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Original Research Paper

Molecular Study of Virulence Factors of *Candida* spp. That Isolated from Cancer Patients.

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Abstract: ***Background****: study of microbes and cancer is considered one of the challenges in sustainable development. Candida is an opportunistic pathogenic fungus, which is inclined to infect the host with faulty immune function involving patients with cancer. A growing many of research have shown that Candida infection rise the host's vulnerability to cancer such as colorectal, gastric, and oral cancer.* ***Aim****: This investigation demonstrates the isolation and identification of Candida spp. from cancer patients of Middle Euphrates Cancer Center in Al- Najaf governorate,* ***Method****: (51) blood specimens were collected for the preparation of serum.*

The serum is cultured on SDA media and the culture is examined for microbial growth, such as culture on SDA media, Chromo agar, microscopic examination, and then by Multiplex- PCR technique. ***Results****: The current investigate showed infection percentage with opportunistic microorganisms was demonstrated as follows: C. tropicalis appeared in 40% in males and 0% in females , C. albicans appeared 10% in males, 5% in females, C. albicans appeared in cancer 10% in males, 5% in females, C. guilliermondii appeared in cancer 0% in males and 2% in females, C. parapsilosis and C. dubliniensis didn't appear in cancer. In cancer isolates the ALS_1 gene was found strongly in C. albicans, and don't found in C. guillermondii, and weakly appeared in C. tropicalis, and the PLB_1gene was found weakly in C. albicans, and don't found in C. guillermondii, and C. tropicalis.* ***Conclusion****: In the normal state, the blood is sterile, but when the immunity is suppressed or weakened as a result of a specific symptom or disease, the whole body and the blood are also vulnerable to any opportunistic microbe that grows and multiplies with it. So the presence of microbes in the blood is a strong indicator of weak immunity in cancer patients to the point of penetration of Candida into the blood. We concluded from our current study that some pathogenic microbes can be isolated from the sera of cancer patients, as some types of Candida were isolated, which include C.albicans, C. tropicalis, ,and C.guillermondii.Our results of this study indicated the capacity of some of Candidia spp. to produced phospholipase enzyme. Not all C. albicans isolates was positive for phospholipase production, we also conclude that some genes of virulence factors don't necessary to express and Candida can be pathogenic without it.*
Keywords: *Candida, Cancer, Multiplex- PCR technique, Virulence factors*

1.Introduction

Molecular techniques are targeted to detect *Candida* species in a short period of time, with a high sensitivity and specificity. For this purpose, several PCR methods have been developed, such as nested PCR, multiplex PCR, Taq-man PCR, Light-Cycler PCR and fluorescent PCR [1, 2]. Pathogenesis of microbes means the ability of microorganisms to cause disease, where the microbes enter the host body and colonize them, virulence factors expression depends on its ability to overcome the immune system of the host this virulence factors

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including ،enzymes, structure, and other factors which may cause the disease and cause harmful effect to the host [3] Fungal infection incidence is different according to several conditions and circumstances such as socioeconomic cultural habits, geographic regions, and other different conditions[4]

Some types of fungi Can inhabit the epithelial tissue of the host of most healthy humans but it can make risk to human life when it reaches to the systems of peoples with Immunocompromised [5] The *C.albicans* is responsible for an unacceptably high number of symptomatic infections each year, ranging from skin and mucous membranes to invasive internal organs. The majority of these infections occur in immunocompromised individuals and originate in the gastrointestinal tract. As a result, it is critical to bridge the knowledge gap regarding *Candida* spp. colonization, commensal lifestyle and transition into a pathogenic state. Surprisingly, *Candida* spp. appear to perform host-beneficial functions [6]

Candida species can cause various diseases in humans and animals [7] Cancer disease act as many immunocompromised diseases, it may decrease the activity of the immune system and decrease the body resistant to the opportunistic microbes like *Candida* and other normal flora have the chance to invade and colonize the blood. The current investigation aimed to isolate and identification of *Candida* species from cancer patients in AL Najaf governorate. reasons that lead to increasing *Candida* infections such as excessive intake of antibiotics, sugars or diabetics , and this immune system during a chemotherapy treatment in cancer patients affects the normal body criteria such as osmolality, the pH, and others ,these changing in conditions are conducive to the growth of opportunistic organisms and allow them to grow ,multiply and transcript the genes of virulence factors and other factors that lead to the settlement of the disease, then the microbes, weakening the immunity and seizing the opportunity to multiply as one of the body's infectious agents and delaying its immune defense, and other causes that inactivate immunity by killing immune cells and paving the way for opportunistic microbes to replace beneficial natural bacteria.

