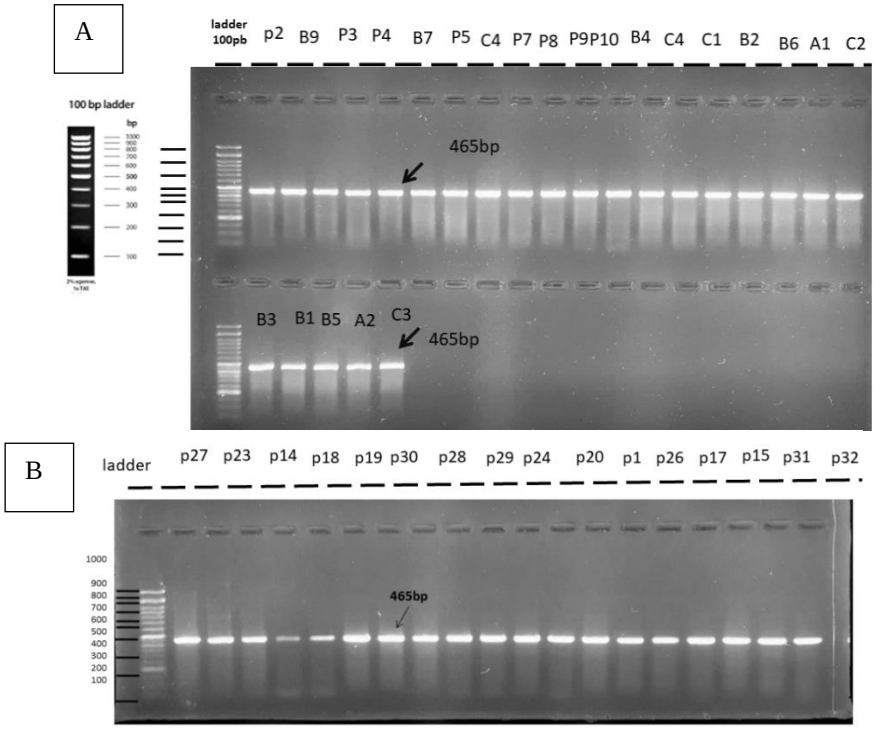

Figure 3: - PCR reactions confirmation of genomic on genomic DNA to detect ABO gene fragment specific band with a perfect product size 465 bp. Bands ladder 100pb were separated on 1% agarose gel at 5 v/cm² for 45 min.

DNA Sequencing analysis Results

Subsequently, sequencing for PCR products for the DNA of severe infection patients COVID-2019 samples. Seven samples were successful. Further multiple-

sequence alignment was submitted to compare between the sequence of PCR fragments of the patient's nucleotide corresponding to the sequence of normal *ABO* gene reference Blast RefSeqGene obtained from NCBI (Accession number: NG_006669). Interestingly, the blast sequence results showed three allele SNPs. Firstly, sequence alignment Blast of DNA for sample P04 compering with RefSeqGene of *ABO* the, as shown in figure 4, This sample was identified as a wild-type genotype (AA). However, due to the fact that the sequence of the RefSeqGene *ABO* is in the beginning flipped. Thus, the complementary alleles are shown and that means the highlighted 'T' in the figure is meant to be 'A'. This financing reflects the gene *ABO* sequence wild type which shows agreement with sequence of control.

Secondly, as shown in figure 5 the sequence alignment showed sequence of a PCR product of sample P15 against a RefSeqGene from NCBI. Interestingly, the sequence for this sample was identified as a mutant heterozygous genotype (AC). SO the study scored a positive mutant SNPS in *ABO* gene of this sample. However, the figure 5 shows a compatible nucleotide due to the fact that the sequence of the RefSeqGene *ABO* is originally flipped. Therefore, the complementary alleles are shown and that means the highlighted 'T' in the figure is meant to be 'A' and the 'G' is meant to be 'C'. Thirdly, the second positive result was shown in figure 6, Blast of DNA sequence for the PCR product sample P15. This sample was identified as a second type of heterozygous genotype

(AC). However, due to the fact that the sequence of the RefSeqGene ABO is originally flipped. Therefore, the complementary alleles are shown and that means the highlighted 'T' in the figure is meant to be 'A' and the 'G' is meant to be 'C'. It is interesting to identify that frequent in five samples cases of significantly, mutant (AC). A possible explanation for these important results may be this type of SNP is common in Iraqi population even limited number of sample. The SNPs results located on re657152 A/C is summarizing in table 3. Ten cases were selected depending on their severity infection blood group type and one control. First. the wild-type genotype (no mutant existed) in sample P02 AA control. Two mutant's allele appears other samples. First mutant SNPs appeared in one sample as one type mutant homozygous CC. Whereas, the two alleles T and C are existed in the other samples AC: The mutant allele C is detected in the sample. Significantly, the mutant type A/C showed the highest 50% number in the COVID-19 patients of the selected cases. In the other hand three samples presented not efficient sequence.

