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Molecular Detection of Risk Genes Susceptibility to Candida Infections of Covid-19 Patients in Babylon Province

A Thesis

Submitted to the Council of the College of Science for Women / University of Babylon in Partial Fulfillment of the Requirements for the Degree of Master in Biology

By

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Supervisors Certification

We certify that this thesis entitles (**Molecular Detection of Risk Genes Susceptibility to Candida Infections of Covid-19 Patients in Babylon Province**) was prepared under our supervision at the department of Biology, College of Science for Women, University of Babylon as a partial requirement for the degree of Master in Biology.

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Dedication

To….. God, the Creator of all things, and He is the Lord of the Great Throne.

To….. the first teacher in the history of mankind, the Messenger of humanity, Muhammad bin Abdullah (PBUH).

To….. souls who left the world and never left my heart

(my grandfather and my brother).

To my brothers , sisters and friends with my love and respect

Zahraa

2022

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Zahraa

2022

Summary :

This study is conducted at the college of sciences for women's at Babylon University. A 146 oral swabs samples were collected 56 samples from patients with Covid-19 infection, 50 samples from recovery persons and 40 samples from healthy people, the swabs with transport media used in this study. And 3ml of blood were collected from 60 humans samples (20 patients, 20 recovery persons and 20 healthy people). Blood injected 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 using in molecular study. Samples were collected over a period of 4 months from Nov.-2021 to Feb-2022. All Clinical samples were collected from patients whom lying in Intensive care department from Marjan Hosptial (Marjan medical city) in Babylon province. Samples were taken from the patients under the supervision of the specialist physician. After diagnosis of Covid-19 infection of the patient, all samples were taken for both sexes and different age groups ranged between 22_89 years old .

This study aimed to isolation pathogenic fungi from oral cavity swabs for patients and health individual, Evaluate the risk factors, Identification of fungal isolates by conventional methods. All sample clinical were culture on SDA and PDA and yeast identified by CHROMagar and detection virulence factor for *Candida* spp. such secretion of the Urease, lipase, phospholipase biofilm formation, and hemolysis . Testing the virulence factors and antifungal activity of fungal isolates. DNA extraction for blood samples for each patients and molecular detection of some genetic risk factors such as (*TLR8* and *CLEC7A*) genes.

Our results showed that the rate of infection in males higher than in females which reached in males (55.4%) of patients group while were the females (44.6%) respectively, on the other hand rate of recovering in

female (64.0%) higher than in male (36.0%). As this study revealed that the most affected age groups between the gender ranges (65-74 years). The proportion of the total positive samples of three categories is 83 (56.84%). Where the percentage of Patients was (29.45%), while positive culture in recovering and control showed 13.7% in both.

Six species of yeast were isolated and diagnosed from patient samples were highest percentage the *C.albicans* (44.6%), C. *parapsilosis* (17.9%) while all of *C. dubliniensis*, C. *glabrata*, and *C. krusei* percentage rate (7.1%) and *C. tropicalis* is (5.4%). In recovering, the rate of *C.albicans* (20.0%) followed by *C. krusei* (6.0%), while in control group, the rate of *C.albicans* (15.0%) followed by C. *parapsilosis* (7.5%). For evaluation of virulence factors of yeasts species under study, the results showed that most of the yeasts possessed high virulence factors. The most effective antifungals were Fluconazole, Clotrimazole and Nystatin has the highest percentage of susceptibility against *Candida* spp. with 72.3%, 67.7% and 61.5% respectively and Amphotericin-B was 58.4%.

The polymorphism of *TLR8* gene was done for 60 samples of blood DNA (20 patients, 20 recovery persons and 20 healthy people) by Tetra-Arm primer. When compared between patients and control, the frequency of genotypes AA, AG and GG of *TLR8* (rs3764880 A/G) gene polymorphism are respectively 40%, 35% and 25% in patients, while respectively 60%, 0% and 40% in control group. The frequency of alleles of *TLR8* gene polymorphism of A allele is 57.5% in patients and 60.0% in control group, while frequency of G allele is 42.5% in patients and 40.0% in control group. While, the results of recovery showed the frequency of genotypes AA, AG and GG of *TLR8* (rs3764880 A/G) gene polymorphism are respectively 25%, 60% and 15% and the frequency of alleles of *TLR8* gene polymorphism of A and G allele are 55.0% and 45.0% respectively.

The polymorphism of *CLEC7A* gene was done for 60 samples of human blood by sequence of PCR results. For checking the polymorphism in this gene two genotypes have been observed, SNPs rs3901532 C/T appeared on position 152 and rs3901533 A/C appeared on position 64. The frequency of genotypes CC, CT and TT of *CLEC7A* (rs3901532 C/T) gene polymorphism are respectively 20%, 45% and 35% in patients, while respectively 20%, 55% and 25% in control group. The frequency of alleles of *CLEC7A* (rs3901532 C/T) gene polymorphism of C allele is 42.5% in patients and 47.5% in control group, while frequency of T allele is 57.5% in patients and 52.5% in control group. While the frequency of genotypes AA, AC and CC of *CLEC7A* (rs3901533 A/C) gene polymorphism are respectively 20%, 50% and 30% in patients, while respectively 25%, 60% and 15% in control group. The frequency of alleles of *CLEC7A* (rs3901533 A/C) gene polymorphism of A allele is 45% in patients and 55% in control group, while frequency of C allele is 57.5% in patients and 45% in control group.

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List of Abbreviations

NO.	Items	Meaning
1.	AB	Amphotericin –B
2.	ACE 2	Angiotensin-converting enzyme 2
3.	ARB	Angiotensin receptor blocker
4.	ARDS	Acute Respiratory distress syndrome
5.	BAL	Bronchoalveolar lavage
6.	CAPA	Corrective and preventive actions
7.	CC	Clotrimazole
8.	CKD	Chronic kidney disease
9.	CLD	Chronic liver disease
10.	CLR	C-type lectin receptor
11.	COPD	Chronic obstructive pulmonary disease
12.	COVID-19	Coronavirus disease 2019
13.	COVs	Coronaviruses
14.	CTLD	C-type lectin like domain
15.	DAMPs	Danger Associated Molecular Patterns
16.	EDTA	Ethylene diamine tetra acetic-acid
17.	EYA	Egg yolk agar
18.	FLU	Fluconazole
19.	IFI	Invasive fungal infections
20.	IGS	Intergenic spacer regions
21.	IT	Itraconazole

22.	ITS	Internal transcribed spacer regions
23.	KT	Ketoconazole
24.	MERs	Middle East respiratory syndrome
25.	MS	Multiple sclerosis
26.	NAC	Non-albicanis candida
27.	NCBI	National center for biotechnology information
28.	NS	Nystatin
29.	OM	Oral mucosa
30.	PCR	Polymerase chain reaction
31.	PDA	Potato dextrose agar
32.	SARs	Sever acute respiratory syndrome
33.	SDA	Sabouraud Dextrose Agar
34.	SDB	Sabouraud Dextrose broth
35.	SF	Saliva flow
36.	SNPs	Single nucleotide polymorphisms
37.	TLRs	Toll – like receptors
38.	WHO	World health organization

Chapter One

Introduction

Introduction:

Invasive mold infection is a serious complication described in patients with severe viral pneumonia (Schauwvlieghe *et al*., 2018). The risk of developing invasive fungal infections (IFI) is high in severe COVID-19 cases, not only because of the patient's clinical condition and the need for invasive care also because of the immune changes caused by SARS-CoV-2 and the treatment used (McNab *et al*., 2015). As the human-to-human transmitted disease, coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARSCoV- 2), has been an emergency global public health events (Gorbalenya *et al*., 2020; Zhou *et al*., 2020).

COVID-19 is initially diagnosed as "pneumonia of unknown etiology" first emerged in December 2019 at Wuhan, Hubei Province, China. The pathogen was proclaimed by the Chinese Center for Disease Control and Prevention (China CDC) on January 08, 2020, to be an unusual coronavirus. Later on, the virus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 in few weeks the contagious virus covid-19 show a wide spread to the whole Hubei Province, the rest of China and abroad (Sariol and Perlman, 2020) .

Covid 19 present with a dry cough; fever, muscle or joint pain, headache/dizziness, diarrhea, nausea and the coughing up of blood some people suffer from breathing difficulty. Viral load reaches its peak within about 10 days after the first signs of symptoms. Some severe covid-19 cases progress to acute respiratory distress syndrome (ARDS) after about two weeks from the disease onset (Tay *et al*., 2020). Infection with SARS-CoV-2 is characterized by increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines, resulting in cytokine storm syndrome status. The control of this inflammatory state can be decreased by dexamethasone Clinical trials performed in the United Kingdom with

dexamethasone, have identified dexamethasone as the first steroid to prevent deaths from serious COVID-19 events, but no influence was seen in patients who did not receive respiratory support. For other causes, this effect was already observed in ARDS (Marcolino *et al*., 2020 Annane *et al*., 2017).

Depletion of membrane ergosterol due to the use of azoles are also shown to disrupt vacuolar ATPase functions resulting in an impairment of the vacuolar acidification and ion homeostasis (Zhang *et al*., 2010). The fungal antigens class of polyene especially Amphotericin B for a long time the most effective by the administration of antibiotics fungal area. Amphotericin B is a fungicide, a pharmaceutical antifungal in the outside and inside of the body and has an effective against *Candida* spp. and *Cryptococcus neoformans* and mold (Lemke *et al*., 2005).

The initiation of an immune response toward fungal pathogens relies on the recognition of fungal pathogen-associated molecular patterns. By using gene-deficient mice, toll-like receptors (TLRs) and C-type lectin-like receptors (CLRs) have been proven to be essential for fungal recognition (Wirnsberger et al., 2016). Receptors of the innate immune system are the first line of defence against infection, being able to recognise and initiate an inflammatory response to invading microorganisms. The Toll-like (TLR), NOD-like (NLR), RIG-I-like (RLR) and C-type lectin-like (CLR) are four receptor families that contribute to the recognition of a vast range of species, including fungi (Plato *et al*., 2015).

Aim of The Study:

This study aims to identify the phenotype by studying the morphological features of the species causing Fungal infection associated with Covid 19 patients and detect for some polymorphism of genetic risk factors of patients, also studying their sensitivity to antifungals of fungal species under study:-

1- Isolation of pathogenic fungi from oral cavity swabs for all study population.

2- Evaluate the risk factors such as age, gender, presence of chronic disease and duration.

3- Identification of fungal isolates by conventional methods.

4- Testing the antifungal sensitivity of fungal isolates by disk diffusion method and determent the inhibition zone .

5- DNA extraction from blood samples for each study population and molecular detection of risk genes such as *TLR8* and *CLEC7A* gene by PCR teqhniqe and sequencing analysis .

Chapter Two

Literatures Review

2. Literature Review:

2.1. Definition of Coronaviruses:

RNA viruses represent one of the most common classes of pathogens behind human diseases (Carrasco-Hernandez *et al*., 2017), with around 180 currently recognized species, and around three new species discovered every year (Woolhouse *et al*., 2014). The harmfulness of these viruses is partially supported by their ability to rapidly evolve and adapt, allowing easier escape of host immune responses and quicker development of resistance towards drugs and vaccines. This ability relies on the low fidelity of viral RNA polymerases. The lack of any proofreading activity results in mutation rates as high as 10−3 (Holland *et al*., 1982), nearly 6–7 orders of magnitude higher than those of bacterial DNA polymerases (Choi, 2012).

Recently, the World Health Organization on March 11,2020 declared Covid-19 a global pandemic, sending a message to world leaders' people all over the world declare that the coronavirus is a fast-moving crisis that needs to be taken seriously. According to WHO Globally, as of 6:46pm CEST, 20 May 2021, there have been 164523894 confirmed cases of COVID-19, including(3,412,032)deaths, reported to WHO. As of 18 May 2021, a total of 1407945776 vaccine doses have been administered .

Coronaviruses are a group of related RNA viruses that cause diseases in mammals and birds, in humans and birds, they cause respiratory tract infections that can range from mild to lethal. Mild illnesses in humans include some cases of the common cold (which is also caused by other viruses, predominantly rhinoviruses), while more lethal varieties can cause SARS, MERS, and covid-19.

Coronaviruses (CoVs), mainly targeting human respiratory system, are responsible for health-threatening outbreaks including severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS) and lastly coronavirus disease 2019 (covid-19) (Rothan, 2020; Tufan and Guler, 2020).

2.2. Historical Background of COVS :

First case of corona virus was notified as cold in 1960. According to the Canadian study 2001, approximately 500 patients were identified as Flu-like system. Occasionally 17-18 cases of them were confirmed as infected with corona virus strain by polymerase chain reaction (PCR). Several cases of severe acute respiratory syndrome caused by corona and their mortally more than 1000 patient was reported in 2003. This was the black year for microbiologist. When microbiologist was started focus to understand these problems. After a deep exercise they conclude and understand the pathogenesis of disease and discovered as corona virus. But till total 8096 patient was confirmed as infected with corona virus. So, in 2004, World health organization and Centers for disease control and prevention declared as “state emergency” (WHO, 2003;Peiris *et al*., 2003).

2.3.Taxonomy and Classification of COVs:

Virus classification (Virus Taxonomy, 2018)

Realm: Riboviria

Kingdom: Orthornavirae

Phylum: Pisuviricota

Class: Pisoniviricetes

Order: Nidovirales

Family: Coronaviridae

Sub family: Orthocoronvirinae and Torovirinae

Orthocoronavirinae is classified into 4 genera:

- Alphacoronavirus
- Betacoronavirus
- Gammacoronavirus
- Deltacoronavirus

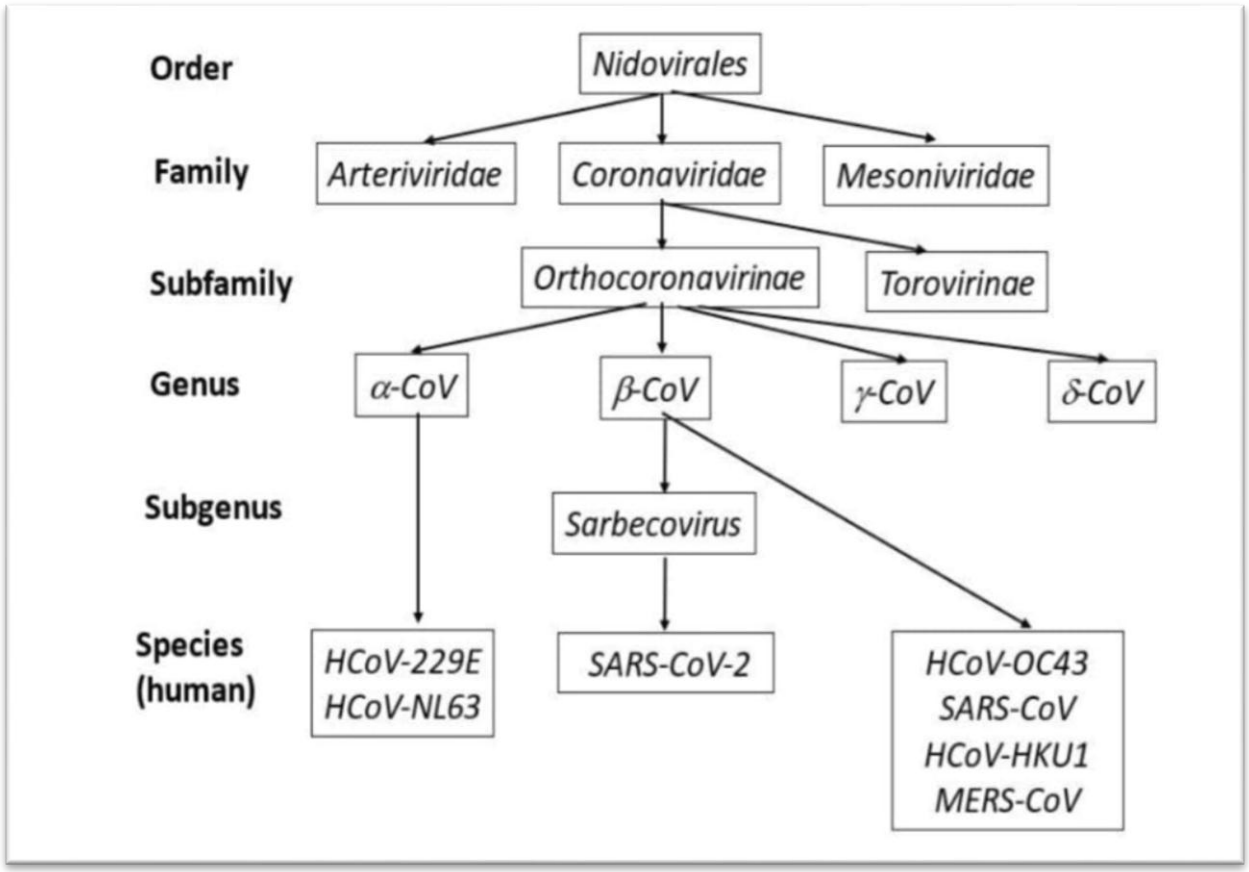


Fig. (2-1): Classification of Human Coronaviruses (Malik, 2020).

Alpha-coronaviruses and Beta-coronaviruses appear to infect mammals and cause human respiratory and gastrointestinal infections, such as SARS (SARS-CoV), MERS (MERS-CoV) and SARS-CoV-2, while Gamma-coronaviruses and Delta-coronaviruses are capable of infecting birds in addition to mammalian (Forni *et al.*, 2017; Zhou *et al.*, 2020).

Beta corona viruses consist of SARS-CoV, MERS CoV, Bat-SARS-like (SL) coronaviruses, human coronaviruses (HCoVs), and finally SARS-CoV-2. SARS-COV-2 has non-segmented, single-stranded positive RNA(+ssRNA) with a 5'-cap and 3'-poly-A tail structure, a typical CoVs genomic structure (Fig. 2-1) (Ashour *et al*., 2019).

2.4.Coronavirus Genome Structure and Replication:

Despite the fact that it is the largest known plus-strand RNA the coronavirus genome is similar to other plus-strand RNA viruses' genomes (With the exception of retroviruses) infectious properties (without using a packaged RNA-dependent RNA polymerase (RdRp)) (Brian *et al.,* 1980). In near proximity to cellular membranes, replication occurs in the cytoplasm (Gosert *et al*., 2002). The genomes of six species of coronaviruses have now been fully sequenced and reported in GenBank (as of November 2002) the following genome structure similarities can be found among the six types of virus The 50 UTRs ranging in length from 209 to 528 nt contain a similarly positioned short, AUG-initiated open reading frame (ORF) relative to the 50 end (Morris and Geballe , 2000).

SARSCov-2 possesses non segmented, single-stranded positive sense RNA (+ssRNA) with 5’-cap structure and 3’-poly-A tail which is a typical genomic structure of CoVs (Ashour *et al*., 2020). The genome analyses have revealed that the genome sequence of SARS-CoV-2 is 96% and 79.5% identical to the bat coronavirus termed Bat CoV RaTG13, and SARS-CoV, respectively (Zhou *et al*., 2020).Therefore, the bat has been suggested as a natural host of SARS-CoV-2 and the transmission route of SARS-CoV-2 could be through unknown intermediate hosts. The genetic analyses of SARS-CoV-2 genomes from 103 Chinese patients demonstrated that this virus has been evolved into two main types; L type

(~ 70%) and S type (~ 30 %). L type is more aggressive and infectious than S type which is the ancestral version (Tang *et al*., 2020).

2.5. Diagnosis:

In most cases of self-limited infection, diagnosis of coronaviruses is unnecessary, as the disease will naturally run its course. However, it may be important in certain clinical and veterinary settings or in epidemiological studies to identify an etiological agent. Serologic assays are important in cases where RNA is difficult to isolate or is no longer present, and for epidemiological studies (Raj *et al*., 2013).

Diagnosis is also important in locations where a severe CoV outbreak is occurring, such as, at present, in the Middle East, where MERS- CoV continues to circulate. RT-PCR has become the method of choice for diagnosis of human CoV, as multiplex real-time RT-PCR assays have been developed, are able to detect all four respiratory HCoVs and could be further adapted to novel CoVs (Emery *et al*., 2004; Gaunt *et al*., 2010).

2.6. Pathology of COVID-19 Disease :

Although less is known about the pathophysiology of COVID-19 and can start with asymptomatic phase when the SARS-CoV-2 which is received via respiratory aerosols binds to the nasal epithelial cells in the upper respiratory tract. The main host receptor for viral entry into cells is the ACE-2, which is seen to be highly expressed in adult nasal epithelial cells. The virus undergoes local replication and propagation, along with the infection of ciliated cells in the conducting airways. This stage lasts a couple of days and the immune response generated during this phase is a limited one. In spite of having a low viral load at this time, the individuals

are highly infectious, and the virus can be detected via nasal swab testing (Hoffmann *et al*., 2020; Wang *et al*., 2020).

However, there are three coronaviruses (severe acute respiratory syndrome coronavirus (SARS- CoV), Middle East respiratory syndrome coronavirus (MERS- CoV) and SARS- CoV-2) that can replicate in the lower respiratory tract and cause pneumonia, which can be fatal. SARS-CoV-2 belongs to the beta coronavirus genus. Its closest relative among human coronaviruses is SARS- CoV, with 79% genetic similarity, However, among all known coronavirus sequences, SARS- CoV-2 is most similar to bat coronavirus RaTG13, with 98% similarity (Fehr & Perlman, 2015; Zhou *et al*., 2020) and coronavirus sequences in the pangolin (a scaly anteater) also share high similarity (Andersen *et al*., 2020).

With 97.5% of symptomatic patients developing symptoms within 5-11 days. At the point of hospital admission, patients with COVID-19 typically exhibit a fever and dry cough; The pathophysiology of SARS-CoV-2 infection closely resembles that of SARS- CoV infection, with aggressive inflammatory responses strongly implicated in the resulting damage to the airways (Wong *et al*., 2004). Therefore, disease severity in patients is due to not only the viral infection but also the host response. The pattern of increasing severity with age is also broadly consistent with the epidemiology of SARS- CoV and MERS- CoV (Guan *et al*., 2020; Huang *et al*., 2020).

2.7. Fungal Infections in COVID-19 Disease:

Oral fungal infections associated with COVID-19 the pandemic severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2) infection caused greatest health and social impact in the world since March 2020 (Faccini *et al*., 2020) A 67-year-old male patient who was tested positive for COVID-19 revealed his medical history of coronary disease and kidney

transplant. Oral examination during hospital stay showed white plaque on the dorsum of the tongue. The patient was diagnosed with oral candidiasis (Dos Santos *et al*., 2020). Salehi *et al*. (2020) stated that COVID-19 patients at high risk included those have or have a history of respiratory distress syndrome, admitted in intensive care units, and used immunosuppressant drugs or corticosteroids are most likely to develop oral candidiasis (Salehi *et al*.,2020).

Another case study on a 47-year-old female patient who was tested positive for COVID-19 infection showed *pseudomembranous* candidiasis (Riad *et al*., 2020). Interestingly, a published report of asymptomatic COVID-19 patient mentioned the presence of oral candidiasis Corchuelo *et al*.,2020). Another published data revealed two patients with oral candidiasis in patients with no risk factors like immunosuppression or prolonged antibiotic use (Barabouti *et al*., 2020).

SARS-CoV-2-associated pulmonary aspergillosis (CAPA) has been the predominant fungal disease adding insult to injury in coronavirus disease 2019 (COVID-19) patients with acute respiratory distress syndrome (ARDS), and while the pathogenesis is incompletely understood, there are several immunological mechanisms that may contribute to the development of CAPA and other fungal diseases. SARS-CoV-2 virus invasion results in the release of danger associated molecular patterns (DAMPs) which act as endogenous signals that exacerbate the immune and inflammatory response leading to lung injury **(**Tolle *et al*., 2013; Arastehfar *et al*.,2020). Importantly, DAMPs are known to play a central role in the pathogenesis of fungal diseases(Cunha *et al*,. 2012).

Moreover, collateral effects of host recognition pathways required for the activation of antiviral immunity may, paradoxically, contribute to a highly permissive inflammatory environment that favors fungal pathogenesis. To date over 100 cases of CAPA have been reported from

many countries in Europe, Asia, Australia and South America (Alanio *et al*.,2020; Arastehfar *et al*.,2020; Helleberg *et al*.,2021). Often occurring in patients with no other risk factor than COVID-19 associated ARDS (Arastehfar *et al*.,2020). and multiple of them proven by autopsy (Alanio *et al*., 2020; Antinori *et al*., 2020; Rutsaert *et al*., 2020; Santana *et al*., 2020).

Contrast, there are fewer reports on other fungal diseases complicating COVID-19, including two cases of *Saccharomyces cerevisiae* fungaemia caused by fungal translocation (Hoenigl., 2020). After administration of probiotic preparations containing Saccharomyces (Ventoulis *et al*.,2020). Cases of invasive Candida infections(Garcia-Vidal *et al*., 2021) and a case of invasive fusariosis (Poignon *et al*., 2020). Now, in this issue of Clinical Infectious Diseases, White and colleagues from the United Kingdom report a 26.7% incidence of invasive fungal disease in a multicenter prospective cohort of COVID-19 intensive care patients, including a 12.6% incidence of invasive yeast infections (White *et al*.,2021).

Factors that may contribute to the differing incidence rates are threefold. First, fungal diseases and specifically CAPA are difficult to diagnose and are likely underestimated, particularly in the setting of COVID-19 associated ARDS, where the clinical picture and radiological findings of CAPA resemble those of severe COVID-19 (Koehler *et al*., 2020; Verweij *et al*., 2020). Blood tests lack sensitivity due to the primarily airway invasive growth of *Aspergillus* in non-neutropenic patients (Jenks *et al*., 2019), and most importantly sampling of the primary site of infection is rarely performed, due to the risk of COVID-19 transmission through bronchoscopies with bronchoalveolar lavage (BAL) or autopsies (due to the overlap of imaging findings between CAPA and COVID-19, postmortem fine needle biopsies alone may not be sufficient to detect focal CAPA (Flikweert *et al*., 2020).