In addition, chemotherapy, radiotherapy, and other treatments for cancer patients can lead to encouragement of inflammation in the body, exaggeration of the mucous membrane of oral cavity, and making it more vulnerable to microbes and any injury, especially challenging ones, including *Candida*, and this is consistent with [8].

2.Methodology

Patients and specimens collection

About 51 specimens were collected from cancer patients, whose ages ranged between (less than 10 years - 60 years) during the period from (January to June 2022), all specimens were collected from cancer patients as blood serum, collected from Al-Najaf governorate. Information from all patients was recorded using proprietary data.

Specimens processing

The specimens were taken transferred to laboratory, Al-Ameen Center for Research and Advanced Biotechnologies, in AL-Najaf Governorate where the specimens were cultured by streaking method on culture media, Sabouraud's dextrose agar and chromagar then incubated at 37C°for (1-7) days for visible growth of Candida colonies, other growth were discarded as a negative.

Identification of Candida spp. by PCR technique

Preparing the primers

The primers that used in this study were received as a lyophilized form, which was prepared according to the manufacturer's instructions by adding deionized distill water in order to get concentration of 100 pmol /µl as stock solution, primers working solution were prepared by adding 10 µlof stock solution to 90 µl of deionized distill water to get final concentration of working solution 10 pmol/µl. [9] .Table (1).

Table (1): Primers pair used in this experiment were Universal primers.

Universal primers	Sequence	Specific primers	Sequence	Reference
ITS_1	(5'TCCGTAGGTGAACCTGCGG-3')	CA3	(5'GGTTTGCTTGAAAGACGGTAG-3')	Tarini et al., 2010
ITS_2	(5'GCTGCGTTCTTCATCGATGC-3')	CA4	(5'AGTTTGAAGATATACGTGGTAG-3').	

Culturing of the isolates

The fungal strains were streaked in the Sabourd dextrose agar (SDA) plate and incubated at 37C° for (24 and 48) hours for Candida species. A single colony of the fungal organism was transferred from SDA plate and cultured in Sabouraud's dextrose broth (SDB) for (24 and 48) hours before proceeding to the DNA extraction.

Fungal DNA extraction

Total DNA was extracted from the culture broth,

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and 1.5 ml of culture broth was pipetted into Eppendorf tubes. Afterward, it was centrifuged at 4.300×g for 5 min, and the supernatant was discarded. Subsequently, 200 ul of TE buffer was added, vortexes well were boiled for 10 min, and then put on ice immediately for1 m in. This was centrifuged again at 6.700×g for 10 min, and the supernatant was collected, which was used as the DNA template [10].

PCR assay

The PCR assay was performed to amplify ITS_1 and ITS_2 sequence for identification of Candida spp. Table (5).

PCR Reaction mixture

PCR Reaction Mixture contents were listed in table (2).

Table (2): PCR Mixture Component Used in the Reaction

PCR Master mix	Volume
DNA template	**5µl**
Forward primer (10pmol)	**1.5µl**
Reveres primer (10pmol)	**1.5µl**
Deionized water	**12µl**
Total volume	**20µl**

Identification of virulence factors of *Candida* spp. by PCR technique , table (3) explain it.

Table (3): Primers coded C. albicans and Virulence Factors Target genes Primer sequences and DNA Sizes [11]**.**

Target genes	Primer sequences	DNA sizes
PLB_1	**Forward** **5'-ATGATTTTGCATCATTTG-3'** **Reverse** **5'-AGTATCTGGAGCTCTACC-3'**	**751 bp**
ALS_1	**Forward5'GACTAGTGAACCAACAAATACCAGA-3'** **Reverse 5'-CCAGAAGAAACAGCAGGTGA-3'**	**318 bp**

PCR conditions

PCR amplification system was used with the following program: initial denaturation for 5 min at 94C°, then 1 min. 94°C, 1 min. at 52C°, 1min. at 72C°, 35 cycles; 5 min at 72C°and for 1 cycle. PCR products were separated by electrophoresis on 1.3% (w/v) agarose gel, and stained in ethidium bromide. General program is listed in Table (4).