Table 3: - Summaries different genotype result located on re657152, AA: Wild-type genotype (the mutation is not existed in the sample). AC: The two alleles T and C are existed in the sample. CC: the mutant allele C is detected in the sample

	rs657152 A/C				
Code of sample	AA	AC	CC		Frequency percentage
P02			CC	Mutant C/C	1%
P04	AA			Wild type A/A	1%

P15		AC		Mutant A/C	50%
P18		AC		Mutant A/C	
P20		AC		Mutant A/C	
P27		AC		Mutant A/C	
C03		AC		Mutant A/C	

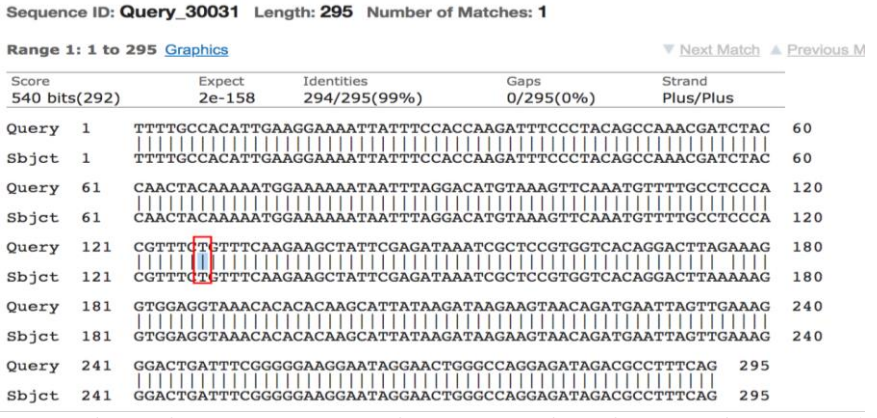

Figure 4: - Blast of DNA sequence of a PCR product for sample P04 against a RefSeqGene of *ABO* deposited in the NCBI (Accession number: NG_006669). This sample was identified as a wild-type genotype (AA). However, due to the fact that the sequence of the RefSeqGene *ABO* is originally flipped. Therefore, the complementary alleles are shown and that means the highlighted 'T' in the figure is meant to be 'A.

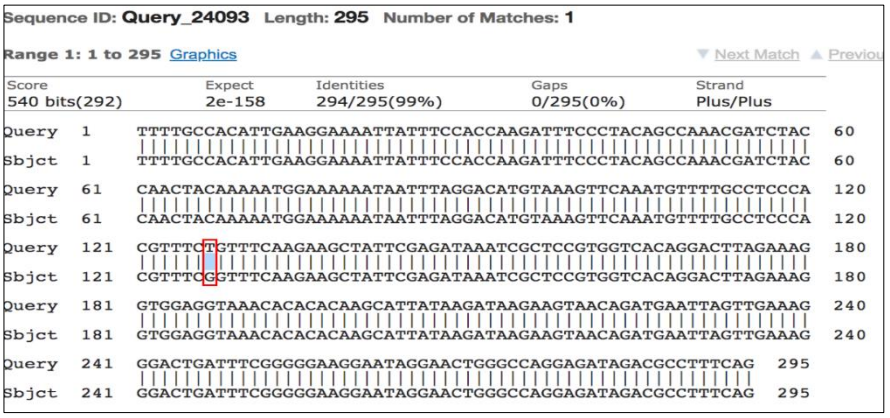

Figure 5: -Blast of DNA sequence of a PCR product for sample P15 against a RefSeqGene of ABO deposited in the NCBI (Accession number: NG_006669). This sample was identified as a hetero-genotype (AC). However, due to the fact that the sequence of the RefSeqGene ABO is originally flipped. Therefore, the complementary alleles are shown and that means the highlighted 'T' in the figure is meant to be 'A' and the 'G' is meant to be 'C'

