University of Kerbala
College of Science
Department of Biology

Study The Role of Some Factors in Genetic Variations and The Rate of COVID-19 Virus

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In partial of fulfillment of requirements for degree Requirements for the Degree of philosophy Doctorate in Biology/ Microbiology

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

وَيَسْأَلُونَكَ عَنِ الرُّوحِ ۖ

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الإسراء - الآية ٨٥

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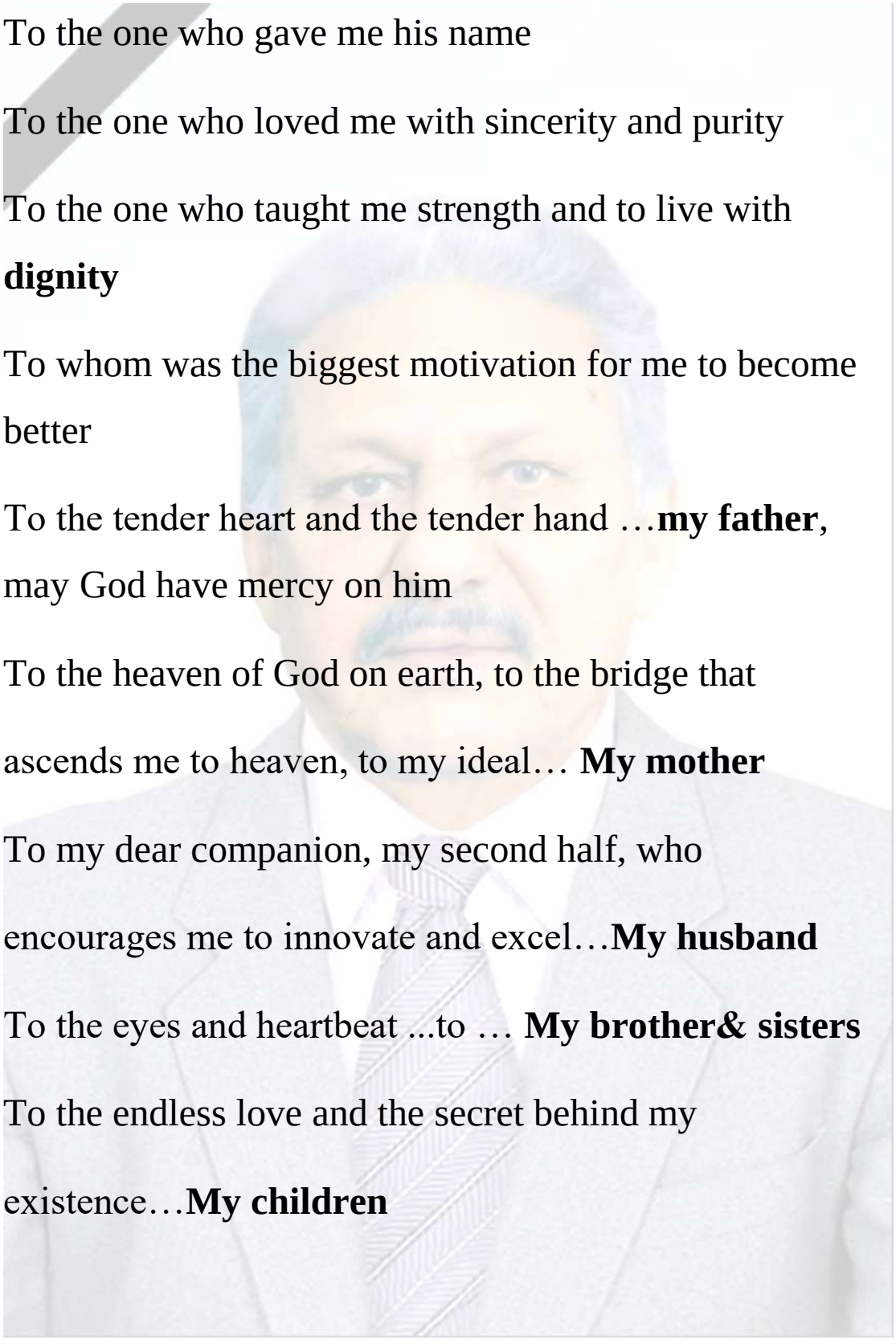
Dedication



To the one who gave me his name

To the one who loved me with sincerity and purity

To the one who taught me strength and to live with **dignity**

To whom was the biggest motivation for me to become better

To the tender heart and the tender hand …**my father**, may God have mercy on him

To the heaven of God on earth, to the bridge that ascends me to heaven, to my ideal… **My mother**

To my dear companion, my second half, who encourages me to innovate and excel…**My husband**

To the eyes and heartbeat ...to … **My brother& sisters**

To the endless love and the secret behind my existence…**My children**

Samar

Summary

Acute respiratory syndrome caused by the Corona virus has spread rapidly, conquering the world and causing the death of more than six million people. Therefore, this study aims to isolate and diagnose the types of fungi associated with infection with the Corona virus, determine the causes of the disease, some symptoms, and some diseases associated with the patients under study, as well as estimate the level of some hematological and immunological parameters and determine the genetic polymorphisms of some genes.

The current study included 167 blood samples and nasal swabs(saliva) distributed as follows: The first group was for 30 patients with Covid-19 and a fungal infection (17 males, 13 females), while the second group was for 107 patients with Covid-19 without a fungal infection. (58 males, 49 females) while the third group wanted 30 healthy people as a control group. The ages of the study samples ranged between 10-70 years.

Clinical samples were collected during the period (January 2022 - April 2022) from patients hospitalized in intensive care units as serious cases from Al-Marjan Teaching Hospital, Imam Al-Sadiq Teaching Hospital in Babil Governorate, and Al-Zahra Hospital in Karbala Governorate. Culturing the samples on SDA Agar and CHROM Agar and testing for hemolysis. The levels of some hematological and immune parameters in the blood were determined by ELISA and CBC... and using the information in the questionnaire to detect some symptoms and associated diseases. In addition, the current study sought to investigate single nucleotide polymorphisms. To the genes IL-17A and IRF-7.

Statistical analysis showed that males were more susceptible to infection than females with ($p = 0.077$), COVID-19 infection increases

with age and that older people were more susceptible to infection than young people, and there was a significant difference ($p \leq 0.0001$) between three groups in Level of WBc, neutrophils, lymphocytes, Ferritin, D-dimer, CRP and suPAR. There was also a significant difference ($p \leq 0.0001$) between three groups in the presence of some symptoms such as fever, cough, shortness of breath and gastrointestinal symptoms. A significant difference was found between three groups in the presence of some Comorbidities.

The statistical analysis of the molecular study showed that the rs2275913 SNP for the IL-17A cellular complex was considered a risk factor for the COVID-19 group with fungal infection, while it was not considered a risk factor for the COVID-19 group without fungal infection, and the rs3819025 gene for the IL-17A group was not considered a risk factor for the group. COVID-19 with fungal infection, while it is considered a risk factor for the COVID-19 with fungal infection group.

Also, the statistical analysis of the polymorphism of the gene RS 17155933 in the interferon-regulating factor-7 showed that it is not considered a risk factor in the presence or absence of a fungal infection, while the genetic polymorphism of rs702965 in the interferon-regulating factor-7 is considered a risk factor for the COVID-19 group without fungal infections only. As for the genetic polymorphism of rs702966 for the interferon-7 regulatory factor, it was considered a risk factor for COVID-19 patients in both groups.

We conclude from the results of the current study that genetic polymorphisms of the cellular complex IL-17A and the protein IRF-7 are associated with the risk of infection with the Coronavirus.

The acute respiratory syndrome caused by the Corona virus, which is one of the respiratory viruses that spread quickly, invaded the world and killed more than six million people. This study searched for several aspect including isolation and diagnosis of fungi species associated with COVID-19, and determined the pathogenic of it ,symptoms and some comorbidities in patients under study, so determined levels of some hematological and immune markers in blood of patients and detection polymorphisms in genes in specific SNPs and role this polymorphisms in immune of patients against diseases, compared this polymorphisms among groups. This case control study included 167 (blood specimens, nasal swaps) distributed as the following: **first group** included 30 patients with COVID-19 with fungal infection was 17 male and 13 female **second group** 107 patients with COVID-19 without fungal infection (after isolation and diagnosis of fungi in nasal swaps) was 58 male and 49 female ,**Third group** included 30 healthy persons as control group was 16 male and 14 female, All with ages (10-70) years .

The blood samples and nasopharyngeal swaps were employed for the detection whether the persons infected by SARS-CoV-2 by SARS-CoV-2 Antigen Rapid Test Kit, that proceeded by collected the specimens between January-2022 to April – 2022, Clinical samples were collected from patients whom lying in Restorative Care Unit as severe case from Marjan Teaching Hospital , AL-Imam AL-Sadiq Teaching Hospital in Babylon province and AL-zahraa Hospital in Karbala Province. After cultivation of specimens in SDA agar ,CHROM agar and hemolysis test, determined levels of some hematological and immune markers in blood by ELISA, CBC,…, By using of information in questionnaire to detection some symptoms and comorbidities finally molecular study .Statistical analysis showed that male ware more prevalence to disease than female with p=0.077, the elderly people were more affected with COVID-19

than young people, there were significant difference with p ≤0.0001, among three groups in level of WBC, Neutrophils, Lymphocyte, Ferritin, D-dimer, CRP and suPAR this is the first study in Iraq that studied the level of suPAR in the blood of COVID-19 patients**,** high significant difference with p ≤0.0001among three groups in presence of some symptoms like fever, cough, dyspnea and gastrointestinal tract GIT symptoms, Significant difference among three groups in the presence of some comorbidities.

Finally, the molecular study statistical analysis found that **rs2275913** SNP on the IL-17A gene was considered a risk factor for COVID-19 with fungal infections patients, while did not considered as a risk factor for COVID-19 without fungal infections group, **rs3819025** on *IL-17A* gene did not considered a risk factor for COVID-19 with fungal infections patients, but considered as a risk factor for COVID-19 without fungal infections group, **rs17155933** on *IRF7* gene did not considered a risk factor in two groups, while **rs702965** on *IRF7* considered as a risk factor for COVID-19 without fungal infections group this is the first study in Iraq and the world which associated polymorphisms in these two SNPs with COVID-19 patients , **rs702966** SNP on the *IRF-7* gene and considered as a risk factor for COVID-19 patients in two groups, Polymorphism in IL-17 A and IRF-7 were associated with the severity of COVID-19 patients.

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List of Abbreviations	
Abbreviate form	**Meaning**
2019nCoV	2019 novel Coronavirus
ACE2	Angiotensin-Converting Enzyme 2
ARDS	Acute Respiratory Distress Syndrome
CBC	Complete blood count
CDC	Centers for Disease Control
COVID-19	Coronavirus Disease 2019
CoVs	Coronavirus
CRP	C-Reactive protein
CSG	Coronaviridae Study Group
ELISA	Enzyme-linked immunosorbent assay
HbA1c	Hemoglobin A1c
HCoVs	Human Coronaviruses
HRP	Horseradish peroxidase
ICTV	International Committee on Taxonomy of Viruses
ICU	Intensive Care Unit
MERS-CoV	Middle East Respiratory Syndrome
MODS	multiple organ dysfunction syndrome
NCBI	National Center for Biotechnology Information
RBD	Receptor-Binding Domain
RNA	RiboNucleic Acid
SARS-CoV	Severe Acute Respiratory Syndrome
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2

SNV	Single nucleotide variation
TMRRSS2	Trans membrane protease, serine2
TNF	Tumor necrosis factor
WHO	World Health Organization
FIA	fluorescence immunoassay
RCU	Restorative Care Unit
MERS	Middle East respiratory syndrome

Chapter One
Introduction

1: Introduction

1-1: Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was the first identified in Wuhan, China in 2019, it caused the spread of a respiratory disease later named COVID-19. The virus usually transmits by air droplets, and spreads rapidly through coughing, talking, sneezing , and contaminating hard surfaces via droplets. Symptoms of this disease may appear in the infected people which can range from mild to critical cases, in addition to the fact that people with comorbidities are more affected, and they are likely to reach the severe stage (WHO, 2020).

Virus that causes severe acute respiratory syndrome coronavirus 2 identified on February 11, 2020, the as (SARS-CoV-2) by The World Health Organization (WHO). Coronavirus disease was named with COVID-19 (Committee, 2020; Zhu *et al.*, 2019). The SARS-CoV-2 is a single-stranded RNA-virus enclosed with capsid protein (Cui *et al.*, 2019). The WHO has classified it as a worldwide pandemic of public health because of its wide geographical impact on an extraordinary high proportion of the world's population (Wilder-Smith, 2021). COVID-19 is an acute disease that can impact numerous organs through the lungs. These organs include the kidney, liver, muscles, nervous system, and spleen (Machhi *et al.*, 2020).

The SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) as its receptor on the surfaces of target cells for Coronaviruses. mainly their matching receptors on targeted cells via S proteins on their surface (Wrapp *et al.*, 2020). Around the world, more than 39.7 million individuals were infected with SARS-CoV-2, and over 1.1 million died in 2020. The number of infections is increasing (WHO, 2020). Coronaviruses often cause minor

respiratory conditions. Human coronaviruses can induce frequent colds and cause lower respiratory tract infections (Li *et al.,* 2019).

Several biomarkers have been identified in connection with the SARS-CoV- count (CBC), high levels of serum C-Reactive protein (CRP), erythrocytes sedimentation rate (ESR), D-dimer, ferritin, lactate dehydrogenase (LDH), and hemoglobin A1c (HbA1c), after a viral infection (Conti *et al.,* 2020).

Early literature reported low rates of bacterial and fungal infection in hospitalized COVID-19 patients but high use of empirical broad-spectrum antimicrobials (Rawson *et al*., 2020). In hospitals, the difficulty in clinically differentiating COVID-19 and its progression from bacterial and fungal infection provides a significant challenge to clinicians (Huttner *et al*., 2020). High-quality evidence to support decision-making on bacterial and fungal infection in COVID-19 is limited. Clinical uncertainty is likely to drive unnecessary antimicrobial prescribing in COVID-19 patients both on and during admission, potentially increasing the selection of drug-resistant infections (Holmes *et al.,* 2016).

COVID-19-associated fungal infections can lead to severe illness and death (Hoenigl,2020) Symptoms of certain fungal diseases can be similar to those of COVID-19, including fever, cough, and shortness of breath, Some patients can have COVID-19 and a fungal infection at the same time(Baddley *et al*., 2021).

COVID-19 likely increases the risk for fungal infections because of its effect on the immune system and because treatments for COVID-19 (like steroids and other drugs) can weaken the body’s defenses against fungi.(

Narayanan *et al*., 2021) . The most commonly reported fungal infections in patients with COVID-19 include aspergillosis, invasive candidiasis, and mucormycosis (sometimes called by the misnomer ”black fungus, Fungal infections resistant to antifungal treatment have also been described in patients with severe COVID-19 (Posteraro *et al*., 2020).

The urokinase-type plasminogen activator receptor (uPAR) is expressed on various cell types, and the complex interaction with its ligand urokinase plasminogen activator (uPA) has been shown to promote tissue invasion in malignant diseases by converting plasminogen into plasmin, resulting in degradation of the extracellular matrix.(Ostergaard *et al*., 2004), Migration of inflammatory cells from the bloodstream into tissues is an essential component of inflammation and the immune response against infection in which the uPAR/uPA system is directly involved(Portelli *et al*., 2014) During inflammatory stimulation, uPAR is cleaved from the cell surface by proteases to create the soluble form of the receptor, suPAR, which can be detected in blood, urine, and cerebrospinal fluid, High suPAR levels were shown to be associated with an undesirable outcome in HIV infection(Lawn *et al*.,2007), active pulmonary tuberculosis, bacterial meningitis, and the pneumococcal bacteremia, suggesting that it may be a good biomarker of inflammation(Eugen-Olsen *et al*., 2010). The role of some cytokines in disease were diagnosed and could be as biomarkers of this disease (Korte and kinney, 2016)

IL-17 is a member of pro-inflammatory cytokines secreted by Th17 cells, which has a crucial role in the recruitment of monocytes and neutrophils to the site of infection. The IL-17 family includes 6 members: IL-17A (known as IL-17) and the related family members, including IL-17B, IL-17C, IL-17D, IL-17E (known as IL-25), and IL-17F. IL-17A is the

most critical member of this family1(McGeachy *et al*., 2019). IL17 can exacerbate inflammatory reactions by activating downstream cytokines, such as IL-1, IL-6, IL-8, TNF-α, and MCP-1 (Rokni *et al*.,2020). Also IRF7 have important role in regulation of target cytokines such as interferon (IFN)beta, and they represent the major players in innate immune responses (Nehyba *et al*.,2009).

1-2: Aim of the Study

The current study was conducted to find out the relationship between fungal infection and certain immunological and hematological promoter as predictors of severity in COVID-19 patients through achieving the following objectives:-

1-Determine species of fungi (causing fungal infection) which associated with COVID-19 infection.

2- Estimation the WBC, Lymphocyte, Neutrophil count .

3-Estimation the level of suPAR (Soluble urokinase-type plasminogen activator receptor) D-Dimer, CBC, FIRRITIN and CRP in the blood among control and patients with SARS-COV-2.

4-Determine the symptoms and comorbidities which associated with COVID-19.

5-Investigate the gene polymorphism in some of SNPs in IL-17A and IRF-7 genes that may be associated with COVID-19 by sequence sanger analysis.

Chapter Two
Literatures Review

2: Literatures Review

2-1: Review Of COVID-19

COVID-19 Initially referred to as 2019 novel coronavirus, the virus has now been designated severe acute respiratory syndrome coronavirus-2 (Wu *et al.*, 2020). As 4 February 2021, there have been 103.989.900 confirmed cases of COVID-19, including 2.260.259 deaths, according to world health organization (WHO) reports (WHO, 2020a). The seafood market in Wuhan, China, is connected to the transfer of zoonotic diseases. Later, it was realized that the outbreak that followed was largely caused by human-to-human transmission. (Li *et al*., 2020a). The Middle East respiratory syndrome coronavirus (MERS-CoV) and the severe acute respiratory syndrome coronavirus (SARS-CoV-1) are the first two lethal coronaviruses to develop in the last two decades, respectively. (Huang *et al.*, 2020) .

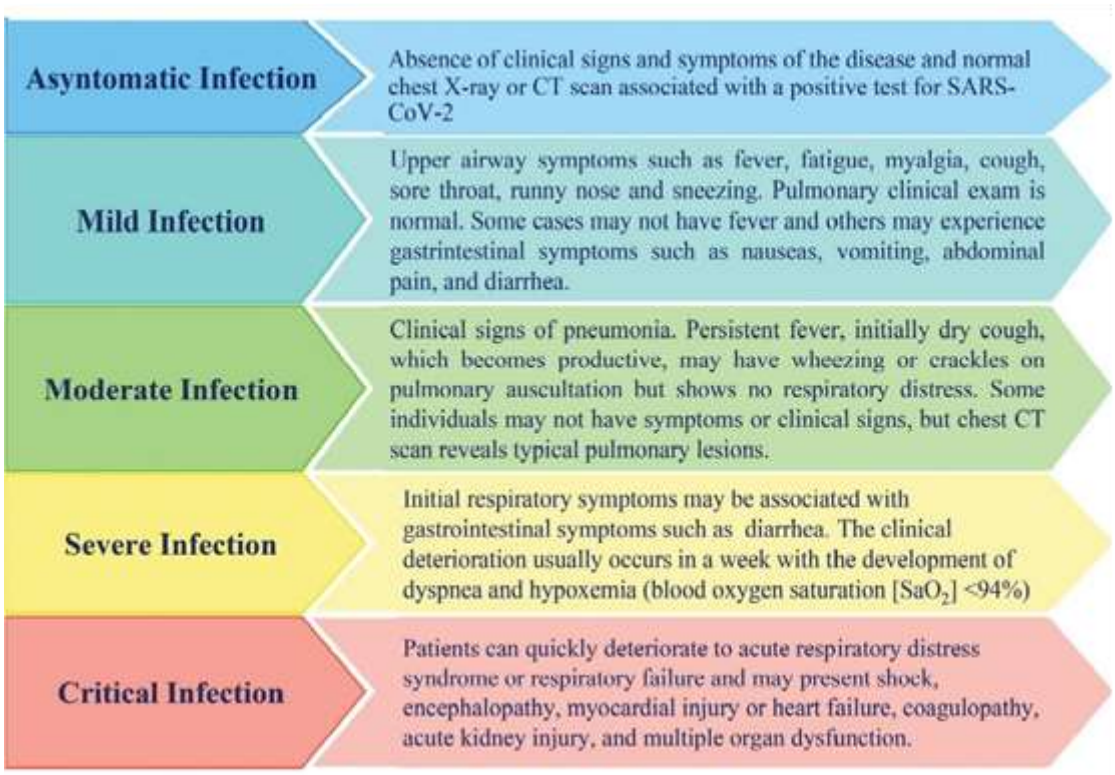
The SARS-CoV-2 can exploit the angiotensinconverting enzyme 2 (ACE2) for priming Spike (S) protein (Balouzard and Whittaker, 2009; Hasan *et al*., 2020). The ACE2 expressed in the esophagus, lungs, liver, and intestinal epithelium (Amico *et al*., 2020 ;Hoffmann *et al*., 2020). SARS-CoV-2 infection can be asymptomatic (without symptoms) or can cause a wide spectrum of symptoms: fever, dry cough, shortness of breath, pneumonia, pulmonary edema, acute respiratory distress syndrome (ARDS), multiple organ failure and death (Wang *et al*., 2019). Some patients had a common symptoms include headache, nausea, and vomiting, and diarrhea is also reported (Chen *et al*., 2019).

The international Committee for the Taxonomy of viruses (ICTV) has approved the naming of more than 40 coronaviruses. Most of which infect only animals. The SARS-CoV-2 virus is a seventh known coronaviruses that infect humans. Of these only four consider as

community acquired and are contagious for human for a very long time, the rest three – SARS-CoV, MERS-CoV and SARS-CoV-2 seem to have recently infected the human population. Sadly, all three of these have a significant death rate (Decaro and Lorusso , 2020).

According to the clinical manifestations, confirmed patients are divided into mild, moderate, severe, and critical types (Table 2-1) (Monassier, 2008; Small *et al*., 2001).

Table (2-1): Clinical presentation of COVID-19 (Carlotti ***et al., 2020)***



Coronavirus is a single-stranded RNA virus of 30 kb. According to it is genomic makeup, the virus is divided into four genera: α, β, γ, and δ (Rabi *et al*., 2020) . The five phases of the SARS-CoV-2 life cycle are attachment, penetration, membrane fusion, biosynthesis, and release. Attachment occurs when the virus binds to the host cell receptors; penetration occurs when the virus enters the host cell; biosynthesis occurs when viral proteins are made using viral mRNA; maturation follows; and

release occurs when the new viral particles are released (Hamming *et al*., 2004).

The virus is composed of a total of four structural proteins, namely Spike (S), Membrane (M), Envelop I and Nucleocapsid (N) (Bosch *et al*., 2003). Spike is made up of two functional subunits, S1 and S2, where S1 would be in charge of binding to the host cell's receptor and S2 facilitates the fusing of the viral and cellular membranes. The functional SARS-CoV-2 receptor has been identified as angiotensin-converting enzyme 2 (ACE-2), and the spike protein binds to this particular receptor (Chen *et al., 2*020).

The spike protein is observed to go through a protease cleavage by two successive steps after attaching to the host receptor, which results in its activation. Here, it can be observed that the first cleavage occurs at the S1/S2 site and is necessary for priming, whereas the second cleavage occurs at the S2 site and is necessary for activation. (Belouzard *et al*., 2009). After cleavage, the S1 subunit's function is to maintain the membrane-anchored S2 subunit in place. But S1 and S2 continue to be bonded non-covalently (Walls *et al*., 2020).

Under the electron microscope, coronaviruses have spike-like projections on their surface that give them the appearance of a crown, giving them the name coronavirus. Coronaviruses are enveloped by positive-sense RNA viruses that range in diameter from 60 nm to 140 nm. They generally cause mild respiratory disease (Singhal, 2020).

CoV is a virus with viral surface proteins that is lipid membrane-wrapped and originated from host cells. The spike [S] protein in particular, which has a distinctive halo-like appearance under the electron microscope, has earned the name corona, which refers to a viral membrane (Bullock *et al.,* 2021). Similar positive RNA (RNA) polarity

has been found in the genomes of CoVs, indicating that the base sequence of the RNA is in the 5' to 3' direction, matching to the later messenger RNA (mRNA). At 26.4-31.7 kilobases, the CoV genome is the biggest of all recognized RNA viruses. (Drosten *et al.*, 2003; Woo *et al.*, 2012).

SARS-CoV-2 consists of four important structural proteins, including spike (S) glycoprotein and small envelope glycoprotein (E). membrane glycoprotein (M) and nucleocapsid (N) as demonstrated by figure (2-1).

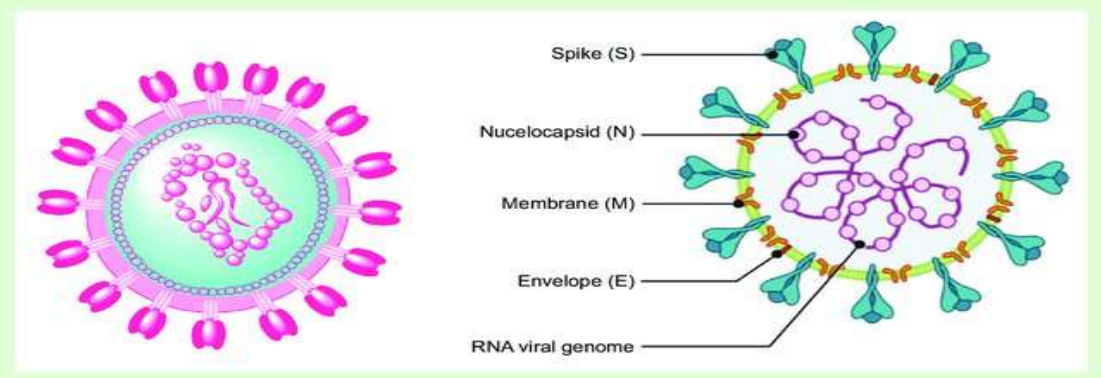


Figure: (2-1). Diagrams showing the structure of the SARS-CoV-2 virus (Amawi *et al.*, 2020)

2-2: Classification of Covid-19

The family Coronaviridae includes coronaviruses. They are separated into two groups: those that can only infect mammals (Alpha and Beta coronaviruses) and those that mostly infect birds (Gamma and Delta coronaviruses) (Cui *et al*., 2019). Seven human coronaviruses (HCoV) have been identified to far; HCoV-229E and HCoV-NL63 are two examples of Alpha coronaviruses. The remaining five Beta coronaviruses are HCoV-OC43, HCoV-HKU1, SARS-CoV-2, Middle East Respiratory Syndrome Coronavirus, and Severe Acute Respiratory Syndrome Coronavirus (Cui *et al*., 2019; Chan *et al.*, 2020) as figure (2-

2). Coronaviridae Study Group (CSG) of the International Committee on Taxonomy of Viruses classify this virus (CSG, 2020).