2.8. Fungi of Oral Cavity:

The majority of oral fungal infections (oral mycosis) are resultant of opportunistic conditions. Host resistance impairment allows for the initiation and progression of pathogenic conditions through local colonization in the oral cavity. The frequency of oral mycosis has remarkably increased globally with the increased use of immunosuppressive drugs and immunodeficiency viral infections (Nagy.,1999; Richardson and Lass- Flörl., 2008).

The predominance of oral candidiasis is being diagnosed probably due to the increasing number of individuals with immunocompromised conditions and dentists who are very experienced in the recognition and differentiation of oral candidiasis from other types of fungal infections (Singh *et al*.,2014; Samaranayake *et al*.,2009). Whereas the clinical presentation of deep fungal infections is varied and not easily recognizable, this results in clinical diagnostic challenges (Hoepelman and Dupont ., 1996). Superficial fungal infections are usually associated with oral discomfort, pain, burning sensation, parageusia, and aversion to food (Samaranayake *et al*.,2009; Carmello *et al.*,2016).

The oral microbes are normal components in healthy people and patients. As the disruption of oral microbes affects the pathway of oral diseases, especially in patients with immunodeficiency (Avila *et al*., 2009). In addition to *Candida* spp, there are other fungi isolated from the oral cavity which previously record includs *Aspergillus*, *Penicillium*, *S. cerevisiae*, *Scopulariopsis*, *Geotrichum*, *Hemispora*, and *Hormodendrum* (Salonen *et al*., 2000). The presence of *Aspergillus, Fusarium*, and some types of yeast such as *Cryptococcus* in the oral cavity of healthy individuals is not expected, as it is not previously recorded that these fungi can colonize the oral cavity. These fungal pathogens can control healthy

individuals in other fungi in the oral microbial community (Ghannoum *et al*., 2010).

The *Candida* spp. are a group of opportunistic yeasts, which is part of the normal flora of the oral mucosa (OM) and the gastrointestinal tract and intestines. About 50% of the world's population, *C. albicans* is carried in the mouths of as a normal component (Priya, 2013; Yang *et al*., 2014). In particular, the risk factors for colonization depend on the properties of fungi such as adhesion to epithelial cells, as well as topical factors of the oral cavity and systemic condition of the host, for example, diabetes or immunosuppression (Gow *et al.*, 2011). *C. albicans* is the most common species in invasion of the OM in healthy and Individuals with immune deficient; nevertheless, *C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, C. guilliermondii* and *C. dubliniensis* (Cannon and Chaffin, 1999; Yang *et al*., 2011).

Oral candidiasis has been divided into two types primary oral candidiasis, with infection occurring in the mouth and surrounding tissues, and secondary oral candidiasis, where the infection spreads approximately to all parts of the body, such as the digestive system, as well as the mouth (Parihar *et al*., 2011). Thorman *et al*., (2009) were described the most common symptoms of these patients are saliva flow (SF), skin tightness, atrophy of the mucosa, in both diabetics without complications and in diabetics with chronic kidney disease (CKD). In addition, there are early symptoms associated with patients with uncontrolled diabetes such as candidiasis and other opportunistic fungal infections (Rosa-Garcia *et al*., 2013).

2.9. Candidiasis*:*

Candida spp. are accomplished of creating mucous membrane and skin infections. Though, cutaneous candidiasis is less common than dermatophytosis. There are about two hundred species in the genus *Candida* and about twenty of them are attendant with human or animal infections, e.g. *C. albicans*, *C.krusei*, *C. glabrata*, *C. parapsilosis, C. tropicalis*, with *C. albicans* accounting for most of the infections. A lessening in the occurrence of *C. albicans* as a reason of infection and an increase in non-*albicans Candida* (NAC) such as, *C. krusei*, and *C. parapsilosis* , C. *glabrata* was found in the last decade. *C. albicans* is the great essential fungal opportunistic pathogen. It generally resides as a common in the gastrointestinal and genitourinary tracts and in the oral and conjunctiva vegetation (Gupta *et al*.,2004; Ayers *et al*.,2005; Bottone and Marro,2011). These infections can be superficial and affect the skin or mucous membrane (Marques *et al*., 2012).

Other *Candida* species found in healthy persons including *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* (MacCallum,2012). All five declared types reason more than 90% of aggressive infections, while the comparative incidence of the species depends on the geographical site, patient population, and clinical settings (Miceli *et al*., 2011; MacCallum,2012).

The genus *Candida* comprises about 150 different species; however, only a few are known to cause human infections . The medically important *Candida* species include the following: *C. albicans*, the most common species identified (50-60%) *C. glabrata* (previously known as *Torulopsis glabrata*) (15-20%), *C. parapsilosis* (10-20%), *C. tropicalis* (6-12%), *C. krusei* (1-3%), *C. kefyr* (<5%), *C. guilliermondi* (<5%) and *C. lusitaniae* (<5%) **(**Abi-Said *et al*., 1997).

2. 10.Virulence Factors:

The disease is defined as the ability of the organism to cause infection in the host and fungus depends on its susceptibility of disease to adapt in the environmental conditions of the tissue and resistance to the effective decomposition by the defenses of the host and these are caused by several factors ferocity possessed by fungi, which gain the ability to multiply and damage to the host (Casadevall , 2007). These fungal factors help to grow at 37 °C, and physiological pH level and facilitate the fungus adhesion, penetration and proliferation and help in resistance against the human body's immune defenses such as phygocytosis, these also facilitate the fungal invasion of deep tissue (Tomee and Kauffman, 2000).

Virulence fungal factors are important for all types of fungi such as their ability to discriminate and respond quickly to external environmental changes and their secretion of hydrolases enzymes. Some of the virulence factors of fungus include extracellular secretions produced by the fungus for the purpose of expanding human infection (Khan *et al*.,2010). The main virulence factors are Lipases and Phospholipases, the genus *Candida* secrete a fat state enzymes such as Esterases , Lipases , Phospholipases these enzymes play a role in the high growth of *Candida* spp. (Khedidja and Abderrahman., 2011).

Lipases production is influenced by several factors such as nutritional materials, nitrogen, carbon sources, temperature, pH, presence of lipids, inorganic salts, and dissolved oxygen concentration (Gupta *et al*.,. 2015). While, Phospholipases works to phospholipid hydrolysis of the host membrane and lead to the alternation of the characteristics of the cell surface and all that facilitate the establishment of infection (Sachin *et al*.,2012). *C. albicans* is secreted essential protease have a role in the occurrence of infections and secreted internal protease are important in the metabolism (Nirmal *et al*.,2011; Rodarte *et al*.,2011).

2.11. Diagnosis of Yeasts:

The clinical presentation of candidiasis is diagnostic, due to the classical white appearance (Hellstein, 2019). The diagnostic methods for oral candidiasis include exfoliative cytology, imprint specimen for microbiology culture, potassium peroxide staining, oral swab specimen for culture analysis, salivary assays, and oral mucosal biopsy. Special staining procedure such as periodic acid–Schiff is helpful in definitive diagnosis (Samaranayake *et al*.,2009).

Oral mucosal biopsy procedure is recommended in chronic hyperplastic candidiasis to differentiate it from leukoplakia and to evaluate the status of dysplasia (Manfredi *et al.,* 2013; Samaranayake *et al*.,2009) mentioned that chronic hyperplastic candidiasis may have a 15% chance of progression to epithelial dysplasia (Verweij *et al*.,2020).

2.11.1. Laboratory Methods:

The conventional methods of yeasts diagnosis include old techniques which may be a time consuming and are missing accurateness, such as these methods is direct microscopic checkup of medical specimens, which can show the presence of *Candida* spp., infection if there are large numbers of *Candida* cells in a fresh clinical specimen (Ridley *et al.*, 2000). Indicative cases is the most useful present-day medium for isolating *Candida* spp and other yeast in a clinical laboratory (Nyirjesy *et al*., 1995).

Since the germ tube are a characteristic morphology detected only in *C. albicans,* confermation of germ tube is available as a rapid way for identifying *C.albicans* (Debbie *et al*., 2004).The ability to form chlamydospore it is anthor test for documentation of *C. albicans,* it has been based on the presence of chlamydoconidia on the corn meal agar medium (Bhavan *et al*., 2010).

2.11.2.Chromogenic Candida Media:

The word "chromogenic" consists of two elements chromo from the Greek word chroma meaning color and genesis meaning creation. Accordingly it can be said that the word "chromogenic" hold as its mean in the idea of producing color or appear pigments (Vosough, 2010). There are several types of Chromogenic candida media as CHROMagar Candida, ChromID candida agar, Bio-rad CandiSelect 4, Oxoid OCCA, and Bismuth sulphite glucose glycine yeast (BiGGY) agar, these medium use to decrease the time required to identify *Candida* species as compared to other traditional methods and so directly, application in clinical specimens, they used by many of authors (Yucesoy and Marol, 2003; Perry and Freydiere, 2007; Adam *et al*., 2010; Daef *et al*., 2014).

A major advantage of these media is that they provide definitive or presumptive identifications in shorter times more than those required for laboratory standard methods. As well as, these media permit the determination of growth of mixed yeast species from a one clinical specimen to differences of colony color, that is reported by several authors (Linares *et al*., 2003; Year *et al*., 2004; Ozcelik, 2006). On CHROMagar, *C. albicans* produce light green colonies, *C. dubliniensis* appear dark green colonies, *C. glabrata* appear purple to pale pink, *C. krusei* appear pink with rough texture, *C. parapsilosis* appear white-cream to pink, *C. tropicalis* appear steel blue that also often brown to purple halo (Fang *et al*., 2007; Ozcan *et al*., 2010). This test is active and rapid in identification of *Candida* spp. On color level after vaccination and the conventional method, technically simple preparation (by boiling), cost effective and rapid compared to conventional methods which expensive and required a long time to accomplish (Momani, 2000; Ozcelik, 2006).

The study of Horvath *et al*., (2003) established that CHROMagar Candida medium is a novel differential culture medium that can be used for the reasonable identification of *Candida* spp. CHROMagar Candida comprises enzymatic substrates that are connected to substrates in Chromogenic, which when acted upon by different enzymes produced by *Candida* spp. results in color differences useful for the reasonable identification of the yeasts (Murray *et al.*, 2005).

2.11.3. Molecular Methods:

Molecular identification, numerous techniques have been suggested to identify and distinguishing fungal species by polymerase chain reaction (PCR), the appearance of PCR is essential for the development molecular methods for recognizing *Candida* spp., that based on extension and recognition of microbial DNA in the background of host DNA (Trtkova and Raclavsky, 2006; Fricke *et al*., 2010) or directly from clinical specimens (Kanbe *et al*., 2002). Molecular methods provide the possibility for more specific and fast identification of *Candida* spp., as compared with conventional phenotypic methods. In general, the laboratory that its phenotypic methods cannot determine the identities of organisms, can be determined by the nucleic acid sequence and may offer the highest benefit to it (Hall *et al*., 2003). Although there are many molecular methods, such as simple PCR assay and sequence methods.

The major part of nucleic acid based systems uses PCR methods for amplifying fungal DNA to be the first stage in the process of identification. PCR primer and suitable DNA targets should be chosen prior to PCR amplification occurrence. The most widespread targets are the very preserved parts of ribosomal DNA and it contain the (5.8S, 18S, and 28S rRNA) genes (Guiver *et al*., 2001). The other targets included the more versatile for (ITS) regions among these genes or the (IGS) region

(Cirak *et al*., 2003; Coignard *et al*., 2004; Massonet *et al*., 2004). The ITS1 and ITS2 regions of RDNA is extensive datasets available, these regions also are used commonly to detect and identify species reported to exhibit a high degree of polymorphism between species and had highly conserved with species and containing valuable genetic markers for species identification and this region most likely will be chosen as one of the universal barcodes for fungal species (Seifert, 2009; Avis *et al*., 2010; Begerow *et al*., 2010).

Recently, several studies have demonstrated that sequence analysis of different regions of rDNA is the golden method for *Candida spp*. identification. Imran and Al-Shukry., (2014) have diagnosed *Candida spp* from patients with vaginal candidiasis by using RAPD-PCR techniques. Fujita *et al*., (2001) have determined the PCR fragments lengths of ITS regions for six of *Candida* species by using both agarose gel electrophoresis and microchip electrophoresis. Mohammadi and Abdi (2015) have diagnosed *Candida* spp. isolated from gastro-oesophageal candidiasis in Tehran, Iran by using restriction fragment length polymorphisms (PCR-RFLP) method of the ITS1-5.8SrDNA-ITS2 region with primer ITS1 and ITS4. While Cornet *et al*., (2011) use primers designed for the IGS2 for identification of closely related *Candida* spp. isolated from medical specimens. Also application nested PCR of *Candida* spp. with universal primer and their PCR product were amplified with species specific primers.

2. 12.Antifungals Drugs:

The limited availability of antifungals is a major impediment for the effective treatment of fungal infections (Vandeputte *et al*. 2012). This is further compounded by the fact that the generation of newer antifungals has lagged behind when compared to the pace of emergence of fungal

infections. The components of the fungal cell wall such as mannans, glucans and chitins; and a few of the enzymes of the ergosterol biosynthetic pathways which are unique to fungal cells are commonly targeted for the development of antifungal agents (St Georgiev 2000; Munro *et al.* 2001; Houst *et al*., 2020).

The number of agents available to treat fungal infections has increased by 30% since 2000, yet still only 15 agents are currently approved for clinical use. The greater number of medications now available allows for therapeutic choices; however, differences in antifungal spectrum of activity, bioavailability, formulation, drug interactions, and side effects necessitates a detailed knowledge of each drug class (Thompson *et al*., 2009). Currently, four antifungal drug classes are used by clinicians and veterinarians for systemic treatment (Carmona and Limper, 2017). But, the two largest groups of antifungal drugs are:-

2.12.1. Azole Group :

Azoles mainly include two subclasses based on the number of nitrogen atoms in a ring; The first class includes imidazoles which consist of miconazole, oxiconazole, econazole, ketoconazole, tioconazole, and clotrimazole with two nitrogen atoms in an azole ring, while another class includes triazoles such as Fluconazole, posaconazole, itraconazole, terconazole, and voriconazole which contain three nitrogen atoms in a cyclic ring (Naeger-Murphy and Pile, 2006; Prasad *et al*., 2016).

Imidazoles are mainly used for the mucosal fungal infections while triazoles are administered both for the systemic as well as for the mucosal infections (Sanglard *et al*., 2009; Vandeputte *et al*., 2012). Depletion of membrane ergosterol due to the use of azoles are also shown to disrupt vacuolar ATPase functions resulting in an impairment of the vacuolar acidification and ion homeostasis (Zhang *et al*., 2010). Since azoles are

fungistatic, their prolonged use poses greater threat of emergence of drug resistance among the surviving fungal population (Shapiro *et al.*, 2011).

Ostrosky-Zeichner *et al*., (2003) used of newer azoles for the treatment of invasive candidiasis. They utilized voriconazole as salvage therapy in 52 patients with invasive candidiasis either refractory to or intolerant of other antifungals (almost all of whom had failed therapy with D-AmB and/or other azoles), and found a 56% favorable response rate in this challenging population. Also, Kullberg *et al*., (2005) studied voriconazole versus D-AmB followed by fluconazole in candidemic patients, with a similar outcome but somewhat better tolerability in the voriconazole arm.

2.12.2. Polyenes Group:

Polyenes are the amphipathic organic natural molecules called macrolides and are generally produced by *Streptomyces* (Vandeputte *et al*., 2012). Polyenes directly bind to ergosterol of fungal cell membranes leading to the formation of pores in membrane, resulting in the loss of ionic balance, membrane integrity and cell death (Sanglard *et al*. 2009).

Polyenes mainly include amphotericin B (AmpB), natamycin and nystatin. AmpB is mostly effective in systemic invasive fungal infections and is used generally against *Cryptococcus*, *Candida* and *Aspergillus* species (Lemke *et al*., 2005) while nystatin and natamycin are preferred for topical infections due to their low absorption (Vandeputte *et al*., 2012). Although polyenes are fungicidal in nature and have been in use for a long time but they show many side effects in humans which limits their use. However, lipid formulations of AmpB are less toxic and are relatively better for the treatment of fungal infections (Shapiro *et al.* 2011).

Amphotericin B is a cyclic heptaene produced by the Gram positive bacterium *Streptomyces nodosus*. It has two mechanisms of action. First, several molecules of AMB incorporate into the fungal lipid bilayer and bind to ergosterol. By ergosterol sequestration, pores are formed, and both the ions **(K^+, $Mg2^+$, $Ca2^+$, and Cl^-)** and electrolyte glucose are released. The rapid depletion of intracellular ions results in fungal cell death. Second, AMB induces the accumulation of reactive oxygen species (ROS), resulting in DNA, protein, mitochondrial, and membrane damage (Mesa-Arango *et al*., 2012).

The structural parts mycosamine and hydroxyl groups at the C8, C9, and C35 positions are critical for the AMB biological activity (Tevyashova *et al*., 2013).

2.13. Antifungal Innate Immunity:

The complex interaction between host and microbe is particularly reflected in the pathogenesis of fungal diseases. Most fungi are ubiquitous in the environment, and our immune system has co-evolved and adapted to their presence over millions of years. Fungi can colonise almost every niche within the human body. Certain fungi, such as *Candida albicans* are commensal, whereas others, such as *Aspergillus fumigatus*, are environmental opportunists, which can cause devastating invasive diseases when immune homeostasis is disrupted (Salazar and brown, 2018). In immunocompromised hosts fungi can colonize and infect the lungs and other organs, causing increases in morbidity and mortality. Alternatively, fungi and their products can cause exaggerated immune responses and pathologic changes in organs in certain subjects (Bartemes and Kita, 2018).

The initiation of an immune response toward fungal pathogens relies on the recognition of fungal pathogen-associated molecular patterns. By using gene-deficient mice, Toll-like receptors (TLRs) and CLRs have been

proven to be essential for fungal recognition. The CLRs dectin-1, dectin-2, and dectin-3, Mincle, and mannose receptor C type 1 (MRC1) recognize various constituents of the fungal cell wall, including β-glucan, hyphal mannose, and glycolipids to initiate protective immunity. Although dectin-1, dectin-2, dectin-3, and Mincle are essential for immune reactions against disseminated candidiasis, mannose-receptor-deficient (*Mrc1*) mice mount a comparatively normal immune response (wirnsberger *et al*., 2016).

Receptors of the innate immune system are the first line of defence against infection, being able to recognise and initiate an inflammatory response to invading microorganisms. The Toll-like Recepter (TLR), NOD-like (NLR), RIG-I-like (RLR) and C-type lectin-like receptors (CLR) are four receptor families that contribute to the recognition of a vast range of species, including fungi. Many of these pattern recognition receptors (PRRs) are able to initiate innate immunity and polarise adaptive responses upon the recognition of fungal cell wall components and other conserved molecular patterns, including fungal nucleic acids. These receptors induce effective mechanisms of fungal clearance in normal hosts, but medical interventions, immunosuppression or genetic predisposition can lead to susceptibility to fungal infections (Plato *et al*., 2015).

2.13.1. Toll-like Receptors (*TLRs*):

Toll_like receptors (TLRs) belong to the family of innate immune receptors, which play an important role in the activation of innate immunity, regulation of cytokine expression, indirect activation of the adaptive immune system, and the recognition of pathogenassociated molecular patterns (PAMPs) (Hedayat *et al*., 2011; Birra *et al*., 2020; Debnath *et al*., 2020). TLRs have ten family members in humans (TLR1–TLR10), and there are twelve TLRs in mice (TLR1–TLR9, TLR11–TLR13). Some of the TLRs are located in the cell membrane, and the

others are situated in endosomes, such as TLR3, TLR7, TLR8, and TLR9 (Lester and Li, 2014).

A total of 10 types of TLRs have been found in human beings, among those some are membrane_bound and some are endosome-specific intracellular receptors. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are cell surface TLRs, while TLR3, TLR7, TLR8, TLR9 are endosomal in nature (Botos *et al*., 2011;Celhar *et al*., 2012; Goulopoulou *et al*., 2016; Moreno_Eutimio *et al*., 2020).

Toll_like receptors (TLRs) are expressed on different immune cells, such as dendritic cells (DCs), macrophages, natural killer cells, and cells of the adaptive immunity – T cells and B cells (Angelopoulou *et al*., 2020). The TLRs can also be classified based on detecting PAMP. TLR4 detects glycoprotein, TLR7 and TLR8 detects viral single_stranded ribonucleic acid (ssRNA), TLR3 detects viral double_stranded RNA (dsRNA), while TLR9 detects viral deoxyribonucleic acid (DNA) (Choi *et al*., 2018). The TLRs play an important function in the innate immune response. Various methods including molecular, metabolic, structural, cell biology, and bioinformatics experiments have explained the exact pathways underlying TLR signaling over the last decade. TLR activation tends to be varied and active in a host of aspects of pathogen innate immune responses (Kawasaki and Kawai, 2014).

Toll_like receptors (TLR7/8) are tandem duplicated genes on the X_chromosome, which are located in the endosome membrane and recognize ssRNA and synthetic oligoribonucleotides, such as imidazoquinoline, imiquimod, and R_848. Therefore, they could be involved in the recognition of the SARS_CoV_2 genome (de Groot and Bontrop, 2020).

Additionally, genetic variation in TLR7 may be an underlying factor in observed sex biases in COVID-19 severity, where males could be predisposed to immunodeficient responses due to the location of TLR7 on the X chromosome (Scully *et al*., 2020). TLR7 also escapes X-inactivation, generally leading to higher basal expression levels and elevated downstream TLR7-induced IFN response in women. Production of IFN-α is also increased in adult females relative to males, and it has been proposed that higher TLR7 expression among women may facilitate more efficient clearing of viral particles – though perhaps to the detriment of higher autoreactivity and increased risk of autoimmunity (Souyris *et al.,* 2019; Spiering and de Vries, 2021; Szeto *et al*., 2021).

Toll_like receptors(TLR7) and TLR8 are considered phylogenetically and structurally similar, but different TLR7 and TLR8 agonists produce different types of cytokines. The elevated level of pro_inflammatory cytokines is also found to be mediated by TLR8, proved by bioinformatic analysis (Moreno_Eutimio *et al*., 2020; Fallerini *et al*., 2021).

2.13.2. Dectin-1 (*CLEC7A*):

Among PRRs, the C-type lectin receptor (CLR) family, including Dectin-1, has been studied mainly in fungal infections and remains less characterized in sterile inflammation and autoimmune disorders, including multiple sclerosis (MS). Dectin-1 induces IL-1β and resulting Th17 responses during fungal infections via Card9/NFkB signaling. Although Dectin-1 is known as a receptor for fungal b-glucans, some studies have identified host-derived endogenous ligands for Dectin-1, as well as functions of Dectin-1 signaling beyond the setting of fungal infections (Taylor *et al*., 2007; Deerhake *et al*., 2019; Roesner *et al*., 2019).

Dectin-1 consists of a single extracellular C-type lectin-like domain (CTLD), a transmembrane region, and a cytoplasmic tail that contains a single tyrosine-based activation motif (Fig. 2-2). In both humans and mice, alternative splicing generates two major Dectin-1 isoforms and a number of minor isoforms. The two major isoforms, which are the only isoforms functional for b-glucan binding, differ with regard to the presence or absence of a stalk region and their ability to bind and induce cellular responses (Heinsbroek *et al*. 2006).

Originally thought to be a dendritic cell-specific receptor, Dectin-1 is now known to be expressed by many other cell types such as monocytes, macrophages, neutrophils, and a subset of T cells (Taylor *et al*., 2002). Dectin-1 (β GR) is also expressed on B cells, eosinophils, and mast cells in humans, and recent reports have also demonstrated expression of this receptor on murine microglia (Olynych *et al*., 2006; Shah *et al*., 2008). Whether the differences in expression between species are functionally significant is currently unclear. Consistent with a potential role in immune surveillance, this receptor is prominently expressed at portals of pathogen entry such as the lung and intestine (Reid *et al*., 2004).

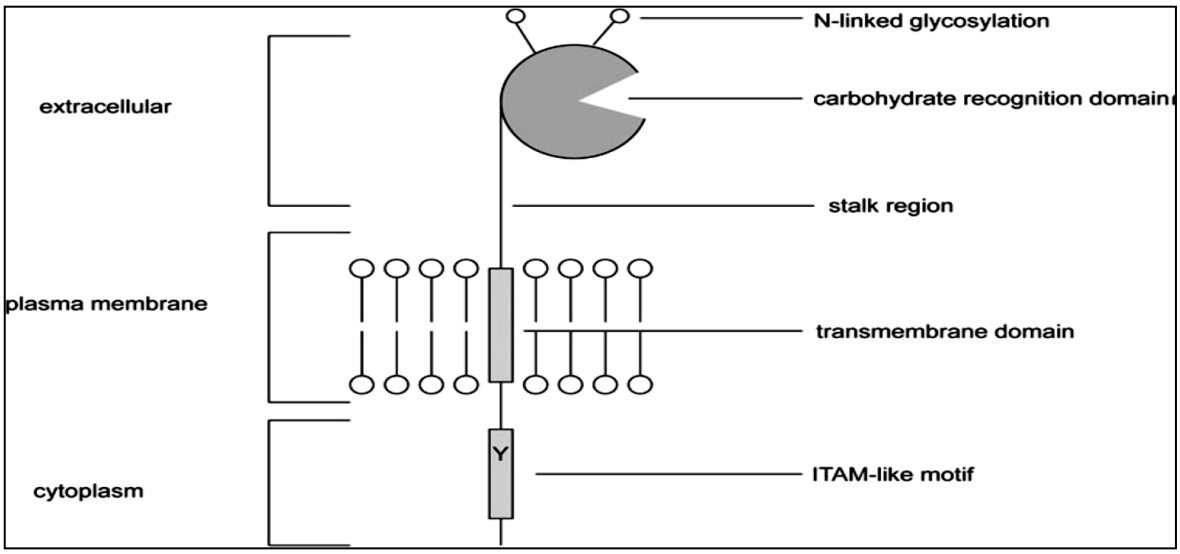


Fig. 2-2: A schematic representation of the structure of Dectin-1 (Marakalala *et al*., 2011).