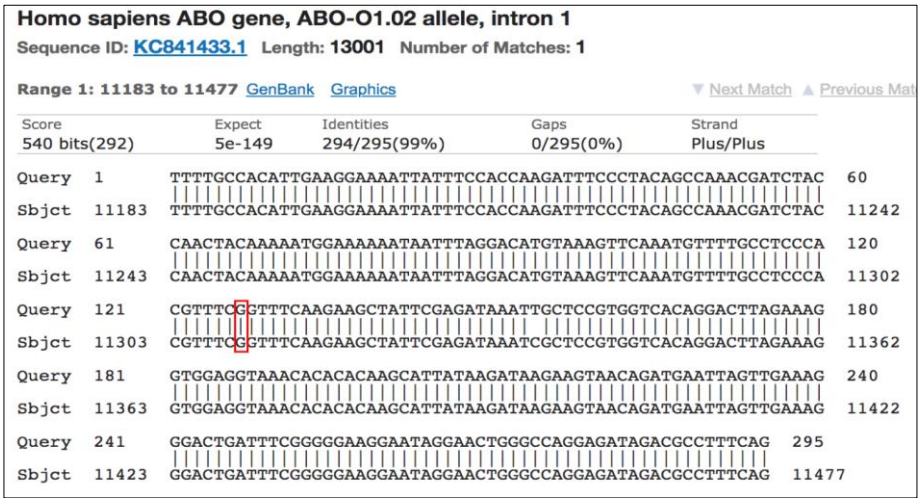

Figure 6: - Blast of DNA sequence of a PCR product for sample P02 against a sequence of gene ABO deposited in the NCBI (Accession number: KC841433.1). This sample was identified as a mutant genotype (CC). However, due to the fact that the sequence of gene ABO is originally flipped. Therefore, the complementary alleles are shown and that means the highlighted 'G' in the figure iS meant to be 'C'

Sequence alignment of the *ABO* gene group analysis and chromatogram by using BioEdit sequence software were done to confirm all previous results of three SNPs types. They are highlighted with yellow box (Figure 7 and 8). Initially, the SNP AA wild-type genotype (the mutation is not existed in the sample as can see in figure 8. AC the two alleles T and C are existed in the sample. CC: The mutant allele C is detected in the sample (Highlighted in a box). (Figure 8). The findings of the current study are consistent with those severe COVID-19 GWAS Genome wide Association StudyGroup which confirmed in (2020) the similar SNP variation which related to COVID-19 susceptibility (Ellinghaus et al., 2020). Subsequently, they confirm also the association specific gene variation at locus 9q34.2 overlapped with the ABO blood group locus. Furthermore, they confirmed A blood-group-as the highest risk than in other blood groups (odds ratio, 1.45; 95% CI, 1.20 to 1.75; P = 1.48×10-4) corresponding to the effect in blood group O as compared with other blood groups (odds ratio, 0.65; 95% CI, 0.53 to 0.79; P = 1.06×10-5). (Ellinghaus et al., 2020)

Moreover, the present study supports the data result obtained from NCBI with mutants associated SNPs in different population (table 4). As table 4 demonstrated the different proportions of mutant alleles and normal alleles A/A (Reference allele) and alternative alleles C/C or A/C. Interestingly, the south country population results show consistency with Iraqi population in this study result in which the dominant of mutant A/C in severe infection corresponding to C/C mutant frequency. A positive correlation was found between our findings of the genetic variation on *ABO* gene position in COVID-19 Iraqi patients. The three SNPs genotype which located on re657152, wild type and two others mutant A/A, and two mutant allele A/C and C/C alleles. Although, our cases sample are smaller than global recent studies (Table 4) as well as our limitation time and

fund our result showed relevant results. In addition, we did an optimization for different kites and material in the begging of our study that give us a powerful to proceed this type of crucial study. Interestingly, our result showed agreement with the reported researches in NCBI data base which also depend (Table 4) in term of variation frequency scores of alleles incidence of the ABO gene in different population.

This study has demonstrated, for the first time, the Iraqi COVID-19 patient's gene variation that increase patient's susceptibility to infect with severe infection more than others. Further investigations of present outcomes need to be done, both as to their effectiveness in clinical risk reporting of patients with COVID-19 and to deep comprehensive the mechanistic of the underlying pathophysiology. According Zhang 2021 the SNP rs657152, located in the intron region of *ABO*, one study found that the ABO gene, chr 9q34.2, which determines blood type, may affect disease severity, although this result has not been reproducible.(Zhang et al., 2021)

Recently Kominato, Sano in 2020 reported a research to find out the transcriptional regulation of the *ABO* gene through sequence analysis of different genetic variation that regulate transcription regions and shape the molecular origin for ABO group phenotypes with variants SNPs in those regions to identify the blood group type (Kominato et al., 2020). Furthermore, the researcher found identified the mutant SNPs rs657152, located in the intron region of the *ABO* and other possible reigns (Kominato et al., 2020). The researchers find the several SNPs specifically SNPs A/C rs657152 is related with blood group A and B. This research confirms our detection of variation SNPs that we detect mostly in sample cases.

To conclude, patients with COVID-19 blood sample showed different genetic SNPs variation which related with ABO blood group detection and detection susceptibility on ABO gene position in COVID-19 Iraqi patients. The three SNPs genotype which located on re657152, wild type and two others mutant A/A, and two mutant allele A/C and C/C alleles. Finally, Further work need to be done to confirm what present study detect by increasing the number of cases to find out the frequent SNPs variation with large scale frequency.