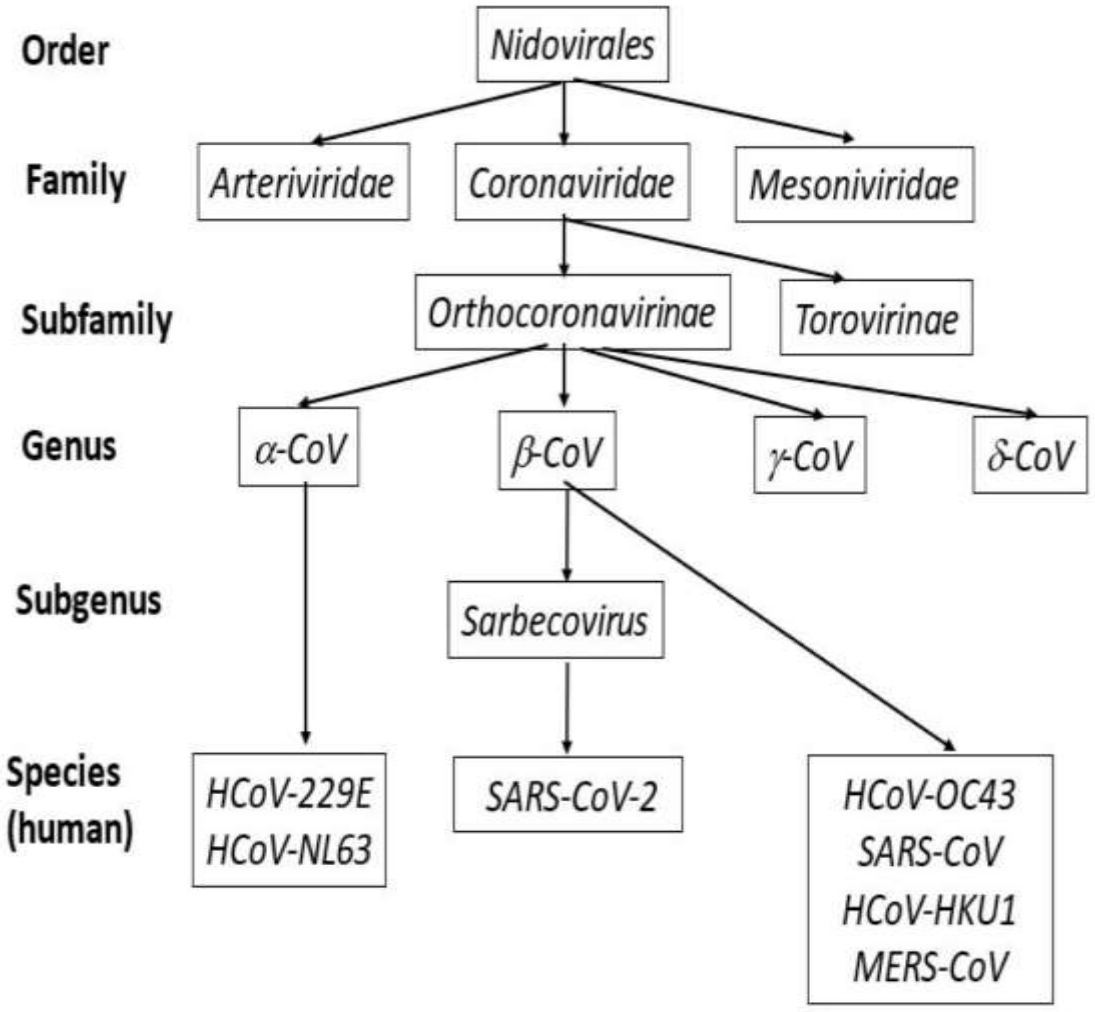


Figure: (2-2). Classification of Coronaviruses (Malik, 2020)

2-3: Pathogenesis of COVID-19

Recently, the following clinical phases for COVID 19 have been proposed: Phases of viremia, acute illness, and recovery (Lin *et al.*, 2020). According to general hypotheses, an infection will progress through the following stages: viral replication, immune system `, multiple organ damage, and recovery (Weiss and Leibowitz, 2011). First, the virus penetrates the host cells, where it multiplies, assembles, and is delivered extracellularly to target cells, directly causing the destruction

and injury of parenchymal cells such alveolar epithelial cells. ARDS, sepsis, and MODS are caused by the simultaneous release of a large number of pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) molecules that activate the innate immune system, cause inflammatory cell infiltration, and release a large amount of cytokines, chemokines, proteases, and free radicals (Hanley *et al*., 2020).

The pathological findings of COVID 19-induced pneumonia have been noted to resemble those seen in SARS-CoV and MERS-CoV infection. These findings include bilateral acute changes with diffuse alveolar damage and vascular congestion, patchy inflammatory cellular infiltration, intra alveolar edema, hemorrhage, proteinaceous exudate, denudation and reactive hyperplasia of pneumocytes, as well as the presence of multinucleated giant cells, but hyaline membrane formation was is not prominent observed (Hanley *et al*., 2020). After the initial critical stage, the inflammatory response gradually subsides, the damaged organ gradually makes a full recovery, and some of the damaged organs progress to the fibrosis and chronic stages, where conditions like chronic critical illness, persistent inflammation, immunosuppression, and catabolism syndrome can develop. It is hypothesized that SARS CoV 2's cytopathic effect and the detrimental immunological responses it triggers may both directly and indirectly contribute to the significant pathological changes that occur in the important organs during COVID 19 (Lescure *et al*., 2020).

SARS-CoV-2 invades host cells as figure (2-3) It is well acknowledged that interactions between the human CoV virus and particular host cells play a major role in both human CoV pathogenesis and transmissibility (Weiss and Navas, 2005). The first stage of viral

infection is receptor recognition and entry, which is also the primary predictor of tissue tropism. It has been hypothesized that increased virus transmissibility and illness severity in humans are correlated with increased binding affinity between SARS CoV 2 and ACE2 (Wan *et al.*, 2020). CoV enters host cells by a multi-step process involving several distinct domains in the S protein which mediates viral attachment to the target cell surface as figure (2-3), membrane fusion, protease processing, and receptor activation. The virus then replicates inside the host cells when the viral DNA is released into the cytoplasm (Letko *et al*., 2020).

Notably, three coronaviruses that bind to the same receptor (ACE2)—human CoV NL63, SARS CoV, and SARS CoV 2—cause illnesses of varied severity, suggesting that other pathogenic mechanisms may be responsible for the discrepancies between these three coronaviruses (Davidson *et al.*, 2020). Additionally, ACE2 is widely expressed in smooth muscle cells and vascular endothelial cells in all organs, which may result in extensive vascular endothelial cell injury. This could be the molecular mechanism underlying the development of numerous organ diseases in COVID 19-infected patients (He *et al*., 2006).

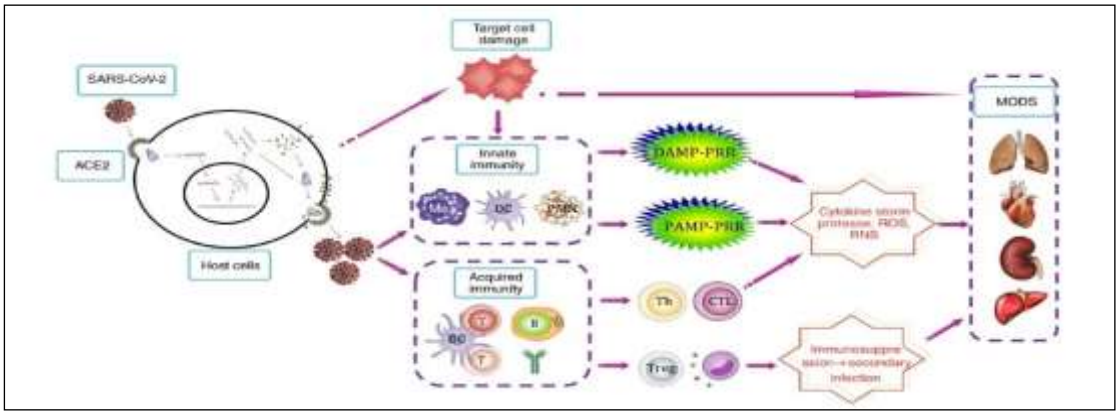


Figure (2-3) 1. Hypothetical pathogenesis of COVID-19 (Li c *et al*., 2021)

2-4: Immunity Against Viral Infection

The human body has three different types of immunity: innate immunity, which is a quick response to infections, adaptive immunity, which is a slower reaction, and passive immunity, which can be either natural immunity acquired from maternal origin or artificial immunity acquired through medication like vaccination. SARS-CoV2 enters the nasal cavity through coughing and sneezing to spread through respiratory droplets to the respiratory system . The primary viral receptor is ACE2 to which the S protein spike is pinched inside the host cell. The host cell's furin enzyme then plays an essential role for the viral entry, where the virus starts to spread and can be detected by nasal swabs. In the respiratory tract, however, the virus encounters a more active innate immune response. An innate response cytokine may be able to anticipate the ensuing clinical course once the disease started to emerge clinically (N.L. Tang, *et al*., 2005).

The Toll-like receptors (TLR), a family of 11 transmembrane receptor proteins, are involved in the activation and development of innate immunity and act by recognizing pathogen-associated molecular patterns (PAMPs). Numerous important body cytokines, including IL-6, IL-1, and TNF, are released in response to a coronavirus. (Conti *et al*., 2020; Conti *et al*., 2020). When a certain TLR recognizes a virus, the body's first line of defense against viral infections is innate immunity. TLR3, which has an unique role in the formation of an immune response to coronaviruses, is associated to both SARS-CoV and MERS viruses (Totura *et al*., 2015). The most prevalent bodily cells that express TLR3 are dendritic cells, placental cells, and pancreatic cells. TLR3 activation through the TRIF (TIR-domain-containing adapter-inducing interferon)

pathway controls the activation of IRF3 (interferon regulatory factor 3) and NF-kB (Uematsu and Akira ., 2007).

Other important cytokines include those involved in adaptive immunity (e.g., IL-2 and IL-4), pro-inflammatory cytokines and interleukins (ILs) (e.g., interferon (IFN)-I, -II, and -III; IL-1, IL-6, and IL-17; and TNF-α); and anti-inflammatory cytokines. Type I interferons (IFN alpha and beta) are produced more frequently (e.g., IL-10) (Totura AL, *et al*., 2015).

IL-1, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, M-CSF, G-CSF, GM-CSF, IP-10, IFN-, MCP-1, MIP-1, hepatocyte growth factor (HGF), TNF-, and vascular endothelial growth factor were all shown to be present in abnormally high levels in COVID-19 patients, according to numerous studies (VEGF) (Huang C, *et al*., 2020; Liu K., *et al*., 2020; Chen C., *et al*., 2020; Costela-Ruiz VJ, *et al*., 2020).

T cells and B cells, which are crucial to adaptive immunity, make up the second element of immunity. While CD8 cells are responsible for actively destroying the virus, CD4 cells direct and develop the immune response to antibodies. Patients with SARS and MERS were shown to have immunogenic CD4 and CD8 T cell epitopes that were primarily localized to structural proteins, particularly the S protein (Li *et al*., 2008; Shin *et al.,* 2019). Dendritic cells in the lung interstitium are able to identify virus particles and/or cell debris damaged by viral infection. After that, they proceed to the lymph nodes where they are exposed to CD4 and CD8 cells, which are crucial for triggering both innate and adaptive immunity.

The macrophages in the lymph nodes create cytokines when antigen enters the lymph nodes, including interferons and interleukins like interleukin-12 (IL-12). In the presence of IL-12, dendritic cells (DCs)

stimulate CD4 T lymphocytes, causing them to differentiate into Th1 effector cells. These generate the cytokines IL-2 and IFN gamma that help CD8 T lymphocytes and B lymphocytes differentiate into cytotoxic cells and immunoglobulin M-producing plasmocytes (Hue *et al.*, 2020; Rydyznski Moderbacher *et al*., 2020; Villas-Boas *et al.*, 2020).

Therefore, it is essential to induce a balanced host immune response using both adaptive and innate immunological responses, as well as processes mediated by the complement system, in order to control and eradicate any infection, including the SARS-CoV-2. On the one hand, unchecked immunity may lead to lung tissue damage, functional impairment, and decreased lung capacity (Kruse R. L., 2020). On the other hand, immunological weakness or confusion may promote viral multiplication and result in tissue damage (Li G. *et al.*, 2020) .

2-5: Review of Respiratory Fungal Infection

Functionally, the respiratory system can be divided into two zones: the conducting zone (from the nose to the bronchioles), which creates a pathway for the conduction of the inhaled gases, and the respiratory zone (from the alveolar duct to the alveoli), which is where gas exchange occurs. The upper (organs outside the thorax, including the nose, pharynx, and larynx) and lower respiratory tracts are separated anatomically by the respiratory tract (organ within thorax - trachea, bronchi, bronchioles, alveolar duct and alveoli) (Patwa and Shah, 2015)

For certain infections, the nose and oral cavity can be thought of as their primary entry points. The phrase "microbiota" refers to the collection of bacteria, fungi, viruses, and archaea that live in the nasal passages and mouth cavity (Zaura *et al*., 2014; Wade, 2013).

Temperature (37C°) and saliva pH (6.5-7) make an appropriate environment for microorganism survival and maintenance (Deo and

Deshmukh, 2019; Lamarre and Talbot,1989). Additionally, the availability of oxygen might affect how an organism grows. The predominant microbiota populations in the nasal cavity are bacterial and fungal. Aspergillus species, Candida species, Cladosporium spp., Penicillium sp., and in healthy cases, other kinds of fungi, make up the majority of the fungal population (Zhang *et al.*, *2*018, Ghannoum *et al.*, *2*010).

Numerous research have looked at the influence of microbiota on disease or human health over the last 20 years. The respiratory system is constantly exposed to fungal spores found in the environment, and investigations have shown that even in healthy individuals, viable fungus can be found in high concentrations in bronchial sputum and sinonasal mucus cultures (Ponikau *et al*., 1999; Buzina *et al*., 2003). Fungal presence is commonly considered as colonization, but it may be an important extrinsic trigger for upper and lower airway allergic diseases especially in patients with asthma, chronic rhinosinusitis (CRS), cystic fibrosis and allergic rhinitis (Kumamoto, 2016).

The huge diversity of fungus, issues designating key allergens, unknown pathophysiology, and the role of fungi as allergens all make it difficult to comprehend how fungi can cause allergic airway disorders. A host's inflammatory response and interactions with microbial communities, particularly fungus, may contribute to or influence the inflammatory process of upper and lower airway disease (Huffnagle and Noverr, 2013) . Each year, invasive fungal diseases brought on by different fungal species, such as Aspergillus, complicate and put millions of people's lives in jeopardy (Hallen and Suhr, 2017).

Numerous infections in humans, including invasive pulmonary aspergillosis (IPA), chronic pulmonary aspergillosis (CPA), allergic

bronchopulmonary *aspergillosis* (ABPA), chronic rhinosinusitis, fungal asthma, A. bronchitis, and others, are caused by the ubiquitous *Aspergillus* genera, most frequently *A. fumigatus*. (Rolling *et al*., 2020;Azoulay *et al*., 2006). IPA, the most severe manifestation of disease from *Aspergillus*, is a serious complication that is frequently seen in people who have profound immunosuppression, such as those undergoing hematopoietic transplantation, as well as people who have structural lung damage and are taking systemic corticosteroids for their underlying condition. Patients with chronic obstructive pulmonary disease are at particular risk for developing this condition because it is linked to high mortality rates (COPD) (Rolling *et al., 2*020).

The majority of the organelles found in fungi are comparable to those seen in other eukaryotes. Fungal nuclei can compress and/or stretch to fit through septal holes and into forming spores. They are typically tiny (2 m in diameter). It has been shown that fungi have between 6 and 21 chromosomes, with 6,000 to almost 18,000 genes on each. In filamentous fungi, genome sizes range from 8.5 megabase pairs (Mb) to little over 400 Mb. (Zolan 1995; Spanu *et al.* 2010; Duplessis *et al.*, 2011), Fungal genomes are among the smallest known eukaryotic creatures on average, measuring only 1. 3 times the size of the largest known bacterial genome and 1% of the size of mammalian genomes ,While some fungi (Ascomycota) have a largely haploid life cycle, others (Basidiomycota) have a protracted dikaryotic phase (Stover *et al.,* 2000).

2-6:Classification of Respiratory Fungal Infection

Contrary to what was previously believed, healthy people's respiratory tracts are really made up of a complex microbial population known as the microbiome (Charlson *et al*., 2010; Erb-Downward *et al.*, 2011) , The term "biome" refers to the entire genome collection of a

particular microbial community, whereas the term "biota" refers to a specific microbial population linked with host tissues or organs. The fungal component of a particular microbial community is thus referred to as the "mycobiota," and the matching genomes are referred to as the "mycobiome" (Iliev *et al.,* 2012; Orgiazzi *et al.,* 2013). Although the idea of the human mycobiome has received attention in recent years (Huffnagle and Noverr, 2013), the most common species or genera found in the respiratory mycobiome were: *Candida spp, Aspergillus spp., Penicillium, Cladosporium spp*. (Cui *et al.,* 2013).

Candida is a genus of yeasts that accounts for the majority of fungal infections in the world (Manolakaki *et al., 2*020). Many species live in hosts, including humans, as innocuous endo symbionts or commensals, but when mucosal defenses are breached or the immune system is weakened, they can invade and spread disease, a condition known as an opportunistic infection. Most mucosal surfaces, particularly those of the gastrointestinal tract, as well as the skin, are home to *Candida, Candida* species be a member of the phylum Ascomycota, subphylum Saccharomycotina, which contains a wide range of fungus that are harmful to humans (Kourkoumpetis *et al., 2*011).

Kingdom: Fungi
Phylum: Ascomycota
Subphylum: Ascomycotina
Class: Ascomycetes
Order: Saccharomycetales
Family: Saccharomycetaceae
Genus: Candida sp. (Lumbsch and Huhndorf, 2007)C. albicans ,C. glabrata ,C. krusei , C. parapsilosis .

Penicillium :- *Penicillium* is well-known and one of the most widespread fungi, found in a variety of habitats, including soil, vegetation, air, interior settings, and many food products. It is widely used and has a significant financial impact on people's lives. Its primary role in nature is the breakdown of organic matter, where specific species harm food crops by causing destructive rots before and after harvest (Samson *et al*., 2010), besides creating a variety of mycotoxins. Select species also have beneficial effects, and the food industry uses some species to produce specialty cheeses like Camembert or Roquefort and fermented sausages (Giraud *et al*., 2010). Due to their capacity for degradation, species are being examined in order to discover new enzymes (Terrasan *et al*., 2010).

Kingdom : Fungi

Division : Ascomycota

Class: Eurotiomycetes

Order: Eurotiales

Family: Trichocomaceae

Genus: Penicillium sp. (Tsang *et al., 2*018)

Aspergillus is a genus made up of a few hundred mold species that can be found in different regions all over the world. Pier Antonio Micheli, an Italian priest and naturalist, first compiled a list of *Aspergillus* species in 1729. When Micheli examined the fungi under a microscope, she was reminded of the aspergillum (Latin for holy water shower), and she named the genus accordingly (Bennett, 2010), While some *Aspergillus* species are known to cause fungal infections, others are of commercial importance, and *A. niger* is the most important species, all *Aspergillus* species share an asexual spore-forming structure called an aspergillum.

About one-third of species are also known to have a sexual stage. (Geiser, 2009).

Kingdom : Fungi

Division :Ascomycota

Class : Hyphomycetes

Order : Hyphotiales

Family: Moniliaceae

Genus: *Aspergellus niger .(Forbes ,1998).*

Cladosporium is a genus of fungi that includes some of the most prevalent molds found both inside and outside. Species have dark-pigmented conidia that grow in simple or branching chains and produce olive-green to brown or black colonies. Both living and dead plant material frequently contain a variety of *Cladosporium* species. Some species parasitize fungi, while others are plant pathogens or endophytes (Khan *et al.,* 2016) Wind disperses *Cladosporium* spores, which are frequently very plentiful in outdoor air. When there is moisture inside, *Cladosporium* species may develop on surfaces. While the ideal stage of fungi (teleomorph) only occasionally forms, these fungi are frequently seen in the conidial stage (anamorph).

However, the findings of molecular investigations allowed their teleomorph to be classified as Davidiella (Kryczyski and Weber, 2011). This genus was previously categorized as belonging to the family Dematiaceae (dark-colored fungi), order Moniliales, class Hyphomycetes, and phylum Deuteromycota (Fungi Imperfecti) (Masclaux *et al.*,1995). This genus is now recognized as belonging to the phylum Ascomycota, class Dothideomycetes, order Capnodiales, and family Davidiellaceae

due to significant modifications in the taxonomy of fungus. (Krzyściak *et al., 2*011).

Kingdom: Fungi

Division: Ascomycota

Class: Dothideomycetes

Order: Capnodiales

Family: Davidiellaceae

Genus: Cladosporium sp. (Krzyściak *et al*., 2011)

2-7:Pathogenesis of Respiratory Fungal Infection

Diseases brought on by fungi continue to pose a serious hazard to public health due to the rising number of immune compromised people. Aspergillus with invasive aspergillosis (Latge, 1999), Cryptococcus with cryptococcosis (Chayakulkeeree and Perfect, 2006), and Pneumocystis with pneumonia (Udwadia *et al.*, 2004) are examples of opportunistic fungus. Endemic fungi are others (Lortholary *et al.*,1999),and endemic fungi (Lortholary *et al.*,1999) are the main causes of fungal infections in the lungs of humans. Despite the fact that these infections are rarely identified in the target organs of healthy individuals, they can cause invasive diseases that are fatal in patients with weakened immune systems. These people include those with immunodeficiency diseases like HIV/AIDS, cancer patients receiving chemotherapy, and people receiving immunosuppressive medication like in bone marrow/stem cell transplants. Invasive mycoses have increased in frequency and infectiously died as a result of pathogenic fungus infections in the lung, especially in individuals with significant host immune response deficiencies (José and Brown, 2012).

As previously indicated, some fungal pathogens, particularly in immune compromised individuals, cause mycosis with numerous tissue

lesions after infecting the host through surface proteins from the pathogen-host contact (McMath and Hussain ,1961).

Aspergillus mold is one of the most widespread fungal species capable of sporulation with airborne conidia discharged. The airborne conidia that are formed are small enough (2 to 3 m) to enter human airways and pulmonary alveoli and cause a variety of illnesses, including fatal infections in people with impaired immune systems and atopic asthmatic patients (Latge ,1999). Alveolar macrophages in healthy people absorb inhaled conidia and destroy them in a phagocyte oxidase-dependent manner (Ibrahim-Granet *et al.,* 2003). In immune compromised individuals, incomplete killing of inhaled fungal conidia results in germination and tissue invasion by fungal hyphae (Latge , 2001).

Cryptococcosis is brought on by exposure to Cryptococcus in the lungs following inhalation of airborne pathogens. According to Kronstad *et al.* (2011), Cryptococcus neoformans is a subtype of the fungus that is widely distributed, especially in soil and avian environments. Cryptococcal meningitis is the most serious consequence of a Cryptococcus infection. Since *C. neoformans and C. gattii* are able to spread from the lung into the brain by piercing the blood-brain barrier (BBB), the fungi directly enter the brain through endothelial cells on blood arteries utilizing a "Trojan horse" technique that involves the movement of phagocytes. (Kronstad *et al.,* 2011).

Counting the quantity of yeast cells in the brain during animal tests revealed that C. neoformans was still present in the CNS, where extensive colonization and tissue damage could still take place despite the host's defense mechanisms (Blasi *et al.*,1993).

PCP (Pneumocystis pneumonia) brought on by a fungus-pathogen species The most prevalent AIDS-defining illness is pneumocystis, which can also be present in non-HIV immune compromised people with a lack of adaptive immunity or those receiving extended high-dose systemic glucocorticoids. (José and Brown, 2012). The Pneumocystis antigens are primary surface glycoprotein (Msg or glycoprotein A) (Lundgren et al.,1991) and Pneumocystis protease, kexin (Kex1, Prt1). As a potential therapeutic target, Kex is supposed to participate in the proteolytic processing of Pneumocystis surface antigens (Morris *et al.,* 2008).

D-Dimer, CBC, FIRRITIN and CRPEndemic mycoses often only occur in a small geographic area, and they can cause serious and even fatal cases that require hospitalization (Queiroz-Telles *et al.,* 2017). Endemisc mycoses can potentially result in more severe, widely distributed illness in immune compromised patients, which increases mortality (Malcolm and Chin-Hong , 2013). A rising number of immune compromised people is associated with a rise in the incidence of endemic mycoses in several countries. Unexpectedly, mortality is also high in hosts who are not immune deficient (Hage *et al.,* 2012).

Candidiasis is range of infections caused by species of the fungal genus Candida; these infections can be acute or chronic, systemic or localized. Disseminated candidiasis is life threatening. The great majority of candidiasis is caused by *Candida albicans* (*C. albicans*) is a typical commensal fungi that lives in the human oropharynx, gastrointestinal system, and vagina, but it can also cause opportunistic infections when the usual flora is upset, the mucocutaneous barrier is breached, or the host's cellular immunity is compromised (Naglik *et al*., 2003).

2-8: Immunity Against Respiratory Fungal Infection

Fungi are common, in and out bodies. Asthma and allergic airway disorders are highly correlated with fungus exposure and/or sensitization. Furthermore, it's conceivable that changes in the global environment may boost the antigenicity and prevalence of fungi (Wheat,1995).

Diseases brought on by fungi continue to pose a serious hazard to public health due to the rising number of immune compromised people. The primary causes of fungal infections in human lungs are opportunistic fungi, such as Aspergillus with invasive aspergillosis (Carvalho *et al.*, 2012), and endemic fungi (Lortholary *et al.*,1999).

There are a relatively poor understanding of the integrated cellular and molecular systems involved in the process of distinct fungal infection in the lung, despite mounting signal mechanisms driving the initiation and development of fungal infection investigated by independent groups being clearly revealed. As a result, there are few viable therapeutic options to treat dangerous pulmonary fungal infection and inflammation. In response to a fungal pathogen challenge, several cell types and chemicals, such as receptors, adaptors, kinases, and transcriptional factors, contribute to the host processes of fungal infection in the lung. A precise network of innate immune cells and adaptive CD4+ Th1 type cells forms the host defense against pulmonary fungal infections. (Cenci *et al*.,1998)

The local alveolar macrophages (AM), DC, and neutrophils in particular frequently function modify the early immune response to pulmonary infection in the first line with an armored defense against pulmonary fungal infections (Osterholzer *et al*., 2009). During the infectious process, neutrophils play a crucial orchestrating role, especially polymorphonuclear neutrophils (PMN). These cells can quickly infiltrate

the lungs and reach inflammatory areas where they serve a crucial function in removing infections and encouraging tissue healing.. At the early phase of *Aspergillus fumigatus* infection (Mircescu *et al.*, 2009). The first line of immune protection in the lung is provided by phagocytic leukocytes such AM, DC, and recruited neutrophils, which lyse yeast when they come into contact with *Aspergillus or Cryptococcus* following inhalation. As a result, the local AM and DC participate in the control of both the early innate immune response and the later adaptive immunological response. Dendritic cells (DCs) are the primary T and B lymphocyte-mediated innate immune cell type that have the potential to initiate and regulate adaptive immunity (Banchereau and Steinman,1998; Zitvogel, 2002). Additionally, lung DCs coordinate innate immune responses to *A. fumigatus* by controlling T cell proliferation and boosting the lung's protective Th1 response (Ramadan, 2004;Bozza *et al*., 2003).