Increasing evidence from in vivo studies, although not entirely consistent, suggests an important role for Dectin-1 in antifungal immunity. Studies in our laboratory have shown that the deletion of CLEC7a, the gene encoding Dectin-1,significantly increased susceptibility of mice to systemic infection with *C. albicans* .Loss of Dectin-1 in this model resulted in increased fungal burdens and much lower survival times. A peritoneal infection model revealed that the Dectin-1 knockout mice had fewer recruited cells than wild types, including neutrophils and inflammatory monocytes, which correlated with defects in the production of cytokines and chemokines such as TNF, IL-6, CCL2, CCL3, and GM-CSF (Taylor *et al*., 2007).

The role of b-glucan recognition by Dectin-1 in antifungal immunity and the requirement for Dectin-1-dependent signaling for the induction of protective immune responses .In contrast to this studyshowed that Dectin-1-deficient mice were not more susceptible than wild-type mice to infection with C. albicans (Saijo *et al.* 2007).

Nevertheless, other studies have supported a role for Dectin-1 in antifungal immunity. In a model of oral candidiasis, Dectin-1-deficient mice showed increased susceptibility, with enhanced dissemination and decreased survival times (Hise *et al*., 2009). Furthermore, mice deficient in the downstream signaling component CARD9 were similarly more susceptible to systemic *C. albicans* infection (Gross *et al*., 2006). Dectin-1 is also required during infection with other fungal pathogens; e.g., Dectin-1-deficient mice displayed increased susceptibility to A. fumigatus infection which correlated with impaired cytokine production and fungal killing (Steele *et al*., 2005; Werner *et al*., 2009).

Furthermore, in a model of intranasal infection with Pneumocystis carinii, Dectin-1-deficient mice were found to be more susceptible than wild-type mice in the early stages of infection (Saijo *et al*., 2007). The study identified and described a polymorphism of human Dectin-1 in four members of a Dutch family who were affected by either recurrent vulvovaginal candidiasis or onychomycosis (fungal infection of the nail), or both (Ferwerda *et al*., 2009).

The polymorphism was characterized by an early-stop-codon mutation (Y238X) in the CTLD of Dectin-1, resulting in defective expression and lack of b-glucan recognition by phagocytes. In addition, the mutation resulted in impaired production of cytokines, including IL-17. However, the uptake and killing of *C. albicans* by neutrophils was not affected. This suggests that alternative receptor pathways can mediate these activities in the absence of Dectin-1, and this is likely to provide protection against systemic fungal infection in these individuals (Ferwerda *et al*., 2009).

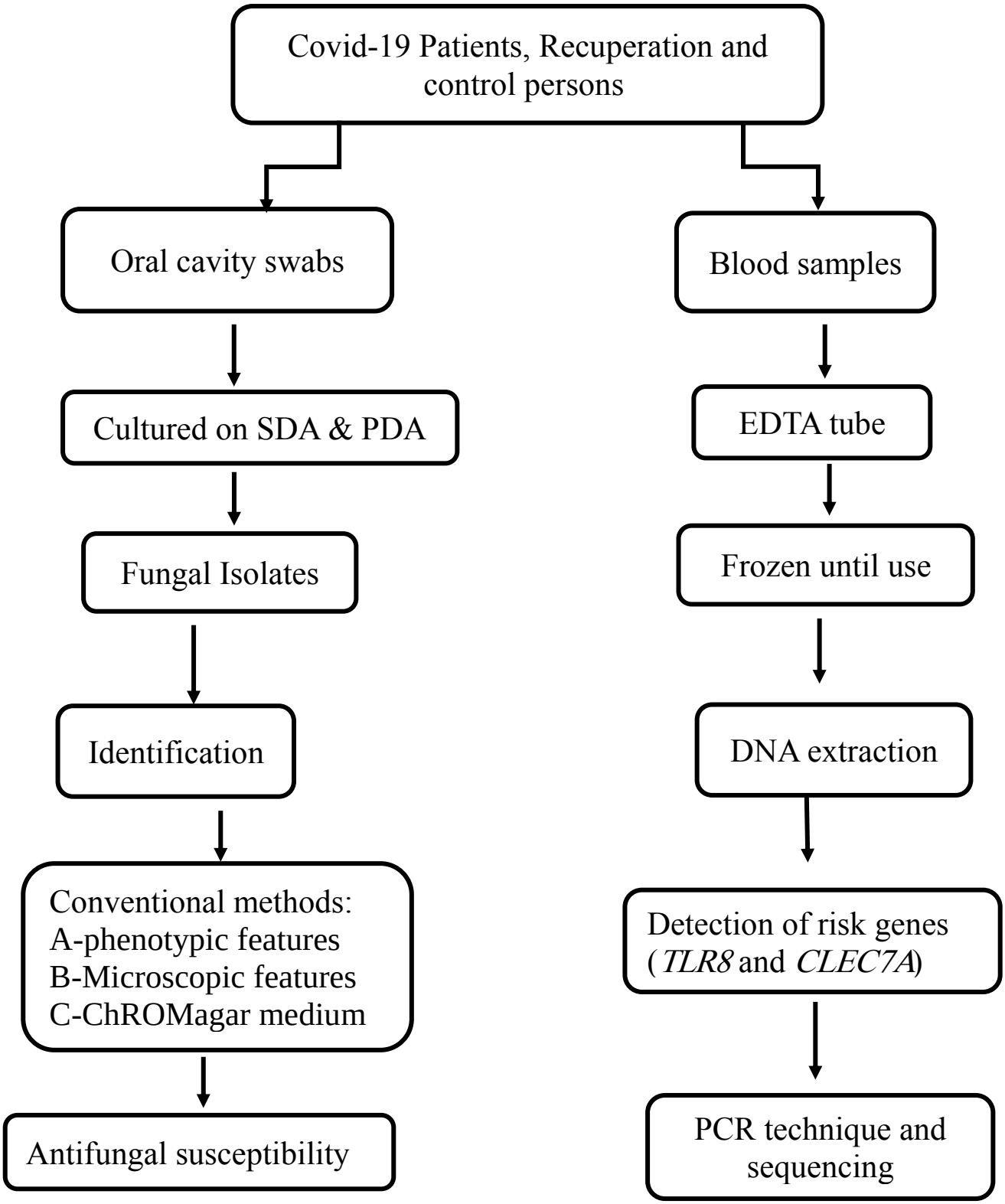
Chapter Three

Materials and Methods

3. Materials and Methods:

3.1. Study Design:

According to our proposal, this study was designed as follow:



3.2. Materials:

3.2.1. Instruments and Equipment:

Table (3-1): The Equipment Used in the Implementation of the Experiments in this Study.

NO.	Equipment	Manufacturing company
1	Autoclave	Hariyama / Korea
2	Benzine burner	Iraq
3	Blue Tips	Afco/ Jordon
4	Centrifuge	Labcco / Germany
5	Compound microscope	Olympus / Japan
6	Digital Camera	Canon / Japan
7	Disposable Gloves	TG Medical/ Malaysia
8	Disposable Syringes	Medico inject/ USA
9	EDTA tubes	Afco-Dispo(Jordan)
10	Electrophoresis meter	Mupid-one / Japan
11	Eppendorf tube1.5ml	Sigma /England
12	Eppendorf centrifuge	Hettich EBA.20 / Germany
13	Flask (250-500) ml	Oxfords
14	Incubator	Memmert/ Germany
15	Micro pipettes	Gillson instruments / France
16	petri dishes	Sterial (Jordan)
17	Plain tube	Afco-Dispo(Jordan)
18	Refrigerator	Concord / Korea
19	Sensitive balance	Denver / Swizer land
20	Slides and Cover slide	Japan
21	Thermo cycle	Labnet / USA
22	Transport media Swab	India

23	UV-Trans illuminator	Desktop Gelimage/scope-21/European
24	Vortex mixture	Memmert/ Germany
25	Water path	Gallen kamp / England
26	Water distillatory	Gallenkamp /England
27	Yellow Tips	Afco/ Jordon

3.2.2. Chemical and Biological Materials:

Table (3-2) : Chemicals and Biological Materials.

NO.	Materials	Industrialization
1	Agar	Himedia/ India
2	Agarose	Shenzhen/China
3	Chloramphenicol	BDH/ England
4	Dextrose	Himedia/ India
5	EDTA	Promega/USA
6	Ethedium bromide	Promega/ USA
7	Ethanol	BDH/ England
8	Extraction kit	Favorgen / Taiwan
9	Formalin	BDH/ England
10	Glucose	BDH/England
11	Isopropanol	BDH/ England
12	Lacto phenol cotton blue stain	Fluke/Switzerland
13	Ladder	Promega/ USA
14	Master Mix	Intron/ Korea
15	Peptone	BDH/ England
16	Phenol	BDH/ England
17	Primers	Pioneer/ China
18	Proteinase k	Favorgen / Taiwan

19	Sodium chloride	BDH/ England
20	Tween 80	Sigma/ USA
21	Urea	BDH/ England

3.2.3. Culture Media:

Table (3-3) : Culture Media .

NO.	Media	Manufacture
1	CHROM agar candida	Liofilchem -Italy
2	Sabouraud Dextrose Agar	Hi media-India
3	Potato dextrose agar	Preparin laboratory
4	Blood agar base	Hi-media /India
5	Egg Yolk Agar	Prepare in laborator
6	Sabouraud Dextrose Broth	Hi-media /India
7	Urea	Hi-media /India
8	Tween 80 opacity	Preparin laboratory

3.2.4. Antifungal:

Table (3-4): Antifungal .

NO.	Antifungal	Abbreviation	Dosage/ disc	Manufacture
1	Amphotericin-B	AP	100 uni	Hi-media /India
2	Clotrimazole	CC	10 mcg	Hi-media /India
3	Fluconazole	FLC	10 mcg	Hi-media /India
4	Itraconazole	IT	30 mcg	Hi-media /India
5	Ketoconazole	KT	10 mcg	Hi-media /India
6	Nystatin	NS	50 mcg	Hi-media /India

3.2.5. The DNA Extraction Kit:

Table (3-5):The Contents of the DNA Extraction Kit (FAVORGEN).

NO.	Material	Volume
1.	FA Buffer	120 ml
2.	FB Buffer	65 ml
3.	TG1 Buffer	45 ml
4.	TG2 Buffer	30 ml
5.	W1 Buffer	44 ml
6.	Wash Buffer	20 ml
7.	Elution Buffer	15 ml
8.	Proteinase K	11 mg

3.2.6. Primers Used in DNA Amplification:

Table (3-6): Primers Used in this Study.

Primers	Primer sequence (5' → 3')	Product size
CLEC A rs3901533*	F: ACCTCATTGAAGCTCTACCCT R: GCAACTGGGCTCTAATCTCCTAA	957 bp
TLR8 SNP rs3764880 A/G**	Fo: AAATCACAAGTTCCCTTCTTTTCATGTA Ro: CATCACTGCATTTGATTTTCAAAATTTA Fi: GGAATGAAAAATTAGAACAACAGAACA Ri: TTTGCTAAAGAAATAGAAGTGGCTTACAAC	423 (wild type) 272 (A allele) 209 (G allele)

***** Designed in this study for CLEC7A rs3901533 (Dectin gene).

****** (Hashemi-Shahri *et al*., 2014).

3.3. Patients and Samples:

3.3.1. Collection of Samples:

Samples were collected over a period of 4 months from (Nov.-2021 to Feb-2022). Clinical samples were collected from patients whom lying in Intensive care department from Marjan Hosptial (Marjan medical city) in Babylon province. Samples were taken from the patients under the supervision of the specialist physician. After diagnosis of Covid-19 infection of the patient, aspiration samples were taken for both sexes and different age groups ranged between 22_89 years old . The questionnaire was used for each patient which included some important information like name, age, gender , the side effect ,antibiotics ,duration of infection , chronic diseases, the nature of the drugs used. The samples were transferred to the laboratory of the college of science for women for the purpose of culturing.

3.3.1.1. Collection of Oral Swabs:

One hundred and forty six oral swabs samples are collected about 56 samples from patients with Covid-19 infection, 50 samples from recovery persons and 40 samples from healthy people by swabs with transport media.

3.3.1.2. Collection of Blood Samples:

Three ml of blood were collected 60 blood samples taken from 20 patients, 20 recovery persons and 20 healthy people. Blood injected 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 using in molecular study.

3.4. Methods:

3.4.1. prepration of Solutions and Stains:

3.4.1.1. Lacto phenol cotton blue stain: This stain is prepared by mixing 20g of phenol nitrate, 20ml of lactic acid and 40ml of glycerol in 20 ml of distilled water. Then mixed well with heating with water bath and then (0.05g) of a blue cotton stain is added. It is used to stain hyphae and chlamydospores and to distinguish the different microscopic structures (Koneman and Roberts, 1985).

3.4.1.2. Normal saline solution: This solution is prepared by dissolving 0.85 gm of Nacl in 90 ml D.W. then completed to 100 ml with D.W. Autoclaved at 121 °C for 15 min (Prough and Bidani, 1999).

3.4.1.3. Tries EDTA buffer (TE buffer): This buffer is prepared by dissolving 0.05 M Tries-OH and 0.001 M, EDTA in 800 ml D.W. The pH is adjusted to 8 and completed to one liter by D.W. Then sterilized by autoclaving and stored at room temperature until used for DNA hydration.

3.4.1.4. Safranin Stain: it is prepared by dissolving 1 g of safranin stain in 100ml of distilled water then sterilized in autoclave. The stain was used information of biofilms test.

3.4.1,5. Urea solution 29%: This solution is prepared by dissolving 29 gm of urea powder in 100ml of distilled water, then sterilized by Millipore filter with 0.22 µm pores.

3.4.2. Prepration of Culture Media:

3.4.2.1. Sabouraud Dextrose Agar medium (SDA):

According to the manufacture's instruction, this medium is prepared by suspending 65 gm of SDA powder in 500 ml of distilled water and homogenized by magnetic stirrer and complete the volume to 1000 ml. 0.05 gm of Chloromenphenicol is added for each to prevent growth of bacteria.

0.5 gm of Cyclohexomide is added to prevent growth of saprophytic fungi. Then mixed and sterilized by autoclave. This medium is used for culturing and maintaining the pathogenic fungi and yeast isolates (Odds,1991).

3.4.2.2. CHROMagar Candida Medium:

According to the manufacture's instruction, this medium is prepared by suspending 30 gm of CHROMagar candida powder in 1000 ml of distilledwater, and then heated until it dissolved completely. This medium is used for preliminary identification of Candida spp. (Horvath et al., 2003).

3.4.2.3. Sabouraud Dextrose Broth medium (SDB):

According to the manufactures instruction this medium is prepared by suspending 30 gm. of medium in 1000 ml of distilled water. Then 0.05 gm of Chloromenphenicol is added and sterilized by autoclave. This medium is used for yeasts ability for biofilm formation (Odds,1991).

3.4.2.4. Egg yolk Agar medium (EYA):

procedures by suspending 65 gm. of SDA and 58.4 gm. of Nacl and 5.5 of Cacl2 in 980 ml of distilled water, and then sterile by autoclave, and taken Egg yolk and centrifuge for 30 minutes 5000 cycle. 2% of supernatant is taken and added to sterilize medium and cooled. This medium is used for determining yeasts viability to produce phospholipase enzyme This medium is prepared according to the Price *et al*.,(1982) and Samaranayake *et al*.,(2006).

3.4.2.5. The Tween 80 opacity test medium:

This medium is prepared by dissolving 10gm of peptone, 5gm Nacl, 0.1gm from $CaCl_2$,15 gm Agar in 1000 ml of distilled water and sterilized in Autoclave, then cooled to about 50˚C and then 5ml of autoclave tween 80 was added to sterilized medium (Aktas *et al.*,2002). This medium is used to determine the viability of yeasts to produce lipase enzyme.

3.4.2.6. Potato Dextrose Agar medium:

According to the manufacture's instruction, this medium is prepared by suspending 39 gm of medium in 1000 ml of distilled water with added 250 mg of chloramphenicol that prevent growth of bacteria and sterilized by autoclave. This medium is used to isolation of yeasts from swabs.

3.4.2.7. Blood Agar medium:

This medium was all set by solution of 40 gm of Sabouraud dextrose agar in 1000ml of distilled water according to the instruction of the manufacturing , after autoclaving and cooling human blood about 50 ml\l add to the medium to detect the capacity of some isolated yeast to causes hemolysis.

3.4.2.8. Urea medium:

This medium is prepared according to manufactured company (Oxoid) by dissolving 2.4gm of Urea agar base in 95 ml of distilled water with pH 6.8 and sterilized by autoclave. After cooling is added 5 ml of urea solution which sterilized by Millipore filter, mixed well and distributed in each test tube 5 ml, this medium is used to determine the viability of yeasts to produce urease enzyme.

3.4.3. Sterilization:

3.4.3.1. Sterilization by autoclave: All the culture media are sterilized using the autoclave device at a temperature of 121°C and a pressure of 15 lbs. for 20 minutes. All the glassware used in the laboratory sterilized using the oven 150°C one hour as well as some of the tools used in the extraction such as the Eppendorf tube tips and PCR tubes are also sterilized.

3.4.3.2. Sterilization by formalin: Sterilization by formalin is performed by adding 15 ml of formalin into Petri dish and left for 24-48 h for the purpose of sterilizing the incubator.

3.4.3.3. Sterilization by heating and alcohol spirit: Sterilization of the indoor of hood pang surfaces by alcohol while needle, tongs other steel tools is sterilized by heating.

3.4.3.4. Sterilization by Millipore filter paper: All the chemicals which denatured by heating are sterilized using Millipore filter paper such as urea.

3.4.4. Cultivation of Specimens:

Clinical specimens are cultured on sabourauds dextrose agar medium. The swabs are cultured on SDA by streaking and incubate at 28-30˚C for (24-48 h) to yeast isolates and for 7 days to molds isolates. Then single colonies from any yeast isolates are pickup and streaking on CHROMagar medium, incubated at 37˚C for 24-48. All isolations (yeast and molds) are isolated in pure culture on PDA medium.

3.4.5. Microbiology Identification:

3.4.5.1. 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.

3.4.5.2.Microscopic examination: Fungi isolates are examined microscopically, taken the fingerprint 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 Leck, A. (1999).

3.4.5.3. CHROMagar 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).

3.4.6. Evaluation of Virulence Factors of Yeasts:

3.4.6.1. Urease Production Assay:

Susceptibility of yeast to produce the urease enzyme is determined by inoculated the urea agar medium which distributes in test tubes with yeasts isolations, then incubate in 28˚C to 24-48 h and the positive result is the change of yellow color in medium to pink color (Barbosa *et al*., 2013).

3.4.6.2. Lipase Production Assay:

Susceptibility of yeast to produce the lipase enzyme is determined by taking the colony of yeasts on the SDA medium for the 24 h then took 10 µl from yeast and inoculated the Tween 80 opacity test medium, then incubate in 37˚C to 72 h and the positive result was the formation of a white precipitate about developing the colony (price et al .,1982).

3.4.6.3. Phospholipase Production Assay:

Yeasts isolations are screened for the production of extracellular phospholipase activity by growing them with egg yolk and measuring the size of the zone of precipitation as follow:

A 10 µl suspension is placed on the egg yolk medium in petri dish and left to dry at room temperature. The culture is then incubated at 37˚C for 48 h, after which the colony diameter plus precipitation zone are measured for each isolation. Calculation of the zone of phospholipase activity is performed according to (price *et al*.,1982) method: Colony diameter/Colony diameter+ zone of precipitation = Pz Pz coefficient (Gokce *et al*.,2007) is classified as:

1- Negative(Pz = 1.00).
2- Positive(0.64 ≥ Pz < 1.00).
3- Strongly positive (Pz < 0.640).

3.4.6.4. Biofilm Formation Test:

Colonies of yeasts isolations are inoculated in saline and incubated overnight at 37°C. 0.5 ml of saline suspension which is added into screw capped conical polystyrene tubes containing 50 ml of Sabouraud dextrose broth enhanced by glucose (the ending concentration of 8%). And the tubes are incubated in 35°C for 48 h. After incubation the broth from the tubes is aspirated gently using Pasteur pipette. The tubes are washed twice with distilled water and stained with 2% safranin. The stain is decanted after 10 minutes. The tubes are rinsed with distilled water to remove excess stain. Presence of visible adhesive film on the wall and at the bottom of the tube indicated biofilm formation. Ring formation at the liquid interface was not considered as an indication of biofilm production (Deorukhkar and Saini, 2014).

3.4.6.5. Hemolysis Test:

Viability of yeasts to hemolysis is 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).

3.4.7. Susceptibility Test to Antifungal Drugs:

3.4.7.1. Preparation of Yeast Suspension:

Yeast suspension is prepared as mentioned by NCCLS M27-A2 (2002). Fresh yeast colonies are transferred from SDA medium into tube containing 5ml from the normal saline, shake gently. Sporulation was assessed by Hemocytometer according to the equation:

The average number of spores\ml = The average number of spores in five Squares * 25 * 10^4 (Sibounnavong *et al.*,2009).

3.4.7.2. Antifungal Activity Against Growth of Yeasts:

The surface of the SDA medium inoculate by using sterile cotton swabs after placing them in the yeast suspension, then remove the excess suspension from the cotton swab by rotating it on the edge of the tube, then it is streaked on the agar with the swab so that the suspension is distributed evenly throughout the agar, leaving the dish for 5- 15 minutes to allow the suspension to be absorbed and until the surface of the agar dries, the tablets of the antifungals under study were placed at equal distances on the surface of the agar using sterile forceps and incubated at a 35-37°C. After 2 days, the inhibition zone by disk deffusion method was measured in mm for each antifungal agent using a metric ruler.

3.4.8. Molecular Study:

3.4.8.1. Solutions and Materials Used in The DNA Extraction:

The extraction and purification of DNA were performed by using FAVORGEN kit, which include the solutions in table (3-5).

Several solvents for extraction and purification were maintained at temperature 2-8˚C until used. In addition to the existing solution in several extraction solvents, other materials were used in the extraction and purification of DNA.

3.4.8.2. DNA Extraction From Blood:

The DNA extraction was carried out according to the company's instructions attached to the FAVORGEN kit as in Appendix 1**.**

3.4.8.3. Solution and Material Used In Electrophoresis:

1- 6X DNA loading dye supplied by promega Corporation.

2- Solution of Ethidium Bromide stain supplied by BDH- Chem –Ltd .boo (0.8) µl.

3- Agarose used from Norgen Biotic Comp.

4- DNA ladder 100 bp supplied by Intron Comp.

5- T.B.E buffer solution supplied by Promega Comp.

3.4.8.4. DNA Electrophoresis:

1- At first, 100 ml of the T.B.E buffer was placed in a beaker.

2- Then 1 g weight of agarose was added to the buffer.

3- the buffer with the agarose was heated on a hot plate to boiling point so that all of its components were solvent.

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

5- Ethidium Bromide dye was added at 0.5 µl to the agarose before solidification of the liquid and mixed it well.

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

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

8- The migration electric basin was filled with the T.B.E buffer solution so that it rose from the gel surface.

9- The samples were placed in the pits with the addition of the agarose gel loading dye so that the dye was linked to the DNA .

10- The electrophoresis was performed in 70 V to 45 min.

11- The agarose gel was exposed to UV trans illuminator for DNA bands visualized and documented.

3.4.9. PCR Assay:

3.4.9.1.Preparation of Primers for the Polymerase Chain Reaction Technique (PCR):

The primers were prepared by adding distilled water free of nuclease in the a different volume according to the manufacturing company instructions to obtain a solution of base stock with a concentration of 100 Pico mole / µl, mixed by vortex, then centrifuged for 10sec at 4000 rpm. Then 10 µl of each primer was taken and putt in the micro centrifuge tube with 90 µl of nuclease free distilled water to prepare the working solution.

3.4.9.2. PCR Mixture:

PCR mixture for all primers used in this study was prepared according to the table (3-7).

Table (3-7): Volumes of chemical materials uses in PCR assay.

Chemical materials	Volumes
Master Mix	5 µl
DNA	1µl
Forward Primer	1 µl
Reveres Primer	1 µl
Deionizer D. W	18 µl
Total	= 26 µl

3.4.9.3. PCR Conditions:

The conditions of PCR reaction for two primers used for polymorphism of human genes as shown in table (3-8).

Table (3-8): The conditions of PCR for two primers.

No.	TLR8 SNP rs3764880 A/G			
	Stages	**Temperature**	**Time**	**Cycles**
1.	Pre denaturation	95°C	5 min	1
2.	Denaturation	95°C	30 sec	30
	Annealing	50°C	30 sec	
	Extension	72°C	30 sec	
3.	Final extension	72°C	5 min	1
4.	Cooling	4°C	∞	
	CLEC7A rs3901533			
	Stages	**Temperature**	**Time**	**Cycles**
1.	Pre denaturation	95°C	5 min	1
2.	Denaturation	95°C	30 sec	30
	Annealing	55°C	30 sec	
	Extension	72°C	40 sec	
3.	Final extension	72°C	5 min	1
4.	Cooling	4°C	∞	

3.4.9.4. PCR Gel Electrophoresis:

The amplified PCR products were detected by agarose gel electrophoresis which was visualized by staining the Ethidium bromide. The electrophoresis result was detected by using gel documentation system. The positive result was distinguished when the DNA band base pairs of sample equal to the target product size (Bartlett and sterling, 2003).

or the size of amplified DNA fragments which were identified by a comparison with molecular size marker DNA (100 - bp DNA ladder).