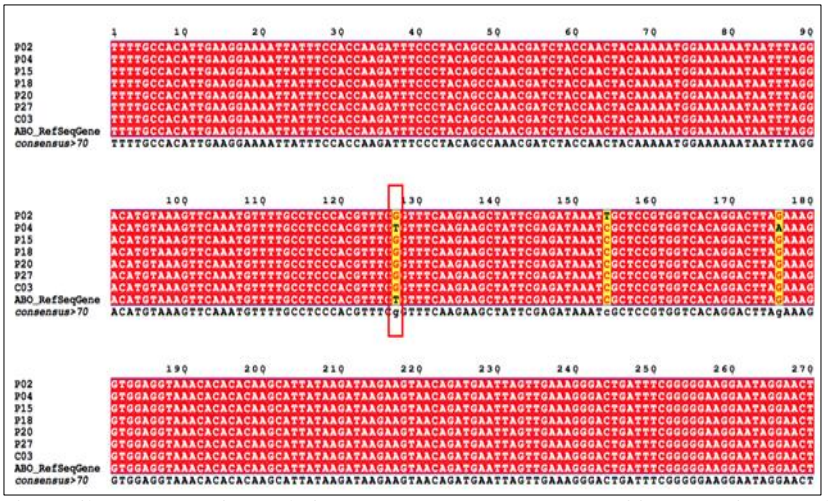

Figure 7: -Alignment of partial ABO gene sequences of 7 samples against a reference ABO gene sequence of Homo sapiens in the NCBI (Accession number: NG_006669) for detection of SNP rs6. There are several hypotheses to explain.

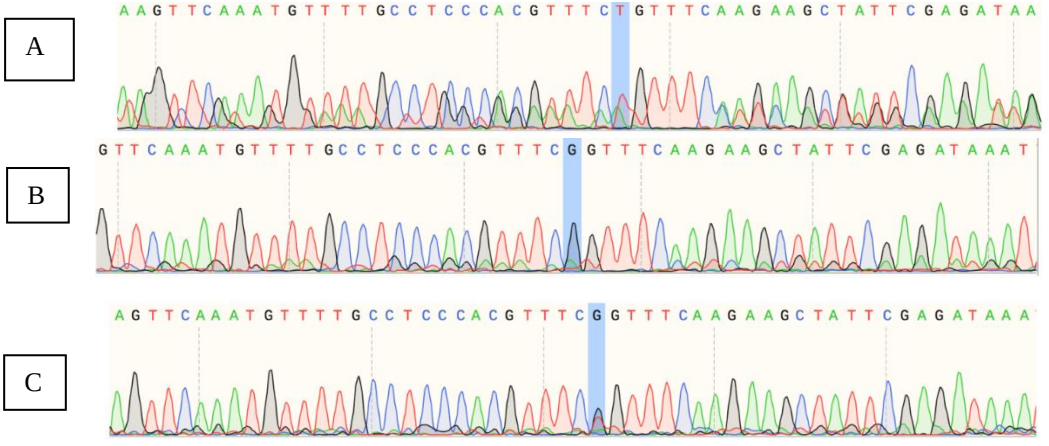

Figure 8: - Chromatogram of the *ABO* gene sequence of PCR product sample of COVID-19 DNA samples (Highlighted in a box. A, wild type AA alleles. B, mutant SNP heterozygous (AC). C, mutant C/C. This figure is provided by Macrogen, Korea using Sanger sequencing method. BioEdit Sequence Alignment Editor software.

Table 4: -Summarizing all globally of different SNPs located the chromosome located rs657152 to assess the relationship of the ABO locus to COVID-19. NCBI

Population	Group	Sample Size	Ref Allele	Alt Allele
Total	Global	250174	A=0.376266	C=0.623734, T=0.000000
European	Sub	219814	A=0.373834	C=0.626166, T=0.000000
African	Sub	5592	A=0.4964	C=0.5036, T=0.0000
African Others	Sub	206	A=0.515	C=0.485, T=0.000
African American	Sub	5386	A=0.4957	C=0.5043, T=0.0000
Asian	Sub	6518	A=0.4396	C=0.5604, T=0.0000
East Asian	Sub	4708	A=0.4565	C=0.5435, T=0.0000
Other Asian	Sub	1810	A=0.3956	C=0.6044, T=0.0000
Latin American 1	Sub	534	A=0.418	C=0.582, T=0.000
Latin American 2	Sub	5292	A=0.2389	C=0.7611, T=0.0000
South Asian	Sub	278	A=0.475	C=0.525, T=0.000
Other	Sub	12146	A=0.38679	C=0.61321, T=0.00000

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