Table (4): Cycling Parameters of Gene´s Amplification for Candida albicans.

PCR step	Temp	Time	Repeat
Initial Denaturation	**94C◦**	**5min**	**1**
Denaturation	**94 C◦**	**1min**	**35 cycle**
Annealing	**52 C◦**	**1min**	
Extension	**72 C◦**	**1min**	
Final extension	**72 C◦**	**5min**	**1**
Hold	**4 C◦**	**Forever**	**-**

3.Results and discussion

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Table (7): Primers coded C. albicans and Virulence Factors Target genes Primer sequences and DNA Sizes

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Annealing	52 C◦	1min	
Extension	72 C◦	1min	
Final extension	72 C◦	5min	1
Hold	4 C◦	Forever	-

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Results and discussion

Cultures and properties of growth on SDA agar

Culture examination on the SDA medium of specimen of cancer patients, where grows rapidly within 24-48 hours. The colony's texture was smooth, glistening, or dry, colored creamy to yellow, and the growth of yeast on Chrom agar is colored according to genus and species, this colony color difference is used to identify the yeast isolates. the table demonstrates the colors of yeast and its identification. these colors' appearance agreed with [10,12,13,14,15]. Figures (1) and (2) explain these properties.

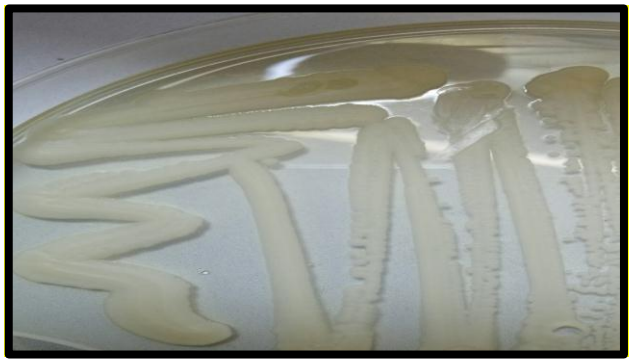

Figure (1): Explain the appearance of colony of *Candida spp*. on SDA after incubation at 37C° for 48 hours.

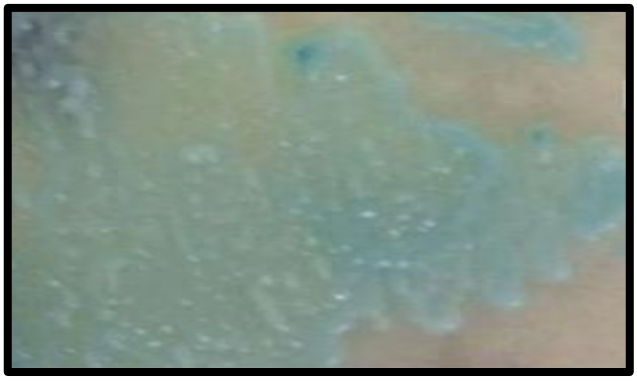

Figure (2): Explain the *Candida spp.* colonies appearance on Chromagar, *Candida parapsilosis*.

Table (9): Explain the negative and positive results of growth of all specimens from cancer patients .

Type of Dise	NO. of speci	Type of speci	Negative	Positive		
				All	*Candi*	Other m.o.
Cancer	51	Blood serum	23	28	15	13

Identification by multiplex PCR technique

DNA amplification with universal fungal primers followed by detection using species-specific probes greatly improved the sensitivity of Candida detection [16]. Molecular techniques are targeted to detect *Candida* species in a short period of time, with a high sensitivity and specificity. For this purpose, several PCR methods have been developed, such as nested PCR, multiplex PCR, Taq-man PCR, Light-Cycler PCR and fluorescent PCR [1, 2].