3.4.9.5. Detection Of Genes Polymorphisms of PCR Products:

3.4.9.5.1.Detection of CLEC7Ars3901533 by Sequencing Analysis:

The 60 PCR products of CLEC7A rs3901533 gene (957 bp) were directly sequenced by Macrogen Company (Korea). After received the sequencing data for PCR products which are compared with gene bank by using NCBI Blast nucleotide database. Only clear chromatographs obtained from ABI sequence files were further analyzed to ensure that themannotation and variations are not due to PCR or sequencing artifacts. 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 BioEdit for multiple alignment sequence based on editor Software version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

3.4.9.5.2.Detection of TLR8 SNP rs3764880 A/G by T- ARMS:

This polymorphism was detected by Tetra- primer Amplification Refractory Mutation System (T- ARMS) of PCR technique. The size of the PCR products of amplified DNA fragments identified by a comparison with the molecular size marker DNA (100-bp DNA ladder) and coded as following:

423 bp = wild type

272 bp = A allele

209 bp = G allele

3.4.10. Statistical Analysis:

Analysis is carried out using SPSS version 22, numerical data was expressed as mean and standard deviation, qualitative data were expressed as frequency and percentage. independent -sample t test used to compare between two groups, chi-square test used to detect if any relation between ordinal and nominal variable in this study. P value of ≤ 0.05 is considered significant (Iuliano and Franzese,2018).

Chapter Four

Results and Discussion

4. Results and Discussion:

4.1.Distribution of Gender According to Study Groups:

Distribution of gender according to study groups show in the table (4-1), that (55.4%) of patients group gender were male, while (60%) of control group and (64%) of recovering were female. Also this table show that a non-significant differences between the three groups regarding to gender. The statistical analysis showed that males are more susceptible to infection than females. The number of infected males was 31 (55.4%), compared to 25 (44.6%) females. As for the numbers of recoveries from the disease, the number of males is less than females. Where the number of males reached 18 (36%), while the number of females reached 32 (64%).

Table(4-1):Distribution of gender according to study groups.

Gender		**Groups**						***p-value**
		Patients		**Control**		**Recovering**		
		N	%	N	%	N	%	
	Male	31	55.4	16	40.0	18	36.0	.107 N.S
	Female	25	44.6	24	60.0	32	64.0	
Total		56	100.0	40	100.0	50	100.0	

**Kruskal-Wallis Test, N.S= non-significance at p > 0.05*

A further factor of heterogeneity in mortality due to COVID19 is gender where large sex differences exist with men having higher risk compared to women, which might have to do with the sex hormones like testosterone and oestrogen that seems to be key in adapting the body's immune response and the presence of other risk factors, e.g. diabetes, hypertension and cardio-vascular diseases,that are affecting more men than women (Dowd *et al*., 2020 ; Caramelo 2020).

4.2. Distribution of Age According to Study Groups:

Table (4-2) show distribution of age according to study groups, that a third (35.7%) of Patients group age within (65-74) years old while more than one quadrant(27.5 %) of control group age and more than two fifth (42 %) of Recovering group were within (25-34) years. Also this table show that a highly significant differences between the three groups regarding to age. As for the age groups shown in this table, it was found that the ages ranging from (65- 74) are more susceptible to Covid-19 disease. The rate of exposure to infection was 20 samples, where their rate was (35.7%).

Table(4-2):Distribution of age according to study groups.

Age groups (years)	Groups						*p-value
	Patients		Control		Recovering		
	N	%	N	%	N	%	
15-24	1	1.8	10	25.0	8	16.0	.001 H.S
25-34	3	5.4	11	27.5	21	42.0	
35-44	4	7.1	9	22.5	5	10.0	
45-54	5	8.9	8	20.0	13	26.0	
55-64	10	17.9	0	0	3	6.0	
65-74	20	35.7	0	0	0	0	
75-more	13	23.2	2	5.0	0	0	
Total	56	100.0	40	100.0	50	100.0	
Mean (SD)	62.98 (15.39)		35.62 (14.73)		34.88 (11.21)		
Minimum – Maximum	22 -90		14- 82		20-55		

**one way- ANOVA, H.S= highly significance at p > 0.05*

The percentage of those recovering from Covid-19 disease and the control percentage was between the age groups (25_34). Where the number of people recovered reached 21 (42%) and the control number was 11 (27.5%) (table 4-2).

Four key demographic factors affect COVID-19 epidemiology, *viz*., advanced age (median age of death—75 years); gender (male sex), immunocompromisation and underlying co-morbidities such as cardiovascular disorders, diabetes mellitus, chronic respiratory disease and hypertension (Zheng *et al*.,2020). High infection and mortality rates highlight the interplay of poor healthcare, poverty, geographical disparity, economic and social risk factors (Egede *et al*.,2020). Old age has been identified as a risk factor to be affected compared to young and middle-age which has mostly to do with the lower immunity due to frailty and higher prevalence of chronic illnesses in the elderly population. A further factor of heterogeneity in mortality due to COVID19 is gender where large sex differences exist with men having higher risk compared to women (Dowd *et al*., 2020 ; Caramelo 2020),

The risk for severe illness with COVID-19 increases with age, the virus is highly contagious for elderly individuals, not only due to a higher rate of mortality (Baud *et al*., 2020), but also due a higher proportion of cases. In essence, aging populations may be at increased risk from a two-fold effect. If a population has a higher proportion of elderly, the proportion of confirmed Covid-19 cases would be higher, accentuated further if no normal tests are made. This is substantially different than what is typically reported for influenza or other pandemics which tend to have higher morbidity for younger individuals (Simonsen *et al*., 1998; Reichert et al., 2004). Older age and history of coronary vascular disease were reported to increase the risk of death from Covid-19 (Porcheddu et al., 2020). Some studies evaluating the risk factors of mortality in patients with

COVID-19 reported higher death rates with increasing age (Casqueiro *et al*., 2012; Al-Khikani, 2020; Zhou *et al*., 2020).

4.3. Distribution of Chronic Disease According to Study Groups:

Table (4-3) show distribution of Chronic disease according to study groups that (14.3%) of patients group and (10%) of control group chronic disease were hypertension, while (8%) of recovering chronic disease were diabetes mellitus. Also this table show that a non-significant differences between the three groups regarding to chronic disease.

This results agrees with (Yin *et al*., 2021), that prevalence of some commodities was lower in COVID-19 patients than that in general population (healthy peoples) such as hypertension (19% vs 23.2%), diabetes (9% vs 10.9%), chronic kidney disease (CKD) (2% vs 9.5%), chronic liver diseases (CLD) (3% vs 24.8%) and chronic obstructive pulmonary disease (COPD) (3% vs 8.6%), while some others including cancer (1% vs 0.6%), cardiovascular disease (6% vs 1.8%) and cerebrovascular disease (2% vs 0.9%) exhibited greater percentage in COVID-19.

It is unclear whether uncontrolled blood pressure is a risk factor for acquiring COVID-19, or whether controlled blood pressure among patients with hypertension is or is not less of a risk factor. However, several organizations have already stressed the fact that blood pressure control remains an important consideration in order to reduce disease burden, even if it has no effect on susceptibility to the SARS-CoV-2 viral infection (Iheanacho *et al*., 2021**).**

In fact, ARBs have been suggested as a treatment for COVID-19 and its complications.13 Increased soluble ACE2 in the circulation could bind SARS-CoV-2, reducing its ability to injure the lungs and other ACE2

bearing organs.14 Using recombinant ACE2 could be a therapeutic approach in COVID-19 to reducing viral load by binding circulating SARS-CoV-2 viral particles and reducing their potential attachment to tissue ACE2. None of these possibilities have however been demonstrated in patients yet (Batlle *et al*., 2020; Fang *et al*., 2020).

Table(4-3): Distribution of Chronic disease among group of study.

Chronic disease		Groups			p-value
		Patients	Control	Recovering	
No disease	N	37	33	40	
	%	66.1%	82.5%	80.0%	
Hypertension	N	8	4	3	
	%	14.3%	10.0%	6.0%	
Cancer	N	4	3	1	
	%	7.1%	7.5%	2.0%	.102
Diabetes mellitus	N	4	0	4	N.S
	%	7.1%	0.0%	8.0%	
Asthma	N	3	0	2	
	%	5.4%	0.0%	4.0%	
Total	N	56	40	50	
	%	100.0%	100.0%	100.0%	

** one way- ANOVA, N.S= non-significance at p > 0.05*

There is as yet no evidence that hypertension is related to outcomes of COVID-19, or that ACE inhibitor or ARB use is harmful, or for that matter beneficial, during the COVID-19 pandemic. Use of these agents should be maintained for the control of blood pressure, and they should not be discontinued, at least on the basis of current evidence at this time.

4.4.Distribution of Signs and Symptoms Among Recovery Group:

Table (4-4) show the distribution of signs and symptoms among recovery group, which revealed that (24%) of sample having shortness of breath, (24%) Coughing, (44%) Tiredness and fatigue, (14%) Joint pain, (10%) Headache, (6%) Amnesia, (12%) Fever , (4%) Loss of smell.

Table(4-4):Distribution of signs and symptoms among Recoverygroup.

Signs and symptoms		N= 50	Percent
Shortness of breath	*Positive*	12	24.0
	Negative	38	76.0
Coughing	*Positive*	12	24.0
	Negative	38	76.0
Tiredness and fatigue	*Positive*	22	44.0
	Negative	28	56.0
Joint pain	*Positive*	7	14.0
	Negative	43	86.0
Headache	*Positive*	5	10.0
	Negative	45	90.0
Amnesia	*Positive*	3	6.0
	Negative	47	94.0
Fever	*Positive*	6	12.0
	Negative	44	88.0
Loss of smell	*Positive*	2	4.0
	Negative	48	96.0

It is noted that most of the injuries were treated at home, and the reason may be due to the overcrowding of hospitals and the lack of the condition for distancing in them. The typical symptoms of covid-19 include fever, sore throat, fatigue, cough, and dyspnoea combined with recent exposure (Gavriatopoulou *et al*., 2020).

An observational study of in 1420 European patients with mild or moderate disease indicated that the most common symptoms were headache (70.3%), loss of smell (70.2%), nasal obstruction (67.8%), cough (63.2%), asthenia (63.3%), myalgia (62.5%), rhinorrhoea (60.1%), gustatory dysfunction (54.2%) and sore throat (52.9%). Fever was reported by on 45.4% recover without hospitalization (Zhang et al., 2021). The five most common symptoms at admission were history of fever, shortness of breath, cough, fatigue/malaise, and confusion (ISARIC, 2020). An analysis of data from 4203 patients mostly from China identified fever, cough and dyspnoea (80.5%, 58.3% and 23.8%, respectively) as the most common clinical symptoms, and hypertension, cardiovascular disease and diabetes(16.4%, 12.1% and 9.8%, respectively) as most common comorbidities (Zhang *et al*., 2021).

This results also, agrees with several studies as (Wang et al., 2020) that found the most common symptoms were fever 87%, cough 55%, and fatigue 42% of patients were confirmed with SARS-CoV-2. Likewise, Chen *et al*., (2020) that found, most Clinical signs and symptoms of patients with 2019-nCoV pneumonia were fever (83%) or cough (82%) and a third of patients had shortness of breath (31%) and other symptoms included muscle ache, headache, confusion, chest pain, and diarrhea.

A study among 20133 hospitalised patients from acute care hospitals in England, Wales and Scotland identified clustering of symptoms with three common group: one respiratory symptom cluster with cough, sputum, shortness of breath, and fever; a musculoskeletal symptom group with

myalgia, joint pain, headache, and fatigue; and a cluster of enteric symptoms with abdominal pain, vomiting, and diarrhoea (Docherty *et al*., 2020).

4.5. Cultivation of Specimens:

The result of cultures are summarized in table (4-5) with 146 specimens of three groups (patients 56 , recovering 50, control 40). The proportion of the total positive samples of three categories is 83 (56.85%). Where the percentage of Patients was 43 (29.45%), the percentage of those who recovered was 20 (13.70%), and the control rate was 20 (13.70%) . While the percentage of the total negative samples was 63 (43.15%) . Where the percentage of the patient was 13 (8.90%), the recovery rate was 30 (20.55%), and the control percentage was 20 (13.70%).

Table(4-5): Distribution fungal infection according of study groups.

Groups / Result	Positive (%)	Negative (%)	Total (%)
Patients	43 (29.45)	13 (8.90)	56 (38.35)
Recovering	20 (13.70)	30 (20.55)	50 (34.25)
Control	20 (13.70)	20 (13.70)	40 (27.40)
Total	**83 (56.85)**	**63 (43.15)**	**146 (100)**

4.6. Isolation and Identification:

4.6.1. Distribution of Fungi Species of Patients Group:

The highest percentage of positive samples for yeasts was *C. albicans* 25 (44.6%) where was the number of its colonies 3,779, and the lowest percentage was *C. tropicals* 3(5.4%) where was the number of its colonies 155. But as for molds, the highest percentage of positive samples was *A. niger* 2 (3.6%), and the lowest percentage was distributed among the *Penicillium* genera, (*P. chrysogenum, P. notatum*) and *C. cladosporioides* 1 (1.8%) where was the number of its colonies 1 (table 4-6). Figure (4-1) show that the highest percentage of isolated fungi from infected group were *C. albicans*.

Table(4-6):Distribution of fungi species that isolated from patients group.

Funal species	Positive samples (%)	Negative samples (%)	N. of colonies	Total (%)
A.niger	2 (3.6%)	54 (96.4%)	2	56 (100%)
C.albicans	25 (44.6%)	31 (55.4%)	3,779	
C.dubliniensis	4 (7.1%)	52 (92.9%)	260	
C.glabrata	4 (7.1%)	52 (92.9%)	685	
C.kruzei	4 (7.1%)	52 (92.9%)	1,150	
C.parapsilosis	10 (17.9%)	46 (82.1%)	880	
C.tropicalis	3 (5.4%)	53 (94.6%)	155	
C.cladosporioides	1 (1.8%)	55 (98.2%)	1	
P.chrysogenum	1 (1.8%)	55 (98.2%)	1	
P.notatum	1 (1.8%)	55 (98.2%)	1	

This results also, agrees with (Wang *et al*., 2020) that, the *C. albicans* was 40% isolated from patients were confirmed to be infected with SARS-CoV-2. Oral pharyngeal candidiasis was studied in hospitalized COVID-19 patients to identify the various *Candida* isolates. The study documented *C. albicans* as the most frequent type of organism (Salehi *et al*., 2020).

Another case study on a 47 year old female patient who was tested positive for COVID-19 infection showed pseudomembranous candidiasis (Riad *et al*., 2020). Interestingly, a published report of asymptomatic COVID-19 patient mentioned the presence of oral candidiasis (Corchuelo and Ulloa, 2020). Another published data revealed two patients with oral candidiasis in patients with no risk factors like immunosuppression or prolonged antibiotic use (Baraboutis *et al*., 2020).

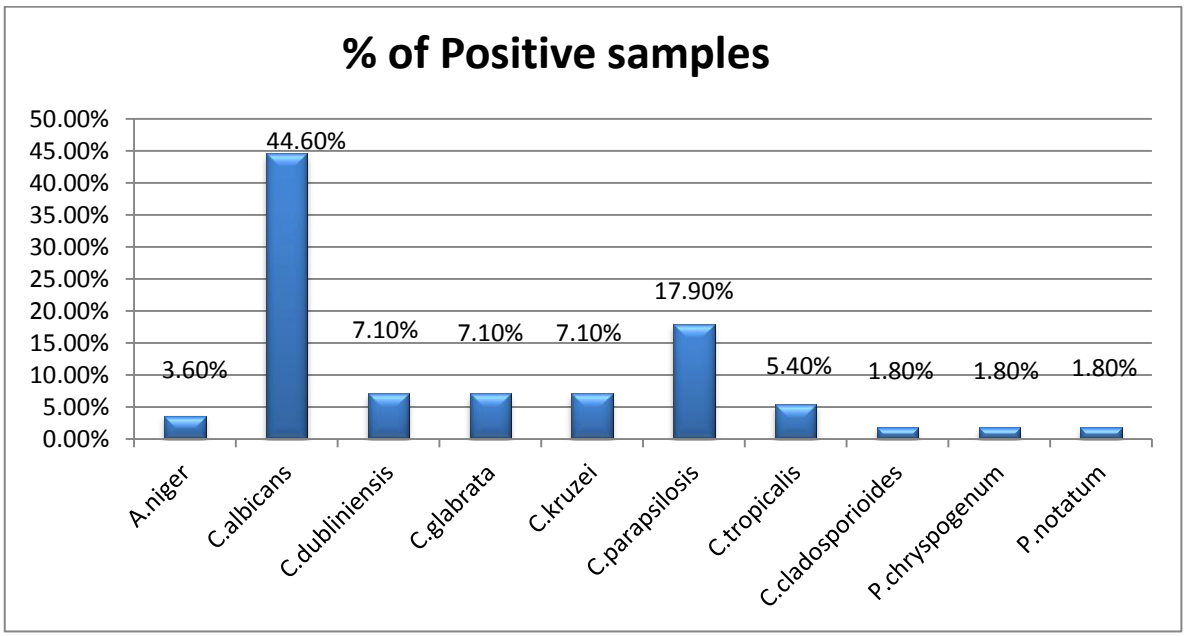


Figure (4. 1): Percentage of fungi that isolated from patients group.

Nucci *et al*., (2021) they studied the incidence of candidemia of hospitalized COVID-19 patients, they found *C. albicans* with percent 55.6% followed by *C. tropicalis* with 22.2% and *C. glabrata* with 11.1%. Similarly, in another study from India during the current pandemic (Meyer et al., 2013) ,64% of candidemia cases were due to non-albicans Candida species. Fungal superinfections have been observed in COVID-19, with COVID-19-associated candidiasis representing one major complication (Lai *et al*., 2020; Mastrangelo *et al*., 2020; Song *et al*., 2020).

Invasive mold infections caused by *Aspergillus* have increasingly been reported in patients with severe COVID-19, the incidence was 26.7% includes 14.1% aspergillosis and 12.6% yeast infections (White *et al*., 2021). Despite the recognition that airborne *Aspergillus fumigatus* is increasingly recognized as an important cause of fungal super-infections among critically ill COVID-19 patients (Arastehfar *et al*., 2020).

4.6.2.Distribution of Fungi Species of Control Group:

The results that were isolated from the control group showed a diversity between(molds and yeasts), where the highest percentage of yeasts was for *C. albicans* 6 (15.0%) and the number of colonies 24 and the lowest percentage was for *C. tropicalis* 1(2.5%) and the number of colonies is 2.

As for the results of molds, the highest percentage was for *A. terreus* 4(10.0%) and the number of colonies 4, and the lowest percentage was distributed between the genera *Penicillium* (*P. chrysogenum, P .notatum*) 1(2.5%) and the number of colonies 1 and the genera *Cladosporium* (*C. cladosporioides*) 3(7.5%) number of colonies 16, *C. herbarum* 2(5.0%) number of colonies 3, and *C. sphaerospormum* 1(2.5%) number of colonies is 20 (Table 4-7; fig. 4-2).

Table(4-7): Distribution of fungi species that isolated from control.

Fungal species	Positive samples (%)	Negative samples (%)	N. of colonies	Total (%)
A .terreus	4 (10.0%)	36 (90.0%)	4	40 (100%)
A.niger	3 (7.5%)	37 (92.5%)	6	
C.albicans	6 (15.0%)	34 (85.0%)	24	
C.glabrata	2 (5.0%)	38 (95,0%)	2	
C.tropicals	1 (2.5%)	39 (97.5%)	2	
C.parapsilosis	3 (7.5%)	37 (2.5%)	3	
C.cladosporioides	3 (7.5%)	37 (92.5%)	16	
C.herbarum	2 (5.0%)	38 (95.0%)	3	
C.sphaerospormum	1 (2.5%)	39 (97.5%)	20	
P.chrysogenum	1 (2.5%)	39 (97.5%)	1	
P.notatum	1 (2.5%)	39 (97.5%)	1	

This results agrees with (Nucci *et al*., 2021) they studied the incidence of candidemia of non- COVID-19 persons, they found *C. albicans* with percent 43.8% followed by *C. Parapsilosis* with 25.0% and *C. tropicalis* with 18.8%. Oral mycological conditions range from superficial to deep fungal infections of the oral tissues. The most frequently diagnosed and reported oral fungal infections are the superficial type and candidiasis (Santosh *et al*., 2021).

The predominance of oral candidiasis is being diagnosed probably due to the increasing number of individuals with immunocompromised conditions and dentists who are very experienced in the recognition and differentiation of oral candidiasis from other types of fungal infections

(Singh *et al*., 2014). Whereas the organism *Candida* may be cytologically/ histological detected in an individual in the absence of a clinical oral condition, i.e., *Candida* can be seen in healthy individuals as a normal commensal. A microbiology report with a positive culture for *Candida* from an oral sample with no clinical presentation should eliminate the diagnosis of oral candidiasis, since *Candida* is a common commensal of the oral cavity (Rajendra *et al*., 2021; Coronado-castellote and jimenez-Soriano, 2013).

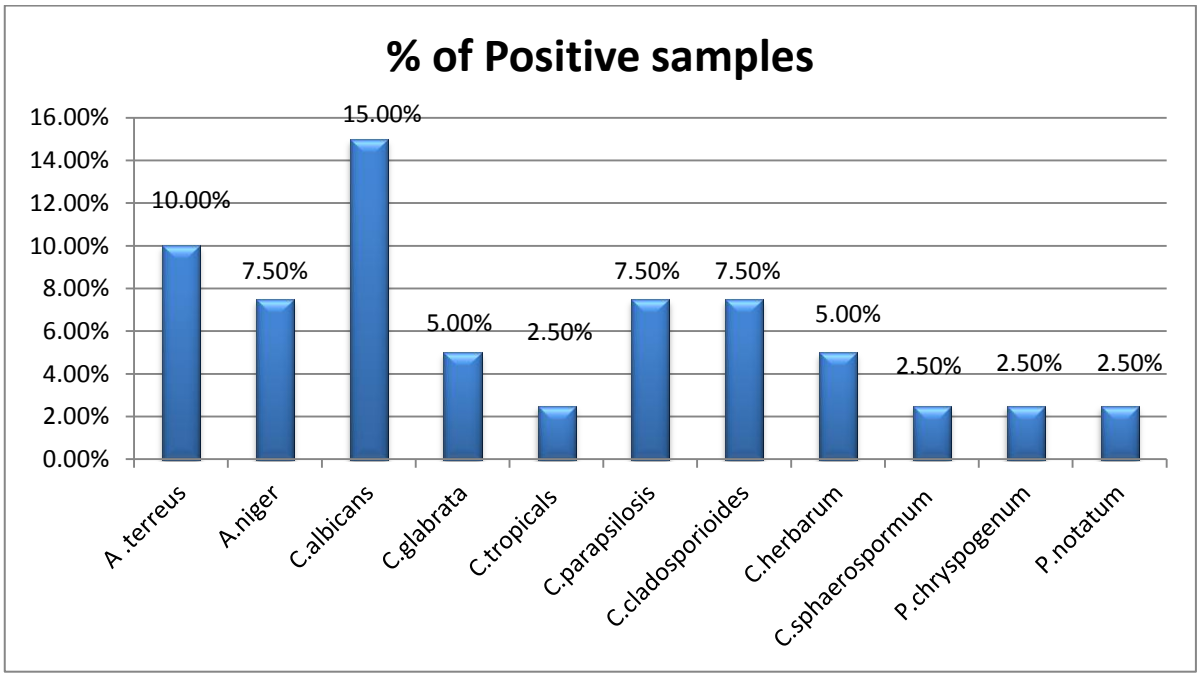


Figure (4. 2): Percentage of fungi that isolated from control group.

In both healthy and immunocompromised individuals, colonization of the oral cavity by *Candida* spp. is a risk factor for initiation of oral candidiasis (Cannon and Chaffin, 2001; Rosa-Garcia *et al*., 2013). Oral candidiasis, also known as oral thrush, is candidiasis that occurs in the mouth (fungal infection) by invading of *Candida* species on the mucous membranes of the mouth (Rao *et al*., 2015).

4.6.3. Distribution of Fungi Species of Recovering Group:

Positive culture results that appeared in the group of the recovered. The highest percentage of yeasts was *C. albicans* 10(20.0%), where the number of colonies was 4, and the lowest percentage was *C. tropicals* 1(2.0%) so that the number of colonies was 2. As for the results of molds, the highest percentage was *A. niger* 3(6.0%) , the number of colonies was 1. and the lowest percentage was *C. cladosporioides* 1(2.0%) the number of colonies of 25 (Table 4-8, Fig. 4-3).

Table(4-8): Distribution of fungi species that isolated from recovering group.

Funal species	Positive samples (%)	Negative samples (%)	No. of colonies	Total (%)
A.niger	3 (6.0%)	47 (94.0%)	3	50 (100%)
C.albicans	10 (20.0%)	40 (80.0%)	40	
C.kruzei	3 (6.0%)	47 (94.0%)	9	
C.parapsilosis	2 (4.0%)	48 (96.0%)	2	
C.tropicals	1 (2.0%)	49 (98.0%)	2	
C.herbarum	2 (4.0%)	48 (96.0%)	2	
C.cladosporioides	1 (2.0%)	49 (98.0%)	25	

There are case reported of mucormycosis after recovery from Covid-19, the patient was a 73 years old woman with a history of chronic kidney disease, diabetes mellitus, hypertension, and dyslipidemia, clinicians should consider mucormycosis and its complications after Covid-19 treatment in diabetic and immunocompromised patients (Alian *et al*., 2022). Another case, a 62-year-old man recovered from COVID-19, but unfortunately, after few days, he contracted a fungal infection in the brain and diagnosed with *Aspergillus* sp. (Gupta *et al*., 2021).

An invasive fungal infection should be suspected in patients who have recently recovered from COVID-19 pneumonia and present with acute destructive rhinosinusitis. There are few cases of Invasive fungal rhinosinusitis (IFRS) reported in Europe during the pandemic of COVID-19, its *Aspergillus niger* was identified (Chatzisouleiman *et al*., 2022).