Immature DCs' pro-inflammatory responses after being exposed to *A. fumigatus* are caused by dectin-1 (Mezger *et al.*, 2008). The neutrophils' ability to mediate lung DC maturation and export is intriguing. (Park *et al*., 2012), while DCs trigger the secreted chemokines for neutrophils/Th1 lymphocytes recruitment, suggesting the cellular interplays among neutrophils, DCs as well as T lymphocytes. In the respiratory tract, increased granulomatous formation, rapid inflammatory infiltration (Gafa *et al.,* 2007) .

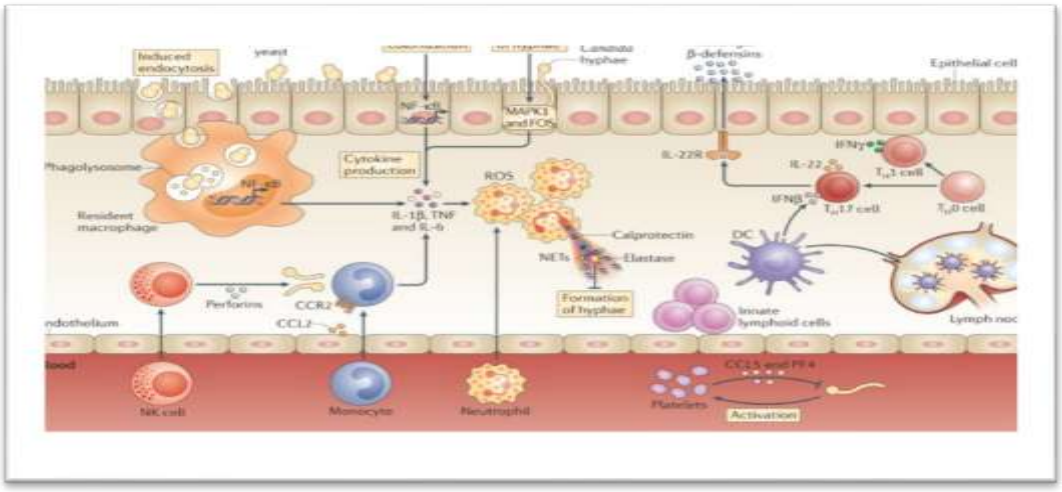


Figure (2-4) Immune defense against *Candida* infections(Mihai *et al.*, *2*015)

2-9:Correlation Between COVID-19 and Respiratory Fungal Infection

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) that caused the coronavirus disease 2019 (COVID-19) to grow globally to pandemic levels throughout the past year (Arastehfar *et al*., 2020). A large fraction of cases advance to severe pneumonia and acute respiratory distress syndrome, needing critical care, even though the majority of cases are asymptomatic or moderate illnesses.

Since the 1918 influenza pandemic, opportunistic illnesses that accompany severe respiratory virus infections have been identified. Particularly secondary fungal infections brought on by *Aspergillus* and *Candida spp*. are more frequently reported in critically ill COVID-19 patients (Hoenigl and Fetalento, 2012). authors have given information on the immune pathogenesis of COVID-19-associated pulmonary aspergillosis (CAPA), This is thought to happen because patients with severe COVID-19 have an ineffective immune response, which causes a hyperimmune state and infections with malfunctioning T cells (Hoenigl, 2020). Paradoxically, collateral effects of host recognition pathways

necessary for the activation of antiviral immunity may paradoxically contribute to a highly permissive inflammatory environment that favors the development of pulmonary mold infections.

The release of danger-associated molecular patterns during severe COVID-19 may contribute to pulmonary epithelial damage. CAPA has been linked to higher mortality, which can only be decreased by starting antifungal treatment sooner rather than later (White *et al*., 2020) Gangneux *et al.* showed that molecular assays to detect *Aspergillus* DNA from blood and respiratory samples resulted in better sensitivity when compared to culture-based approaches, which may aid in the early identification of CAPA, demonstrating the importance of early detection. (Gangneux *et al*., 2020).

Importantly, reports of yeast infections in seriously unwell COVID-19 patients are also available. Additionally, *candida* blood stream infections can happen in patients with traditional clinical risk factors such prolonged ICU hospitalizations, vascular devices that need to be left in place, and using antibiotics and corticosteroids. (Araslehfar *et al*., 2020).

2-10: suPAR as Immunological Signal for Covid-19 Severity

Soluble urokinase-type plasminogen activator receptor (suPAR) is a soluble receptor that is produced as a result of the enzymatic cleavage of the membrane-bound uPAR receptor on the surface of blood mononuclear and endothelial cells in response to a wide range of inflammatory and immune modulatory stimuli, including viral infections (Huang,Q. *et al*., 2020). In addition to its well-established function as a bioactive factor linked to several types of endothelial dysfunction and sepsis (Huang, Q. *et al*., 2020).

SuPAR plasma levels can forecast the likelihood of developing acute and chronic renal failure (Faubel, 2020). Recent data also hints at a

potential function for this biomarker in a number of thrombotic disorders. In a sizable segment of the population (Engström *et al*., 2016) .

High levels of suPAR were found to independently predict the risk of developing venous thromboembolism in the Malmö Diet and Cancer (MDC) trial, which included over 5,000 participants. In a different study, suPAR levels were found to be higher in people with paroxysmal nocturnal hemoglobinuria (PNH) compared to healthy controls, and levels were also linked to an increased risk of having thrombotic events. Patients with suPAR values below 2 ng/mL had shorter thrombosis-free survival times (Sloand *et al*., 2008). due to kidney damage (Menon *et al*., 2012), and various forms of venous thrombosis are commonplace in patients with severe coronavirus disease 2019 (COVID-19) (Lippi *et al*., 2019;Vadasz *et al*., 2005),, as figure(2-5).

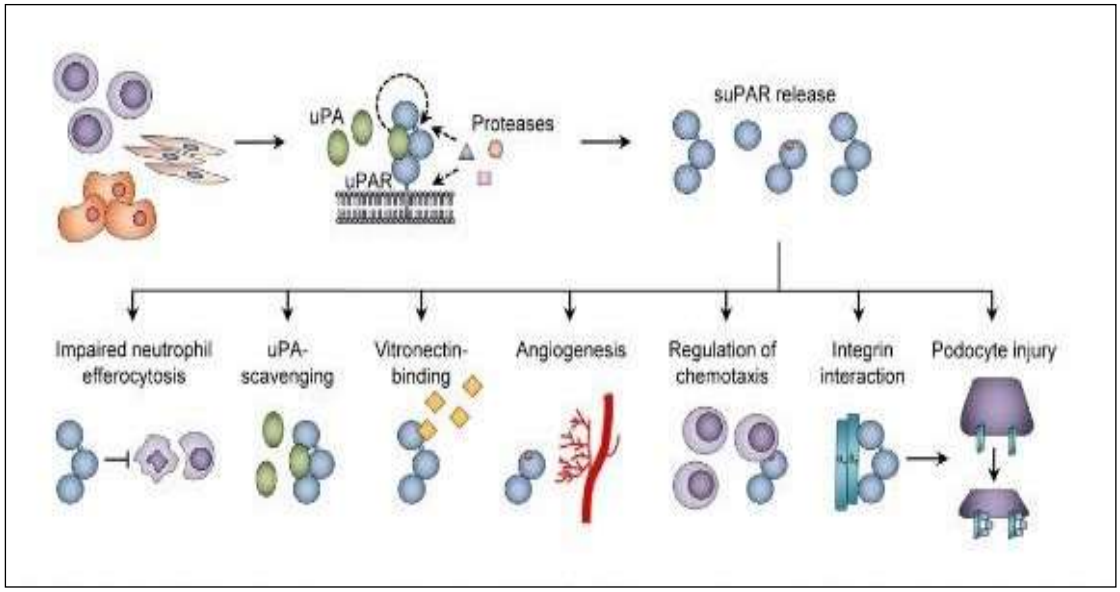


Figure (2-5) functions of suPAR (Rasmussen, 2018)

2-11:Cytokines and chemokine networks

2-11-1: Cytokines related to Viral and Fungal Infections

Communication between cells is carried out by cytokines and chemokine (chemotactic cytokines). cytokine and chemokine signals

control the immunological response to infection, When inflammation begins and continues, the small, low-molecular weight proteins cytokines regulate the intensity and duration of response. Genetic transcriptional regulation of nuclear factor kappa-B must be activated to regulate the release of pro inflammatory cytokines from various cell types for specific pathogenassociated molecular patterns, such as lipopolysaccharide, through the Toll like receptor pathway(Hanada and Yoshimura, 2002), as well as epithelial and fibroblast cells, phagocytes (neutrophils and macrophages) are responsible for the production of cytokines during acute and early chronic phases of inflammation, while lymphocytes are responsible for the production of cytokines during established and advanced diseases(Ara, *et al*., 2009).

Cytokines like as interleukin-1 and IL-6 were originally discovered in the context of periodontal infection (Fonseca *et al., 2*009). Tumor necrosis factor alpha has been linked to inflammatory cell migration and osteoclastogenesis. TNF- is a multi-effect cytokine with numerous functions: TNF- stimulates RANKL as well as the release of IL-1 beta and IL-6, impacts cell motility and extravasation by encouraging neutrophil rolling and adherence through increased adhesion molecules, and cell migration to infected and inflamed sites is further facilitated by chemokine synthesis (Kwan, *et al*., 2006; Kindle, *et al*., 2006). TNF-α, IL-1α, IL-1β, IL-6 and IL-11 have all been shown to stimulate bone resorption while others such as IL-4 , IL-5, IL-10, IL-13 and IL-18 inhibits bone resorption as well as transforming growth factor TGF-1β (Takayanagi, *et al., 2*005).

IL-23 stimulated T-helper 17 cells enhance osteoclastogenesis primarily through the production of interleukin-17 (Sato,*et al*., 2006). Therefore, the discovery of Th17 cells and relative IL-17 cytokines

family gave a new impulse to the immunology field, bridging the gap and giving not only “a wider vision” of both innate and adaptive immunity, but also to identify this ”unique” cytokine as a silent amplifier of the immunity process (Acquisto *et al., 2*010).

2-11-1-1:Interleukine-17A (IL-17A) Silent Amplifier of COVID-19

The scientific community was able to gain a better understanding of the immunopathology of inflammatory illnesses in humans in the 1990s thanks to the discovery of two unique subsets of helper T cells, IFN-producing Th1 cells and IL-4-producing Th2 cells)Noack and Miossec, 2014). However, the discovery that Th1 and Th2 subsets were not responsible for experimental autoimmune and auto-inflammatory illnesses led the researchers to label a specific subset of helper T cells as Th17 (Miossec and Kolls, 2012).

As a result, the identification of this "unique" cytokine as a quiet amplifier of the immune process provided a fresh impetus to the science of immunology, bridging the gap and providing not just "a larger view" of both innate and adaptive immunity (Acquisto *et al*., 2010). The Th17 subtype of T helper cells, which specifically generates this cytokine, determines IL-17A's distinctiveness. The study of immunity has undergone a revolution thanks to the discovery of IL-17A and its biological role. And it has fundamentally altered how we see a number of immune- and inflammation-based disorders (Maione, 2016). Chronologically, the identification of IL-17A as an inflammatory cytokine associated with arthritis was made several years before the description of Th17 cells. It was identified on chromosome 6. However, a substantial role for this cytokine in host defense, as well as in the setting

of acute and chronic inflammation, has been conclusively determined since the discovery of Th17 cells. (Maione *et al*., 2009).

The IL-17A immune axis is unquestionably characterized by distinctive biological effects that vary among diseases, according to data from both fundamental research and clinical trials. Chronic and acute inflammation and IL-17A The scientific world has focused emphasis on IL-17A in recent years because of its crucial involvement in the continuous processes that are typical of various inflammatory-based chronic illnesses (Lubberts, 2015). This cytokine is in fact connected to the processes that control cell activation, expansion, and proliferation (Kehlen *et al., 2*002). IL-17A plays a main role in neutrophils maturation and differentiation. This is due to its ability to increase granulocyte-colony stimulating factor (G-CSF) release , thereby fostering the differentiation of the progenitors hematopoietic CD34+ towards neutrophils (Fossiez *et al*.,1996).

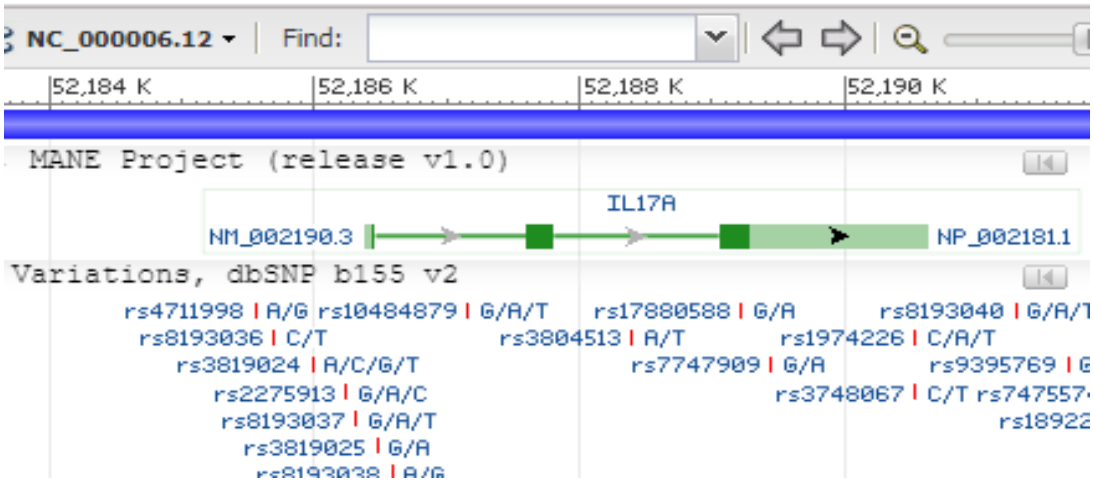


Figure (2-6) The site of gene IL17A on chromosome 6 with reference strain NO. NC_000006.12.

It can also induce other granulopoiesis markers and chemokines, such as growth-regulated oncogene-α (GRO-α), that regulate neutrophil penetration into tissues (Schwarzenberger *et al*.,1998). Due to its

chemotactic action, IL-17A is indeed important as a regulator of PMN infiltration, as demonstrated by both preclinical and clinical studies. In this respect, it has been demonstrated that IL-17A is crucial for the maturation and differentiation of neutrophils. This is because it promotes the production of granulocyte-colony stimulating factor (G-CSF), which in turn encourages the development of hematopoietic CD34+ progenitors into neutrophils (Witsowski *et al.,* 2020).

IL-17A's synergistic activity with other pro-inflammatory "inducers" is another of its biological effects. IL-17A increases the inflammatory response in cartilage, synovium, and meniscus when combined with IL-1 and TNF-α (Moseley *et al.*, 2003). IL-17A is also associated with the degradation of articular cartilage and destruction of bone (due to the production of the matrix metalloproteinase-(MMP-) 1 and MMP-13 collagenases in chondrocytes), the synovium's fibroblast-like cells exhibit proteoglycan breakdown, IL-6 expression, and leukemia inhibitory factor expression. (Kehlen *et al*., 2003). IL-17A can be defined as “not canonical” pro-inflammatory cytokine, considering the variety of its actions. Indeed, By escalating cellular and metabolic activities activated during the acute phase of the inflammatory response, it plays a special function in the setting of persistent inflammatory disorders. Additionally, IL-17A can circulate in the blood stream even though it primarily affects the local area, which means it may indirectly affect the function of endothelial cells, causing vascular inflammation and raising the risk of atherosclerosis and/or cardiac and thrombotic events in patients with certain inflammatory-based diseases (Beringer and Miossec, 2019).

Moreover, IL-17A, in combination with TNF-α, it is also responsible for a pro-coagulant and pro-thrombotic state , thus providing

evidence for its implication in the cardiovascular events associated with autoimmune diseases (Casillo *et al., 2*020)

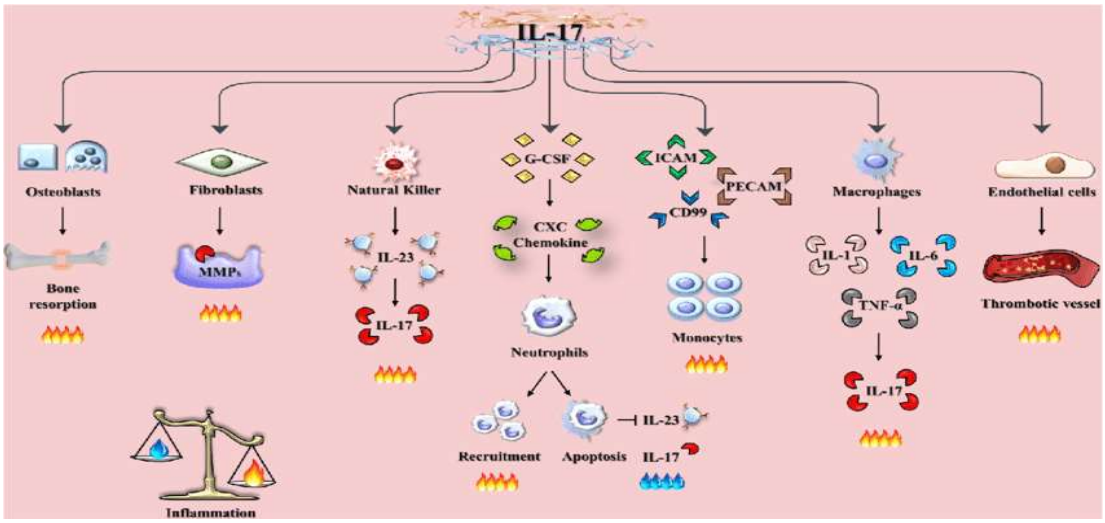


Figure (2-7) Biological function of IL-17(Beringer and Miossec 2019)

2-11-1-2:Interferon regulatory factor 7(IRF7)

In mammalian cells, the interferon regulatory factors (IRFs) family of transcription factors (IRF1-9) consists of nine members. IRF10's DNA homolog is not expressed in humans; it has only been discovered in avian species. (2002) Nehyba *et al.* Genes encoding IRFs are found in all major metazoan groups, albeit the family numbers vary. The first member, IRF1, was discovered in 1988 (Nehyba *et al*., 2009). Interestingly, the IRF family has coevolved with the nuclear factor (NF)kB family, both of which share some evolutionary characteristics (Nehyba *et al*., 2009) both families are activated by pathways signaling from the same pathogen recognition receptors (PRRs) and by the same kinase family IkB kinases (IKKs); both cooperate extensively in regulation of target cytokines such as interferon (IFN)b, and together they represent the major players in innate immune responses (Hiscott, 2007) .

The conserved N-terminal DNA-binding domain (DBD), which contains a distinctive tryptophan pentad that is necessary for DNA

binding, has substantial commonality among the members of the IRF family. (2008) Chen and Royer The consensus DNA sequences, which typically contain at least two GAAA repeats, are recognized by the helix-turn-helix shape that DBD generates, according to crystal structure studies (Chen *et al.*, 2008)

The viral IRFs are deficient in numerous tryptophan residues, which prevents them from binding to DNA and causing them to act as dominant-negative mutants. IRFs have various C-termini that give each member unique roles (Ghosh and Hayden, 2008) They typically have a nuclear export sequence, an autoinhibitory domain, an IRF-association domain, a signal responsive domain, and critical serine residues that are phosphorylated during infection with pathogens (Chen and Royer, 2008). The IRF family is the important player in multiple facets of host defense systems (Honda and Taniguchi, 2006). Also IRFs have pivotal roles in immune cell development and regulation of oncogenesis (Savitsky *et al*., 2010).

The IRF7 gene was originally cloned in 1997, in the context of latent Epstein–Barr virus (EBV) infection where the encoded protein binds to and regulates the EBNA1 Q promoter(Zhang and Pagano,1997).

our isoforms of the human IRF7 gene, IRF7A, -B, -C, and -D (-H), are encoded by the gene, which is found on chromosome 11p15.5 (Zhang and Pagano, 2002). The human IRF7A protein has 503 amino acids and a molecular weight of 55 kD. IRF7 must be activated in order to function as a transcription factor. IRF7 is present in the cytoplasm in a "latent" state. IRF7 is phosphorylated and translocated into the nucleus in response to pathogenic infection, where it joins forces with additional co-activators to create a transcriptional complex that binds to the promoter regions of target genes to activate transcription (Wathelet *et al*.,1998).

Additionally, studies with truncated mutants have shown that IRF7's C-terminus contains a number of additional functional domains that control IRF7 activity. Importantly, IRF7 activation requires the human IRF7A virus-activation region, which spans amino acids 278–305 (Lin *et al.*, 2000).

IRF7 is a lymphoid-specific factor, which is constitutively expressed in the cytoplasm in B cells, pDCs and monocytes in the spleen, thymus, and peripheral blood lymphocytes, and is potently inducible by type I IFNs, virus infection and other stimuli such as 12-o-tetradecanoylphonol-13-acetate, TNFa and lipopolysaccharide in various cell types (Zhang and Pagano,1997) The primary source of IRF7 expression in the cell is the constructive regulatory feedback between IRF7 and type I IFNs during antiviral immune responses (Marie *et al.*,1998), At the early 'priming' stage of virus infection, the low level of endogenous IRF7 in the cell is phosphorylated and activated by signaling triggered from PRRs, and together with NFkB and IRF3, which are also activated by the same pathways, binds to the virus-responsive elements in the Ifna and Ifnb promoters and induces small amounts of type I IFNs, which bind to IFNA receptors in the other cells. IRF7 has important role in the priming (Zhang and Pagano, 2002).

Binding of IFNs to IFNA receptor results in the activation of the IFN Janus kinase-signal transducers and activator of transcription signaling cascade, leading to phosphorylation and activation of signal transducers and activator of transcription 1 and -2 (Honda *et al.*, 2006). The activated signal transducers and activator of transcription 1/2 after that bind with IRF9 as a complex named 'IFN-stimulated gene factor 3', which binds to the IFN-stimulated response element on the IRF7 promoter and induces synthesis of more IRF7. Later, the newly

synthesized IRF7 is activated and induces more IFNs so that more and more IRF7 there are more IFNs are produced, but IRF3 at late stages is degraded by virus infection (Honda *et al*., 2006).

A variety of cellular functions are regulated by IRFs. Besides their roles in IFN-mediated immune responses, IRFs also have important role in leukemia and other malignancies in many cell types, and in regulation of apoptosis and cell growth, therefore they affect susceptibility to and the progression of cancer. Another important function conferred by IRFs is regulated of immune cell differentiation and activation , IRF7 is a positive regulators of type I IFNs in different cell types and it required for efficient IFN production in most immune cells (Ozato *et al*., 2007).

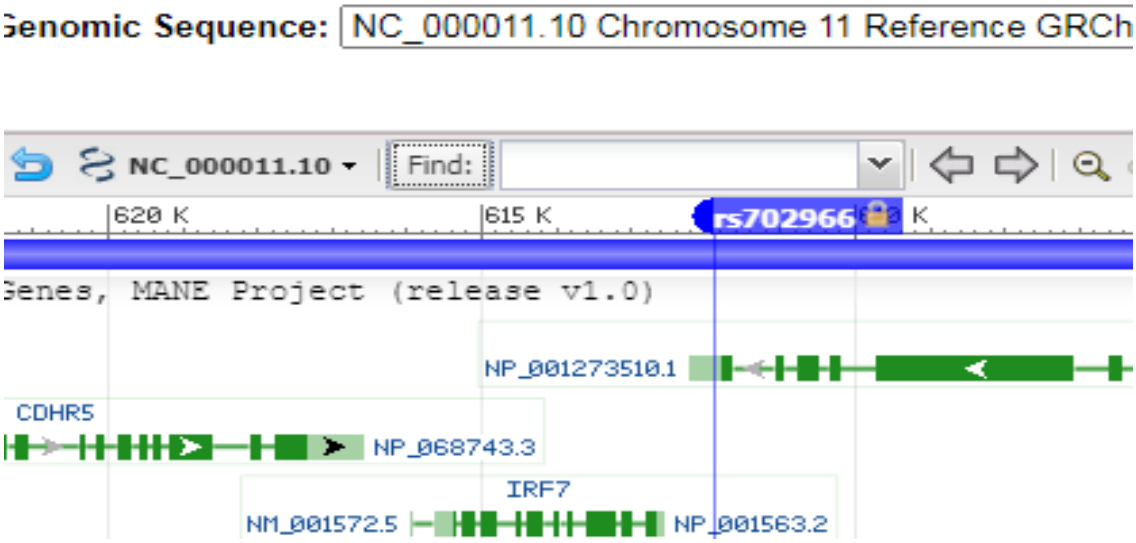


Figure (2-8) The site of gene IRF7 on chromosome 11 with reference strain NO. NC_000011.10.

Chapter Three
Methodology

3:Methodology

3-1: Materials

3-1-1: Equipment and Tools

Many apparatuses and equipment were used in this study in order to performed many tasks for each experiment under interest (Table 3-1)

Table (3-1) Apparatuses and equipment used in the implementation of the experiments in this study

NO.	Equipment and Tools	Manufacturing company
1	Camera	Canon / Japan
2	Centrifuge	Labnet / USA
3	Deep freeze	Royal China
4	EDTA Tube ,Gel Tube, Eppendorf tubes	China
5	Electric dry hot	USA / USA
6	Electrophoresis	Mupid-one / Japan
7	ELISA printer	Bio Tek USA
8	ELISA reader	Bio Tek USA
9	ELISA washer	DIALAB Austria
10	I Chroma	Korea
11	Micro Pipettes	Gillson Instruments / France
12	Mindray BC-5000	China

13	Nanodrop2000c spectrophotometer	Canada
14	Nasopharyngeal swab	China
15	Petry dish	China
16	PCR-Thermo cyclic	Labnet / USA
17	PH Meter	Philips / Holland
18	Pipette Tips	Bioneer/ Korea
19	Refrigerator	Concord / France
20	Specimens Transport box	India MART India
21	UV-Trans illuminator	Desktop Gel image
22	Vortex Mixture	Memmert / USA

3-1-2-Biological ,Chemicals Material and Kits:

The Biological and chemicals material were used to perform our experiments targeted in this study (Table 3-2).

Table (3-2): biological , chemicals material and kits used in this study

NO.	Chemicals	Manufacturing
1	Agarose	Bio Basic/USA
2	I chroma™ CRP	Boditech /Korea
3	Ichroma™ D-Dimer	Boditech /Korea
4	Ichroma™ Ferritin	Boditech /Korea
5	Master Mix	Promega USA
6	Proteinase K	Himedia/India

7	Primers	Macrogen Korea
8	SARS-CoV-2 Antigen Rapid Test Kit	Elabscience USA

The component of extraction kit were illustrated in the following Table (3-3).

Table (3-3):Genomic DNA purification kit and Buffer (FAVROGEN Korea).