The phenotypic description of *Candida* may insufficient due to the similarities of morphological, biochemical with other *Candida* spp. [17]. Therefore, in recent years, the identification of *Candida* has been done by modern genetic identification methods. In particular, PCR is the most common diagnostic method that used for this purpose.

In this study according to Identification of all specimens that appear positive growth for *Candida* species by multiplex PCR technique, we notice that some *Candida* species appeared, while other disappeared, all these results will detail as following:

Candidiasis may have an association with a variety of malignancies. In cancer *C. tropicalis* appeared in 40% in males and 0% in females, *C. albicans* appeared 10% in males, 5% in females, *C. guilliermondii* appeared in cancer 0% in males and 2% in females, *C. parapsilosis* and *C. dubliniensis* didn't appear in cancer. these results concurred with [18]. All cancer patients are with immunodeficiency so as a result of immunity inhibition, all normal flora may be opportunistic, some of these opportunistic fungi microbes, which is characterized by *Candida* species, are yeast like fungi, eukaryotic opportunistic organisms that be a flora on the mucosa of the digestive tract as well as genital tract [19] . All these results explain in Tables (7-10) and Figure (3).

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Table (10): Explain the appearance percentage of *Candida* species by multiplex PCR technique of specimens from cancer patients

Cancer / Blood Serum		
Type of yeast	**Percent %**	
	Male	**Female**
C. albicans	**10%**	**5%**
C.guillermondii	**0%**	**2%**
C.tropicalis	**40%**	**0%**
Other growth (non-*Candida* growth)	**43%**	

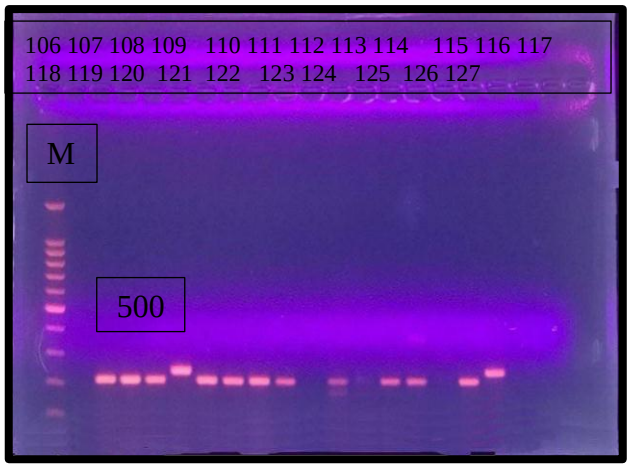

Figure (3): Agarose gel electrophoresis of ITS regions PCR product by pair primers (ITS_1-ITS_2) of different yeast spp.(1.3g agarose gel, 80 volts for 1 hr.) (M: DNA ladder; lane106-127: *Candida* spp. isolated from cancer patients).

Identification of virulence factors of *Candida* spp. of specimens from cancer and candidiasis patients by Multiplex- PCR technique

These genes are the most important virulence factors of *C. albicans* [8]. According to this study the results of identification of virulence factors of *Candida* spp. of specimen from cancer patients by Multiplex- PCR technique appeared as the following:

In cancer isolates the ALS_1 gene was found strongly in *C. albicans*, and don't found in *C. guillermondii*, and weakly appeared in *C. tropicalis.* Till now nine ALS proteins have been identified in *C. albicans* isolates ALS_1 proteins are responsible for adhesion and biofilm formation [20] While the $CALB_1$gene was found strongly in *C. albicans*, and don't found in *C. guillermondii*, and weakly appeared in *C. tropicalis.*And the PLB_1gene was found weakly in *C. albicans*, and don't found in *C. guillermondii*, and *C. tropicalis.* Therefore, these *C. albicans* isolates could be found as normal flora. In fact, isolation of *C. albicans* from healthy ones has been reported, but the presence of virulence factors could not be detected in these isolates.