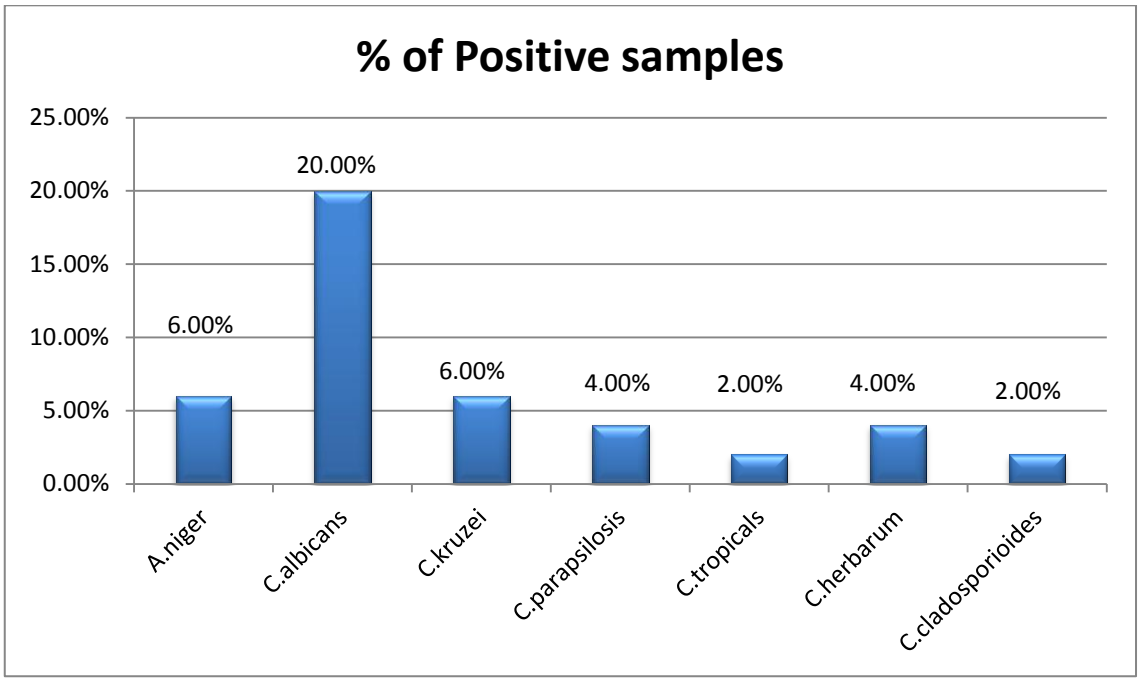


Figure (4. 3): Percentage of fungi that isolated from recovering group.

It has been suggested that the incidence of IFRS is higher in post COVID-19 patients than in non-COVID-19 patients, notably, in immunocompetent patients (Ebeid *et al*., 2021; Ismaiel *et al*., 2021). Research on SARS-CoV and SARS-CoV-2 showed remarkable similarities in their mechanisms, both increasing the incidence of fungal invasion. Aspergillosis of sinuses is reported among recently recovered patients from COVID-19 (30.6%) (El-Kholy *et al*., 2021).

Direct damage of the respiratory epithelium by COVID-19 virus associated with anoxia induced by pneumonia and pulmonary thromboembolism predisposes the lung to invasive *Aspergillus* infection. Although a direct causation cannot be established in this case, immune modulation by COVID-19 infection, coexisting comorbidities and steroid use are considered to be responsible for spread of fungal infection to the brain (Chauvet *et al*., 2020).

Recent case reports have indicated the incidence of mucormycosis And other fungal infections in COVID-19 serious cases after recovery (Maini *et al*., 2021; Waizel-Haiat *et al*., 2021; Kumar, 2022)

4.7. Identification by CHROMagar Medium:

CHROMagar a quick test to diagnose the *Candida* species; colonies' development is visible within 24 to 48 hours. Identification of *Candida* species and their proliferation; When compared to conventional techniques, it has the advantages of being technically easy, quick, and affordable (Vijaya *et al*., 2011). The principle of using this medium of chromogenic substrate that are connected with metabolic enzymes specific for certain species of yeasts and results colonies with various color that are useful for the reasonable identification of the yeasts (Horvath *et al*., 2003; Daef *et al*., 2014).

The results of this test of the colonies produce different color on CHROMagar medium. Current results of *C. albicans* produce the light green color on this medium while *C. tropicalis* colonies produce metallic blue color , the colony of *C. krusei* produce light pink to purple color on this medium (Fig. 4-4), current results are coincided with Shaba'a (2011). CHROM agar Candida contains enzymatic chromogenic substrates, which combine with certain enzymes secreted by the types of *Candida* when they grow on this medium, which leads to different colors depending on the

Candida species, this test is useful in the laboratory diagnosis of yeast (Murray *et al*.,2005).

Current result of this test of the *C. parapsilosis* colonies produce cream to white or purple with halo on this medium while *C. dubliniensis* colonies produce dark green color on CHROMagar medium, *C. glabrata* colonies produce purple on this medium our results are considered as with (Hospenthal *et al.*, 2006; Nadeem *et al.*, 2010; Vignesh Kanna *et al* 2017).

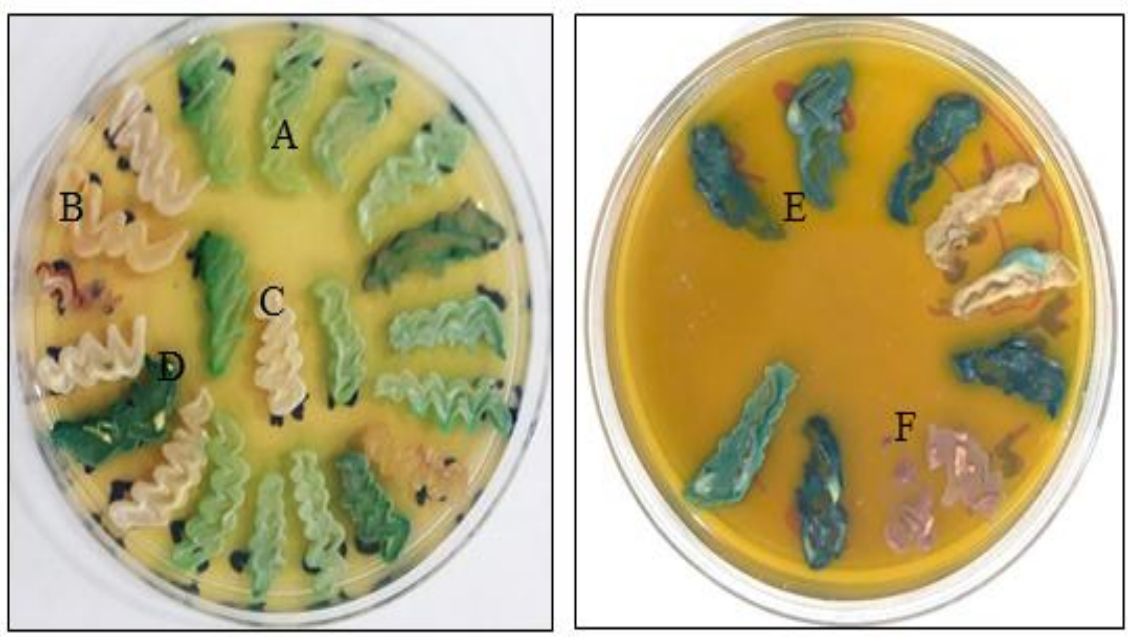


Fig. (4.4): Colonies color of *Candida* species on CHROM agar medium at 30˚C for 24-48h. A: *C. albicans,* B: *C. krusei* C: *C. parspsilosis* D: *C. dublinensis* E: *C. tropicalis* F: *C. glabrata*

4.8 Evaluation of Virulence Factors of Yeast:

4.8.1 : Urease Assay:

The result of this test show a positive result of most isolates, but *C. albicans* was variable**,** *C. dubliniensis*, *C. krusei*, strong positive result, *C. glabrata* , *C. parapsilosis* gave a weak positive result, and *C. tropicalis* negative result after 48 hours by turning the color of the medium from yellow to pink color in complete analysis or partially (Fig. 4-5). This result is consistent with Barbosa Júnior *et al.*, (2013) believed that *Candida* yeast lost this enzyme. (Mirbod-Donovan *et al.*, 2006) were mentioned that it is not only yeast *Cryptococcus* that secrete this enzyme but the genus of candida spp. of the ability to produce urease.

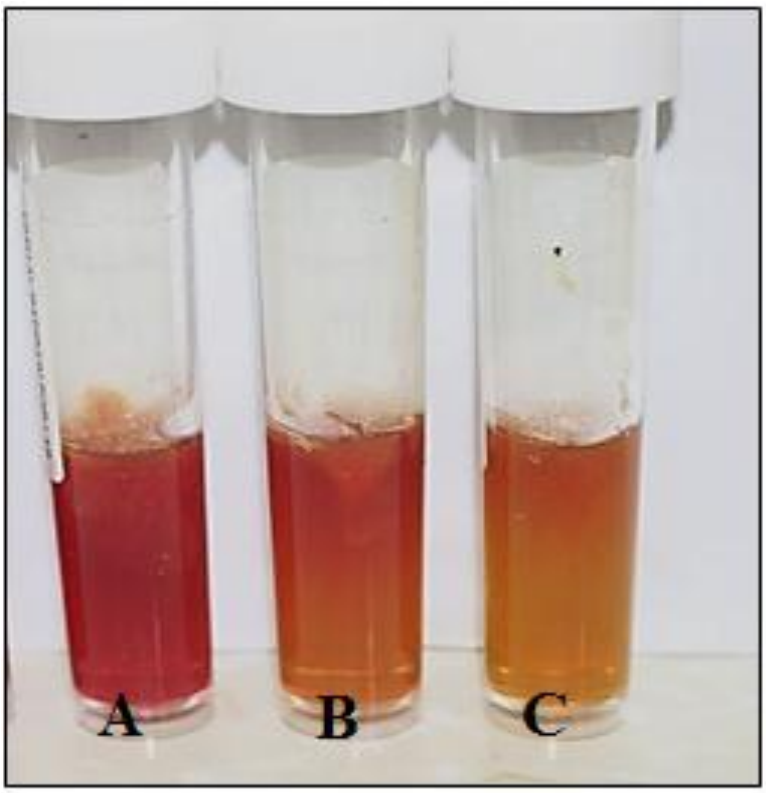


Fig.(4-5): Urease production of yeast species.

A: Complete analysis; B: Partially analysis; C: Negative result

4.8.2 : Production of Lipase Test:

The result of this test showed that produce lipase enzymes by some of *Candida* spp .positive result by producing white sediments around the grow colonies after incubating their colonies after 72 h at 30℃ as show in figure (4-8) observed *C. krusei* and *C. glabrata* have a moderate potential for the production of the enzyme while *C .albicans* and *C. dubliniensis* a higher production of the enzyme and *C. tropicalis* is negative. These results were in agreement with many studies (Paraje *et al*., 2008; Ramesh *et al*., 2011) . *Candida parapsilosis* has no ability to produce this enzyme and this result is similar to that reported by Alkhazraji (2015). Lipase has very important role in *Candida* species pathogenicity because it is able to digest lipid for nutrient acquisition, adhesion to host cells and host tissues (Schaller *et al*.,2005).

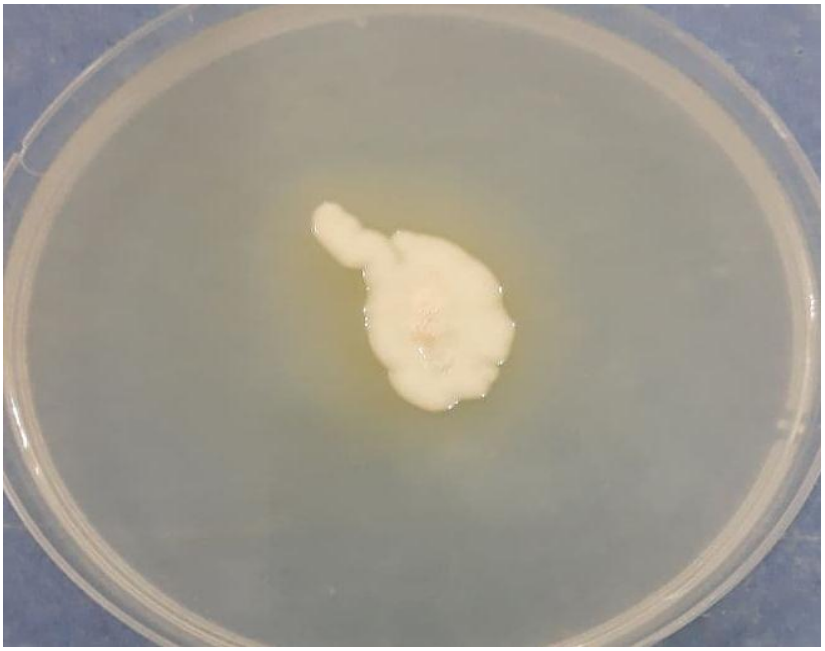


Fig. (4.6): Lipase Production of *C. albicans*.

4.8.3 : Phospholipase Production Test:

The result this test that showed the ability of some *Candida* species to produced Phospholipase enzyme was positive result the isolation *C. tropicalis, C. prapsilosis ,C. albicans* show through observation a clear deposition zone around the colony developing on egg yolk agar as show in figure (4-9) it measured sedimentation coefficient according to the equation (measure the diameter of the colony/ diameter of the colony + diameter halo deposition). This is consistent with the Hakim *et al*.,(2013). *C. glabrata, C. dubliniensis, C. krusei* negative .

Our result of *C. albicans* is high phospholipase enzyme produce because of the behavior disease. This is in consistent with the Tsang *et al*.,(2007) and Sachin *et al*., (2012), Where it signals a number of studies that secret this Chapter Four Results and Discussion 69 enzyme from *C. albicans*, it has an important role in pathogenicity and penetrate the epithelial cells (Samaranayake *et al*., 2006).

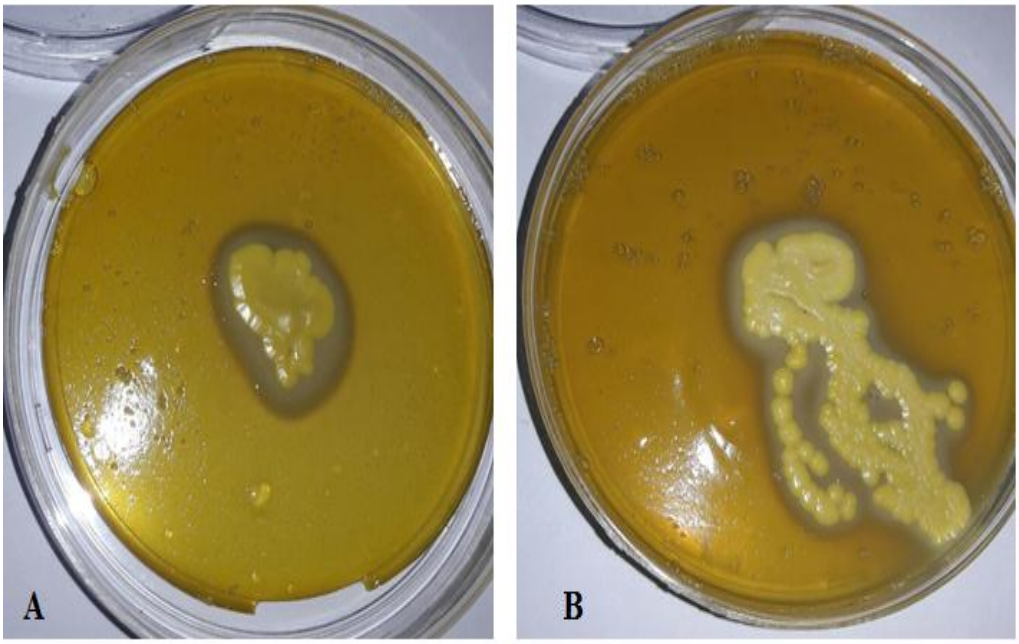


Fig. (4-7): Phospholipase Production of Yeast Species.

A: *C .albicans* **B: *C. parapsilosis***

4.8.4 : Formation of Biofilm Test:

Biofilm is essential for the growth of microbes that cause a wide range of clinical infections in humans, as well as the progression of clinical infections**.** The result of the biofilm test showed that the yeast isolates have ability to form biofilm and in different proportion as *C. parapsilosis* and *C. dubliniensis* were variable, while, *C. albicans* has ability to the formation of biofilm low (+ = few adhesive colonies on tube wall) as well as, *C. glabrata, C. krusei* , *C. tropicalis* is strong (++) as in figure (4-6). Report that all type of *candida* have the ability to from biofilm and the biofilm is an essential form of microbial growth and is responsible for a wide range of clinical infection of human.

Malm *et al*., (2011) and Shin, *et al*.,(2002) reported that all types of *Candida* have the ability to form biofilm and the biofilm is an essential form of microbial growth and is responsible for a wide range of clinical infections of human, and some of medically important fungi are form biofilms including *Candida* (Finkel and Mitchell, 2011). High level for producing biofilm by *C. albicans* is coincided with results of Kuhn *et al.*,(2004). *C. prapsilosis* is tends to form biofilms on the surface and lumens of catheters (Shin *et al*.,2002), *C. prapsilosis* isolates can be divided into three groupe on the basis of molecular studies, none of the clinical *C. prapsilosis* isolates were found to produce biofilm in vitro (Kean, *et al*., 2018).

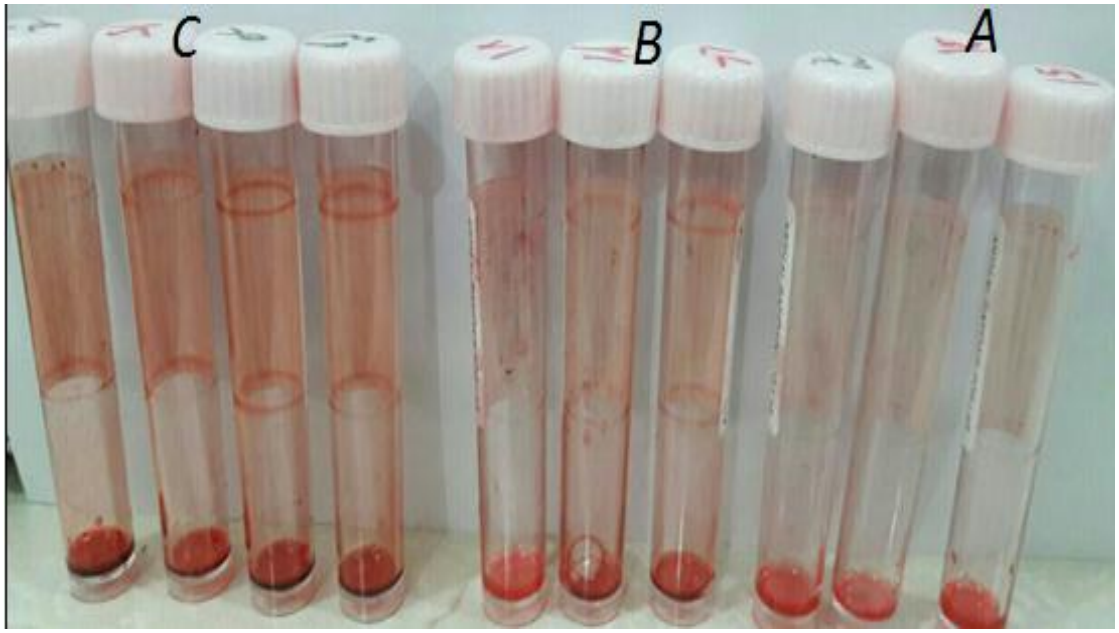


Fig. (4-8): Biofilm formation in yeast species.

A: negative , B: positive , C: strong positive.

4.8.5 : Hemolysis Test:

The results of the study show the ability of all yeasts under study on blood decomposition (Fig., 4-7). The isolations are given positive results through halo around the developing colonies in the blood agar media. This is consistent with Luo *et al*., (2001), can Candida yeast can dissolve blood, this is consistent Malcok *et al*.,*(*2009). while the *C. tropicalis* and Sporothrix were given negative result.

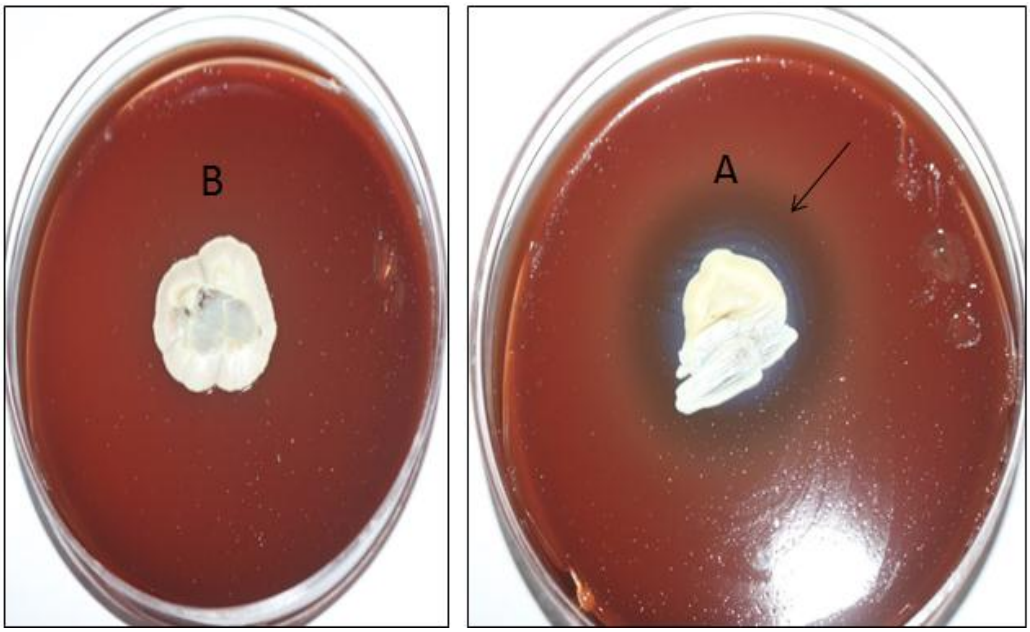


Fig. (4-9): Hemolysin production of yeast species.

A: *C. albicans*; B : *C. krusie*.

The evaluation of virulence factors, which included each of the urease, lipase, phospholipase, biofilm and hemolysis for yeast species under study is shown of table (4-9).

Table(4-9): Evaluation of virulence factor of yeast species.

Yeast species (n.)	Urase	Lipase	Phospholipase	Biofilm	Hemolysis
C. albicans **(10)**	V	++	++	+	++
C. dubliniensis **(7)**	++	++	_	V	+
C. glabrata **(5)**	+	+	_	++	++
C. krusei **(7)**	++	+	_	++	_
C. parapsilosis **(10)**	+	_	++	V	++
C. tropicalis **(5)**	_	+	++	++	+

V: variable , - : negative result ,+:weak result ,++: strong result .

4.9. Antifungal Activity of Against Growth of Yeasts:

In this study, the antifungal activity are used including Amphotericin-B (AP), Nystatin (NS), Fluconazole (FLC), Ketoconazole (KT), Itraconazole (IT), and Clotrimazole (CC) by using disc diffusion method against growth of *Candida* species under study. Depending on the number of samples studied for each species of yeast (table 4-10), it was the most effective antifungals were Fluconazole, Clotrimazole and Nystatin has the highest percentage of susceptibility against *Candida* spp. with (72.3%, 67.7% and 61.5%) respectively and Amphotericin-B was 58.4% , while Itraconazole and Ketoconazole were 53.8% and 52.3% respectively.

Table(4-10): Number and rate of isolates susceptibility to antifungal activity.

Fungal isolates (n)	NS S (%)	AP S (%)	FLC S (%)	KT S (%)	IT S (%)	CC S (%)
***C. albicans* (17)**	13(76.5)	9(53.0)	15(88.2)	15(88.2)	13(76.5)	15(88.2)
***C.dubliniensis* (9)**	8(88.8)	5(55.5)	9 (100)	7(77.8)	7(77.8)	9(100)
***C. glabrata* (7)**	0	5(71.4)	0	0	3(42.8)	5(71.4)
***C. krusi* (12)**	6(50.0)	9(75.0)	8 (66.6)	5(41.6)	5(41.6)	8(66.6)
***C.parapsilosis* (15)**	8(53.3)	5(33.3)	10(66.6)	7(46.6)	7(46.6)	7(46.6)
***C. tropicalis* (5)**	5(100)	5(100)	5(100)	0	0	0
Total No. 65	40(61.5)	38(58.4)	47(72.3)	34(52.3)	35(53.8)	44(67.7)

Table (4-11) shows the effect of variation type of antifungal drugs of yeast species isolations by inhibition zone measured with cm, the highest inhibition zone appears with first Fluconazole against *C. krusei* (3.25 cm) followed by *C. prapsilosis* (3 cm), *C. glabrata* (2.95 cm), *C. dubliniensis* (2.75 cm) and *C. albicans* (2.25 cm), while do not effect on *C. tropicalis*.

The second antifungal was Ketoconazole with inhibition zone against *C. albicans* (2.75 cm) followed by *C. prapsilosis* (2.4 cm), and *C. dubliniensis* (2.3 cm). While Itraconazole and Nystatin appear inhibition zone against *C. krusei* with 2.35 cm and 2.15 cm respectively.

Table(4-11): Evaluation of antifungal activity against growth of yeast species by inhibition zone measured with cm.

Fungal isolates (n.)	NS	AP	FLC	KT	IT	CC
***C. albicans* (17)**	1.5	1.0	2.25	2.75	0.75	0.75
***C. dubliniensis* (10)**	1.9	1.0	2.75	2.3	1.75	2.3
***C. glabrata* (7)**	0	0.9	2.95	0	0.5	0
***C. krusei* (12)**	2.15	0.95	3.25	2.35	1.8	2.75
***C. parapsilosis* (15)**	1.75	0.9	3	2.4	0.6	0
***C. tropicalis* (5)**	1.75	1.0	0	0	0	2.5
Average of inhibition zone	1.5	0.95	2.36	1.54	0.99	1.83

According to this results, the antifungal drug that gave the largest percentage of inhibition zone for the studied yeasts is Fluconazole with average of inhibition zone is 2.36 cm followed by Ketoconazole and Nystatin with 1.54 cm and 1.5 cm respectively (Figure 4-10). As for the yeasts, the most yeasts that were sensitive to antifungals drug and gave the largest percentage of inhibition zone is *C. krusei* with average of inhibition zone is 2.1 cm followed by *C. dubliniensis* with 1.95 cm (Figure 4-11). These results prove that there are significant differences between yeast species used and significant differences between antifungal drugs used in this study. Fig. (4-12) show the inhibition zones of *Candida* species by different of antifungal drugs.