NO.	Genomic DNA extraction kit and Buffer
1	FABG
2	FAGB
3	Binding Buffer
4	Wash **1** Buffer
5	Wash **2** Buffer
6	TE buffer

The Primer list included primer names, Sequence of nucleotides, PCR product size, Annealing temperature(Tn), Gene names and source of primers Table (3-4).

3-1-3- Primers pairs used in DNA amplification

Table (3-4) the primers that used in this study

Primers	DNA sequences	Size	Tn	Gene	References
F(IL-17A)	AATCAAGGTACATGACACCAG	694bp	56	IL17A	Designed in current study
R(IL-17A)	TTAGCCCCAATATAGCTATCTT			IL17A	
F(IRF7)	CTCCAGATCTGCCACAGCAA	534bp	56	IFR7	Designed in current study
R(IRF7)	CGGGAGGCCACCTAGAGATT			IFR7	

3-2:Methods

3-2-1:Study Design

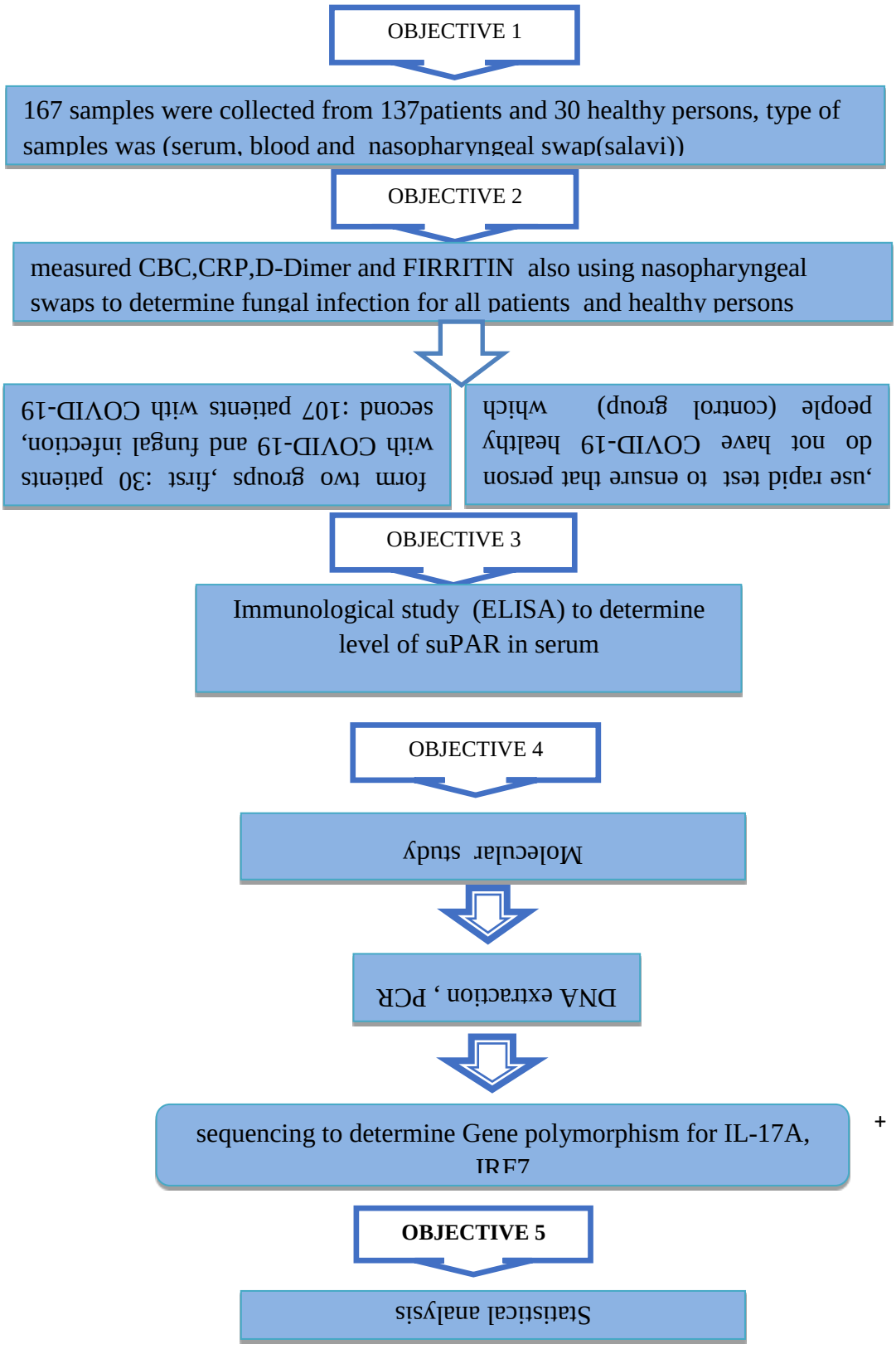


Figure (3-1) study design

3-2-2:Case–Control Study, Collection and sample size

This case-control study included (167) case, 137 of it was for patients who have needed ventilators and lying in the respiratory care unit (RCU) with severe respiratory distress, respiratory rate ≥30 breaths/minute and pulse oxygen saturation (SpO2) ≤ 93% on resting state, the case was considered to be in severe illness with RT-PCR positive , (75 male and 62 female) with COVID-19, age range between (10 to 70 years) the specimens was blood samples and nasopharyngeal swabs, selected from them two groups first group was(30case) for patients with COVID-19 with fungal infection, second group was(107case) for COVID-19 without fungal infection(after isolation and diagnosis of fungi in nasopharyngeal swaps) ,Third group was from 30 healthy persons as control group also specimens was blood samples and nasopharyngeal swaps in addition to nasal swap specific to detection if the persons infected by SARS-CoV-2 by(SARS-CoV-2 Antigen Rapid Test Kit), it was proceeded by collected the specimens between January-2022 to April- 2022.

Clinical samples were collected from patients whom lying in RCU from Marjan Teaching Hospital (Marjan medical city), AL-Imam AL-Sadiq Teaching Hospital in Babylon province and AL-zahraa hospital in Karbala province. Samples were taken from the patients under the supervision of the specialist physician. After diagnosis of COVID-19 infection of the patients, The questionnaire was used for each patient which included some important information like name, age, gender , housing, presence of comorbidities, and symptoms . patients out years and patients with COVID-19 who are not considered severe cases were excluded .

3-2-2-1: Collection of Nasopharyngeal Swabs

One hundred sixty seven nasopharyngeal swabs samples with transport media. are collected , One hundred thirty seven samples from patients with COVID-19 infection, and 30 samples from control (in addition to take 30 samples to detection the infection with COVID-19 in control group only). nasopharyngeal swap

One hundred thirty seven samples (patients) divided in two groups the first for 30 patients with COVID-19 and respiratory fungal infection, the second group with COVID-19 without respiratory fungal infection(107), and 30 samples from healthy people by swabs with transport media.

3-2-2-2: Collection of Blood Samples

One hundred sixty seven blood samples were collected ,This samples taken from 137 patients with COVID-19 patients in addition to 30 samples taken from healthy people, Blood divided in two groups : the first collected directly in EDTA tube. Cold box was used to transfer samples for the purpose of transferring them from hospital to laboratory and saved -20C˚ in deep freeze for extraction of human DNA (Gustincich *et al*., 1991).for using in molecular study ,the second collected in gel tube stay for 20 minutes then put in centrifuge ,put it in Eppendorf tubes to hematological and immunological tests, others storage in -20C˚for ELISA test to determine level of suPAR.

3-2-2-3:Determine if the Control Group with COVID-19

SARS-CoV-2 Antigen Rapid Test Kit

This SARS-CoV-2 Antigen Rapid Test Kit (Colloidal Gold) uses the sandwich Immunecapture method and colloidal gold immune chromatography to qualitatively determine the presence of SARS-CoV-2

antigens in human oropharyngeal swabs, nasal swabs and nasopharyngeal swabs. It is helpful as an aid in the screening of early mild, asymptomatic, or acute patients for identification of SARS-CoV-2 infection.

Procedure:-

1-. the pouch was brought to room temperature before use.

2- the cassette was Took out, put it on a horizontal table.

3- sample was took from nasal by nasal swap with Kit, put swap in treatment solution in vials , put Tube cap.

4- Three drops was Added of the processed sample vertically into the sample well and start the timer.

5- the result was Observed after 10 minutes, the result was valid within 30 minutes, read results after 30 minutes was invalid.

Testing Results:-

Positive: Both the detection line (T line) and the quality control line (C line) appear colors.

Negative: The test line (T line) does not appear color, only the quality control line (C line) appears color

Invalid: The quality control line (C line) does not appear color, which means that the test is invalid and the test should be repeated.

3-2-2-4:Cultivation of Specimens

Clinical specimens are cultured on Sabourauds dextrose agar medium. The swabs are cultured on SDA by streaking and incubate at 37˚C for 24-48h to yeast isolates and for 7 days to molds isolates. Then single colonies from any yeast isolates are pickup and streaking on CHROM agar medium, incubated at 37˚C for 24-48. All isolations (yeast and molds) are isolated in pure culture on PDA medium.

After incubation and identification the percentages of frequency and appearance of isolated fungi were calculated according to the following equation:-

Percentage of appearance = Number of isolate that appeared in the same species/ Total number of samples * 100

Percentage of frequency = Number of isolate per species/ Total number of isolates of all species * 100

3-2-2-5: Microbiology Identification

3-2-2-5-1: Morphological Examination

After appearance growth as well as examining colonies of fungi from respect colony color, shape and texture (Powdery, Granular, Cottony) as recorded pigments is examined on foundation at surface of colony, appearance (Paritpokee *et al*.,2005**).**

3-2-2-5-2:Microscopic Examination

Fungi isolates were examined microscopically, taken the imprint of the fungus in the colony by Adhesive tape, it is used transparent adhesive tape, it is touching with the surface of the fungal colonies and then paste the tapeon a glass slide containing a drop lacto phenol cotton blue. Slides examined under magnification 10X, 40X and 100X as described by Astrid (1999).

3-2-2-5-3: CHROM agar Test

This test is performed by inoculating CHROM agar *Candida* medium which is prepared previously from Candida isolate culture grown on SDA for 24 h, and then incubated at 30˚C for 24-48 h (Paritpokee *et al*.,2005). CHROM agar test is used for the presumptive identification of *Candida* species by production of different colors on this medium (*C. albicans*= green/ blue green, *C. dubliniensis*= dark green, *C. tropical*=

blue, *C. parapsilosis*= cream white, and *C. krusei*=pink) (Horvath *et al.*,2003).

3-2-2-5-4:Hemolysis Test

Viability of yeasts to hemolysis was determined by inoculated the blood agar medium with yeasts isolates, then incubate in 37˚C to 48 h and the positive result is the formation of a clear hallo about developing the colony (Sachin *et al*., 2012).

3-2-2-6:Hematological study

3-2-2-6-1:Determination of W.B.Cs, Lymphocyte and Neutrophils

Hematological parameters were performed on EDTA blood using device to estimate numbers and percentages of white blood cells. Whole blood was collected via an EDTA tube to measure, Total WBCs, Lymphocytes Neutrophils and others parameters.

3-2-2-6-2: Ferritin measurement

Serum ferritin levels were measured using a kit from Boditech Med Inc. In Korea, the test is designed to be used on I-chroma™ devices. It is a fluorescence immunoassay (FIA) for determining the quantity of human ferritin in serum. This assay is designed to help doctors quantify human ferritin levels.

Principle of I-chroma™ Ferritin

The test utilizes the immunodetection technique for sandwiches. In the buffer, the protein detector binds to sample antibodies and builds complex recombinant protein-antibody into a nitrocellulose matrix to be collected on the test line with the other immobilised antigens. The more antibodies in the sample, the more recombinant protein-antimicrobial

complexes are formed and the intensity of the fluorescent signal on the recombinant protein detector is increased, which is processed in the sample by I-chroma™ Instrument to show ferritin concentrations.

Procedure of Ichroma™ Ferritin

1- Thirty microliter of the human serum sample was transferred to a tube containing detection solution using a transfer pipette.

2- The cap was closed and the sample was mixed well by shaking the detection tube about 10 times.

3- Seventy-five ml of the sample mixture was withdrawn and loaded into a sample well on the cartridge.

4- The sample-loaded test cartridge was Inserted into the i-Chamber slot.

5- the cartridge was Leave in the i-Chamber for 10 minutes.

6- The cartridge was inserted in the cartridge holder for I-chroma™ testing to scan the cartridge loaded with the sample.

7- The scanning process was started, pressed the 'Start' button for I-chroma™ tests.

8- The sample-loaded cartridge was started scanning immediately with the instrument for I-chroma™ testing.

9- The test results was read for the I-chroma™ test on the display screen.

Result of Ichroma™ Ferritin

The Instrument for Testing Ichroma™ automatically calculates the test result and displays the concentration of ferritin in the test sample as ng/mL

3-2-2-6-3: D-Dimer level measurement

Serum ferritin levels were measured using a kit from Boditech Med

Inc. In Korea, the test is designed to be used on I-chroma™ devices. It is a fluorescence immunoassay (FIA) for determining the quantity of human D-Dimer in serum. This assay is designed to help doctors quantify human D-Dimer.

Procedure of Ichroma™ D-Dimer

1- Thirty microliter of the human serum sample was transferred to a tube containing detection solution using a transfer pipette.

2- The cap was closed and the sample was mixed well by shaking the detection tube about 10 times.

3- Seventy-five ml of the sample mixture was withdrawn and loaded into a sample well on the cartridge.

4- The sample-loaded test cartridge was inserted into the i-Chamber slot.

5- The cartridge was leave in the i-Chamber for 10 minutes.

6- The cartridge was inserted in the cartridge holder for I-chroma™ testing to scan the cartridge loaded with the sample.

7- scanning process, pressed the 'Start' button for I-chroma™ tests.

8- The sample-loaded cartridge will started scanning immediately with the instrument for I-chroma™ testing.

9- The test results was read for the I-chroma™ test on the display screen.

Result of Ichroma™ D-Dimer

The Instrument for Testing Ichroma™ automatically calculates the test result and displays the concentration of D-Dimer in the test sample as ng/mL.

3-2-2-7: Immunological Study

3-2-2-7-1: C-Reactive Protein Level Measurement

Serum ferritin levels were measured using a kit from Boditech Med Inc. In Korea, the test is designed to be used on I-chroma™ devices. It is a fluorescence immunoassay (FIA) for determining the quantity of human CRP in serum. This assay is designed to help doctors quantify human CRP.

Procedure of Ichroma™ CRP

1- Thirty microliter of the human serum sample was transferred to a tube containing detection solution using a transfer pipette.

2- The cap was closed and the sample was mixed well by shaking the detection tube about 10 times.

3- Seventy-five ml of the sample mixture was withdrawn and loaded into a sample well on the cartridge.

4-The sample-loaded test cartridge was insertsd into the i-Chamber slot.

5- The cartridge was leave in the i-Chamber for 10 minutes.

6- The cartridge was inserted in the cartridge holder for I-chroma™ testing to scan the cartridge loaded with the sample.

7- scanning process, pressed the 'Start' button for I-chroma™ tests.

8- The sample-loaded cartridge was started scanning immediately with the instrument for I-chroma™ testing.

9- The test results was read for the I-chroma™ test on the display screen.

Result of Ichroma™ CRP

The Instrument for Testing Ichroma automatically calculates the test result and displays the concentration of CRP in the test sample as ng/mL.

3-2-2-7-2: suPAR Level Measurement

Test principle ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human suPAR. Samples (or Standards) are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human suPAR and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human suPAR, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color.

The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human suPAR. You can calculate the concentration of Human suPAR in the samples by comparing the OD of the samples to the standard curve.

Reagent preparation

1. All reagents was brought to room temperature (18-25℃).
2. Thirty mL of Concentrated Wash Buffer was diluted with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer.
3. The standard was Centrifuged at 10,000×g for 1 min. Added 1.0 mL of Reference Standard &Sample Diluent, left it stand for 10 min and inverted it gently several times. After it dissolved fully, mixed it thoroughly with a pipette. This reconstitution produced a working solution of 10 ng/mL, Then made serial dilutions as needed. The recommended dilution gradient is as follows: 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0 ng/mL.

4.Biotinylated Detection Ab working solution: Calculated the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuged the Concentrated Biotinylated Detection Ab at 800×g for 1 min, then diluted the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent(Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1: 99).

5.Concentrated HRP Conjugate working solution: Calculated the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuged the Concentrated HRP Conjugate at 800×g for 1 min, then diluted the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent(Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99).

Assay procedure

1. Wells was determined for diluted standard, blank and sample. Added 100 μL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate). Coverd the plate with the sealer provided in the kit. Incubated for 90 min at 37℃. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.

2. The liquid was decanted from each well, do not wash. Immediately added 100 μL of Biotinylated Detection Ab working solution to each well. Covered the plate with a new sealer. Incubated for 1 hour at 37°C.

3. The solution was decanted from each well, added 350 μL of wash buffer to each well. Soaked for 1 min and aspirateed or decanted the solution from each well and pat it dry against clean absorbent paper. Repeated this wash step 3 times. Note: a micro plate washer can be used

in this step and other wash steps. Made the tested strips in use immediately after the wash step. Do not allow wells to be dry.

4. 100 µL of HRP was added Conjugated working solution to each well. Covered the plate with a new sealer. Incubated for 30 min at 37°C.

5. The solutionwas decanted from each well, repeated the wash process for 5 times as conducted in step 3.

6. 90 µL was added of Substrate Reagent to each well. Covered the plate with a new sealer. Incubated for about 15 min at 37°C. Protected the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Micro plate Reader for about 15 min before OD measurement.

7. 50 µL was added of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution.

8. The optical density (OD value) of each well was determinated at once with a micro-plate reader set to 450 nm.

Calculation of Results

The standard curve was created by plotting the mean OD value on the y-axis against the concentration on the x-axis and a best fit curve through the points on the graph was drawn as figure (3-2).

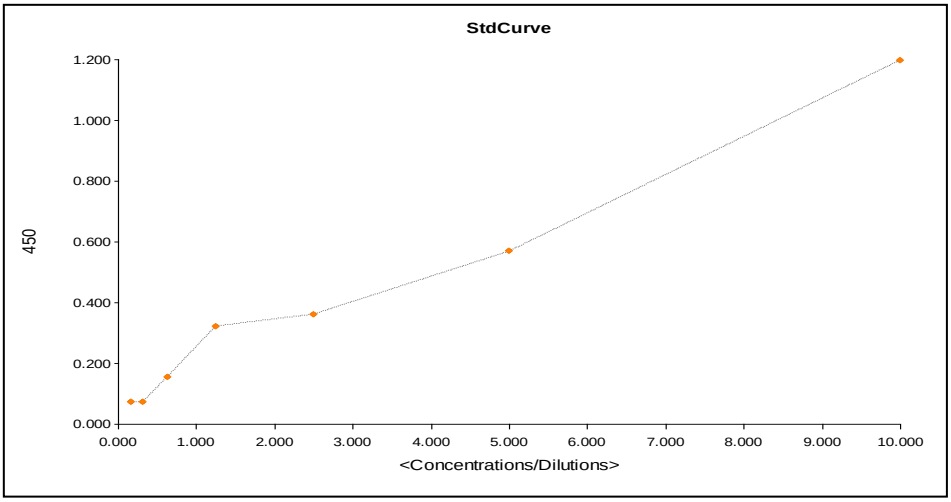


Figure (3-2) Standard curve of suPAR level

3-2-2-8:Molecular Study

3-2-2-8-1: DNA Extraction from Blood

1. Frozen human blood was collected in an anticoagulant-treat collection tube and waited at room temperature until it thawed and gently mixed.

2.300µl of blood was transferred to a microcentrifuge tube for each sample .

3. 30ul of Protinase K was added for each sample and mixed gently .

4. Incubated for 20 minutes at 56°C temperature .

5. A 200µl FABG Buffer added and vortex combined .

6 .Incubated at 70°C to for 15 minutes. Invert the sample every 3 ~ 5 minutes, during incubation .

7. The sample was supplemented with 200µl ethanol (96 ~ 100 per cent). Mix vigorously for 10 seconds with overtaxing 30 min.

8- Spin column tubes was pick up in their collection tubes

9. All lysate was transverse into spin column of samples for each .

10. The micro centrifuge was spin for 1minute.dischaed the filtrate lysate and replace the collection tubes.

11. FABG Column was washed with 400µl W1 Buffer (ethanol added) by centrifuge for 30 seconds.

12.FABG Column was washed with 600µl Wash Buffer (ethanol added) by centrifuge for 30 seconds.

13. Centrifuged for an additional 3 min to dry the column.

14. The FABG Column tubes was putted to a new 1.5ml microcentrifuge tubes for each.

15. 100µl of Preheated Elution Buffer or TE was added to the membrane center of FABG Column. Stand FAGB Column for 3~5 min or until the buffer is absorbed by the membrane.

16. Centrifuged for 30 seconds to elute the pure DNA .

17.The DNA fragment was stored at 4ºCor-20ºC (Al-Jubory and Imran 2020).

3-2-2-8-2: Estimation the DNA Concentration and Purity

The DNA concentration of samples was estimated by using the Nanodrop by putting 1 µl of the extracted DNA in the machine to detect concentration in ng/µl and the purity detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The accepted 260/280 ration for purifying DNA was between 1.7-1.9 (Sambrook and Russell, 2001).

3-2-2-8-3:Dissolving Lyophilized Primers

The designed primer pairs were synthesis in Macrogen as pellet in a lyophilized; our primers were arrived as a lyophilized film at the bottom of a cryo-tube. To use them, we must re-suspend them in dH_2O.toMade a concentration stock by re-suspending the lyophilized primer to a standard 100 pmol/µl concentration by added 300 µl dH_2O to each tube based on Macrogen manufacture. For PCR reaction the primer concentration required 10pmol/µl. To prepare a pmol/µl low-concentration as working solution, take 10ul stock solution and add to 90ul water.

3-2-2-8-4: Prepared the Reaction Mixture

Amplification of DNA was carried out in a final volume of 25 µl reaction mixture for detection of under interested genes for performed polymorphism as mentioned in table (3-5) as mentioned adding (1.1µl) from each diluted primer.

Table (3-5): Contents of the Reaction Mixture (Promega) of performed the PCR under interested genes polymorphism

No.	Contents of reaction mixture	Volume µl
1.	Master mix G2(Promega)	12.5
2.	Forward primer	1
3.	revers primer	1
4.	DNA template	3
7.	Nuclease free Water (Promega)	7.5
Total volume		25

3-2-2-8-5:Thermal Cycling Conditions (PCR conduction)

The Protocol for technique Polymerase Chain Reaction (PCR) was consists of four steps:

1-Required reagents was added included master mix, water, primers forward and reverse and template to PCR tubes.

2-Mixed and centrifuged .

3. The PCR tubes was putted in a thermal cycler (PCR machine).

4-The favorable PCR program was steed up for each gene under amplification (3-6)

Table (3-6): Thermal cycling conditions for Specific Primers for genes

Step Type	Temperature °C	Time	Cycling
Initial Denaturation	93	3 min.	1
Denaturation	93	30 Sec.	30
Annealing	56	30 Sec.	
Extension	72	30 Sec.	
Final Extension	72	3 min.	1
Hold	4	α	1

3-2-2-8-6: DNA Electrophoresis

1- A 100 ml of the T.B.E buffer was placed in conical flask (250ml).

2- A 1 g. of agarose was added to the buffer and heated on a hot plate to boiling point so that all of its components were solvent.

3- The agarose mixture was cooled by leaving it between 50-60˚C.

4- Pre-staining with Ethidium bromide is a DNA interchelator dye by added 0.5 µl to the agarose-TBE buffer before solidification.

5- The comb was put into one of the ends of the agarose gel template.

6- Agarose was poured into the template gently to prevent the bubbles formation and left it to cool at room temperature for 30 minutes.

8- The electrophoresis tray was filled by 350ml of T.B.E buffer solution.

9- Five µl of DNA product were mixed with one µl loading stain and loaded into the agarose -gel wells.

10- The electrophoresis was performed at 70 V for 45 min.

11- The agarose gel was exposed to UV light illuminators for DNA bands visualized and documented (Al-Jubory and Imran 2020).

3-2-2-8-7:SNP Selection and Genotyping

Candidate SNPs in this study were selected according to the literature review of previous studies and in silico functional prediction from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/snp). SNPs were selected if they were reported to be associated with disease and/or predicted to have effects on function.

3-2-2-8-8:Analysis of sequencing data

About 18-20 µl of PCR products of all gene under interest in this study samples were sent to the macrogen company in South Korea to performed the sequence of DNA for detection SNPs under interest. Through 15 days the data of sequencing received by email in three formula; pdf file, text document sheet and AB1 file which requires sequencing reading program by Geneious prime purchased version. In addition, the NCBI data tools were used for alignment the gene sequence by BLAST tool of NCBI.

3-2-2-8-9:Pairwise alignment of text files sequence

The reserved A FASTA sequence of text file(from Macrogen Lab.) of two genes under interest for each, were copy and paste on Align Sequences Nucleotide BLAST field, and press **BLAST** to Search nucleotide sequence using Megablast (Optimize for highly similar sequences) based on link: https://blast.ncbi.nlm.nih.gov/Blast.cgi.

After verifying that the sequence belongs to the gene of interest, the identity percentage, the E. value, and the coverage ratio are checked, provided that they achieve identity percentage of at least 97-100%, and E

value = 0, a coverage ratio not less than 98-100%, the accuracy of the sequence analysis is adopted. Sequence analysis results are considered contaminated and sequences outside the three criteria are excluded.

3-2-2-8-10:Interpretation of Sequencing Data

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using reading program by Geneious prime purchased version. In addition, the NCBI data tools were used for alignment the gene sequence by BLAST tool of NCBI. The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

3-2-2-8-12:Checking the Validity and Novelty of SNPs

The first step of checking, must visit gene bank and detection the present SNP(s) under interest on specific site previously determined on it chromosome by NCBI. The observed SNPs were submitted to the dbSNP database to check their originality. Each particular SNP was re-positioned according to its place in the reference genome subsequently; the determination of the presence of previous SNP was performed by viewing its corresponding dbSNP position. Then, all dbSNPs positions for all observed SNPs were documented respectively. https://www.ncbi.nlm.nih.gov/snp/.

3-2-2-8-13:SNP Validity

Any mutations(s) frequently in our sequences samples at the same site and this mutation site similar to the same site of reference SNP on targeted gene on related chromosome was considered as valid SNP and termed as Valid SNP(s). Otherwise any high frequent mutation(s) or variant(s) occurred on the same site in any sequence samples, but it not

present on similar site of gene on related chromosome, this variant termed as new variant and must send to gene bank to get a new **r**eference **s**train number (rs). Sometimes on mutation may be appeared in sequence chart, but not frequent in the other sequence samples, this variant considered as bad proof reading to Taq polymerase.

3-2-2-8-14:Statistical Analysis

All statistical calculation was performed by the using of a statistical package for social science (SPSS) software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. USA) and Microsoft Excel (2010, Microsoft Corp. USA). The mean and standard deviation (SD) of quantitative data were provided. Frequency and percentage were used to show qualitative data.