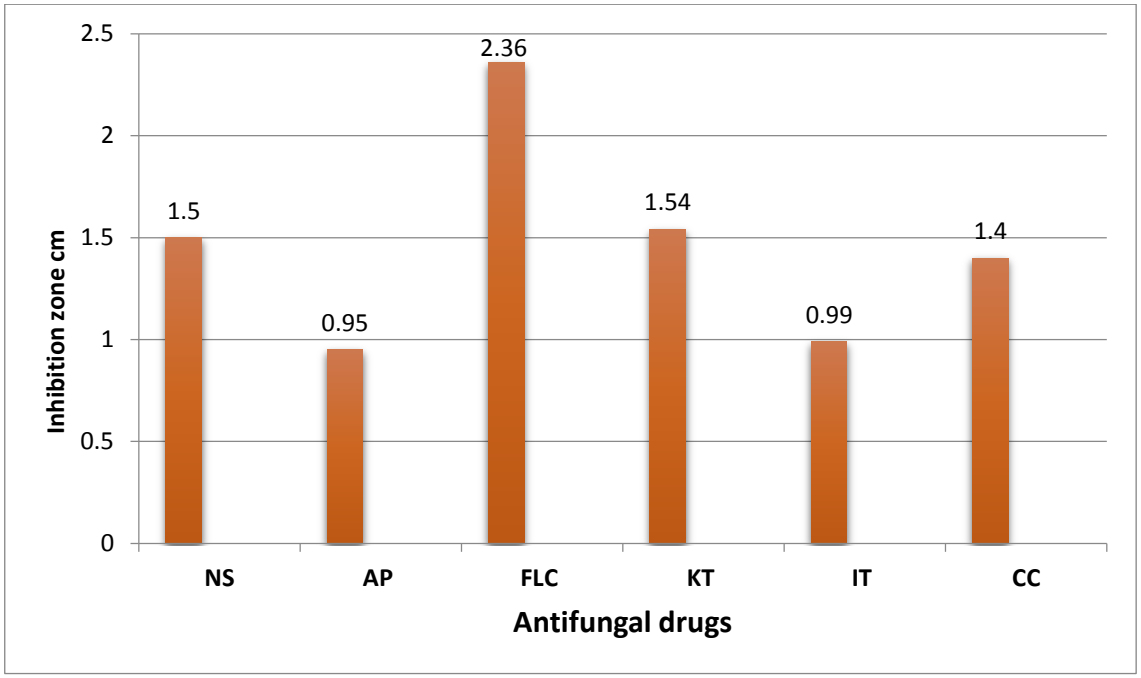


Fig. (4-10): Average of Inhibition zone of antifungal drugs on *Candida* species.

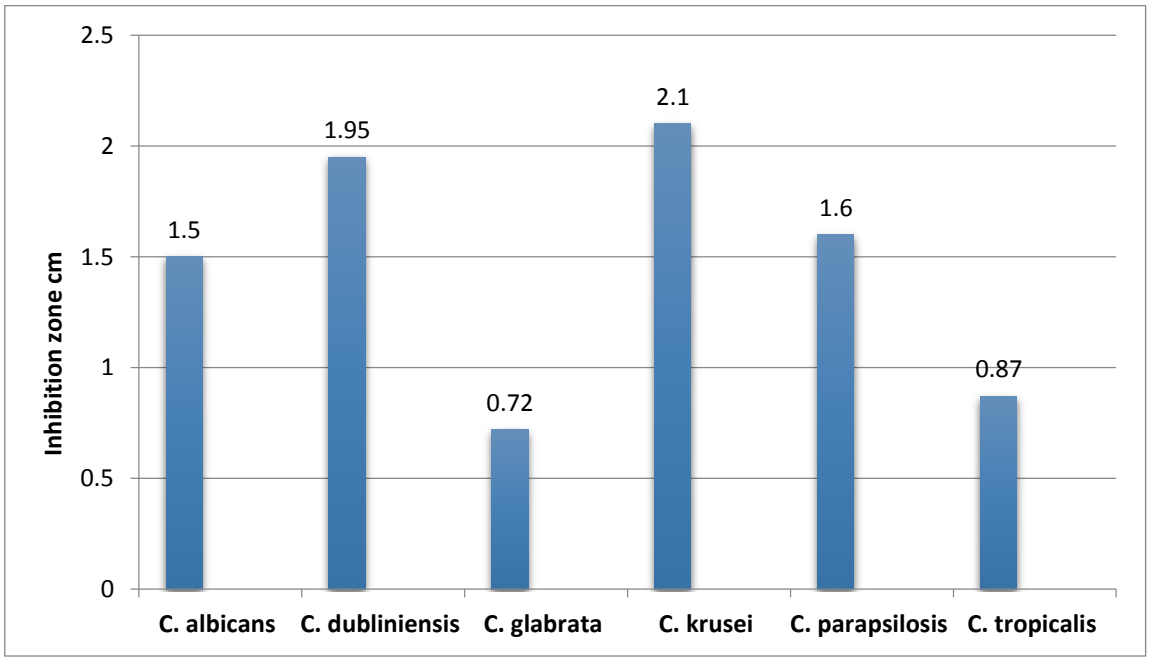


Fig. (4-11): Inhibition zone of *Candida* spp. effecteded by antifungal drugs.

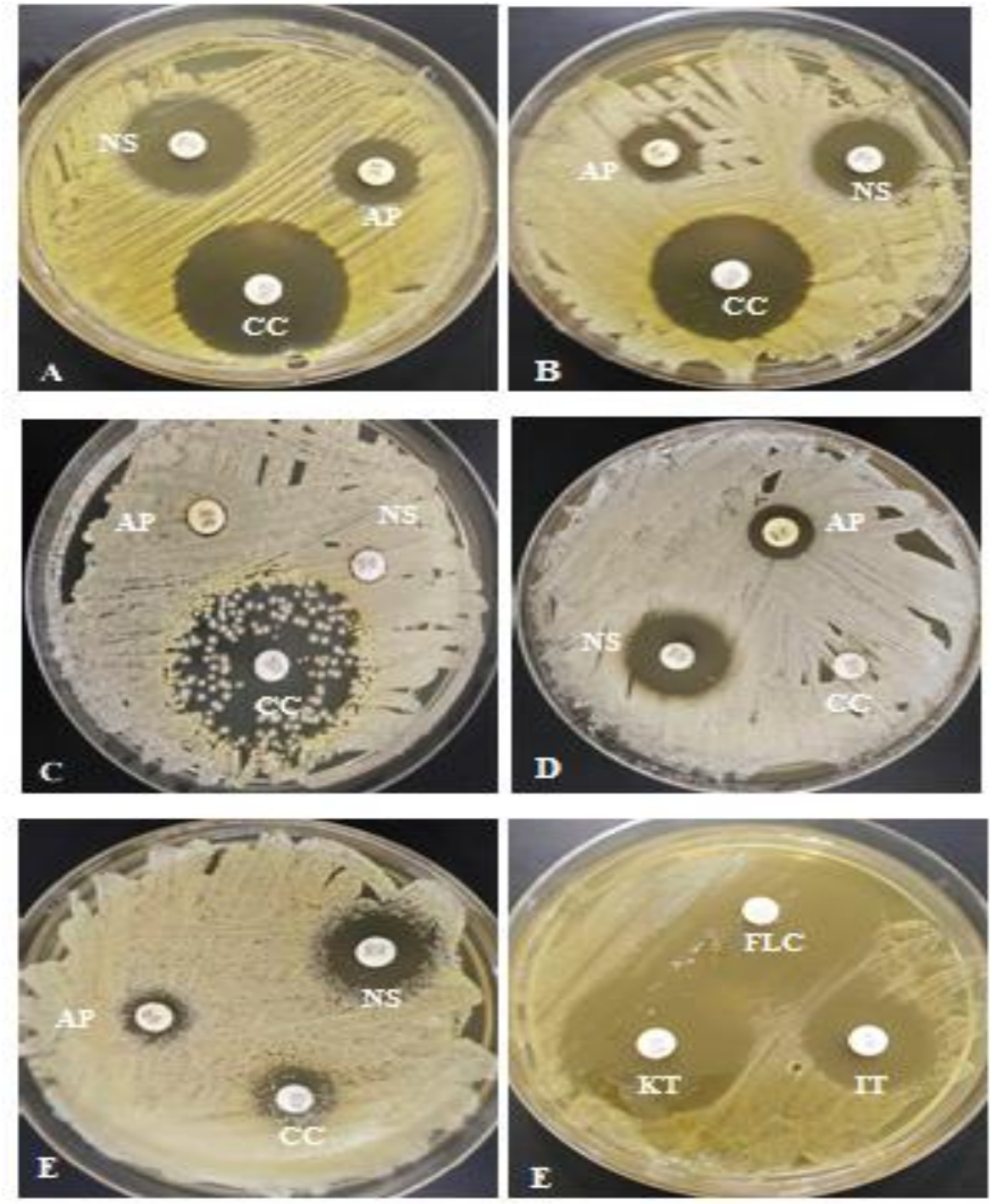


Fig. (4-12):Inhibition zones of *Candida* species by different of antifungal drugs: Amphotericin-B (AP), Nystatin (NS), Fluconazole (FLC), Ketoconazole (KT), Itraconazole (IT), and Clotrimazole (CC). A= *C. tropicalis*, B= *C. dubliniensis*, C= *C. krusei*, D= *C. parapsilosis*, E= *C. albicans*.

Our results are consistent with those of Meneses *et al.*, (2022) who calculate the Minimum inhibitory concentrations (MICs) of silver nanoparticles and clotrimazole for *Candida* species and their interaction by the adaptation of standarized methods who reported that MICs for this combination were 106, 53, and 26.5 ug/ml for *C. krusei, C. albicans, and C. parapsilosis* respectevily.

These results in accordance with, Costa *et al*., (2016) who reported that, 138 *C. glabrata* isolates, coming from two major Portuguese hospitals, were screened for Fluconazole and Clotrimazole drug resistance, regarding the MIC50 values, most isolates (93.5%) were found to be susceptible-dose dependent to FLC, while 9 (6.5%) were found to be resistant to FLC. As for CLT, most isolates (64.5%) were found to be resistant to CLT while (35.5%) were susceptible. Clotrimazole and bifonazole are highly effective antifungal agents against mucosal *C. albicans* infections. Here we examined the effects of low levels of clotrimazole and bifonazole on the ability of *C. albicans* to adhere, invade, and damage vaginal epithelial cells. Although adhesion and invasion were not affected, damage was greatly reduced upon azole treatment. This clearly indicates that low levels of azoles influence specific activities of *C. albicans* during distinct stages of vaginal epithelium infections (Wachtler *et al*., 2011).

Although the appearance and development of azoles and echinocandins antifungal agents, which had better tastes and less gastrointestinal adverse reactions, provided more clinical options, topical therapy, such as nystatin, is still one of the main recommended treatments for oral candidiasis due to its high efficacy, low cost, and less side effects, especially in developing countries (Lyu *et al*., 2016).

Nystatin suspension was not a good choice for infants, children, and HIV/AIDS patients with oral candidiasis, probably because of its short-term action on the oral mucosa. Nystatin shows a remarkable postantifungal effect, which is defined as the delay in fungal regrowth that persists after a brief exposure to an antifungal agent.16 Therefore, nystatin in the topical pastille form seems to be more effective in treating oral candidiasis than oral nystatin suspension. (Lyu *et al*., 2016).

Also, some studies combined between two antifungals or antifungals and a plant extract to show the synergistic effect of them on some fungal species. In study by da silva *et al*., (2020) stated that combination of nystatin and punicalagin (isolated from pomegranate) increased antifungal efficacy by increased the candidal inhibition compared with compounds tested alone. Wei *et al*., (2021) who identify that fingolimod (FTY720), an immunomodulatory drug used for oral treatment of relapsing-remitting multiple sclerosis, can potentiate the efficacy of AMB against *C. albicans* growth synergistically. Furthermore, they observed an antifungal efficacy of FTY720 in combination with AMB against diverse fungal pathogens. Therefore, the combination of AMB and FTY720 provides a promising antifungal strategy. Li *et al*., (2019) demonstrated for the first time that D-penicillamine (PCA) combined with fluconazole showed a synergistic effect against *C. albicans*. PCA combined with fluconazole not only showed synergistic effects against planktonic cells of *C. albicans*, but also showed synergistic effects against *C. albicans* biofilms formed within 12 h in vitro.

4.10: Molecular Study:

4.10.1: DNA Extraction:

Sixty samples of human blood (20 samples of Covid_19 patients, 20 samples of recovery persons and 20 samples of healthy people) of this study are subjected for DNA extraction. Figure (4-13) is shows agarose gel electrophoresis of DNA extraction for samples of human blood.

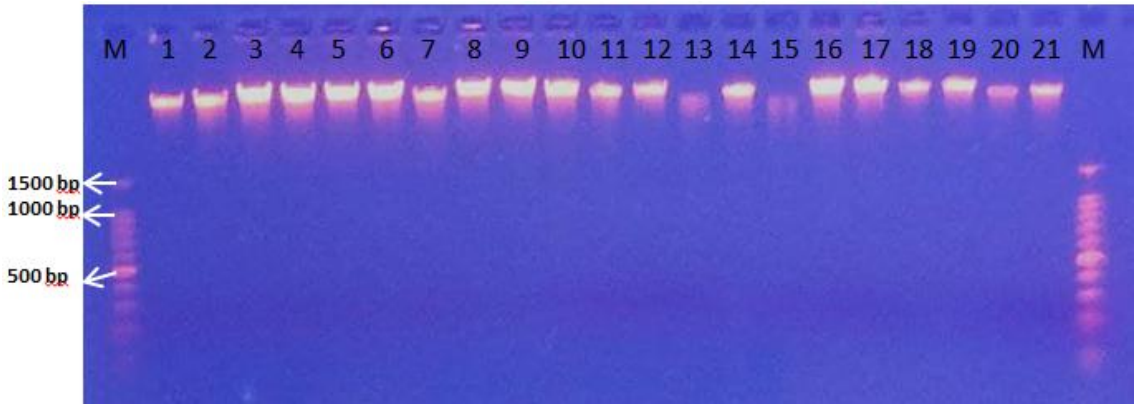


Fig. (4-13): Agarose gel electrophoresis of DNA extraction for human blood samples. 1% agarose gel at 70 volt for 45 min.

4.10.2:The Polymorphism of *TLR8* Gene:

The frequency of genotypes and alleles of *TLR8* (rs3764880 A/G) gene polymorphism were amplified by Tetra- Amplification Refractory Mutation System (T- ARMS) technique, with tetra primers (two outer primers, and two inner primers forward and reverse). The electrophoresis result for *TLR8* Tetra primer is coded as follows (423 bp = wild type, 272 bp= A allele, and 209 bp= G allele). Figure (4-14) show agarose gel electrophoresis of PCR products .

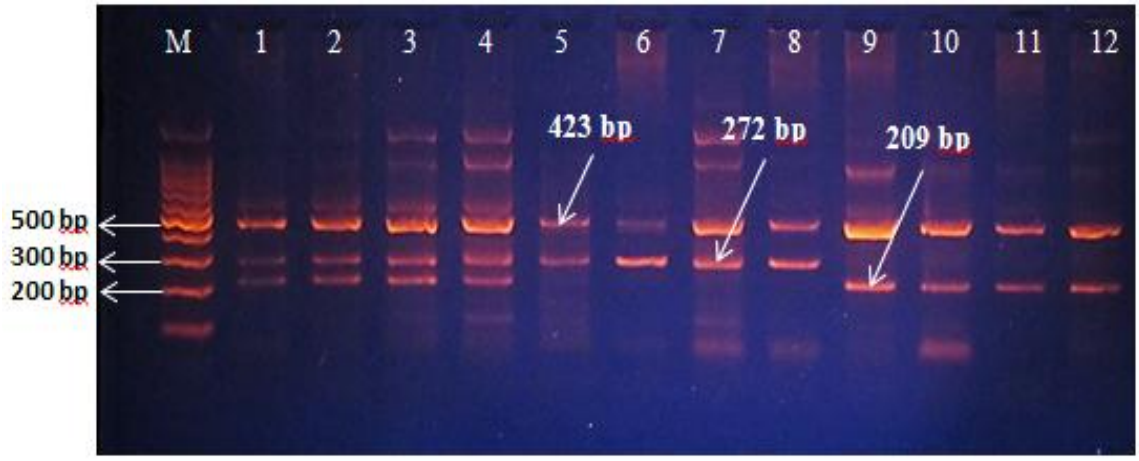


Fig. (4-14): Agarose gel electrophoresis of PCR products for (*TLR8*) gene polymorphism of human blood for patients and control. Lane M= molecular marker 100 bp. Line 1-4= heterozygous AG, Line 5-8 = homozygous AA and Line 9-12= homozygous GG. 1% agarose gel at 70 volt for 45 min.

The polymorphism of *TLR8* gene was done for 60 samples of blood DNA (20 patients, 20 recovery persons and 20 healthy people). When compared between patients and control, the frequency of genotypes AA, AG and GG of *TLR8* (rs3764880 A/G) gene polymorphism are respectively 40%, 35% and 25% in patients, while respectively 60%, 0% and 40% in control group. The frequency of alleles of *TLR8* gene polymorphism of A allele is 57.5% in patients and 60.0% in control group, while frequency of G allele is 42.5% in patients and 40.0% in control group (table 4-13).

Table(4-12):Genotype and allele distribution *TLR8* gene polymorphism in patients and control, shown the Odd Ratio value.

TLR8 (rs3764880 A/G) genotypes	Genotypes frequency (%)			
	Patients n=20 (%)	Control n= 20 (%)	OR (95% CI)	P- value
AA	8 (40%)	12 (60%)	Reference group	
AG	7 (35%)	0 (0%)	22.7(1.19-432.6)	0.03
GG	5 (25%)	8 (40%)	0.5 (0.13- 1.9)	0.3
TLR8 alleles	Alleles frequency (%)			
A	23 (57.5%)	24 (60%)	0.9 (0.23-2.19)	0.8
G	17 (42.5%)	16 (40%)	1.11 (0.46-2.7)	0.8

When compared between recovery and control, the frequency of genotypes AA, AG and GG of *TLR8* (rs3764880 A/G) gene polymorphism are respectively 25%, 60% and 15% in recovering, while respectively 60%, 0% and 40% in control group. The frequency of alleles of *TLR8* gene polymorphism of A allele is 55.0% in recovering and 60.0% in control group, while frequency of G allele is 45.0% in recovering and 40.0% in control group (table 4-14).

The results for *TLR8* (rs3764880 A/G) gene polymorphism have shown that AA and AG genotype frequency 8 (40%) and 7 (35%) respectively were closed among the Covid 19 patients, which is more than GG genotypes 5 (25%), while in recovering was AG agenotype frequency 12 (60%) which is more than other two genotypes AA was 5 (25%) and GG was 3 (15%). Also, in healthy control subjects was AA genotype 12 (60%) more than GG genotype 8 (40%%) and AG genotype 0 (0%), which is did not appear in control subjects. Therefore, *TLR8* (rs3764880 A/G) AG heterozygous genotype was significantly associated with increased susceptibility to Covid 19 disease.

Table(4-13):Genotype and allele distribution *TLR8* gene polymorphism in Recovering and control, shown the Odd Ratio value.

TLR8 (rs3764880 A/G) genotypes	Genotypes frequency (%)			
	Recovering n=20 (%)	Control n= 20 (%)	OR(95%CI)	P-value
AA	5 (25%)	12 (60%)	Reference group	
AG	12 (60%)	0 (0%)	60.29 (3.2-1137)	0.006
GG	3 (15%)	8 (40%)	0.26 (0. 05-1.2)	0.08
TLR8 alleles	Alleles frequency (%)			
A	22 (55%)	24 (60%)	0.8 (0.33 -1.9)	0.6
G	18 (45%)	16 (40%)	1.23 ((05-2.9)	0.6

However, the results for *TLR8* (rs3764880 A/G) gene polymorphism have shown that A allele frequency was 23 (57.5%) in patients, 22 (55%) in recovering and 24 (60%) in control subjects, whereas G allele 17 (42.5%) in patients, 18 (45%) in recovering and 16 (40%) in control subjects without significant difference (P value = 0.8 and 0.6 respectively), as shown in Table (4-13 and 4-14).

Based on the formula of odd ratio of allele A and G: When the odd ratio of allele G (OR_G) > 1 implies that the allele G associated with disease. While the allele A (OR_A) < 1 implies that the allele A protect against the disease. Based on our results in table (4-8 and 4-9) the odd ratio of alleles are 0.9 (95% CI = 0.23-2.19) and 0.8 (95% CI = 0.33-1.9), that refers to the A allele protect against the Covid 19 disease.

TLRs have a critical role in pathogen recognition and activation of innate immunity and act in multiple cellular processes such as cytokine secretion, modulation of the adaptive immune response and apoptosis (Kawai *et al.*, 2007; Thada *et al.*, 2013). TLR8 is located on X chromosome and is encoded by two exons(de Groot and Bontrop, 2020). TLR8 has a role in immunity against mycobacterium through IRF-7 and induced production of IFN (Cervantes *et al*., 2012).

This study is agreement with Wang *et al*., (2018), they reported genotypes frequencies at TLR8 (rs3764880 A/G) in PTB females patients and that control subjects did show significant differences in Chinese Han population. In contrast to our findings, Dalgic *et al*., (2011) and Hashemi *et al.*, (2014), they reported genotypes frequencies at TLR8 (rs3764880A/G) in PTB patients and control subjects did not show significant differences in Turkish population and Southeast Iran respectively.

Activation of TLR pathways leads to secretion of pro-inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α, as well as type 1 interferon. Different TLRs, like TLR2,TLR3, TLR4, TLR6, TLR7, TLR8, and TLR9 are potentially important in COVID-19 infection. It is also worth mentioning that we should bear in mind both the beneficial and harmful effects of TLR in confronting COVID-19 infection. TLRs could be a potential target in controlling the infection in the early stages of disease and production of vaccine against SARS-CoV-2 (Khanmohammadi and Rezaei, 2021).

Manik and Singh (2022) referred to that the cytokine storm migrates into the other organ through the systemic circulation. The inflammation and the organ damage occur due to the TLR mediated NF-κB, MAPK pathway. Hence blocking these specific TLRs may alleviate the chance of SARS-COV-2 infection.

4.10.3:The Polymorphism of *CLEC7A* Gene:

The polymorphism of *CLEC7A* gene was done for 60 samples of human blood DNA (20 patients, 20 recovery persons and 20 healthy people). this gene locate on Chromosome 12 arranged from right to left started from scale number between (10,124,386 kb) to (10,125,342 kb). All samples in this study comparing the observed DNA sequences of these local samples with the retrieved DNA sequences (Gen Bank NC _ 000012.12). Figure (4-16 and 4-17) a shows the sequence and chromatogram for forward and reverse primer for *CLEC7A* gene and determines the location of SNPs rs3901532 C/T and rs3901533 C/T on chromosome 12 and several of SNPs are recorded by the NCBI genebank at this location.

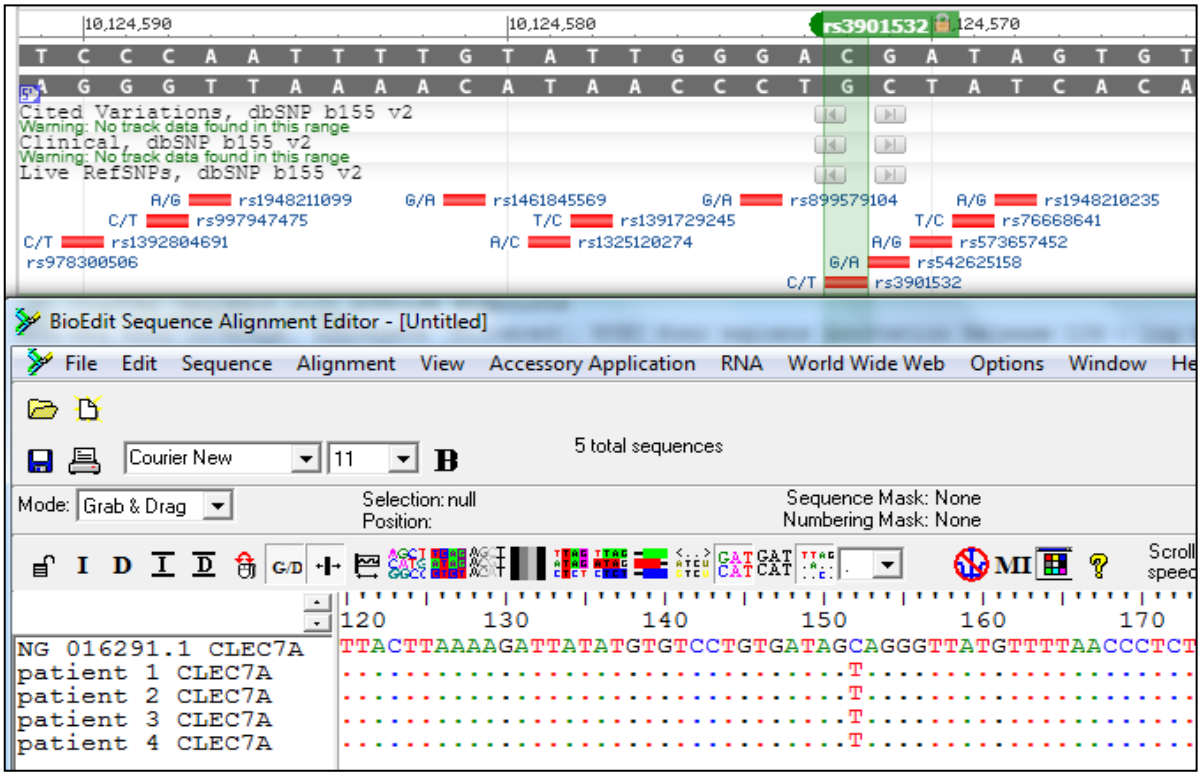


Fig. (4-15): Illustration the sequence and chromatogram for forward and reverse primer for *CLEC7A* gene and determined the location of SNPs rs3901532 C/T on chromosome 12 and several of SNPs in this location.