We utilized the $\chi 2$-test, analysis of variance test, and T test to compare groups. A $p < 0.05$ was considered statistically significant. Chi-square test to assess the categorically association variables and genetic association, according to (Kang & Shin, 2004).Allele frequencies of genes were calculated by direct gene counting methods, while a significant departure from Hardy-Weinberg (H-W) equilibrium was estimated using H-W calculator for two alleles, which is available free onlin:athttp://www.had2know.com/academics/hardyweinbergequilibrium calculator-3-ale;es.html.Hardy-Weinberg equilibrium is the expected frequencies of genotypes if mating is non –as hortative and there are no mutations from one allele to another. Significant differences between the observed and expected frequencies are assessed by Pearson‟s Chi-square test (Ad'hiah, 1990).

Chapter Four
Results and Analysis

4-Results and Analysis

4-1:Distribution of COVID-19 Patients by presence of fungal co infection

As a figure (4-1) most of (nasal swap) samples which taken from patients was have species of fungi but Only 30 samples from total number of patients samples was have invasive (pathogenic fungi).

Figure (4-1): Fungal isolated of nasal cavity samples on SDA from COVID-19 patients A: *Candida albicans* B: *Penicillium* sp.

As showed in table(4-2) the number of samples of and percentage of appearance and frequency was deferent from species to another in patients samples, where larger percentage of appearance and frequency was (41.3% ,64.1%) respectively for same species *Candida albicanes*, while the lower percentage of appearance was (5.3%) for *C. glabrata*, and lower percentage of frequency was (3.79%) for *Cladosporium sp*. Whereas yeast species which appear in healthy samples was only *Candida albicanes* and *C. krusei* with high significant deference between patients and healthy samples.

CHROM agar test is used for the presumptive identification of *Candida* species by production of different colors on this medium (*C. albicans*= green/ blue green, *C. dubliniensis*= dark green, *C. tropical*= blue, *C. parapsilosis*= cream white, and *C. krusei*=pink) (Horvath *et al.*, 2003) as figure (4-2).

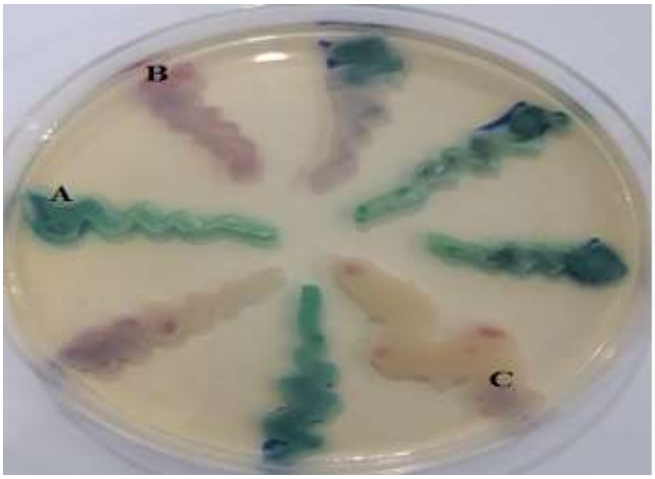
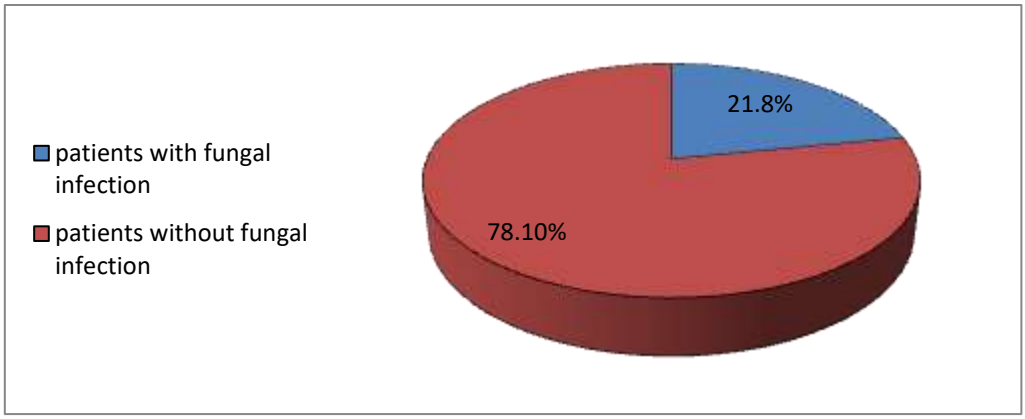


Figure (4-2): Colonies color of *Candida* species on CHROM agar medium at 37˚C for 24-48h. A: *C. albicans*, B: *C. glabrata* C: *C. krusei*

Table (4-1): Distribution of fungi species in nasal cavity isolated from COVID-19 patients and healthy persons

	COVID-19 patients (137)		**Control (30)**
Molds species	**No. of samples (%) Appearance**	**No. of colony (%) Frequency**	**No. of colony (%) Frequency**
Aspergillus niger	**15 (10)**	**17 (1.53)**	**-**
***Cladosporium* sp.**	**16 (10.6)**	**42 (3.79)**	**-**
***Penicillium* sp.**	**21 (14)**	**63 (5.6)**	**-**
Candida albicanes	**62 (41.3)**	**709 (64.1)**	**5 (71.42)**
C. glabrata	**8 (5.3)**	**80 (7.23)**	**-**
C. krusei	**19 (12.6)**	**115 (10.39)**	**2 (28.75)**
C. parapsilosis	**9(6)**	**80 (7.23)**	**-**
Total no.	**150 (100)**	**1106 (100)**	**7 (100)**

The percentage of fungal infection in COVID-19 patients was (21.8%) as figure (4-2), After examined for fungi species respective responses in vitro hemolytic test, A group *Candida species* only which included *Candida albicans* (76.65), *C. glabrata* (11.3%), C. parapsilosis (6.6%) *C. krusei* (4.7%), demonstrated both alpha and beta hemolysis at 48 h post inoculation .



Figure(4-3) percentage of fungal infection in COVID-19 patients

4-2:Distribution of COVID-19 Patients and Control Group by Gender and Age group

One hundred thirty seven patients (75 males and 62 females) participate in this research. The range of their age was from (10-70) years old and the healthy persons include 30 (18 males and 12 females) . They are considered the healthy controlwith age range(10-70), There are significant differences between the percentage of infection between male and female, among the three age group as table (4-3).

Table (4-2):Distribution of patient with COVID-19 and control based on age and gender

Characteristics		**Patients with fungal infection(30)**	**Patients without fungal infection(107)**	**Controls (30)**
Gender No.(%)	**Male**	17 (56.6%)	58 (54.2%)	18 (60%)
	Female	13 (43.3%)	49 (45.7%)	12 (40%)
Age M±SD(%)	**10- 29**	17.9±6.14 (16%)	17.63±5.67(14.6%)	20.39±5.76 (22%)
	30- 49	37.2±6.25 (30%)	37.16±6.17(37.5%)	41.07±6.42 (30.4%)
	50- 69	64.63±11.91(54%)	64.96±10.6(47.8%)	62.15±11.72 (46.6%)
P.value ≤0.05				

This table show that male was more likely to be affected than women, percentage of infection in male was (56.6%) while female was

(43.3%) in patients with COVID-19 and fungal infection group , Male was (54.2%) whereas female was(45.7%) in patients with COVID-19 without fungal infection group. Also it showed That the infection with COVID-19 increases with age and that elderly people were more affected by the disease than young people or younger people.

4-3:Distribution of COVID-19 Patients and Control Groups by Hematological and Immunological Parameters

Complete blood count is one of the essential widely available investigations for COVID-19 infection diagnosis and severity assessment .Statistical analysis of blood variables for COVID-19 patients showed that white blood cells increased in both groups of patients (patients with fungal infection and patients without fungal infection) the mean of the patients' white blood cells was (16.38±1.43 cell× $10^3/mm^3$ and 16.01±1.42 cell× $10^3/mm^3$) Consequently, compared to the mean of the healthy control sample was (8.42±3.34 cell× $10^3/mm^3$). The neutrophil cells were also observed to increase in both groups that is the mean of patients(patients with fungal infection and patients without fungal infection) is(15.36±7.27 cell× $10^3/mm^3$and 10.73±3.30 cell× $10^3/mm^3$) Consequently compared with the mean of the healthy control sample, which is (4.63±2.51 cell× $10^3/mm^3$).

Lymphocytes showed a significant decrease between patients groups and control group, where the mean of it in COVID-19 patients(patients with fungal infection and patients without fungal infection) was (0.66±0.57 cell× $10^3/mm^3$ and 0.53±0.54 cell× $10^3/mm^3$) Consequently, while the mean of the control sample was(2.16±0.84 cell× $10^3/mm^3$). Whereas level of Ferritin increased as the mean of it patients groups(patients with fungal infection and patients without fungal infection) was (796.46±14.75ng/ml and 823.90±16.89 ng/ml)

consequently , while the mean of it in the healthy control sample was (126.68±19.12 ng/ml).

D-dimer and CRP also increased as The mean of these which shown in table (4-4). The current investigation discover a significant rise of suPAR levels in patients serum when compared to healthy controls. Additionally, very significant differences in suPAR levels are seen between patient groups(COVID-19 patients with fungal infection and without fungal infection) and control group in serum at (p=0.003,0.007) consequently.

parameters	Patients with fungal infection (30)	Control (30)	P .value
WBC	16.38±1.43cell×10^3/mm^3	8.42±3.34 cell×10^3/mm^3	0.000***
Neutrophils	15.36±7.27cell×10^3/mm^3	.63±2.51 cell× 10^3/mm^3	0.000***
Lymphocyte	0.66±0.57cell×10^3/mm^3	2.16±0.84 cell×10^3/mm^3	0.000***
Ferritin	796.46±14.75ng/ml	126.68±19.12ng/ml	0.000***
D-dimer	879.48±13.96ng/ml	318.73±13.87ng/ml	0.000***
CRP	93.07±10.44IU/ml	4.60±2.47IU/ml	0.001**
suPAR	6.55± 1.21ng/ml	1.20±0.77ng/ml	0.003**

parameters	Patients without fungal Infection(107)	Control (30)	p. value
WBC	16.01±1.42cell×10^3/mm^3	8.42±3.34 cell× 10^3/mm^3	0.000***
Neutrophils	10.73±3.30cell×10^3/mm	4.63±2.51 cell× 10^3/mm^3	0.000***
Lymphocyte	0.53±0.54cell×10^3/mm^3	2.16±0.84 cell× 10^3/mm^3	0.01*
Ferritin	823.90±16.89ng/ml	126.68±19.12ng/ml	0.000***
D-dimer	896.684±15.64ng/ml	318.73±13.87ng/ml	0.000***
CRP	97.03±11.51IU/ml	4.60±2.47 IU/ml	0.001**
suPAR	6.42±1.09 ng/ml	1.20±0.77 ng/ml	0.007**

Table(4-3) :Distribution of (COVID-19 patients with fungal infection and without fungal infection) and control group based on Hematological and Immunological Parameters

4-4: Distribution of COVID-19 Patients and Control Group by Presence of some symptoms

After statistical analysis of the results, it was noted that there were high statistically significant differences (P≤0.001*) between the patient groups and the control group depending on presence of some symptoms

which associated with COVID-19 as fever, Cough ,Dyspnea and GIT symptoms , percentage of fever was(63.3 and 77.75)in(COVID-19 patients with fungal infection and without fungal infection) Consequently. Whereas this and all symptoms was not presence in control group persons as shown in table (4-5)

Table(4-4):Distribution of COVID-19 patients with fungal infection and without fungal infection and control group based on presence of some symptoms

Symptoms	**Patients with fungal infection (30) No. (%)**	**Patients without fungal infection (107) No. (%)**	**Control (30) No. (%)**	**P.value**
Cough	15 (50%)	75 (70.09%)	0 (0.0)	≤0.001*
Fever	19 (63.3%)	83 (77.75%)	0 (0.0)	≤0.001*
Dyspnea	13 (43.3%)	70 (65.42%)	0 (0.0)	≤0.001*
GITsymptom	5 (16.6%)	30 (28.03%)	0 (0.0)	≤0.001*

4-5: Distribution of COVID-19 Patients and Control Groups by Presence of Comorbidities

After statistical analysis of the results, it was noted that there were statistically significant differences (P= 0.001) between the patient groups and the control group, percentage of hypertension was(43.3% and 32.7%)in(COVID-19 patients with fungal infection and without fungal infection) respectively.

Also, there were significant differences for diabetics between the patient groups and the control group, as the percentage of diabetics COVID-19 patients with fungal infection and without fungal infection was (36.6 and 28.03) consequently , Also with the rest of the diseases, their numbers and percentages are shown in Table (4-6), where the

groups significantly (P<0.001) between patients groups and the control for the rest of the diseases .

Table(4-5):Distribution of (COVID-19 patients with fungal infection and without fungal infection) and control group based on presence of comorbidities

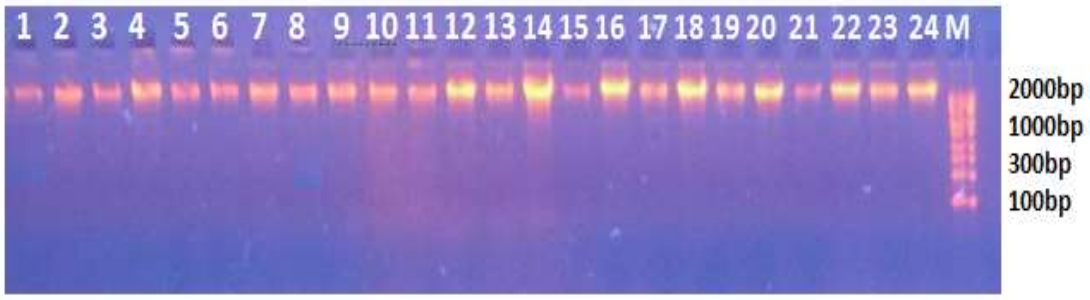
comorbidities No. (%)	Patients with fungal infection (30)	Control(30)	P. value
Hypertension	13(43.3%)	0 (0%)	0.000***
Diabetes	11(36.6%)	1(3.3%)	0.000***
Hype.+Dia.	7(23.3%)	2(6.6%)	0.001**
Cardiovascular	5(16.6%)	0(0%)	0.001**
Cardio + hype + dia	2(6.6%)	0(0%)	0.001**
comorbidities No. (%)	Patients without fungal infection(107)	Control(30)	P. value
Hypertension	35(32.7%)	0 (0%)	0.000***
Diabetes	30(28.03%)	1(3.3%)	0.000***
Hype.+Dia.	15(14.01%)	2(6.6%)	0.001**
Cardiovascular	10(9.34%)	0(0%)	0.001**
Cardio + hype + dia	5(4.67%)	0(0%)	0.001**

4-6:Molecular study

4-6-1: Interleuken-17A

4-6-1-1:Human DNA Extraction

The extracted human DNA genome from whole blood of all samples included: 30 samples of COVID-19 patients with fungal infection patients group, 30 samples COVID-19 patients without fungal infection (patients group), and 30 samples of healthy (control group). The estimation of DNA concentration revealed from (50-100ng) with purity (1.7-1.8) (Figure 4-5) the gel preparation and it condition consistent with Lee *et al.*,2012).

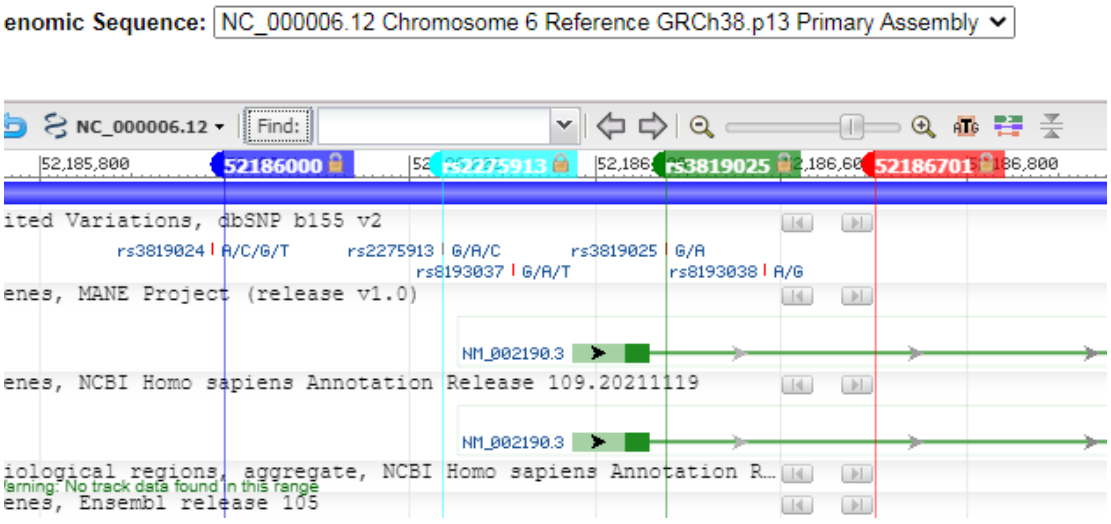


Figure(4-5): Gel-electrophoresis profile illustration quality of DNA extracted from COVID-19 patients group, M=molecular marker 100bp for each step.1.5% agarose-TBE gel, pre-staining with 0.5ul Ethidium, bromid,45min.,100 voltage

4-6-1-2:PCR Products Detection

A DNA samples under interest for each patients groups , and then subjected to PCR amplification. The targeting specific region in the DNA was delimited by using specific primer pair .The primer pair designed in this study covering SNPs under interest span sequence region 69 of chr6, the amplicon length with flanking regions of primers equal 694bp.

The targeted region of partial sequence of IL17A covering the SNPs: rs2275913 and rs3819025 as figure (4-6). then enrolled for detection of single nucleotide polymorphisms (SNPs) by using amplification polymerase chain reaction (simple PCR), and sequencing techniques.



Figure(4-6): The targeted region of partial sequence of IL17A amplified by primer pair IL-17A covering the SNPs: rs2275913 and rs3819025.

4-6-1-2-1: PCR products of Amplification of The targeted region of partial sequence of IL-17A of COVID-19 with fungal infection

The Figure (4-7) was illustrated result of amplification IL17A F,IL17A R region as target DNA region of IL-17A results, the amplification region with flanking primers, PCR products 694bp for COVID-19 patients with fungal infection group.

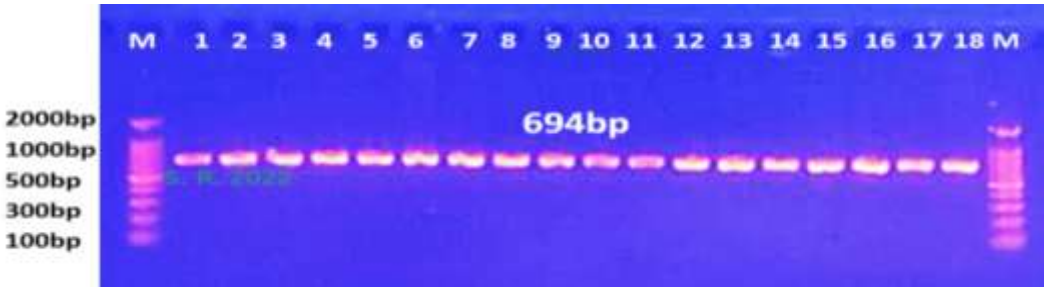
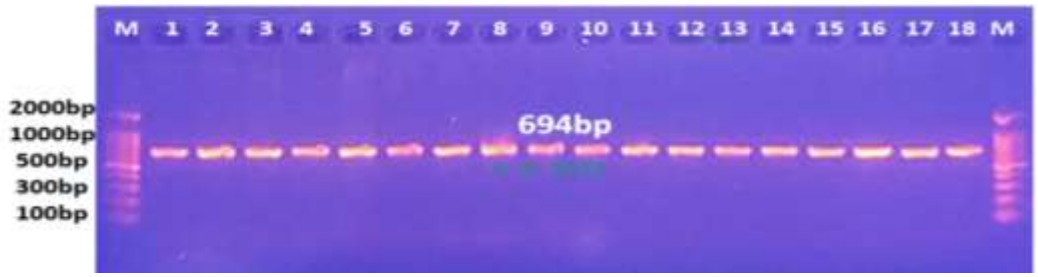


Figure (4-7): Gel electrophoresis of chr6 as target DNA region of IL17A(COVID-19 patient with fungal infection group) amplification region with flanking primers, number of patients PCR products 694bp, M= molecular marker 100bp for each step

4-6-1-2-2:PCR products of Amplification of The targeted region of partial sequence of IL-17A of COVID-19 without fungal infection

The results shown success the primer pair efficiency to amplification region as target DNA region of IL-17A, the amplification region with flanking primers, PCR product 694bp for patients COVID-19 without fungal infection group (Figure 4-8).



Figure(4-8):Gel electrophoresis IL17A F, IL17A R 69 of chr6 as target DNA region of IL17A(COVID-19 patient without fungal infection group)amplification region with flanking primers, number of patient PCR products 694bp,M= molecular marker 100bp for each step

4-6-1-2-3: PCR products of amplification of the targeted region of healthy group

The Figure (4-9) illustrated electrophoresis profile result of amplification IL17A F, IL17A R region as target DNA region of IL-17A results, the amplification region with flanking primers, PCR products 694bp for control group.

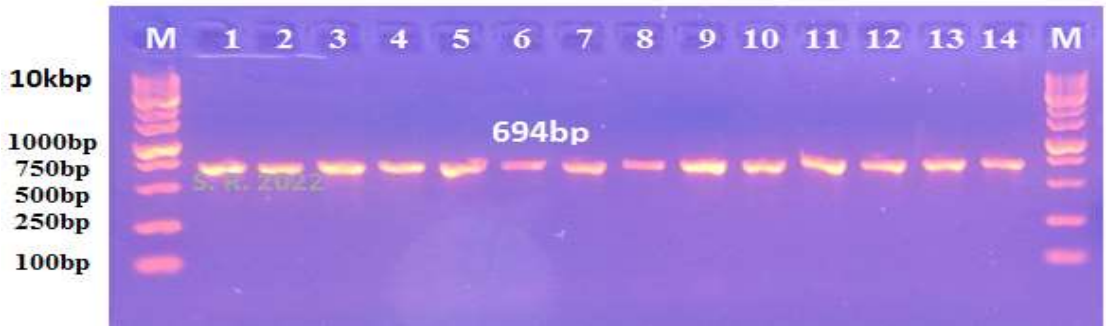


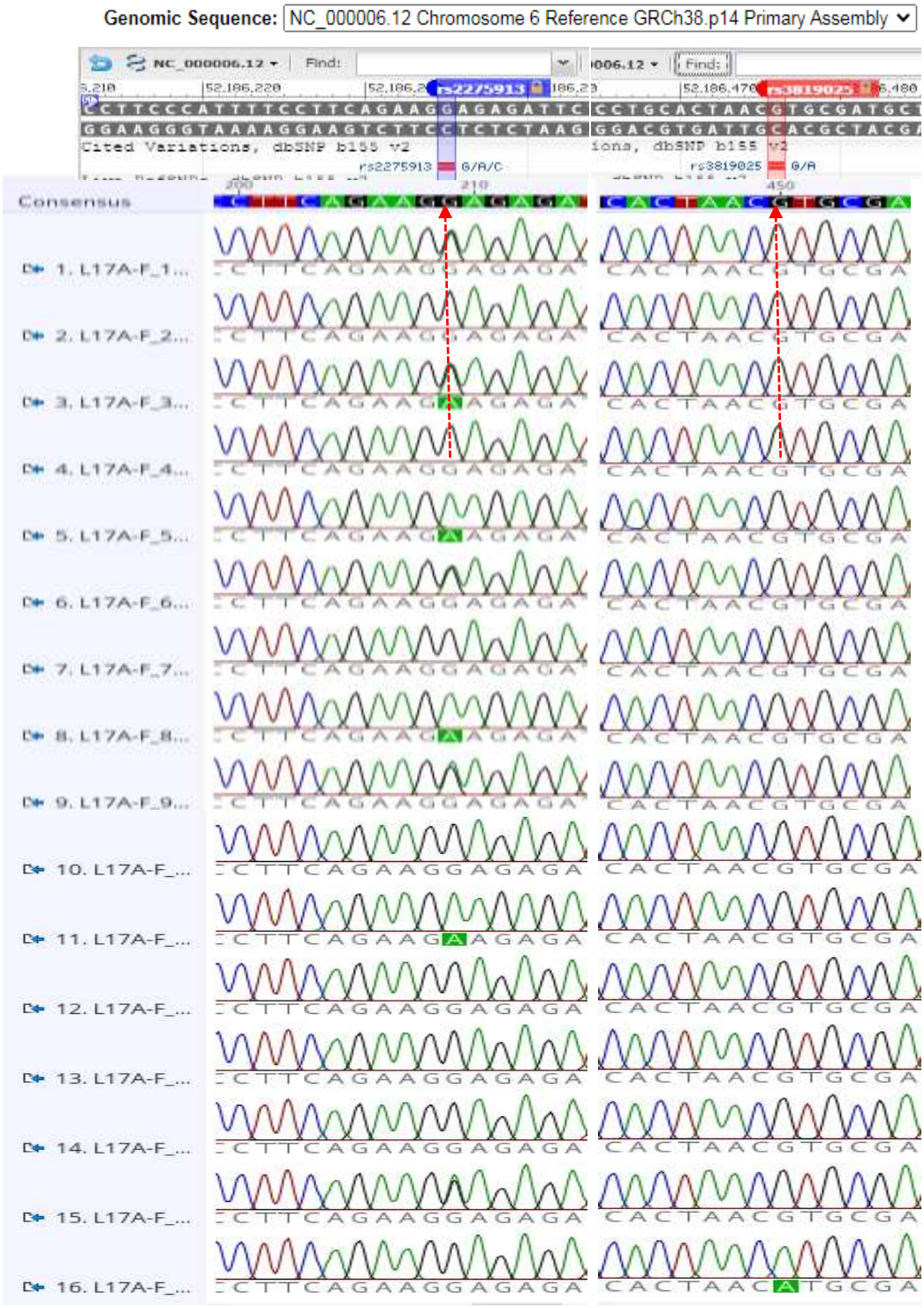
Figure (4-9): Gel electrophoresis IL17A F, IL17A R 69 of chr6 as target DNA region of IL17A(healthy group) amplification region with flanking primers, number of healthy (Control group) PCR products 694bp, M= molecular marker 100bp for each first step.

4-6-1-3:Polymorphism screening and genotyping

Screening of IL-17A polymorphisms was performed by direct sequencing of genomic DNA from blood of COVID-19 patients and healthy(control). To amplify the partial sequence IL-17A gene by polymerase chain reaction (PCR), one primer pair was designed in this study. Direct sequencing was conducted by send 18-20ul of PCR of samples of three groups for each. After reserved sequence charts from macrogen. The text file was used for pair wise alignment with deposited NCBI database. The Chromatograme file in rare which out of 4 files enclosed were import to genius software for alignment.

4-6-1-3-1:Multiple alignments of IL-17A of COVID-19 patients without fungal infection

The multiple alignments of chromatograms data show the genotyping findings for patients with COVID-19 showing the two SNPs: rs2275913 and rs3819025 . The genotypes for the SNP rs2275913 were (GG, GA&AA) and the SNP rs3819025 were (GG, GA &AA). Figure (4-10).