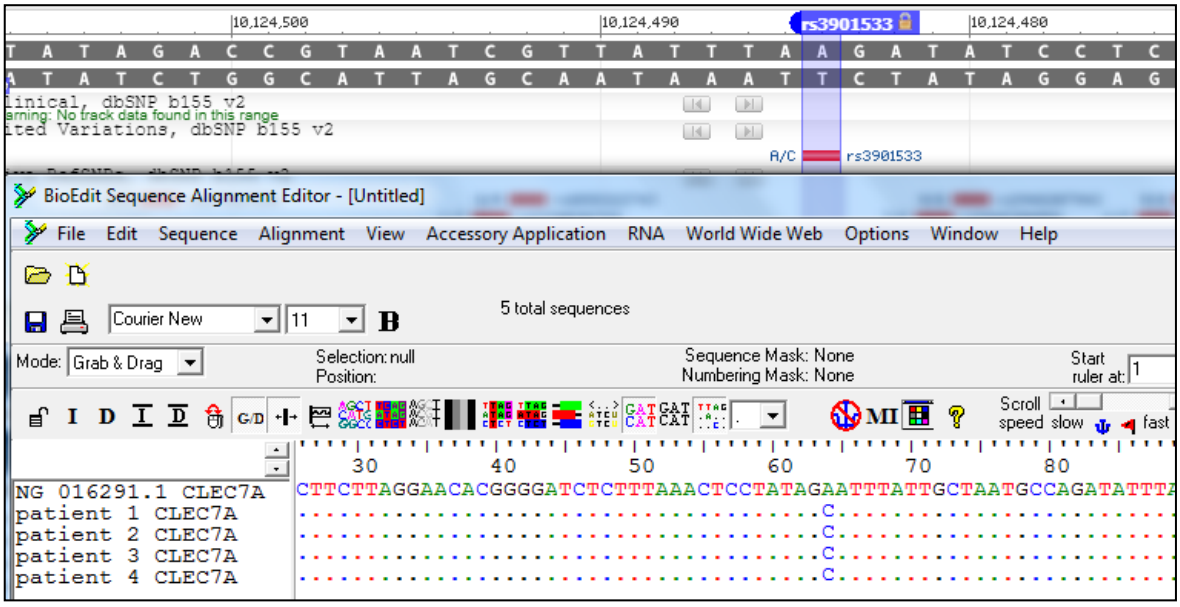


Fig. (4-16): Illustration the sequence and chromatogram for forward and reverse primer for *CLEC7A* gene and determined the location of SNPs rs3901533 A/C on chromosome 12.

The primer pair for the gene of *CLEC7A* was successfully amplified the target under amplification. The PCR products of *CLEC7A* gene shown 524bp. Figure (4-17) shows agarose gel electrophoresis of PCR products of CLEC7A pair primer.

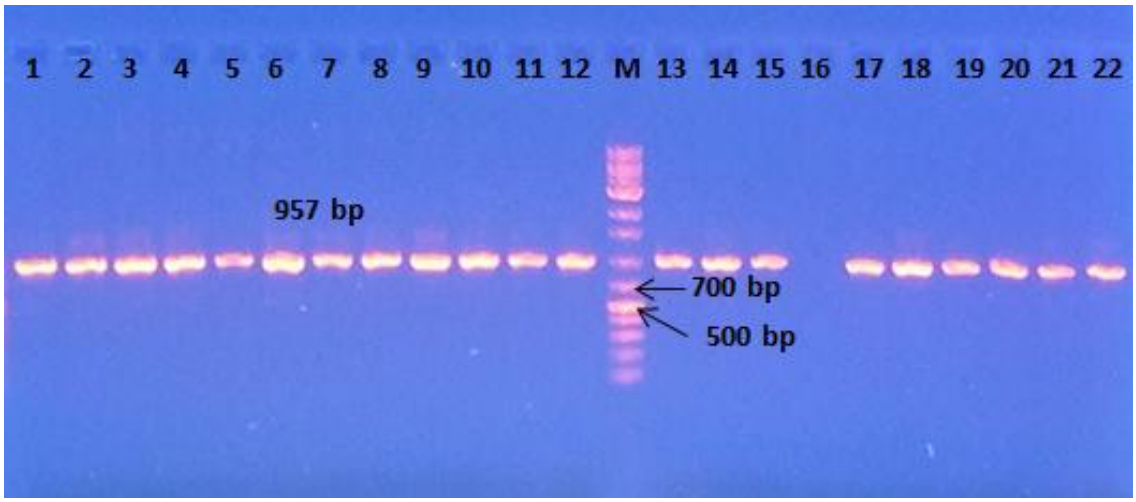


Fig. (4-17): Agarose gel electrophoresis of PCR products 957 bp of *CLEC7A* gene. Lane M= molecular marker, Lanes: 1-12= patients, 13-17 = recovery, and 18-22= control. 1% agarose gel at 70 volt for 45 min.

For checking the polymorphism in this gene in patient samples under interest must been subjected to sequence analysis. Sixty PCR products (20 patients, 20 recovery persons and 20 healthy people) of *CLEC7A* gene (957bp) are directly sequenced by Macrogen Company (Korea). The results of sequences were aligned with the reference sequence in National Center for Biotechnology Information (NCBI) using Chromas pro software. The sequencing chromatogram of each observed substitution mutation (Heterozygous or Homozygous allele) as well as its detailed annotations were documented, and the chromatogram details of observed SNP are shown according to its position in the PCR amplicon.

The results of sequences were aligned with the reference sequence in National Center for Biotechnology Information (NCBI) using Chromas pro software. The sequencing chromatogram of each observed substitution mutation (Heterozygous or Homozygous allele) as well as its detailed annotations were documented, and the chromatogram details of observed SNP are shown according to its position in the PCR amplicon.

After subjecting the sequence of the amplicon of the 957bp of *CLEC7A* gene and flanking primers region, and multiple alignment for 60 samples comparing with leading sequence of reference sequence of the same gene locus (NC_000012.12) by the BioEdit software. In the *CLEC7A* gene, two genotypes have been observed, SNPs rs3901532 C/T appeared on position 152 and rs3901533 A/C appeared on position 64.

In target rs3901532 C/T, the nucleotide Cytosine was substituted to Thymine on the upstream sequence of the promoter of *CLEC7A* gene. There are Heterozygous or Homozygous allele appeared of most sample in patients , recovery persons and control in appendix 2 (Fig. 1 , 2 , and 3) respectively.

When compared between patients and control, the frequency of genotypes CC, CT and TT of *CLEC7A* (rs3901532 C/T) gene polymorphism are respectively 20%, 45% and 35% in patients, while respectively 20%, 55% and 25% in control group. The frequency of alleles of *CLEC7A* (rs3901532 C/T) gene polymorphism of C allele is 42.5% in patients and 47.5% in control group, while frequency of T allele is 57.5% in patients and 52.5% in control group (table 4-14).

Table(4-14):Genotypes of SNP:rs3901532 of Patients group with Odd Ratio and Allele frequency based on using Hardy-Weinberg Equation.

rs3901532 C/T	patients N=20		Control N=20	OR (95%CI)	P-value
genotypes	CC	4 (20%)	4 (20%)	Reference group	
	CT	9 (45%)	11 (55%)	0.66 (0.19-2.3)	0.53
	TT	7 (35%)	5 (25%)	1.6 (0.4-6.3)	0.49
Allele Frequency	C	17(42.5%)	19(47.5%)	0.8 (0.33-1.97)	0.65
	T	23(57.5%)	21(52.5%)	1.22 (0.50-2.9)	0.65

When compared between recovery persons and control, The frequency of alleles of CLEC7A (rs3901532 C/T) gene polymorphism of C allele is 47.5% in both recovery persons and control group, while frequency of T allele is 52.5% in both recovery and control group (table 4-15).

Table(4-15): Genotypes of SNP:rs3901532 of recovery group with Odd Ratio and Allele frequency based on using Hardy-Weinberg Equation.

rs3901532 C/T		Recovery N=20	Control N=20	OR (95%CI)	P-value
genotypes	CC	5 (25%)	4 (20%)	Reference group	
	CT	9 (45%)	11(55%)	0.67(0.19-2.3)	0.5
	TT	6 (30%)	5 (25%)	1.28 (0.32-5.17)	0.7
Allele Frequency	C	19(47.5%)	19(47.5%)	1.0 (0.41-2.4)	1.000
	T	21(52.5%)	21(52.5%)	1.0 (0.41-2.4)	1.000

The results for *CLEC7A* (rs3901532 C/T) gene polymorphism have shown that CT and TT genotype frequency 9 (45%) and 7 (35%) respectively were closed among the Covid 19 patients, which is more than CC genotypes 4 (20%), while in recovering was CT genotype frequency 9 (45%) which is more than other two genotypes TT was 6 (30%) and CC was 5 (25%). Also, in healthy control subjects was CT genotype 11 (55%) more than TT genotype 5 (25%%) and CC genotype 4 (20%). Therefore, *CLEC7A* (rs3901532 C/T) that TT homozygous genotype was significantly associated with increased susceptibility to Covid 19 disease.

Regarding the evaluation of alleles, the results for *CLEC7A* (rs3901532 C/T) gene polymorphism have shown that T allele frequency was 23 (57.5%) in patients, while 21(52.5%) in both recovering persons and control subjects with significant difference between patients and control (P value = 0.65), whereas C allele 17 (42.5%) in patients, and 19(47.5%) in both recovering persons and control subjects without significant difference (P value = 1.00), as shown in Table (4-15 and 4-16).

Based on the formula of odd ratio of allele T and C: When the odd ratio of allele T (OR_T) >1 implies that the allele T associated with disease. While the allele C (OR_C) < 1 implies that the allele C protect against the disease. Based on our results in table (4-10) the odd ratio of alleles are 0.8 (95% CI = 0.33-1.97) and 1.22 (95% CI = 0.50 -2.9), that refers to the T allele associated with disease while C allele protect against the Covid 19 disease.

In target rs3901533 A/C, the nucleotide Adenine was substituted to Cytosine on the upstream sequence of the promoter of *CLEC7A* gene. There are Heterozygous or Homozygous allele appeared of most sample in patients , recovery persons and control in appendix 2 (Fig. 4 , 5 , and 6) respectively.

When compared between patients and control, the frequency of genotypes AA, AC and CC of *CLEC7A* (rs3901533 A/C) gene polymorphism are respectively 20%, 50% and 30% in patients, while respectively 25%, 60% and 15% in control group. The frequency of alleles of *CLEC7A* (rs3901532 A/C) gene polymorphism of A allele is 45% in patients and 55% in control group, while frequency of C allele is 57.5% in patients and 45% in control group (table 4-16).

Table(4-16): Genotypes of SNP:rs3901533 of patients group with Odd Ratio and Allele frequency based on using Hardy-Weinberg Equation.

rs3901533 A/C	Patients N=20		Control N=20	OR (95%CI)	P-value
genotypes	AA	4 (20%)	5 (25%)	Reference group	
	AC	10(50%)	12(60%)	0.67(0.19-2.6)	0.53
	CC	6 (30%)	3 (15%)	2.4(0.51-11.5)	0.24
Allele Frequency	A	18(45%)	22(55%)	0.67(0.27-1.6)	0.37
	C	22(55%)	18(45%)	1.14(0.62-3.6)	0.37

When compared between recovery and control, the frequency of genotypes AA, AC and CC of *CLEC7A* (rs3901533 A/C) gene polymorphism are respectively 25%, 55% and 20% in recovery, while respectively 25%, 60% and 15% in control group. The frequency of alleles of *CLEC7A* (rs3901533 A/C) gene polymorphism of A allele is 52.5% in recovery and 55% in control group, while frequency of C allele is 47.5% in recovery and 45% in control group (table 4-18).

Table (4-17): Genotypes of SNP:rs3901533 of recovery group with Odd Ratio and Allele frequency based on using Hardy-Weinberg Equation.

rs3901533 A/T	Recovery N=20		Control N=20	OR (95%CI)	P-value
genotypes	AA	5 (25%)	5 (25%)	Reference group	
	AC	11 (55%)	12(60%)	0.81(0.23-2.86)	0.74
	CC	4 (20%)	3 (15%)	1.42(0.27-7.34)	0.67
Allele Frequency	A	21(52.5%)	22(55%)	0.90(0.37-2.2)	0.82
	C	19(47.5%)	18(45%)	1.1(0.46-2.66)	0.82

The results for *CLEC7A* (rs3901533 A/C) gene polymorphism have shown that AC and CC genotype frequency 10 (505%) and 6 (30%) respectively were closed among the Covid 19 patients, which is more than AA genotypes 4 (20%), while in recovering was AC agenotype frequency 11 (55%) which is more than other two genotypes AA and CC were 5 (25%) and 4 (20%) respectively . Also, in healthy control subjects was AC genotype 12 (60%) more than other two genotypes AA and CC were 5 (25%) and 3 (15%) respectively. Therefore, *CLEC7A* (rs3901533 A/C) that CC homozygous genotype was significantly associated with increased susceptibility to Covid 19 disease.

Regarding the evaluation of alleles, the results for *CLEC7A* (rs3901533 A/C) gene polymorphism have shown that A allele frequency was 18 (45%) in patients, while 21(52.5%) in recovering persons and 22 (55%) in control subjects, whereas C allele 22 (55%) in patients, and 19(47.5%) and 18 (45%) in both recovering persons and control subjects respectively, with significant difference between recovering persons and control (P value = 0.82), as shown in Table (4-17 and 4-18).

Based on the formula of odd ratio of allele A and C: When the odd ratio of allele C (OR_C) > 1 implies that the allele C associated with disease. While the allele A (OR_A) < 1 implies that the allele A protect against the disease. Based on our results in table (4-12 and 4-13) the odd ratio of allele A is 0.67 (95% CI = 0.27-1.6) and 0.9 (95% CI = 0.37 -2.2), while odd ratio of allele C is 1.14 (95% CI = 0.62-3.6) and 1.1 (95% CI = 0.46 -2.6), that refers to the C allele associated with disease, while A allele protect against the Covid 19 disease.

CLEC7A gene is an abbreviation of the term (C- type lectin), alternate name is Dectin- 1, Dectin-1 has been shown to recognize several fungal species, including *Saccharomyces, Candida, Pneumocystis, Coccidiodes, Penicillium* and *Aspergillus*, but not *Cryptococcus*. Recognition of these organisms by Dectin-1 triggers many protective responses, such as fungal uptake by phagocytosis and killing via the respiratory burst (Huysamen and brown, 2009).

Several study confirming the association between dectin-1 variation and susceptibility to IA was performed by Cunha *et al*., (2010) they revealed that dectin-1 receptor (*CLEC7A* rs16910526) variation is a predisposing factor for IA in high risk patients. This confirms the suspicion that such a receptor has a role in controlling resistance and immune tolerance to *Aspergillus* spp. While Sainz *et al*. (2012) was used SNPs rs7309123 and rs3901533, the level of significance for the association was

similar to the previous study (OR = 4.91, 95% CI = 1.52-15.9, p = 0.05) and (OR = 5.59, 95% CI = 1.37- 22.7, p = 0.012) respectively.

The association between variability in the gene encoding dectin-1 and IA was also found in a study by Chai *et al*., (2011), they used *CLEC7A* rs16910526 with (OR = 1.79, 95% CI = 0.77–4.19, p = 0.017), which described that the studied polymorphism increased the susceptibility to IA. Similar results were observed by Smith *et al*., (2014) but used *CLEC7A* rs7309123, who investigated the genetic association of 112 biologically plausible patients with IA and 279 healthy controls.

there is a significant association between genetic polymorphisms and the development of IA. Such studies are important because the identification of concrete genetic polymorphisms associated with diseases will enable the identification of patients at high risk of developing the pathology. As a result, efficient diagnostic procedures can be developed using the polymerase chain reaction technique (Cunha *et al.*, 2018).

In humans, eQTLs regulating CLEC7A (Dectin-1) gene expression have been identified and CLEC7A is expressed in active MS lesions Despite the well-known inflammatory role of Dectin-1, some studies have suggested tolerogenic and anti-inflammatory functions of Dectin-1 signaling (Gour *et al*., 2018; Bode *et al*., 2019; Voskuhl *et al*., 2019). The dectin-1 play a crucial role in pathogen recognition and phagocytosis by macrophages, several studies indicate that they contribute differently to the activation of inflammatory signaling pathways. Indeed, it has been shown that dectin-1, by recognizing carbohydrates on the surface of pathogens, promotes production of reactive oxygen species (Lefevre *et al.*, 2013; Rahabi *et al*., 2020).

CONCLUSION

AND

RECOMMENDATIONS

Conclusions :

1. The proportion of males was higher than the rate of females infected with COVID-19.

2. Age groups ranging from (65-74) were more likely to be infected with COVID-19.

3. The highest percentage of samples positive for fungal species was *Candida albicans* in the three groups (patients, recovery persons, and healthy people)

4. The most effective antifungals were Fluconazole, Clotrimazole and Nystatin has the highest percentage of susceptibility against *Candida* spp. respectively and followed by Amphotericin-B, Itraconazole and Ketoconazole respectively.

5. The polymorphism of *TLR8* gene by Tetra-Arm primer . (patients, recovery persons, healthy people) appeared relationship to sensitivity to patients infected with COVID-19.

6. The polymorphism of *CLEC7A* gene by sequence of PCR results. For checking the polymorphism in this gene have been observed two SNPs rs3901532 C/T appeared on position 152 and rs3901533 A/C appeared on position 64.

Recommendations:

1. Intensive study of the reason for existence the *Candida* spp. and their frequency in sputum sample of patient with COVID-19.

2. Give attention for candidiasis in Iraq by collection of a larger number of blood samples from patients with COVID-19 in the intensive care unit for identifying by genetic profiling of those.

3. Complete study the rest of virulence factors such as mycotoxins and doubling heat and the ability to adhesion.

4. *CLEC7A* gene requires more sequence analysis studies to verify more SNPS and variant with deficiency of *CLEC7A* gene function.

5. Study of the fungi that associated with COVID-19 infection and, as it provides clues to the etiology of this infection.

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Appendices

APPENDIX (1)

3.4.8.2. DNA Extraction From Blood:

Step 1: RBC Lysis:

1. Collected frozen human blood in an anticoagulant-treat collection tube and waited at room temperature until it thawed.

2. Transferred to a microcentrifuge tube, up to 300µl of blood .

3. Added RBC Lysis Buffer volume 600 and combined by inversion .

4 .Incubated for 10 minutes, at room temperature .

5. Centrifuge for 5 minutes at 3000 x g, and extract the supernatant completely .

6. Resuspended the pellet with 100 µl RBC lysis buffer and added 20 µl protenase k Mix thoroughly by Incubate water bath at 56ºC for 15 minutes.

Step 2: Cell Lysis:

7 . 200µl FABG Buffer applied and vortex combined .

.Incubated at 70oC to lyse the sample for 15 minutes. Invert the sample every 3 ~ 5 minutes, during incubation .

9 .Spin the tube quickly to extract droplets from within the cap .

10 Preheat the buffer with the elution in a 70oC water bath (DNA Elution for step 5 .

11. 5µl of RNase A was applied to the sample and vortex, then incubated at room temperature for 5 minutes.

Step 3: Binding:

12. The sample was supplemented with 200µl ethanol (96 ~ 100 per cent). Mix vigorously for 10 seconds using pulse vortexing.

13. Spin the tube quickly to extract droplets from within the cap.

14. The sample collection tube mixture (including any precipitate) was carefully transferred to FABG Column. 5 Minute centrifuge.

Step 4: Washing:

15. Washed FABG Column with 400µl W1 Buffer (ethanol added) by centrifuge for 30 seconds.

16. Washed FABG Column with 600µl Wash Buffer (ethanol added) by centrifuge for 30 seconds.

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

Step 5: Elution:

18. Putted FABG Column to a new 1.5ml microcentrifuge tube.

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

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

21. Stored the DNA fragment at 4ºC or -20ºC.

APPENDIX (2)

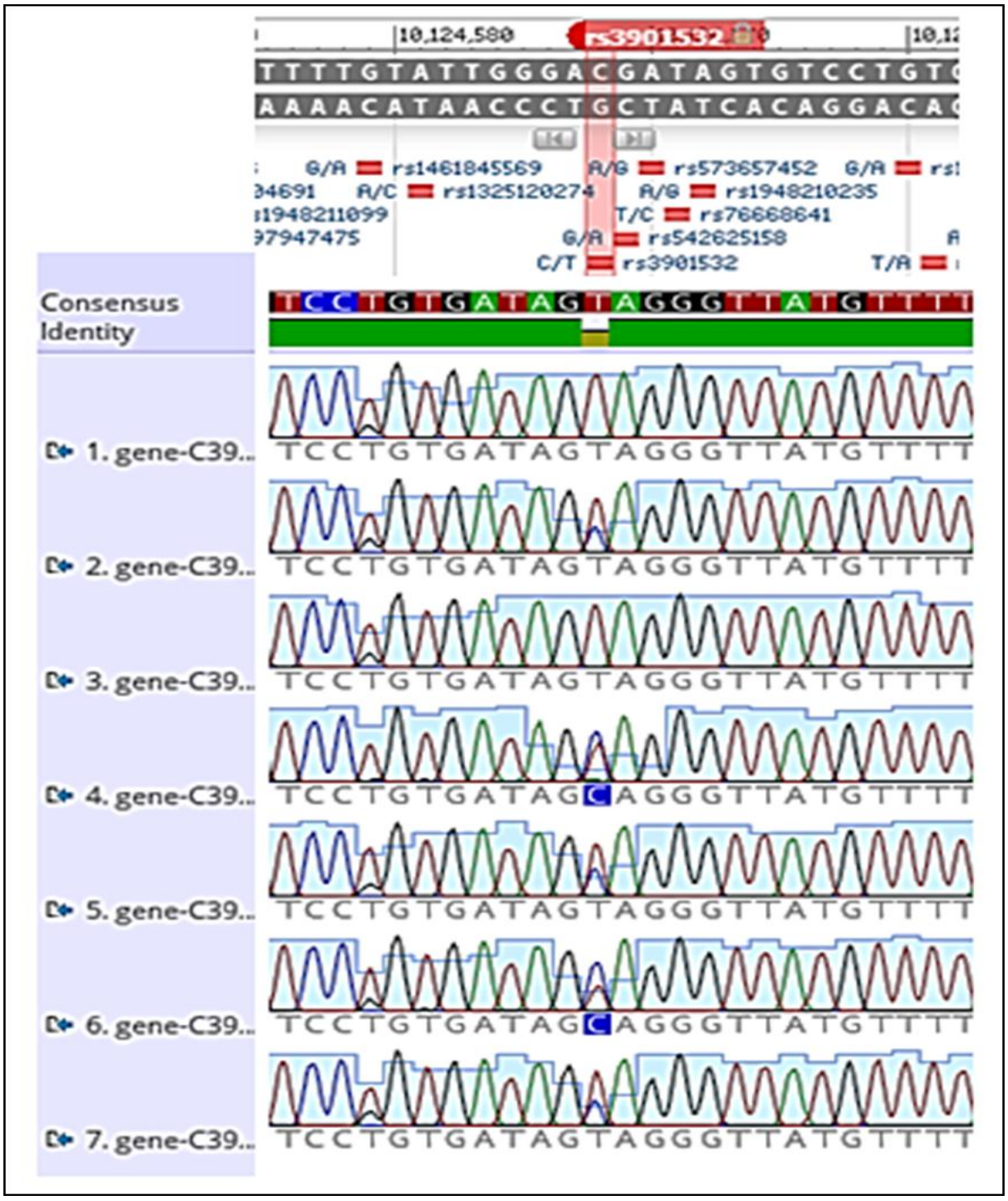

Fig. (1): The multiple alignment of chromatograms of targeted region of gene *CLEC7A* Chr12 SNP : rs3901532 C/T, of patients group. alignment performed by Geneious prime software.

APPENDIX (2)

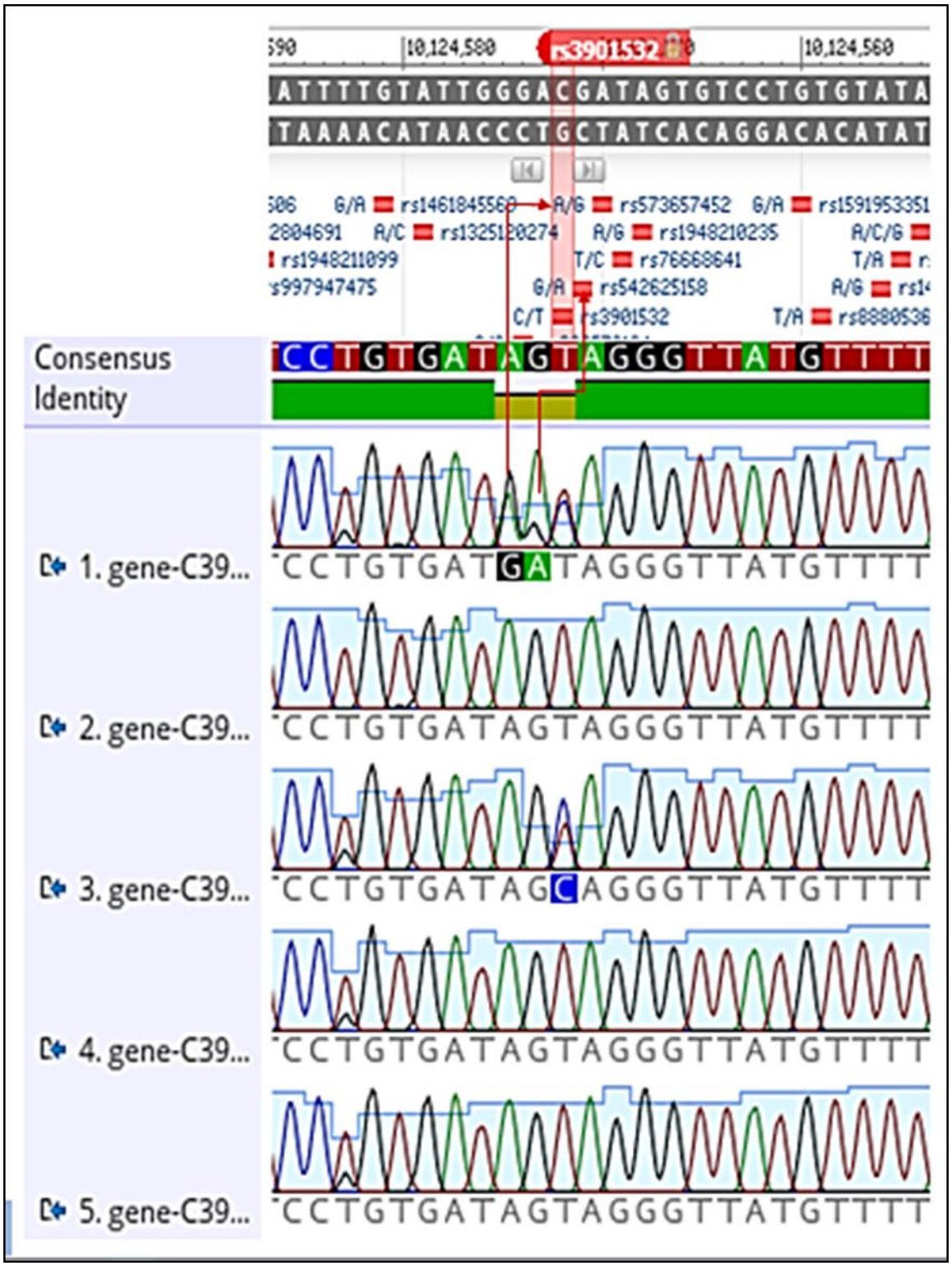

Fig. (2): The multiple alignment of chromatograms of targeted region of gene *CLEC7A* Chr12 SNP : rs3901532 C/T, of recover group. alignment performed by Geneious prime software.

APPENDIX (2)

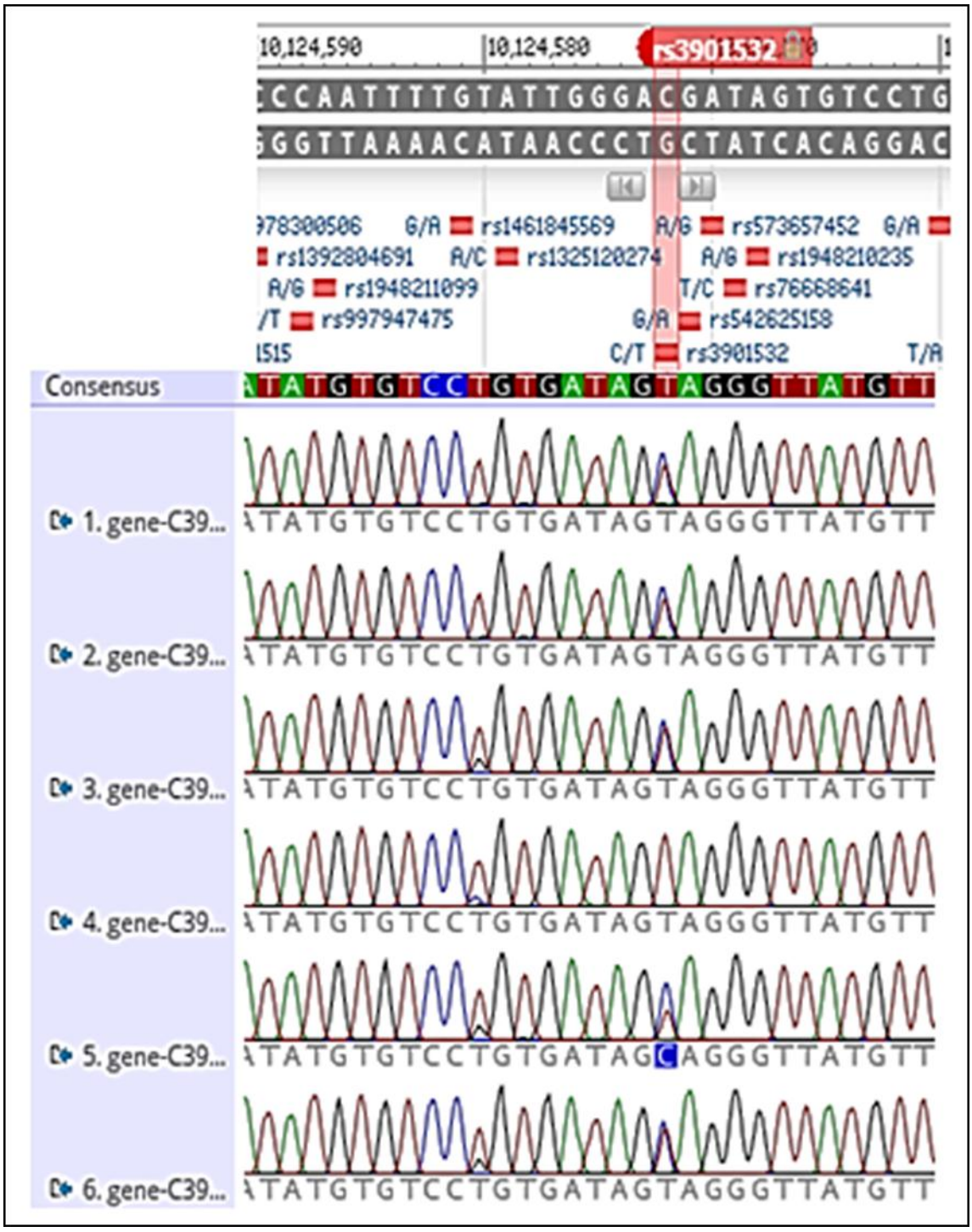

Fig. (3): The multiple alignment of chromatograms of targeted region of gene *CLEC7A* Chr12 SNP : rs3901532 C/T, of control group. alignment performed by Geneious prime software.

APPENDIX (2)

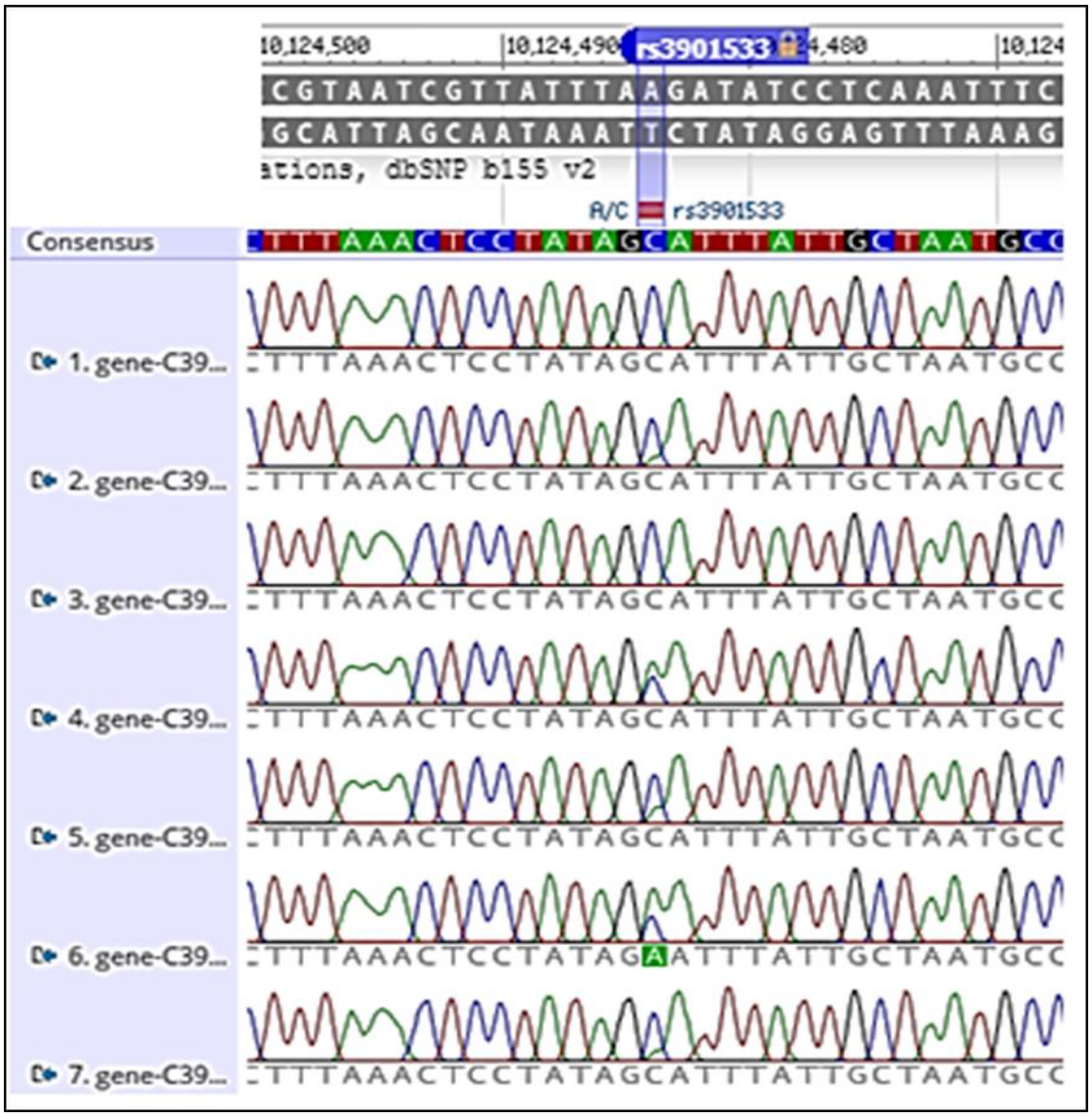

Fig. (4): The multiple alignment of chromatograms of targeted region of gene CLEC7A Chr12 SNP: rs3901533 A/C, of patient group. alignment performed by Geneious prime software.

APPENDIX (2)

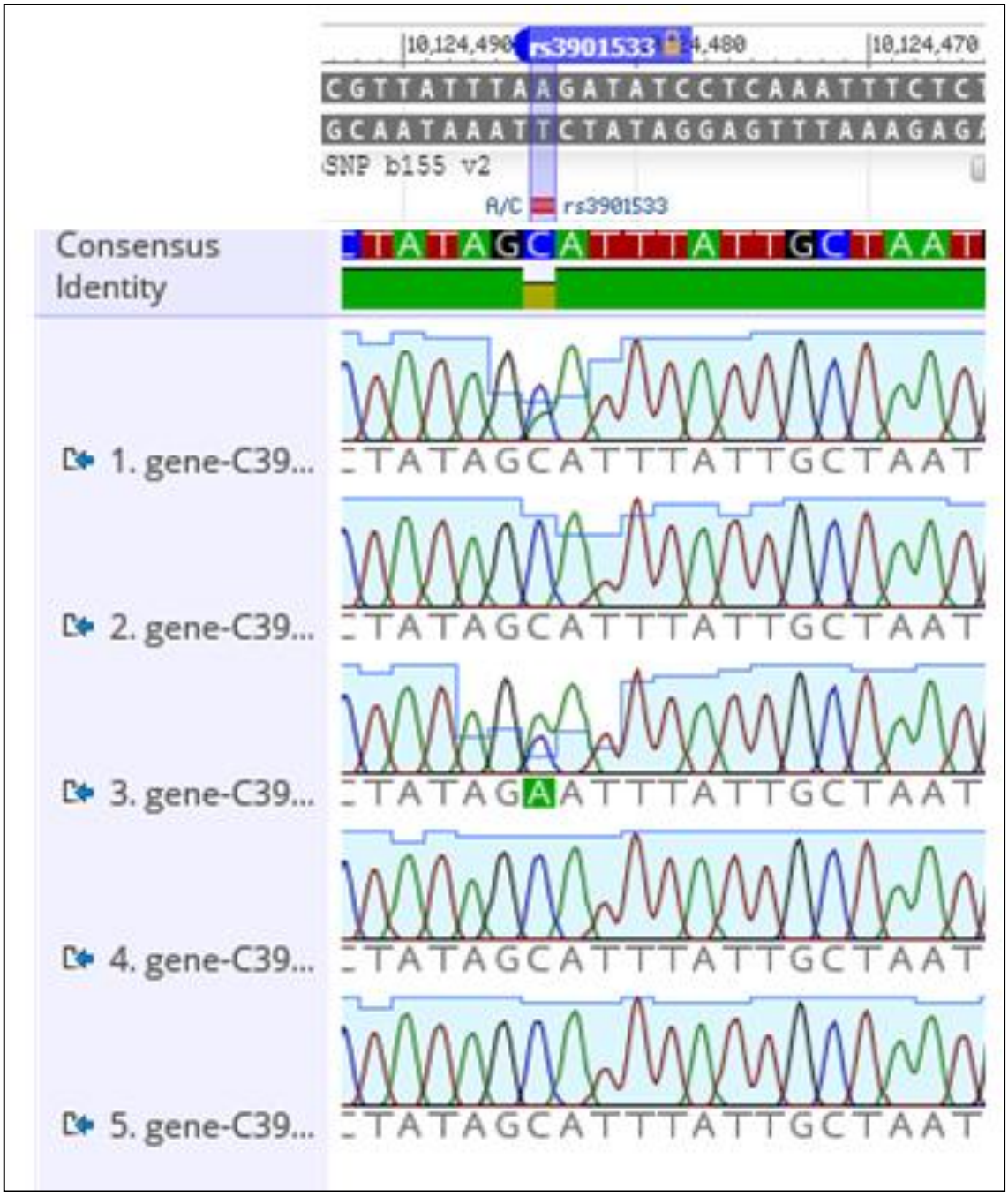

Fig. (5): The multiple alignment of chromatograms of targeted region of gene *CLEC7A* Chr12 SNP: rs3901533 A/C, of recovery group alignment performed by Geneious prime software.

APPENDIX (2)

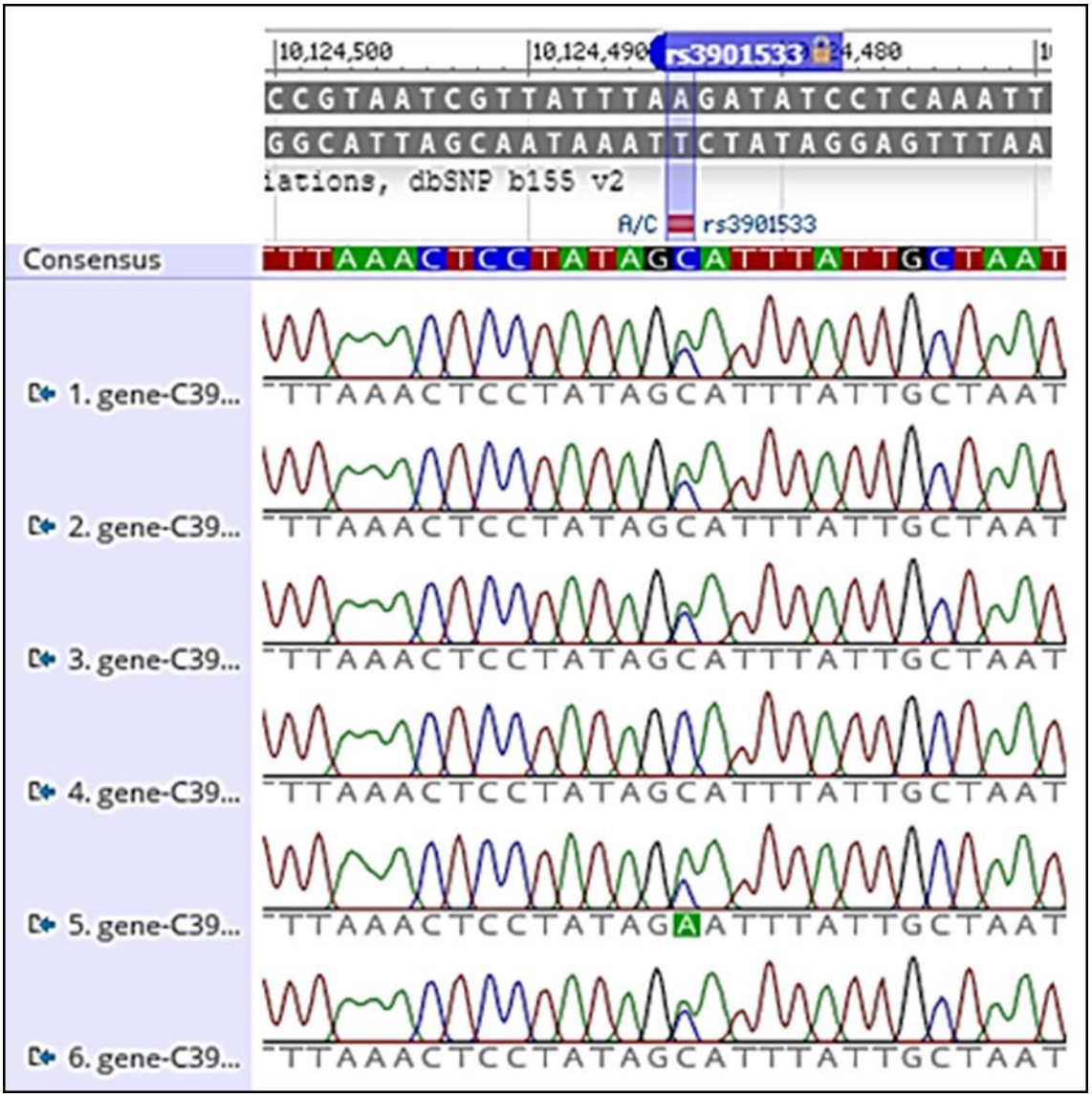

Fig. (6): The multiple alignment of chromatograms of targeted region of gene *CLEC7A* Chr12 SNP: rs3901533 A/C, of control group alignment performed by Geneious prime software.

الخلاصة:

أجريت هذه الدراسة في كلية علوم للبنات بجامعة بابل. تم جمع 146 عينة من مسحة الفم وكانت 56 عينة من مرضى مصابين بعدوى Covid-19 ، و 50 عينة من الأشخاص الذين تعافوا و 40 عينة من الأشخاص الأصحاء عن طريق المسحات ذات الوسط الناقل. وتم سحب 3 مل من الدم بواقع 60 عينة دم منها 20 مريضا و 20 شخصا تماثلوا للشفاء و 20 من الاصحاء. تم حقن الدم مباشرة في أنبوب EDTA. تم استخدام صندوق بارد لغرض نقل العينات من المستشفى إلى المختبر وحفظها تحت درجة -20 درجة مئوية في حالة التجميد العميق لاستخدامها في الدراسة الجزيئية. تم جمع العينات على مدى 4 أشهر من نوفمبر 2021 إلى فبراير 2022. جمعت جميع العينات السريرية من المرضى الذين يرقدون في قسم العناية المركزة من مستشفى مرجان (مدينة المرجان الطبية) في محافظة بابل. تم أخذ عينات من المرضى تحت إشراف الطبيب المختص. بعد تشخيص إصابة المرضى بفيروس كوفيد -19 ، تم أخذ كل العينات لكلا الجنسين وفئات عمرية مختلفة تراوحت بين 22_89 سنة.

هدفت هذه الدراسة إلى عزل الفطريات المسببة للأمراض من خلال مسحات الفم للمرضى والأفراد الاصحاء والمتشافين ، تقييم عوامل الخطر ، تحديد العزلات الفطرية بالطرق التقليدية. اختبار عوامل الضراوة وفعالية المضادات الفطرية للعزلات الفطرية. استخراج الحمض النووي لعينات الدم لكل مريض والكشف الجزيئي لبعض عوامل الخطر الجينية مثل جينات (*CLEC7A* و *TLR8*). تم زرع جميع العينات السريرية على SDA و PDA والخميرة التي تم تحديدها بواسطة CHROM Agar والكشف عن عوامل الضراوة لخميرة *Candida* spp. مثل إفراز الفوسفوليباز والليباز وتكوين الأغشية الحيوية وانحلال الدم.

أظهرت النتائج أن نسبة الإصابة عند الذكور أعلى منها عند الإناث حيث بلغت عند الذكور (55.4%) من المرضى بينما كانت الإناث (44.6%) على التوالي، بالمقابل كانت نسبة المتشافين عند الاناث (64.0%) اعلى مما عند الذكور (36.0%). وقد كشفت هذه الدراسة أن السن الأكثر تضرراً بين الجنسين تتراوح أعمارهم (65-74 سنة). وبلغت نسبة إجمالي العينات الإيجابية من الفئات الثلاث (56.84%) 83. حيث كانت النسبة المئوية للمرضى (29.45%)، بينما أظهرت الزرع الإيجابية في المتشافين ومجموعة السيطرة 13.7% لكل منهما.

الخلاصة

تم عزل وتشخيص ستة أنواع من الخميرة من عينات المرضى كانت أعلى نسبة لخميرة *C. albicans* حيث بلغت (44.6%)، والمرتبة الثانية لخميرة C. *parapsilosis* بنسبة مئوية بلغت (17.9%)، اما كل من الخمائر *C.dubliniensis* ، C. *glabrata* و *C. krusei* بمعدل النسبة المئوية (7.1%) و C. *tropicalis* بلغت (5.4%). في المتشافين بلغت نسبة خميرة *C. albicans* (20.0%) تلتها بالمرتبة الثانية *C.kruzei* بنسبة (6.0%) بينما في مجموعة السيطرة بلغت نسبة خميرة *C. albicans* (15.0%) تلتها بالمرتبة الثانية C. *parapsilosis* بنسبة (7.5%). لتقييم عوامل الضراوة لأنواع الخمائر قيد الدراسة ، أظهرت النتائج أن معظم الخمائر تمتلك عوامل ضراوة عالية. أكثر مضادات الفطريات فاعلية هي فلوكونازول ، كلوتريمازول ونيستاتين ولها أعلى نسبة حساسية ضد المبيضات بنسبة 72.3% و 67.7% و 61.5% على التوالي ، وبلغت نسبة الأمفوتريسين-ب 58.4%.

تم إجراء تعدد الأشكال لجين *TLR8* على 60 عينة من الحمض النووي للدم (20 مريضًا و 20 شخصًا يتعافى و 20 شخصًا سليمًا) بواسطة بادئ رباعي. عند المقارنة بين المرضى والمجموعة السيطرة ، كان تردد الأنماط الجينية AA و AG و GG لتعدد الأشكال الجيني *TLR8* (rs3764880 A / G)) هو على التوالي 40٪ و 35٪ و 25٪ في المرضى ، بينما على التوالي 60٪ و 0٪ و 40٪ في مجموعة السيطرة. بلغ تردد أليلات للجين *TLR8* تعدد الأشكال الجيني للأليل A 57.5٪ في المرضى و 60.0٪ في المجموعة السيطرة ، بينما تردد الاليل G بلغ 42.5٪ في المرضى و 40.0٪ في المجموعة االسيطرة. بينما أظهرت نتائج التردد للاشخاص المتعافين لتعدد الأشكال الجينية AA و AG و GG لـ A / G (rs3764880) *TLR8* هو على التوالي 25٪ و 60٪ و 15٪ وتردد أليلات للأليل A و G. هي 55.0٪ و 45.0٪ على التوالي.

تم إجراء تعدد الأشكال لجين *CLEC7A* على 60 عينة من دم الإنسان بواسطة تتابع نتائج PCR. للتحقق من تعدد الأشكال في هذا الجين ، لوحظ نمطين وراثيين ، ظهرت SNPs rs3901532 C / T في الموقع 152 وظهرت rs3901533 A / C في الموقع 64. تردد الأنماط الجينية CC و CT و TT لجين C / T (rs3901532) *CLEC7A* على التوالي 20٪ ، 45٪ و 35٪ في المرضى ، بينما على التوالي 20٪ ، 55٪ و 25٪ في المجموعة السيطرة. وبلغ تردد أليلات للجين C / T (rs3901532) *CLEC7A* بلغ للأليل C نسبة 42.5٪ في المرضى و 47.5٪ في المجموعة السيطرة ، بينما تردد الاليل T بلغ في المرضى و 52.5٪ في المجموعة السيطرة. في حين أن تردد الأنماط الجينية AA و AC و CC لتعدد الأشكال الجيني *CLEC7A* A / C (rs3901533) هي على التوالي 20٪ و 50٪ و 30٪ في المرضى ، بينما على التوالي

الخلاصة

25٪ و 60٪ و 15٪ في المجموعة السيطرة. وبلغ تردد أليلات للجين *CLEC7A* (rs3901533) A / C للأليل A 45٪ في المرضى و 55٪ في المجموعة السيطرة ، بينما تردد الاليل C هو 57.5٪ في المرضى و 45٪ في المجموعة السيطرة.

وزارة التعليم العالي والبحث العلمي
جامعة بابل/كلية العلوم للبنات
قسم علوم الحياة

الكشف الجزيئي لجينات الخطورة المتحسسة لعدوى الخمائر لمرضى كوفيد _19 في محافظة بابل

رسالة مقدمة الى
مجلس كلية العلوم للبنات، جامعة بابل
وهي جزء من متطلبات نيل درجة الماجستير
في علوم الحياة

من قبل

زهراء علي عبد الأمير

(بكالوريوس علوم الحياة/ كلية العلوم للبنات/ جامعة بابل،2018)

بإشراف

أ.د. كوثر محمد علي حسن

2022 م **1444هـ**