Figure(4-10): forms of Multiple alignment of chromatograms IL17-AF IL17-ARof targeted region of Il-17A as shown two SNPs: rs2275913 and rs3819025. (COVID-19 patient without fungal infection) alignment performed by Geneious prime software

4-6-1-3-2: Multiple alignments of IL-17A of COVID-19 patients with fungal infection

The multiple alignments of chromatograms data show the genotyping findings for patients with COVID-19 showing the two SNPs: rs2275913 and rs3819025 . The genotypes for the SNP rs2275913 were (GG, GA&AA) and the SNP rs3819025 were (GG, GA &AA). Figure (4-11)

Figure(4-11): forms of polymorphism of chromatograms IL17-AF IL17-ARof targeted region of Il-17A as shown two SNPs: rs2275913 and rs3819025. (COVID-19 patient with fungal infection) alignment performed by Geneious prime software

4-6-1-3-3: Multiple alignments of IL17A of healthy group

The multiple alignments of chromatograms data show the genotyping findings for patients with COVID-19 showing the two SNPs: rs2275913 and rs3819025 . The genotypes for the SNP rs2275913 were (GG, GA&AA) and the SNP rs3819025 were (GG, GA &AA). Figure (4-12).

Figure(4-12): forms of Multiple alignments of chromatograms IL17-AF IL17-ARof targeted region of Il-17A as shown two SNPs: rs2275913 and rs3819025. (control group) alignment performed by Geneious prime software.

4-6-1-4 :IL-17A gene rs2275913 G> A polymorphism

The genotyping findings for COVID-19 patients with fungal infection and control group which was clearest in Table (4-7) , showing the three genotypes (GG, GA &AA),(56.6% , 18.4, 25%) respectively and allele frequency of (G 65% ,A 35%) respectively for the patients and genotypes (GG, GA &AA),(70%,10, 20%) respectively and allele frequency (G 75% ,A 25%) respectively for control persons ,Heterozygous genotype GA was more frequent in the patients group in comparison with the control group in less significant manner (p= 0.5) In terms of odds ratio (OR) the risk attributed to GA was 1.8, on other hand , genotype AA show no significant association with disease risk (p= 0.5) with an OR of 1.4. In allele analysis , allele A, G less significant risk factor (p = 0.2)OR (1.6 and 0.61).

In compartmented with genotyping rs2275913 G>A in COVID-19 patients without fungal infection, the results showed three genotypes (GG,GA and AA), but the deference in their percentage 75%,6.7 and 20%, the allele frequency G=76.7% while A=23.3%), In Heterozygous genotype GA was no deference in frequent in the control group and patients group in less significant manner (p= 0.6) , Also odds ratio (OR) the risk attributed to GA was 0.6 . on other hand , the homozygous GG genotype was also no deference in frequent in patients group and control group, genotype AA show no significant association with disease risk (p= 1) with an OR of 1 also. In allele analysis ,in allele A and G there are no significant risk factor (p = 0.8)OR (1.09 , 0.9) respectively .This SNP:rs2275913 C>G was recorded in NCBI SNP blas thttps://www.ncbi.nlm.nih.gov/variation/view

Table (4-6): Genotypes distribution and allele frequency of wild type allele and mutant allele of SNP: ss2275913, OR values and p values for COVID-19 patients with fungal infection and COVID-19 patients without fungal infection.

Rs2275913 G>A	Covid19 Patients with fungal infection (30)		Control (30)	OR(95%CI)	P-value
genotypes	GG	17(56.6%)	21(70%)	Reference group	
	GA	5(18.4%)	3(10%)	**1.8 (0.38-8.3)**	0.5
	AA	8(25%)	6(20%)	**1. 4(0.43-8.4)**	0.5
Allele Frequency	G	39(65%)	45(75%)	**1.09(0.28-1.**3)	0.2
	A	**21(35%)**	15(25%)	0.9 (0.73-3.6**)**	0.2
Rs2275913 G>A	Covid19 Patients without fungal infection (30)		Control (30)	OR(95%CI)	P-value
genotypes	GG	22(75%)	21(70%)	Reference group	
	GA	2(6.7%)	3(10%)	0.6(0.09-4.1)	0.5
	AA	6(20%)	6(20%)	**1 (0.23-3.4)**	1
Allele Frequency	G	46(76.7%)	45 (75%)	0.6(0.4-2.5)	0.8
	A	14(23.3%)	15 (25%)	**1.6(0.39-2.1**)	0.8

*, OR, odds ratio; CI, confidence interval

4-6-1-5 :IL-17A gene rs3819025G> A polymorphism

The detail information was showed in Table (4-8). The genotyping findings for COVID-19 patients with fungal infection and control group, showing the three genotypes (GG,GA and AA),(93.3% , 0, 6.6%) respectively and allele frequency of (G 93.3% ,A 6.6%) respectively for the patients and genotypes (GG GA and AA),(90%,0%, 10%) respectively and allele frequency (G 90% ,10%) respectively for control subjects, Heterozygous genotype GA was no frequent in both control group and patients group in less significant manner (p= 1) In terms of odds ratio (OR) the risk attributed to GA was 1 . on other hand , the homozygous GG genotype was more frequents in control group, genotype AA show no significant association with disease risk (p= 0.6) with an OR of 0.6

In allele analysis , allele A no significant risk factor (p = 0.5)OR (0.6). In compartmented with genotyping rs3819025 G>A in COVID-19 patients without fungal infection, the results showed three genotypes (GG,GA and AA), but the deference in their percentage(66.6%, 20% and 13.3%), the allele frequency G=76.6% while A=23.3%).

Heterozygous genotype GA was more frequency in patients group than control group in less significant manner (p= 0.06) In terms of odds ratio (OR) the risk attributed to GA was 16.2 . on other hand , the homozygous GG genotype was more frequents in control group and can be regarded as protective factor , genotype AA show no significant association with disease risk (p= 0.6) with an OR of 1.3 , In allele analysis , allele A more significant risk factor (p = 0.05) OR (2.7). this SNP **rs3819025** C>G was recorded in NCBI SNP blast.

Table (4-7): Genotypes distribution and allele frequency of wild type allele and mutant allele of SNP:rs3819025, OR values and p values for COVID-19 patients with fungal infection and COVID-19 patients without fungal infection.

Rs3819025 G>A	Covid19 Patients with fungal infection (30)		Control (30)	OR(95%CI)	P-value
genotypes	GG	28(93.3%)	27(90%)	Reference group	
	GA	0(0%)	0(0%)	**1(0.01-52)**	1
	AA	2(6.6%)	3(10%)	0.6 (0.09-4.1)	0.6
Allele Frequenc	G	56(93.3%)	54(90%)	**1.5(0.41-5.8)**	0. 5
	A	4(6.6%)	6(10%)	0.6(0.17-2.4)	0.5
Rs3819025 G>A	Covid19 Patients without fungal infection (30)		Control (30)	OR(95%CI)	P-value
genotypes	GG	20(66.6%)	27(90%)	Reference group	
	GA	6(20%)	0(0%)	**16.2(0.8-301.6)**	0.06
	AA	4(13.3%)	3(10%)	**1. 3(0.28-6.7)**	0.6
Allele Frequenc	G	46(76.6%)	54(90%)	0.36(0.12-1.02)	0.05
	A	**14(23.3%)**	6(10%)	**2.7(0.97-7.7)**	0.05

*, OR, odds ratio; CI, confidence interval

4-6-2:Interferon administrative factor -7 (IRF-7)

4-6-2-1:Human DNA Extraction

The extracted human DNA genome from whole blood of all samples included: 30 samples of COVID-19 with fungal infection patients group, 30 samples COVID-19 without fungal infection patients group, and 30 samples of healthy control group. The estimation of DNA concentration revealed from (50-100ng) with purity (1.7-1.8) ,the gel preparation and it condition consistent with (Lee, 2012).

4-6-2-2:PCR Products Detection

DNA samples for each patients groups were selected randomly from whole samples under interest, and then submitted to PCR amplification. The targeting specific region in the DNA was delimited by using specific primer pair . The primer pair designed in this study, which covering SNPs under interest span sequence region from 611628 to 612161 of chr11 illustrated the amplicon length with flanking regions of primers equal 534bp. then enrolled for detection of single nucleotide polymorphisms (SNPs) by using amplification polymerase chain reaction (simple PCR), and sequencing techniques.

4-6-2-3:The PHRFI/ IRF7 gene- gene interaction

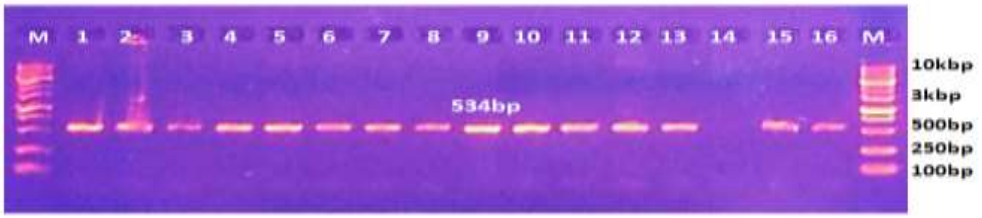
This study was gave attention to present case of gene-gene interaction, the two gene were IRF7 and PHRFI , both of them laid closed to each other Figure (4-12). The IRF7 gene was well known on Chr11, many literature referred to it role activation interferon alpha, and the SNP under interest (rs702966) was located in IRF7 gene based on many reports like (Salloum *et al*., 2010; Fu *et al*., 2011; Kawasaki *et al*., 2012), the PHRFI was described as nonfunctional gene to date, some studies considered that SNP rs702966 occurred in intergenic region (Figure 4-

13), so be under action of the two gene PHRFI/ IRF7. We followed a mode included the SNP(s) in IRF7 gene along all text of this thesis for facilitate, explanation and justification, at the same time we hope from reader to gives attention to our option.

Figure (4-13): NCBI Gene-gene interaction of IRF7/PHRFI site, validity of SNP rs702966 occurred in intergenic region and attached with schematic diagram summarized location of two genes and intergenic region

4-6-2-3-1:Target amplified in patients COVID-19 Patients without fungal infection

The results shown success the primer pair efficiency to amplification region 611628 to 612161 as target DNA region of IRF7, the amplification region with flanking primers, PCR product 534bp for patients COVID-19 without fungal infection group (Figure 4-14).



Figure(4-14): Profile of PCR products of target of IRF7 shown PCR products 534bp for PCR products for patients without fungal. Number of patient samples, M=molecular marker first step100bp. 1.5% agarose -TBE gel, pre-staining with 0.5ul Ethidium, bromide, 45min., 100 voltages.

4--62-3-2:Target amplified in patients COVID-19 with ffungal infection

Figure(4-15) was illustrated result of amplification region 611628 to 612161as target DNA region of IFR7 results, the amplification region with flanking primers, PCR products 534bp for COVID-19 patients with fungal infection group.

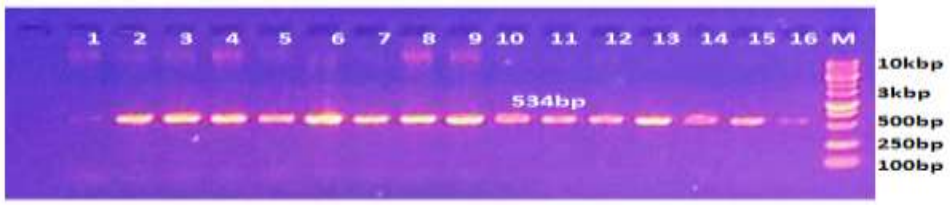


Figure (4-15): Profile of PCR products of target of IRF7 shown PCR products 534bp for PCR products for patient with fungal. Number of patient samples, M=molecular marker first step100bp. 1.5% agarose-TBE gel, pre-staining with 0.5ul Ethidium, bromide, 45min., 100 voltages.

4-6-2-3-3:Target amplified in control group

The Figure(4-16) illustrated electrophoresis profile result of amplification region 611628 to 612161as target DNA region of IRF7 results, the amplification region with flanking primers, PCR products 534bp for control group.

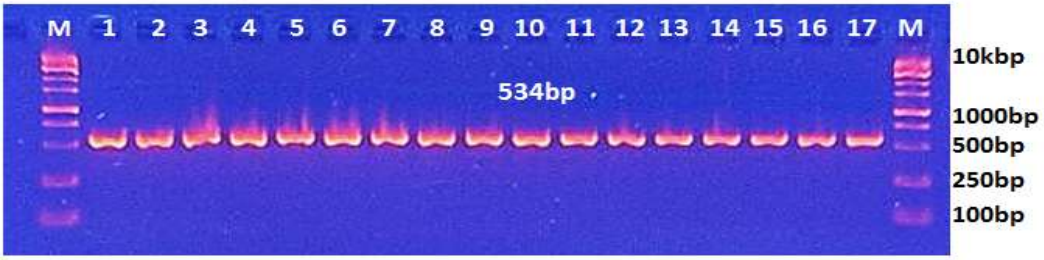


Figure (4-16): Profile of PCR products of target of IRF7 shown PCR products 534bp for PCR products for control group. Number of samples, M=molecular marker first step100bp.1.5% agarose-TBE gel, pre-staining with 0.5ul Ethidium, bromide, 45min.,100 voltages.

4-6-2-4:Polymorphism screening and genotyping

Screening of IRF7/PHRFI polymorphisms was performed by direct sequencing of genomic DNA for COVID-19 patients and healthy group. To amplify the partial sequence IRF7 gene by polymerase chain reaction (PCR), one primer pair was designed in this study (Table1). Direct sequencing was conducted by send 18-20ul of PCR of samples of three groups for each. After reserved sequence charts from macrogen, the sequences were checked they familiarity to IRF7/PHFRI intergenic region based pairwise alignment for each. The text file was used for pair wise alignment with deposited NCBI database. The Chromatograme file in rare which out of 4 files enclosed were import to genius software for alignment.

4-6-2-4-1:Multiple alignments of IRF7 gene in COVID-19 without fungal infection

The multiple alignments of chromatograms data show the genotyping findings for patients with COVID-19 showing the three SNPs: rs17155933, rs702965 and rs702966. The genotypes for the SNP rs17155933 were (CC, CT &TT), for the SNP rs702965 were (CC, CT &TT), and rs702966 (CC, CG, GG). Figure (4-17).

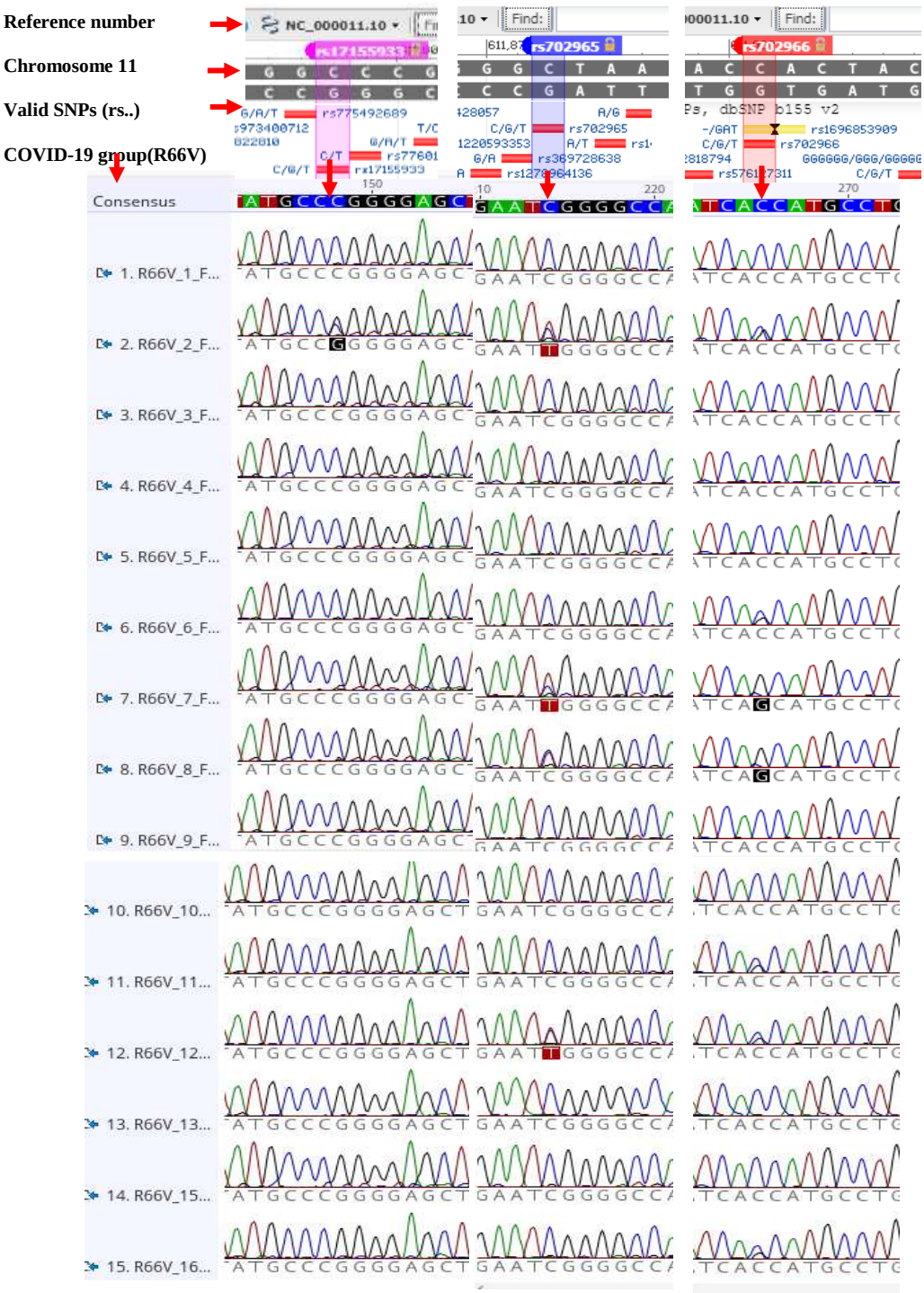


Figure (4-17): Forms of multiple alignment of chromatograms of targeted region 611628 to 612161 of IRF7 as shown three SNPs: three SNPs: rs17155933rs702965 and rs702966. (COVID-19 patient without fungal infection) alignment performed by Geneious prime software.

4-6-2-4-2: Multiple alignments of IRF7 gene in COVID-19 with fungal infection

Multiple alignment of chromatograms of targeted region 611628 to 612161 of IRF7 as shown three SNPs : three SNPs: rs17155933, rs702965 and rs702966. (COVID-19 with fungal infection) alignment performed by Geneious prime software. Figure (4-18).

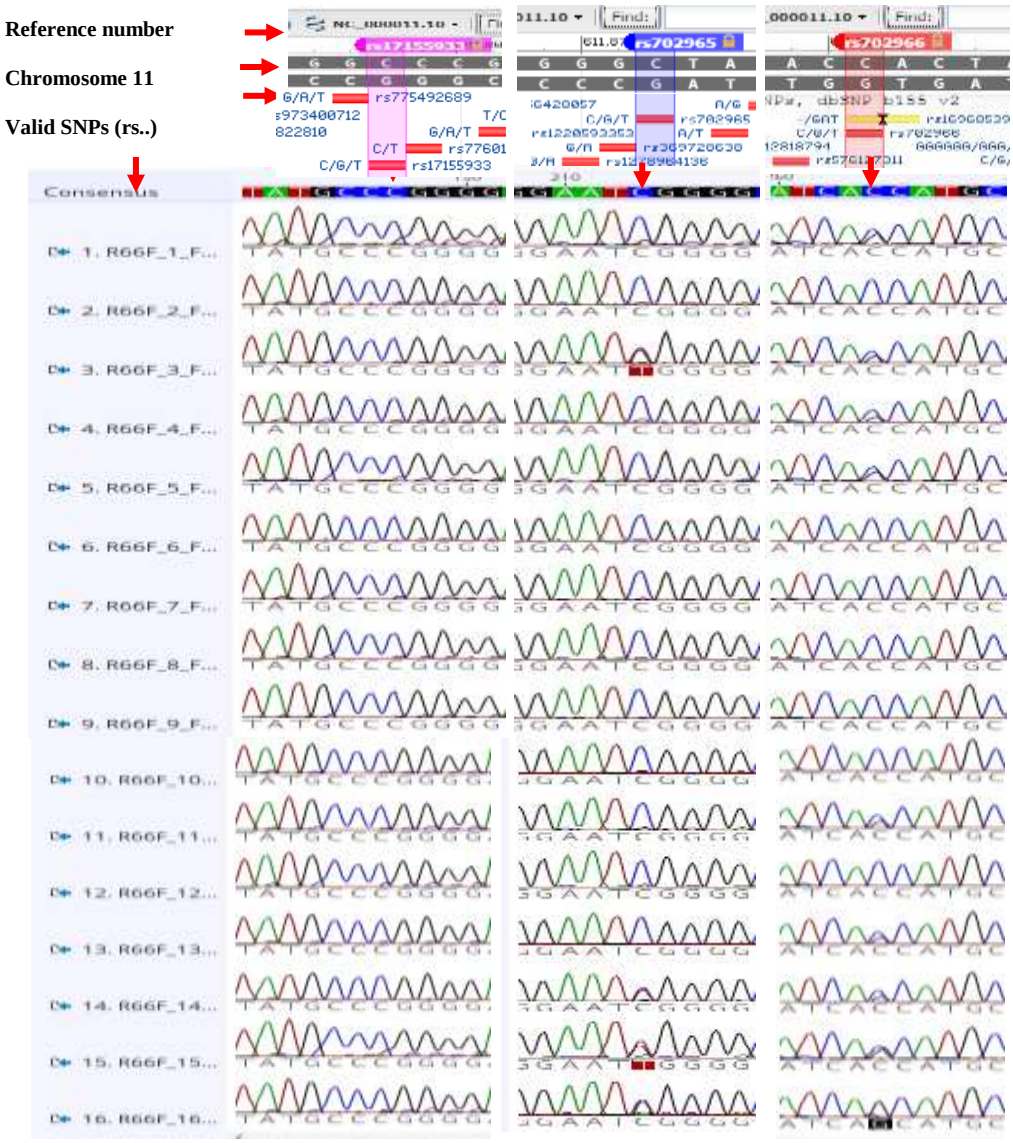


Figure (4-18): forms of multiple alignment of chromatograms of targeted region 611628 to 612161 of IRF7 COVID-19 patient with fungal infection, as shown three SNPs: three SNPs: rs17155933, rs702965 and rs702966. alignment performed by Geneious prime software.

4-6-2-4-3: The multiple alignment of chromatograms of targeted region of IRF7 of control group

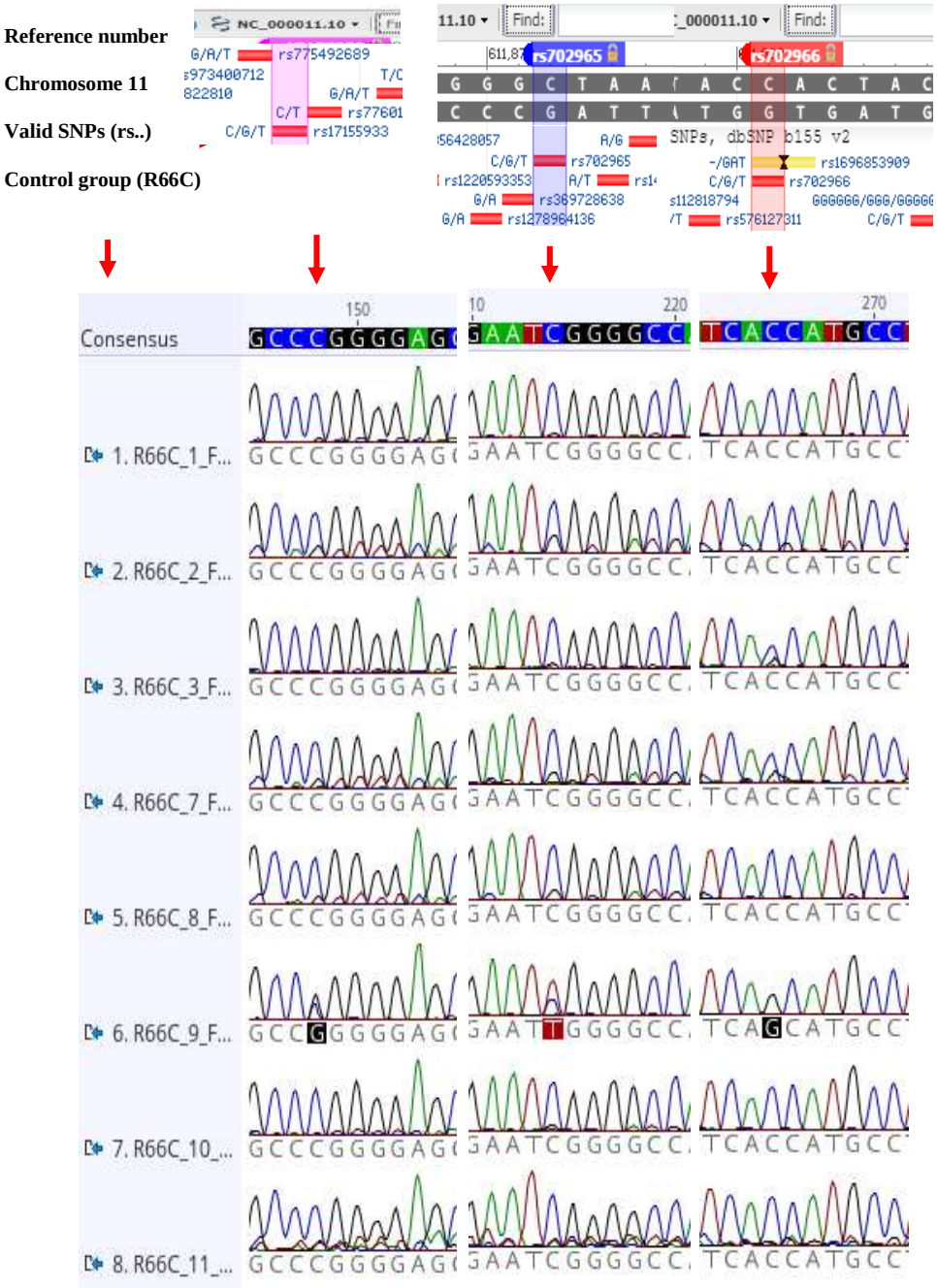


Figure (4-19): forms of multiple alignment of chromatograms of targeted region 611628 to 612161 of IRF7 as shown three SNPs : rs17155933, rs702965 and rs702966. (Control group), alignment performed by Geneious prime software.

4-6-2-5-1:IRF7 gene rs17155933 C> G polymorphism

The detail information was clearest in Table (4-9). The genotyping findings for COVID-19 patients with fungal infection and control group, showing the three genotypes (CC, CG &GG),(93.3% , 0, 6.7) respectively and allele frequency of (C 93.3 ,G 6.7%) respectively for the patients and genotypes (CC, CG &GG),(53.3%, 36.7, 10) respectively and allele frequency (C 71.6% ,G 29.31) respectively for control subjects, Heterozygous genotype CG was more frequent in control group in significant manner (p= 0.01) In terms of odds ratio (OR) the risk attributed to CG was 0.026 . on other hand , the homozygous CC genotype was more frequents in COVID-19 patients with fungal infection group, genotype GG show no significant association with disease risk (p= 0.6) with an OR of 0.62, In allele analysis , allele C frequency was significant risk factor (p = 0.03) OR =5.8 .

In compartmented with genotyping rs17155933 C>G in COVID-19 patients without fungal infection, the results showed three genotypes (CC,CG and GG), but the deference in their percentage 100%,0 and 0%, the allele frequency C=100% while G=0). Also allele C more significant risk factor (p = 0.002), OR =2 , with significant deference between allele G in two groups (p=0.002).

Unfortunately, no previous studies referred to no relation of this SNP with COVID-19. This study proven the validity of this SNP in studied population under interest for the first time at the same time this SNP:rs17155933 C>G was recorded in NCBI SNP blast .

Table (4-8) Genotype and allele frequency of *IRF7* rs17155933 C>G*)* associated with COVID-19 patients with /without fungal infection and control

Rs17155933 C>G	COVID-19 Patients with Fungal infection .(30)		Control (30)	OR(95%CI)	P-value
genotypes	CC	28(93.3%)	16(53.3%)	Reference group	
	CG	0(0%)	11(36.7%)	0.026(0.001 to 0.44	0.01
	GG	2(6.7%)	3(10%)	0.62(0.09 to 4.0)	0.6
Allele Frequenc	C	56(93.3%)	43(71.6%)	**5.8(1.8 -18.5)**	0.003
	G	4(6.7%)	17(29.31%)	0.17(0.05-0.5)	0.003
Rs17155933 C>G	COVID-19 Patients without Funga infection(30)		Control (30)	OR(95%CT)	P-value
genotypes	CC	30(100%)	16(53.3%)	Reference group	
	CG	0(0%)	11(36.7%)	0.07(0.004-1.4)	0.09
	GG	0(0%)	3(10%)	1(0.18-5.4)	0.5
Allele Frequenc	C	60(100%)	43(71.6%)	**2.0(0.6-5.8)**	0.002
	G	0(0%)	17(29.31%)	0.5 (0.17-1.4)	0.002

*, OR, odds ratio; CI, confidence interval

4-6-2-5-2:IRF7 gene: rs702965C>T polymorphism

The Table (4-10) summarized the genotyping findings for COVID-19 patients with fungal infection and control group, showing the three genotypes (CC, CT &TT),(80% , 13.4, 6.6) respectively and allele frequency of (C 86.7 ,T 13.3%) respectively for the patients and genotypes (CC, CT &TT),(63.3%, 26.7, 10) respectively and allele frequency (C 76.6% ,T 23.3) respectively for control subjects, Heterozygous and homozygous genotype CT,CC and TT appeared no significant frequent ,Allele C risk allele with OR=1.97, In compartmented with genotyping rs702965 **C**/T in COVID-19 patients without fungal infection, the results showed three genotypes (CC,CT and TT), but the deference in their percentage 70%, 10 and 20, the allele frequency C=75% while T=25).This SNP:rs702965 not shown significant appeared

in OR and allele frequency in the case of patients with fungal infection group, this SNP not correlated with disease based on the value of allele (T) in genotype TT with value less than 1 and has low allele frequency of allele T in patients with fungal infection than in control group. while, the homozygous allele TT shown significant OR= 1.3(0.35-6.96) without fungal infection. The frequency of T allele shown high OR value=1.09(0.47-2.5). The OR values of TT allele indicated on impact of TT allele on disease incidence based on value of OR >1. Unfortunately, no previous studies referred to the relation of this SNP with COVID-19. This study proven the validity of this SNP in studied population under interest for the first time at the same time this SNP: rs702965 C>T was recorded in NCBI SNP blast at 9-4-2021 based on data of NCBI(https://www.ncbi.nlm.nih.gov/snp/rs702965).

Table (4-9) Genotype and allele frequency of *IRF7* rs702965 C>T associated with COVID-19 patients with/without fungal infection and control

rs702965 C>T	COVID-19 Patients with Fungal infection. (30)		Control (30)	OR(95%CI)	P-value
genotypes	CC	24(80%)	19(63.3%)	Reference group	
	CT	4(13.4%)	8(26.7.6%)	0.35(0.09-1.3)	0.7
	TT	2(6.6%)	3(10%)	0. 52(0.1-4.1)	0.507
Allele Frequency	C	52(86.7%)	46(76.67%)	**1.97(0.7-5.1)**	0.1
	T	8(13.3%)	14(23.33%)	0.51(0.19-3.1)	0.1
	COVID-19 Patients without Funga infection (30)		Control(30)		
genotypes	CC	21(70 %)	19(63.3%)	Reference group	
	CT	3(10%)	8(26.7.6%)	0.29(0.06-1.2)	0.09
	TT	6(20%)	3(10%)	**1.3(0.35-6.96**	0.5
Allele Frequency	C	45(75%)	46(76.67%)	0.9(0.39-2)	0.8
	T	15(25%)	14(23.33%)	**1.09(0.47-2.5)**	0.8

*, OR, odds ratio; CI, confidence interval

4-6-2-5-3:IRF7 gene: rs702966C> G polymorphism

Table (4-11) shows the genotyping findings for COVID-19 patients with fungal infection and control group, showing the three genotypes (CC, CG &GG),(53.3% , 36.7, 10) respectively and allele frequency of (C 71.6,G 28.3) respectively for the patients and genotypes (CC, CG &GG),(63.3%, 23.3, 13.4) respectively and allele frequency (C 75% ,G 25%) respectively for control subjects, In the SNP:rs702966, the heterozygous allele GC shown high appeared in OR and allele frequency in the case of patients OR= CG=1.9 with fungal infection. The frequency of G allele shown high OR value=1.18. The OR values of CG and GG allele indicated on impact of CC allele on disease incidence based on value of OR >1.

In compartmented with genotyping rs702966 **C**/**G** in COVID-19 patients without fungal infection, the results showed three genotypes (CC,CG and GG), but the deference in their percentage 55%, 31.7 and 13.3, the allele frequency C=68.7% while G=31.3). The OR and allele frequency in the case of patients OR= CG=1.4 and GG= 1.89 with fungal infection.

The frequency of G allele shown high OR value=1.2. The OR values of CG and GG allele indicated on impact of CC allele on disease incidence based on value of OR >1. This study proven the validity of this SNP in studied population under interest for the first time at the same time this SNP: rs702966 C>T was recorded in NCBI SNP blast at 9-4-2021 based on data of NCBI(https://www.ncbi.nlm.nih.gov/snp/rs702965).

Table (4-10) Genotype and allele frequency of *IRF7* rs702966 C>G associated with COVID-19 patients with/without fungal infection and control

rs702966 C>G	COVID-19 Patients with Fungal infection. (30)		Control (30)	OR(95%CI)	P-value
genotypes	CC	16(53.3%)	19(63.3%)	Reference group	
	CG	11(36.7%)	7(23.33%)	**1.9(0.6-5.8)**	0.2
	GG	3(10%)	4(13.4%)	0.89(0.17-4.5)	0.9
Allele Frequency	C	43(71.67%)	45(75%)	0.84(0.37-1.8)	0.6
	G	**17(28.33%)**	15(25%)	**1.18(0.5-2.6)**	0.6
	COVID-19 Patients without Fungal infection(30)		(Control)		
genotypes	CC	16(55%)	19(63.3%)	Reference group	
	CG	9(31.7%)	7(23.33%)	**1.4(0.4-4.4)**	0.5
	GG	5(13.3%)	4(13.4%)	**1.3(0.3-5.4)**	0.7
Allele Frequency	C	45 (68.7%)	45(75%)	0.78(0.3 -1.7)	0.5
	G	**19(31.3%)**	15(25%)	**1.2(0. 5-2.7)**	0.5

*, OR, odds ratio; CI, confidence interval

In current study, accession number for eleven isolates were added to the gene bank NCBI :

1-LC780104

2-LC780105

3-LC780106

4-LC780107

5-LC780108

6-LC780109

7-LC780110

8-LC780111

9-LC780112

10-LC780113

11-LC780114

Chapter Five

Discussion, Conclusions and Recommendations

5-1: Discussion

COVID-19 established its prevalence from December 2019, it quickly spread all over the world, and then it became complex public health challenge negatively affecting humanity and business.

5-1-1:Demographic Characteristics of the Studied Groups

5-1-1-1:Distribution of COVID-19 Patients by presence of fungal co infection

It became known how fungal infection have adverse effects on immune compromised bodies. Also, this has a high risk of mortality in the body where the steroids and other drugs were taken during the treatment. there is a high risk of mortality when steroids and cytokines are used during treatment. Here, it could be speculated that over-activation of the immune system could cause misfunction in the regulation of defense mechanisms against other pathogens, resulting in the spread of coinfections. Patients with other disease histories such as diabetes and respiratory problems are at high risk.

The percentage of fungal infection in COVID-19 patients in current study was (21.8%), and this agree with (Zhu *et al*., 2020) in China where they found that the percentage of fungal infection in COVID-19 patients was(23.3%) , Also this result was near to result of (White *et al*., 2020) in UK which percentage of fungal infection was (26.7%), but disagree with (Hughes *et al., 2*020) in UK where fungal infection in there study was (3.2%) and disagree with (Nasir *et al*., 2020) in Pakistan which fungal infection was (6.1%). These increase of fungal infection in COVID-19 patients may be came from used of immune suppressants which decreased or increased the risk of infection in COVID-19 patients (Thng *et al*., 2021), Also In COVID-19 infection, persistent lymphopenia is

induced significantly, which is responsible for the increase in generating other infections (Pasero *et al*., 2021).

In a laboratory test of different patients, for COVID-19, 85% showed lymphopenia, indicating a significantly lower T lymphocyte concentration (Yang *et al*., 2020). It is well known that lymphocytes are a significant contributor to balancing immune homeostasis; the lower concentration of lymphocytes in COVID-19 patients induced fungal infection (Salehi *et al*., 2020). Many factors have also been linked with the spread of fungal infection, such as long term use of antibiotics, vitamins, zinc, and steroids (Gandra *et al*., 2021). In addition, long-term use of low-quality medical equipment and contaminated oxygen storage increase the risk of fungal infection. In addition, fungal bioaerosols are present in the air inhaled via the respiratory tract, and because the lung tissues and the alveoli-interstitial lesion have already been damaged. (Pushparaj *et al*., 2022)

5-1-1-2:Distribution of COVID-19 Patients and Control Group by Gender and Age group

According to result of this study most infection was noticed in males 17 (56.6%) and 58 (54.2%) than infection in females 13 (43.3%) and 49 (45.7%) in COVID-19 patients with fungal infection and COVID-19 patients without fungal infection groups consequently and statistically significant (Table 4-3). This result is comparable with result of several studies done neighboring countries such as four studies in Saudi Arabia done by Al-Omari *et al*., (2020) who report that 80% from confirmed cases were males, Alsofayan *et al.,* (2020) found that among 1519 confirmed SARS-COV-2 the rate of infection in males were 54.4%, Barry *et al*., (2020) who found 66% from infection with SARS-CoV-2 were males, Alamri *et al.,* (2021) found among total confirmed cases, the

infection rate in male was 51.1% and Shahriarirad *et al.*, (2020) who found that 62.8% of infection in males and significantly higher than female in Iran. Also, this result agrees with many studies done in Wuhan-China, and reported that male have significantly high rate of infection than female such as (Chen *et al*., 2020b; Jin *et al*., 2020). The same result was reported in United States of America such as (Albitar *et al*., 2020).

But , the result of present study disagreed with the result of current study, also unlike were report by Lee *et al*., (2020) who found that among 632 confirmed SARS-COV-2 the number of infected females 430 was more than half number of total confirmed cases in South Korea. Suleyman *et al.*, (2020) report that among 463, 259 (55.9%) were female in USA also in other report from USA found that female had high percentage of infection than males (Gebhard *et al*., 2020).

These differences in rate of infection between male and female may contribute to one of the following reasons: SARS-CoV-2 enters the body through the angiotensin-converting enzyme 2 (Hoffmann *et al*., 2020). Difference in expression of ACE2 due to hormonal factor may contribute to these sex-related differences as SARS-COV-2 spike receptors have high affinity with ACE binding in male than affinity in females because female have high degree of heterodimer assembled than males (Yan *et al*., 2020). Angiotensin-converting enzyme 2 genes lays on the X-chromosome, thus allowing females to be potentially heterozygous and differently assorted compared to men who are definitely hemizygous (Gemmati *et al*., 2020).

There is strong humeral and cell mediate immunity among female in response to viral infection than male (Syrett *et al*., 2019). Female crude the infection more rapidly and more effectively than male (Klein, 2012). Also, sex hormone plays an effective role in this process by binding to specific receptors on immune cells. They can increase their activity by

estrogen or inhabit it by testosterone (Schurz *et al*., 2019). Estrogen increase antibody response This may be due to hormones factor as estrogen has positive effect and promote IgG and IgM expression in opposite hand testosterone has suppressed effect (Ruggieri *et al*., 2016).

On the other hand, in current study the lowest percentage of infection with COVID-19 was 14.9% in age group(10-30y) , largest percentage was 53.3% in age group(51-70) , these results agreed with result of study done by Xue (2020) show that elderly people more susceptible to the more severe forms of the disease, Wu and McGoogan (2020) demonstrate that the case-fatality rate of patient within aged 70 to 79 years was 18.0% compared to patient within aged 40 years and older where it was 12.8%, Yang *et al*., (2020a) reported that older patients (> 65 years) with comorbidities and ARDS are at increased risk of death. Also, Bialek *et al*., (2020) report that SARS-COV-2 occurs more frequently in old age individuals and those in high susceptibility to ICU admission and mortality in United States. The high infection rate of SARS-COV-2 among old age may be due to several comorbidities among them was observed (Pahan and Pahan, 2020). In addition to poor clinical outcome (Wortham, 2020).

The poor out was observed among elderly with cardiovascular disease (Gebhard *et al*. 2020), These differences in rate of infection between age groups and increase it in elderly persons may contribute to one of the following reasons: Aging is associated with reduced in adaptive and innate immunity activity (Golomb *et al*., 2015).

Adverse age the body is fair to protect itself from viral and other infection (Van Deursen, 2014). Dendritic cell which is way of liked between innate and adaptive immunity their function is decreased with advance age (Gupta, 2014). Indeed, increased CD5+ B lymphocytes in the elderly population play a key role as producers of autoantibodies that

lead to an imbalance of the mechanism controlling the immune response against self-antigens (Bulati *et al*., 2011). Also, specific CD8+ T cell response against influenza in old age patient was strongly associated with low TNF-α production and lower DC maturation (Liu *et al*., 2012).

Regarding production of B and T cell Elderly people characterized with decrease in number of these cell (Stervbo *et al.*, 2015). Also, impair in B cell response and antibody production against viruses and bacteria (Visentini *et al*., 2011, Buffa *et al*., 2013). Many studies reported that respiratory syncytial virus (RSV) infections in adults is significantly higher than those below 50 years old as RSV is detected in 12% among old age with respiratory tract infection (Colosia *et al*., 2017).

5-1-1-3:Distribution of COVID-19 Patients and Control Groups by Hematological and Immunological Parameters

The results of the statistical analysis of some aspects of the blood and Immunological indicators studied in this study, which included (W.B.C, neutrophils, Lymphocyte ,Ferritin, D-Dimer, CRP, suPAR,), in SARS-CoV-2 virus patients and compared to the control group of the uninfected, showed that the values of hematological indicators in patients with SARS-CoV-2 virus have changed. the hematological results of this study showed that there were significant differences after statistical analysis of the results in the number of lymphocytes, as their numbers decreased significantly in patients infected with SARS-CoV-2. Percentage of lymphocytes in patients was (0.66±0.57 and 0.53±0.54) when compared with the control group (2.16±0.84).

The results of the current study indicated a decrease in the number of lymphocytes compared to the control group, our study agreed with the results of studies by (Tan *et al.*, 2020; Terpos *et al.,* 2020), which indicated a low percentage of lymphocytes in patients, and it can be

considered a reliable indicator in patients with COVID-19.The lack of lymphocytes in patients with COVID-19 can be attributed to several reasons, including the fact that the SARS-CoV-2 coronavirus may directly attack lymphocytes, leading to the death of lymphocytes. Lymphocytes also express the angiotensin-converting enzyme 2 (ACE2) receptor, which is specific to the coronavirus, which is the direct target of the virus (Fathi & Rezaei, 2020).

SARS-CoV-2 infection can directly damage lymphatic organs, such as the spleen and thymus. Also, many inflammatory cytokines released during COVID-19 infection may lead to apoptosis of lymphocytes. Also, cytokines secreted during infection may lead to a deficiency of lymphocytes. Finally, complications of the severe form of COVID-19 may inhibit lymphocyte proliferation (Tan *et al.,* 2020). also The inflammatory cytokine storm is most likely one of the causes of lymphopenia. The presence of pro-inflammatory cytokines like TNF- and IL-6 in the blood has been linked to lymphopenia, while healed patients had near-normal levels of these cytokines (Tavakolpour et al., 2020).

Munshi and Montgomery (2000) illustrated that viral infections are common causes of neutropenia, due to either bone marrow suppression or peripheral destruction. The agents commonly implicated include Epstein-Barr virus, cytomegalovirus, hepatitis A and B viruses, parvovirus, Influenza virus species, and measles (Munshi & Montgomery, 2000). But through this study, a significant increase in the number of neutrophil cells or neutrophilia was seen as a results in this study. Huang *et al* (2020) highlight that increased neutrophil counts in the blood of severely affected people were discovered to be a prominent clinical feature of SARS COV 2 disease (Huang, Wang, Li, Ren, Zhao, *et al.,* 2020). Some COVID-19 patients have an increasing in neutrophil count and dropping lymphocyte count through severe stage, according to Wang *et al*., 2020.

patients with severe symptoms compared to moderate symptoms, this resulted in elevated WBCs because neutrophils represented the largest proportion of total WBCs (36-66%). This increase in neutrophils shows the extent of damage in patients with SARS-CoV-19 due to the defect in the immune response that exacerbated the hyper inflammation (Huang, Wang, *et al.,* 2020; Ruan *et al.,* 2020). Severely symptomatic patients suffer from a cytokine storm during viral infection that produces large amounts of inflammatory factors such as IL-1, TNF alpha, IL6, all of which control neutrophil activity (Bordon *et al*., 2013).

Also Ferritin level in COVID-19 patients, it was (796.46±14.75) (823.90±16.89) in patients with fungal infection and without fungal infection consequently compared to the control group (126.68±19.12), Our results agreed with the findings of (Al Meani *et al.*, 2020) in the Anbar Governorate. As the percentage of ferritin was higher in the patients' group compared to the control group.

Our study also agreed with the findings of (Cheng *et al.,* 2020; Szarpak *et al.,* 2020), where they indicated that ferritin levels were significantly elevated in SARS-CoV-2 patients compared to the levels in the control group. The increase in ferritin levels in infected subjects was greater than in non-infected subjects.

In addition, the results of the current study recorded a significant increase in CRP levels in the serum of COVID-19 patients compared to the control group. the results showed that patients with severe symptoms had higher levels of CRP compared to patients with mild symptoms and control group. CRP is one of the first markers to be considered in response to SARS-CoV-2 infection. Elevated levels of CRP in serum are major markers of disease progression and a risk factor for severe COVID-19 patients and an indication of the development of a cellular storm in COVID-19 patients (Gao *et al.,* 2021). . CRP levels were increased in

COVID-19 patients to different degrees. The highest degree of increase in cases of severe and critical diseases was greater than that of mild cases (Yuan *et al.*, 2020).

Thus, our current study agreed with the study by (Khraise *et al.*, 2020) that CRP acts as an indicator of acute phase inflammation, infection, and tissue damage. Our study indicated that CRP levels were positively correlated with COVID-19 severity. Serum CRP levels were increased during the inflammatory response. As indicated by disease severity, the index might be elevated by viral infections.

Our current study agreed with studies of (Liu *et al.*, 2020e and Qin *et al.*, 2020), where they showed that CRP levels were significantly elevated in critical and severe cases compared to patients without severe disease, which suggests that CRP levels might be a marker of disease severity and progression in COVID-19 patients. We reported that severe cases of COVID-19 expressed significantly higher levels of CRP than control cases. Significantly increased serum CRP levels in COVID-19 patients may be associated with secondary bacterial infection or associated with poor clinical prognosis (Zhou *et al.*, 2020b).

As for the mean of D-Dimer level in patients was higher than in the control group .The results of our current study agreed with the study conducted by (Al Meani *et al.*, 2020) in the Anbar Governorate and by (Vidali *et al.*, 2020) in Italy, as the average level of D-dimer in COVID-19 patients was significantly higher than that of the control group. Increased ferritin levels in COVID-19 could indicate a strong inflammatory response, and recent studies by Tang *et al* in 2020 which suggest that elevated ferritin levels play a critical role in the formation of a cytokine storm. Abnormal coagulation function, including elevated D-dimer , has been demonstrated to be involved in the disease progression of COVID-19 (Han *et al*., 2020). Increased D-dimer levels have been

linked to the development of COVID-19 disease in recent studies recording laboratory improvements in patients with confirmed COVID-19. The level of D-dimer in COVID19 patients admitted to the ICU (Intensive Care Unit) was found to be significantly higher (Huang *et al.*, *2*020).

Finally, mean of suPAR level in patient groups was significantly higher than in the control group . This aspect of our research that correlates the level of suPAR in the blood of patients with COVID-19 is considered the first in Iraq and its agreed with the study conducted by Oulhaj *et al.,* in 2021 investigated the association between suPAR level and the incidence of severe complications in COVID-19 patients, including ARDS, ICU admission, and death from any cause. Our study's results demonstrate that patients who experienced these complications had higher baseline suPAR level than those without (Azam *et al*., 2020). Furthermore, a competing risk analysis showed that the higher the level of suPAR at baseline, the higher the risk of experiencing COVID-19 complications, even after adjusting for potential demographic, clinical and laboratory parameters. More specifically, for every increase of 1 ng/mL in suPAR level at baseline, there is a corresponding increase of 58% in the hazard of experiencing COVID-19 complications.

SuPAR levels have previously been shown to be significantly higher in patients with fatal outcomes than survivors in a myriad of critical illnesses, including systemic inflammatory response syndrome and invasive bacterial bloodstream infections (Hoenigl *et al*., 2013; Koch *et al.,* 2011),They were also found to be immune mediators for developing acute and chronic kidney disease, and more recently predictive of in-hospital acute kidney injury and the need for dialysis in COVID-19 hospitalised patients (Hayek *et al.,* 2020). A potential role for suPAR in triaging patients attending emergency departments has been

highlighted (Schultz *et al*., 2019)

5-1-1-4: Distribution of COVID-19 Patients and Control Group by Presence of some symptoms

Several signs and symptoms were reported in this study as shown in (Table 4-5). Most clinical feature were measured as following fever (63.3%,77.75%) , cough (50%, 70,09%), Dyspnea (43.3%, 65.42%) and GIT symptoms (16.6%, 28.3%) in patients group (with fungal infection and without fungal infection)consequently . This is an agreement with several neighboring countries as Barry *et al,* (2020) recorded that the most common clinical sign was fever (67.7%), cough (60.6%), dyspnea (43.4%), upper respiratory symptoms (27.3%), fatigue (26.3%), diarrhea (19.2%) and loss of smell (9.1%) among 99 hospitalized SARS-COV-2 patients in Saudi Arabia, Nasiri *et al*, (2020) showed that the most common symptoms in COVID-19 patients were fever (83.0%) and cough (65.2%) in Tehran Iran. Also, this result agrees with Guan *et al*., (2020a) who found that fever is the most common symptoms 88.7% and the second common symptom is cough (67.8%), whereas diarrhea (3.8%) were uncommon. Chen *et al,* (2020) found the common clinical features of patients with SARS COV-2 include fever (83%), cough (82%) were most common, shortness of breath (31%), and muscle ache (11%).

Du *et al.*, (2020) were most patients had fever 78 (91.8%) and dyspnea 60 (70.6%), shortness of breath and fatigue 50(58.8%) and almost one-half of the patients had anorexia, and 32 (37.6%) of the patients had expectoration 32 (37.6%) while other uncommon symptoms included dry cough, diarrhea, myalgia, headache, vomiting, abdominal pain, chest pain, and pharyngalgia in China. Tian *et al.*, (2020) found that the most common symptoms was fever (82.1%), cough (45.8%), fatigue

(26.3%), dyspnea (6.9%) and headache (6.5%) among 262 cases in China.

A Study done by Alsofayan *et al.*, (2020) founded that cough constituted 89.4% of cases (429 out of 480), fever in 85.6% (333 out of 389), sore throat in 81.6% (257 out of 315), and runny nose in 72% (139 out of 193) was most prominent. less common symptoms were myalgia 28.6% (202 out00 of 707), headache 27.3% (193 out of 707) and gastrointestinal symptoms 14.3% (101 out of 707) among confirmed SARS-COV-2 infection in Saudi Arabia.

Kapoor *et al.,* (2014) in United States demonstrated that MERS-COV also had the same symptoms as following fever 98%, dyspnea (55%), and dry cough 47% the most common symptoms. On the other hand, another symptoms observed in few number of cases of study done by Pan and Guan (2020) which diarrhea constituted 26% and sore throat in 21%. While in SARS-COV-1 the most common symptoms were fever (99%-100%), dyspnea (40%-42%), dry cough (29%-75%), sore throat (13%-25%) and diarrhea (20-25%).

From all these studies in different regions and different stage of severity fever was the most common sign. This may be due to multiple reasons, fever during SARS-COV-2 is mainly caused by the immune response to the viral infection. Furthermore the high incidence of vascular thrombosis and of extra-cerebral organ injuries may increase expression damage-associated molecular patterns (DAMPs) concentrations.

This lead to production pyrogenic mediators and as result hypothalamus dysfunction and fever, this may indicate fever is a good prognosis because fever in many viral infections promotes a better viral clearance. These in concern go in line with last report among SARS-COV-2 patients which records those patients with body temperature below 36°C are at increased risk of development to sever infection

(Evans *et al*., 2015; Tharakan *et al*., 2020). On the other hand, fever may increase cellular metabolism due to high temperature resulting in an increased oxygen consumption and carbon dioxide production, and this may lead to hypoxia, hypercapnia and there for serve as a poor prognosis (Jeong *et al*., 2020). Or may be due to difference in region, lifestyle, means age and epidemiology factor in clinical sign differences (Wang *et al*, 2021).

5-1-1-5: Distribution of COVID-19 Patients and Control Groups by Presence of Comorbidities

From the results of the current study of patients, it was found that almost patients have comorbidities, and the severity of COVID-19 infection was related to the presence of comorbidities. It was noted that the percentage of people with high blood pressure was higher than the rest of the diseases (43.3%and 32.7%), followed by diabetes was (36.6% and 28.03%) and cardiovascular diseases was (16.6% and 9.34%)in patients with fungal infection and without fungal infection consequently, that those with comorbid health conditions or comorbidities had an increasingly rapid and severe progression of the disease (Bajgain *et al.*, 2021).

Patients who had been COVID-19 and had comorbidities, such as high blood pressure or diabetes, can have a more severe and progressive disease course. Furthermore, elderly patients, especially those 65 years of age and older with comorbidities and co-infected with COVID-19, have significant changes in the levels of immunological and hematological indicators due to the impact of COVID-19 disease (Sanyaolu *et al.*, 2020). Patients with comorbidities should take all necessary precautions to avoid infection with SARS-CoV-2, as they usually have the worst prognosis (Sanyaolu *et al.*, 2020).

COVID-19 patients with high blood pressure, diabetes, cardiovascular disease (CVD), and other comorbidities can develop a life-threatening condition. The SARS-CoV-2 virus uses ACE-2 receptors on the surface of host cells to enter the cell. Some comorbidities are associated with strong ACE2 receptor expression and higher release of an adapter protein that promotes viral entry into host cells (Ejaz *et al.*, 2020).

The results of this study agreed with the results of (Abbas *et al.*, 2020), in the Baghdad governorate, which indicated the most important comorbidities, especially high blood pressure, diabetes, and cardiovascular diseases, and the disease rates were (30%, 19%, 8%), respectively, for patients infected with COVID-19. The results of (Taher, 2020) in Wasit Governorate were higher than the results of our study, as it confirmed that the most important diseases associated with COVID-19 patients were high blood pressure (15%), followed by diabetes and coronary heart disease.

The results of the current study were consistent with the study conducted by researchers in Iran where patients with COVID-19 had one or more co-existing medical conditions. The most common diseases were high blood pressure (19.5%), diabetes (14.2%), and finally cardiovascular diseases (Shahriarirad *et al.*, 2020). Also, the results of this study agreed with (Yang *et al.*, 2021) in New York City in the United States of America, where there is an increase in comorbidities, such as hypertension or diabetes, usually associated with increasing age in Western society.

Also, the results of our current study agreed with studies conducted by researchers (Zhang *et al.*, 2020c) in Wuhan, China, where high blood pressure (30.0%) and diabetes (12.1%) were the most common comorbidities. The results of our study were lower than those of

(Richardson *et al.*, 2020), and that the most common comorbidities were high blood pressure (56.6%) and diabetes (33.8%).

5-1-2:Molecular study

5-1-2-1: Interleuken-17A

IL-17A is the most widely studied member of the IL-17 family, a group of proteins that have a highly conserved C-terminus containing a cysteine-knot fold structure (Weaver *et al.*, 2007). IL-17A plays a critical role in host defense against various microbial pathogens as well as tissue inflammation. IL-17A and IL-17 receptor signaling has been shown to be play a protective role in host defenses against many bacterial and fungal pathogens (Chen and Kolls, 2013).

Our study shed lighting on role of polymorphism of gene IL-17A by study two SNPs: rs2275913 and rs3819025, any mutation occurred in this gene IL-17A effect on level of it and effect in host defense against microbial pathogens as well as tissue inflammation. ,in (rs2275913) SNP analyzed was an SNV 2KB Upstream Variant located in a promoter region (rs2275913 G/A). The allele G frequency is 65% in the 1,000 genome database for the European population.
https://www.ncbi.nlm.nih.gov/snp/rs2275913.

This results of OR high values of mutant allele: heterozygous GA and homozygous AA for each, OR = 1.8(0.38-8.3), 1.4(0.43-8.4) and 1(0.23-3.4) for homozygous AA ,the A allele had OR= 0.9(0.73-3.6) and the G allele 0.61 (0.4-2.5) in both patients groups (Table 4-7), also the allele frequency of A was 35% in patients with fungal infection ,while it was 25% in control group while it was 23.3% , 25% in patients without fungal infection and control group consequently, compared the wild allele G with OR values= 1.09(0.28-1.3) in patients with fungal infection

group and A allele with OR= 0.9 (0.39 -2.1) in patients without fungal infection group.

this results of genotyping and allele frequency of heterozygous and homozygous allele GA and AA were shown high values ,G allele as protect allele in patients with fungal infection . Azevedo *et al* in (2021) also found that The allele G was presented with a higher frequency in all groups. For this polymorphism, the G allele could also be observed associated with the higher tissue expression of IL-17A in both COVID and H1N1 groups. These results were consistent with the study which found that the G allele's presence increases infection risk with the influenza virus (Keshavarz *et al*., 2019). Although the study's focus was COVID-19, They also indicated an association between rs2275913 and higher IL-17A tissue expression .

Our result was agree with Kadhim *et al.,* (2021), when they referred to the Allele frequency of A allele genotype in rs2275913 in patients with oral fungal infection was higher than control group which correlated with high level of IL-17A in serum .

Mikacenic *et al*. measured circulating IL-17A in ARDS and showed that elevated circulating and alveolar levels of IL-17A are associated with increased percentage of alveolar neutrophils, alveolar permeability and organ dysfunction in ARDS. However, there is no detailed information about the association between frequency of polymorphisms of IL-17A a gene of COVID patients among the populations. And Xie *et al.* study in 2019 showed the positive significant correlation between the prevalence and mortality rates in COVID-19 and GG genotype of rs2275913 SNP in IL-17A gene, additionally, a negative correlation between those prevalence and mortality rates and AG genotype. AA genotype also gave a significant negative correlation with the prevalence of disease among countries, our study showed that A allele is risk allele , this result agree

with study of Karcioglu and Hekim in 2019in that referred to the significant association of IL-17A AG (rs2275913) SNP with international severity and death outcome due to the COVID-19, but disagree with Xie *et al.* study in 2019 which showed that Individuals carrying the mutant A-allele of rs2275913 had decreased ARDS risk and better 30-day survival outcome**,** also Karcioglu Batur, & Hekim in 2021 found that the prevalence (per million) and mortality rates (per million) of COVID-19 among populations of China, Japan, India, Iran, Spain, Italy, Mexico, Netherlands, Sweden, Turkey, Finland, Brazil, Czechia, Russia, Poland. AG and GG genotypes of rs2275913 in IL-17A was found to be correlated with prevalence and mortality rates, especially in Spain and Brazil populations also found The variations in the prevalence of COVID-19 and its mortality rates among countries may be explained by cytokine storm differed by the polymorphisms of rs2275913 locus in IL-17A gene. However, the prevalence of infection differs from severity of COVID-19, based on many factors such as public awareness, behaviors and antiviral policy of countries. Yet, the severity of disease induced by viral infection might be associated with genetic host factors including immune profiling.

On the other hand, our results of Rs3819025 G>A OR high values of mutant allele: heterozygous GA, OR = 1(0.01-52), 16.2 (0.8-301.6) and AA OR=1.3 (0.28-6.7) ,the A allele had OR= 2.7(0.97-7.7) in both patients groups (Table 4-8), also the allele frequency of A was 76.6% in patients without fungal infection ,while it was 10% in control group, compared with allele A with OR values= 0.6 (0.17-2.4) patients with fungal infection group and G allele with OR=0.36 (0.12-1.02) in patients without fungal infection group .

The rs3819025 has also been studied in different types of disease (Rafiei, 2013). This SNP (rs3819025) is a Single Nucleotide Variation

(SNV), and the change is G/A in an intronic region located near exon one in the short arm of chromosome 6 , The allele G frequency is 94% in the 1,000 genome database for the European population https://www.ncbi.nlm.nih.gov/snp/rs3819025.

In our results, the GA genotype (for rs3819025) was present only in the COVID without fungal infection group, and this fact could indicate a risk association for this disease (compared to the COVID-19 patients with fungal infection and control group). whereas in Azevedo *et al* in (2021) found that the GG genotype (for rs3819025) was present only in the COVID-19 group (compared to the H1N1 and control group) Besides, the GG/GA genotypes of this SNP are associated with a higher tissue expression of IL-17A in the COVID-19 group. This same GG genotype has been associated with higher serum levels of IL-17A for graft versus host disease after allogeneic hematopoietic stem cell transplantation (Karimi *et al*., 2014). 0ur result appear that G allele was a risk allele in COVID-19 with fungal infection whereas in COVID-19 without fungal infection group A allele was a risk allele compare with control group also in Azevado *et al* study in (2021) which it would be reasonable to consider the G allele in rs3819025 for COVID-19 group a risk allele (compared to the CONTROL group) associated with highest tissue expression of IL-17A . Previously, all research related to this SNP rs3819025 polymorphism and its relationship with COVID-19, our study in this aspect was the first in Iraq.

5-1-2-2:Interferon administrative factor -7 (IRF-7)

The IRF7 gene considered a pivotal part in the enlistment of type I IFN. IRF7 has been believed to be areas of strength for a helplessness gene to disease based on activation high level of IFNα to overcome many diseases, with our perception; relationship of IRF7 SNPs has been accounted for in Iraqi people. In the present study, in which particular

determine the polymorphism IRF7/PHRF1 and evaluated the interaction with IFNα with COVID-19 disease. We performed sequencing analysis of 534bp as partial sequence of IRF7/PHRF1 based on the idea of Salloum *et al*.,(2010), when they emphasis to performed future large-scale mapping and sequencing in this region IRF7/PHRF1 will help clarify the true causal genomic elements between the present study and the previous works with the IRF7 locus, would make any polymorphism of IRF7 gene a more likely causal candidate gene to disease incidence (Katherine *et al., 2*003).

Our study shed lighting on role of polymorphism of gene IRF7 as important activator for interferon alpha by study three SNPs: rs17155933, rs702965 and rs702966, any mutation occurred in this gene IRF7 effect on level of IFNα., In spite of the SNP rs17155933 which shown validity and recorded for first time in COVID-19 patients in Iraq and on the world level , but unfortunately the mutant allele heterozygous CG and homozygous GG of this SNP(rs17155933)was shown low values of OR except GG allele OR=1(0.18-5.4) and allele C frequency more than in control group with OR=5.8(1.8-18.5)with high significant in comparison between patients with fungal infection and control group, GG and CG was not present in patient group, C allele more frequent in patient group than control with OR=2.0(0.6-5.8) and significant deference P=0.002 as Table (4-9), that mean C allele is a risk allele. (no previous studies sharing to rs17155933 in literature, except recorded it on chromosome11). (https://www.ncbi.nih.gov/snp/rs17155933).

Based on the OR values in Rs702965 C>T The results of table (4-10) shown that this SNP not correlated with disease based on the value of allele (T) in genotype TT with value less than 1 and has low allele frequency of allele T in patients with fungal infection than in control group. Whereas The results of part 2 from same Table shown that this

SNP correlated with disease based on the value of allele risk(T) in genotype TT with value 1.3(0.35-6.96) and has high allele frequency of allele risk T with OR=1.09 (0.47-2.5), these data was enable us to candidate T allele to be risk allele with COVID-19 patients without fungal infection.

This results of OR high values of mutant allele: heterozygous CG and homozygous GG for each, OR= 5(1.5-16.5), 1.03(0.19-5.6) and 2.14(0.6-7.4) ,1.8(0.38-8.3), the G allele had OR= 1.59(0.65-3.9) and 1.88(0.8-4.8) in both patients groups (Table 4-11), also the allele frequency of G was 14 and 19 in two patients groups respectively while 11 in control group, compared the wild allele C with OR values= 0.62(0.25-1.5) and 0.53(0.22 -1.2) in both patients groups.

In Table 4-11 illustrated the results of genotyping and allele frequency of heterozygous and homozygous allele CG and GG were shown high values, These results were consistent with Salloum *et al.*, (2010), when they referred to the high frequency of wild allele CC genotype in rs702966 which correlated with high level serum of IFNα based on high value, This result of low OR of CC allele led to decline IFNα in serum of patients under interest and led to increase the disease incidence was boosted by the results of Salloum *et al.,(*2010) .

This study shed light on the sequences sequence analysis that sequence of amplicon under interest (534bp) were checked they familiarity to IRF7/PHFRI inter genic region based pairwise alignment for each. This issue was observed by Harley et al.,(2008). Our finding that the results was contestant with previous reports which considered a new examinations that revealed the relationship of PHRF1 SNPs found near IRF7. Nonetheless, proof for relationship with the event of disease was noticed neither for PHRF1 SNPs nor IRF7 SNPs. Examination for gene-gene interaction among SNPs and every disease risk alleles lay out

in many countries like Japanese and Chinese population in Asia (Carmona *et al.*, 2012). These outcomes upheld the referred to it enclosed within PHRF1 gene IRF7; while others going to share two neighbor both gene IRF7/PHRF1. This issue made argument on these SNPs in inter genic genes (Kawasaki *et al*., 2012). Due to the essential role of IRF7 gene in IFNα activity, any SNPs occurred in it led to shifted gene function, while the gene PHRFI considered unknown functional (Harley *et al*., 2008).

The results of our study was shared the attention with study of Nln, *et al*. (2021). Specifically, the G allele of the IRF7 SNP rs702966 gene was linked to increased mortality and is also associated with decreasing type I IFN response in patients with lupus.

The COVID-19 pandemic allowed an unprecedented opportunity to test this theory. The type I IFN response is a key player in the immune system's defense against the severe acute respiratory coronavirus 2 (SARS-CoV-2) (Nln, *et al*. 2021). However, in this study, single nucleotide polymorphisms appear to linked SNPs with inhibit IFN response and are associated with increased mortality risk from COVID-19 disease. The important of study the genetic polymorphism IRF7/PHRFI on from their role in change the expression of IRF7 and effected on interferon activities against COVID-19. This conclusion consistent with Idea of Nln *et al*.,(2021), when they mention to the PHRF1 SNP could tag a functional genetic element that modulates the adjacent IRF7 gene to influence type I IFN, as PHRF1 is not thought to function in the type I IFN pathway," explained the researchers (Honda *et al., 2*005).

Based on explanation of Salloum *et al.,* (2010). The IRF-7 is a transcription factor that can induce transcription of IFNα and IFNα-induced genes downstream of endosomal TLRs, similar to IRF5 (Barnes

et al., *2*004). A SNP near IRF7 was found to be associated with systemic lupus erythematosus based on idea of Harley *et al*.,(2008), they referred to associated the SNP rs702966 SNP in IRF7 was located at 0.6 kb telomeric to IRF7 in a gene of unknown function named PHD and RING-finger domains 1 (PHRF1). This SNP was in high linkage disequilibrium with the PHRF1 gene contains PHD-finger and RING-finger domains, and has not been functionally characterized to date (Bienz, 2006).Several studies have demonstrated gene–gene interaction may play an important role in etiologically complex disorders such as NSCL/P (Letra *et al.*, 2012; Li *et al*., 2015).

Individuals were genotyped at the rs171155933, rs702965 and rs702966 SNPs in the IRF7/PHRF1 gene regions to tag genotype present in our results. These 3 SNPs capture >60% of common genetic variation (minor allele frequency >0.05) with greater in this 0.6-kb region (534bp) in studied populations. This partial sequence 0.6kb region(534bp) includes in both IRF7 and PHRF1 genes and the genotypes block associated with COVID-19.

Our Finding about the risk allele G in SNP rs702966 in Iraqi COVID-19 patients was supported by the results of Nln, *et al*.,(2021), when they found that G allele of the IRF7 SNP rs702966 was associated with mortality in COVID-19 patients (OR=2.09, p=0.015) in African-American patients.

In this study, we followed new approach to this evaluate the relationship between fungal infection and polymorphism in combined with I IFN. Tracking the present of fungal infection in patient group and how induced transcription of inflammatory gene like IFN that are commonly induced by other inflammatory stimuli. We emphasized the importance of the type I IFN pathway for defense against the fungal host

in humans by immunological and genetic studies in both healthy volunteers and patients with or chronically ill with systemic candidiasis. Our results consistent with results of Smeekens *et al*.,(2013), when they show that the polymorphisms in type I IFN genes modulated *Candida*-induced cytokine production and were correlated with susceptibility to systemic candidiasis. In in-vitro experiments, type I IFNs skewed *Candida*-induced inflammation from a Th17-response toward a Th1-response. Patients with chronic mucocutaneaous candidiasis displayed defective expression of genes in the type I IFN pathway. These findings indicate that the type I IFN pathway is a main signature of *Candida*-induced inflammation and plays a crucial role in anti-*Candida* host defense in humans (Fu *et al*., 2011).

This study followed an approach to validate the role of the type I IFN pathway for host defense against fungi by detection the genetic variation in IRF7 gene and correlated with fungal infection. The study of Smeekens *et al*., 2013 followed the same style and mentioned to release of TNF-α, IL-1β, IL-8, IL-6, IL-10, IFN-γ , IL-17 and CARD9 with fungal cases infections (AL-Jubory and Imran 2020).

This study shed light on the relationship between Chronic fungal infection patients with polymorphism in IRF7 gene and the OR values shown significant mutant homozygous and heterozygous allele in SNP rs702966 (Table 4-11). Our result come consistent with result of Smeekens *et al*., 2013, when they detection the expression of type I IFN pathway genes (including IRF5, IRF7) that are induced downstream of STAT1-STAT2 showed patterns of defective expression in fungal infection patients compared to healthy controls, supporting a role of the type I IFN pathway in this immunodeficiency, our results consistent with report of (Izaguirre *et al*., 2003), they conclude that Plasmacytoid dendritic cells (PDC) are uniquely preprogrammed to respond rapidly and

effectively to a range of viral pathogens with high levels of IFN-alpha production due to the high levels of constitutively expressed IRF-7.

One of the challenges faced this study; the samples collection period by researcher may have affected the study's results. During the COVID-19 pandemic wave in Iraqi Cities in 2021, the researcher successfully enrolled a high group of people for the study. However, since COVID-19 cases have decreased and remained low later, it has been challenging to increase the sample size a similar cohort more than 30 patients for each groups.

5-2: Conclusions:

Depending on the current study results, the works concluded the following:

1. male was more likely to be affected than female.

2.the infection with COVID-19 increases with age and that elderly people were more affected by the disease than young people.

3.COVID-19 affected in some hematological and immunological markers and change their levels in serum of patients.

4. Patients with comorbidities was more affected with COVID-19 and fungal infection And vice versa.

5.IL-17A and IRF-7 play a role in pathogenesis.

6.**rs2275913** SNP on the IL-17A gene did not considered a risk factor for COVID-19 with fungal infection patients, but consider as a risk factor for COVID-19 without fungal infection group .

7. **rs3819025** on **IL-17A gene** did not consider a risk factor for COVID-19 with fungal infection patients, but considered as a risk factor for COVID-19 without fungal infection group.

8. **rs17155933** on **IRF7 gene** did not consider a risk factor in two groups, **rs17155933** in this study showed validity and recorded for first time in COVID-19 patients in Iraq and on the world level.

9. rs702965 on **IRF7 gene** considered a risk factor in COVID-19 patients without fungal infection group, **rs702965** in this study showed validity and recorded for first time in COVID-19 patients in Iraq and on the world level.

10. **rs702966** SNP on the IRF-7gene and considered as a risk factor for COVID-19 patients in two groups.

11.Polymorphism in IL-17 A and IRF-7 may contribute to severe infection.

5-3: Recommendations:

The results of this study encouraged the investigator to propose the following recommendations:

1. Use of level of suPAR in serum as early detector of infection of COVID-19

2. Detection of other cytokines such as interferon-α and interferon-γ and determining their specificity and their polymorphism to detect if they contribute to the severity of COVID-19. And further studies are required to detect IL-6 serum levels and determine their specificity and their polymorphism to detect if they contribute to the severity of COVID-19 and especially can be served as an early detection marker.

3. Use of level of IL-17 and IRF-7 in serum for early detection of infection with COVID-19.

Appendices

Questionnaire for Covid-19 Patients

Patient name: NO: Date:

Age: Gender: profession:

Address:

Mobile number:

No	Clinical Symptoms	Answer	Notes
1	Do you have a fever?		
2	Do you have a cough?		
4	Are you have Dyspnea ?		
5	Do you have diarrhea or any GIT symptoms?		
6	Do you have Hypertension		
7	Do you have Diabetes?		
8	Do you have Cardiovascular ?		
9	Presence of oral fungal infection		
10	Oxygen concentration:		
11	respiratory rate (breaths/minute)		
12	WBC		
13	Lymphocyte		
14	Neutrophil		
15	D-Dimer		
16	Ferritin		
17	CRP		
18	suPAR		
19	Treatment		

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Gen

Homo sapiens C4 PHRF1 gene, partial sequence

GenBank: LC780110.1

FASTA Graphics

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  AUTHORS   Raffia,S., Ali,H. and Hameed,Z.
  TITLE     PHRF1 gene polymorphisms in COVID-19 patients in Hilla and Karbala
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  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 534)
  AUTHORS   Isam,Z.
  TITLE     Direct Submission
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ILA17 gene for interleukin 17, partial cds7Homo sapiens C

GenBank: LC780104.1

FASTA Graphics

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  AUTHORS   Raffia,S., Ali,H. and Hameed,Z.
  TITLE     IL17 gene polymorphisms in COVID19 patients in Hila and Karbala
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REFERENCE   2 (bases 1 to 684)
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  TITLE     Direct Submission
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ILA17 gene for interleukin 17, partial cds8Homo sapiens C

GenBank: LC780105.1

FASTA Graphics

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  TITLE     IL17 gene polymorphisms in COVID19 patients in Hila and Karbala
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  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 684)
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  TITLE     Direct Submission
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GenBank: LC780111.1

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REFERENCE   1
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  TITLE     PHRF1 gene polymorphisms in COVID-19 patients in Hilla and Karbala
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  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 534)
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GenBank: LC780112.1

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GenBank: LC780113.1

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Homo sapiens V10 ILA17 gene for interleukin 17, partial cds

GenBank: LC780106.1

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Homo sapiens V11 ILA17 gene for interleukin 17, partial cds

GenBank: LC780107.1

FASTA Graphics

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  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 684)
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  TITLE     Direct Submission
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Homo sapiens V12 ILA17 gene for interleukin 17, partial cds

GenBank: LC780108.1

FASTA Graphics

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REFERENCE   1
  AUTHORS   Raffia,S., Ali,H. and Hameed,Z.
  TITLE     IL17 gene polymorphisms in COVID19 patients in Hila and Karbala
           provinces
  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 684)
  AUTHORS   Isam,Z.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-SEP-2023) Contact:Zahraa Isam Babylon university,
           Biotechnology; 40 street, Babel, Hilla 51001, Iraq
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Homo sapiens VF-3 PHRF1 gene, partial sequence

GenBank: LC780114.1

FASTA Graphics

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REFERENCE   1
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  TITLE     PHRF1 gene polymorphisms in COVID-19 patients in Hilla and Karbala
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  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 534)
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  TITLE     Direct Submission
  JOURNAL   Submitted (22-SEP-2023) Contact:Zahraa Isam Babylon university,
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Homo sapiens VF-14 ILA17 gene for interleukin 17, partial cds

GenBank: LC780109.1

FASTA Graphics

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REFERENCE   1
  AUTHORS   Raffia,S., Ali,H. and Hameed,Z.
  TITLE     IL17 gene polymorphisms in COVID19 patients in Hila and Karbala
           provinces
  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 684)
  AUTHORS   Isam,Z.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-SEP-2023) Contact:Zahraa Isam Babylon university,
           Biotechnology; 40 street, Babel, Hilla 51001, Iraq
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الخلاصة:

انتشرت متلازمة الجهاز التنفسي الحادة الناجمة عن فيروس كورونا بسرعة ، اذ غزت العالم وتسببت بوفاة أكثر من ستة مليون شخص. لذلك تهدف هذه الدراسة عزل وتشخيص أنواع الفطريات المصاحبة للخمج بفايروس كورونا وتحديد مسببات المرض وبعض الأعراض وبعض الأمراض المصاحبة للمرضى قيد الدراسة وكذلك تقدير مستوى بعض المعايير الدموية والمناعية وتحديد تعدد الأشكال الجينية لبعض الجينات.

شملت دراسة الحالية 167 (عينة دم ومسحة انفية) وزعت على النحو التالي: المجموعة الأولى كانت لـ 30 مريضًا مصابًا بـ Covid-19 وعدوى فطرية(17 ذكر،13 انثى) ، اما المجموعة الثانية كانت لـ107 مرضى مصابين بـ Covid-19 بدون عدوى فطرية (58 ذكر،49 انثى) في حين تمنت المجموعة الثالثة 30 شخصًا سليمًا كمجموعة سيطرة . وقد تراوحت اعمار عينات الدراسة بين10-70 عاما .

تم جمع العينات السريرية خلال الفترة (كانون الثاني 2022-نيسان 2022) من المرضى الراقدين في وحدات العناية المركزة كحالة خطيرة من مستشفى المرجان التعليمي (مدينة المرجان الطبية) ومستشفى الامام الصادق التعليمي بمحافظة بابل ومستشفى الزهراء بمحافظة كربلاء. زراعة العينات في SDA Agar و CHROM Agar واختبار انحلال الدم و تم تحديد مستويات بعض المعلمات الدموية والمناعية في الدم بواسطة ELISA و CBC ... وباستخدام المعلومات في الاستبيان للكشف عن بعض الأعراض والأمراض المصاحبة بالاافة الى ذلك تمنت الدراسة الحالية التحري عن تعدد الاشكال النيوكليوتيد المفرد الى الجينات IL-17A and IRF-7 .

أظهر التحليل الإحصائي أن الذكور كانوا أكثر عرضة للإصابة من الإناث مع ($p = 0.077$) ، وتزداد الإصابة بـ COVID-19 مع تقدم العمر وأن كبار السن كانوا أكثر عرضة للاصابة من الشباب ، وكان هناك فرق كبير ($p\leq0.0001$) بين ثلاث مجموعات في مستوى WBc ، العدلات ، الخلايا الليمفاوية ، Ferritin ، D-dimer، CRP و suPAR وايضا هنالك فرق كبير ($p\leq0.0001$) بين ثلاث مجموعات في وجود بعض الأعراض مثل الحمى والسعال وضيق التنفس وأعراض الجهاز الهضمي ووجد فرق معنوي بين ثلاث مجموعات في وجود بعض الأمراض المصاحبة.

أخيرًا ، اظهر التحليل الإحصائي للدراسة الجزيئية أن SNP rs2275913 للمركب الخلوي IL-17A يعتبر عامل خطر لمجموعة COVID-19 مع العدوى الفطرية ، بينما لم يعتبر عامل خطر لمجموعة COVID-19 بدون عدوى فطرية ، rs3819025 على IL-17A

لم يعتبر الجين عامل خطر لـمجموعة COVID-19 مع العدوى الفطرية ، في حين يعتبر عامل خطر لـمجموعة COVID-19 مع العدوى الفطرية .

كذلك بين التحليل الاحصائي لتعدد الاشكال للجين RS 17155933في العامل المنظم للانترفيرون -7 انه لا يعتبر عامل خطورة بوجود او عدم وجود عدوى فطرية، في حين ان تعدد الاشكال الجيني لل702965rs في العامل المنظم للانترفيرون-7 يعتبر عامل خطر لمجموعة COVID-19بدون اصابات فطرية فقط ،اما تعدد الاشكال الجيني لل rs702966 للعامل المنظم للانترفيرون-7 فقد اعتبر عامل خطورة لمرضى COVID-19 في المجموعتين.

استنتجت نتائج الدراسة الحالية ان تعدد الاشكال الجيني للمركب الخلوي IL-17A والبروتين IRF-7يكون مرتبط مع خطورة الاصابة بفايروس كورونا .

جامعة كربلاء
كلية العلوم
قسم علوم الحياة

دراسة دور بعض العوامل في التغايرات الوراثية ونسبة الاصابة بفايروس COVID-19

أطروحة مقدمة
إلى مجلس كلية العلوم/ جامعة كربلاء
وهي جزء من متطلبات نيل درجة دكتوراه في علوم الحياة/احياء مجهرية

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