These results partially agreed with [21]. Our results of this study indicated the capacity of some of *Candidia spp.* to produced phospholipase enzyme. Not all *C. albicans* isolates was positive for phospholipase production, this result disagreement with [22]. who found positive in yeast *Candida albicans* and *Candida krusei*, while negative results appear in other species. And agree with [23]. Which explain some study suggest that direct host cell injury and lysis are the chief mechanisms contributing to virulence of fungi without needing to these lyses enzymes. Also some of negative results of the PLB_1gene may don't express at this state agree with [24], which explained that some *C. albicans* produces all (4) extracellular phospholipases (A to D) depending upon (64) the sites of attack, all of which able either to lyse membranes or shifting the nature of the host cell surface, possibly simplifying adherence and settlement. All these results explained in Table (11) and Figures (4-6).

Table (11): explain the identification of virulence factors of *Candida* spp. by multiplex PCR technique of specimen from patients (cancer and candidiasis).

Type of disease	**Type of *Candida***	**Type of genes**	
		ALS_1	**PLB_1**
Cancer	***C. tropicalis***	**+***	**-**
	C. albicans	**+**	**+***
	C.guillermondii	**-**	**-**

+:positive (presence of gene) , - :nagative (absence of gene) * weak positive

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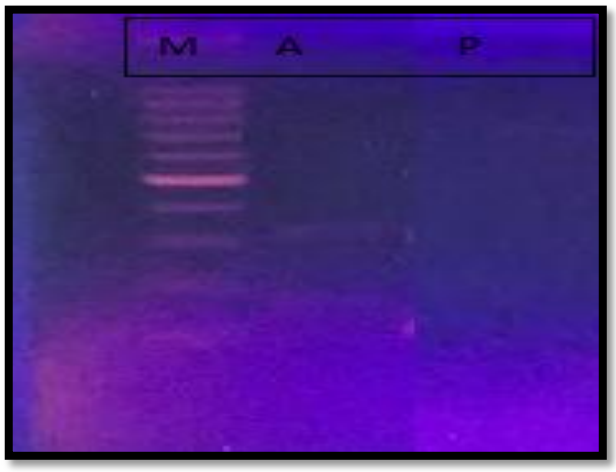

Figure (4): Explain diagnosis of virulence factors genes by multiplex-PCR technique in *Candida tropicalis* isolate [M: Marker, Isolate: *C. tropicalis*]: Genes: A:ALS1and P:PLB_1

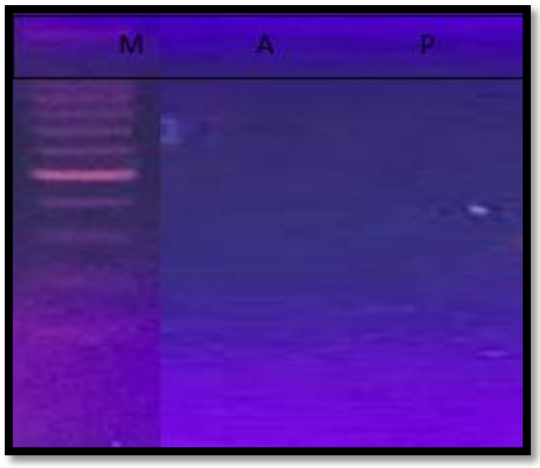

Figure (5): Explain diagnosis of virulence factors genes by multiplex-PCR technique in *Candida guillermondii* isolate [M: Marker, Isolate: *C. guillermondii*]: Genes: A:ALS1and P:PLB_1

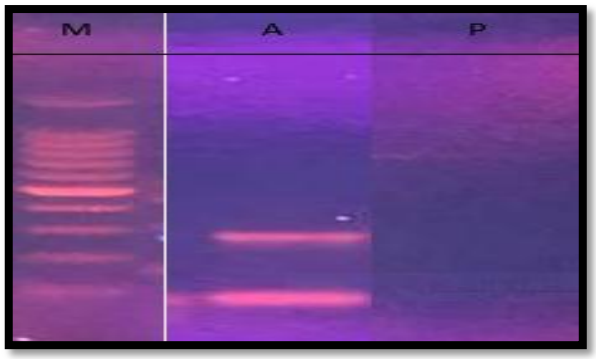

Figure (6): Explain diagnosis of virulence factors genes by multiplex-PCR technique in *Candida C. albicans* isolate [M: Marker, Isolate: *C. albicans*]: Genes:ALS_1, and P:PLB_1

Conclusion

In the normal state, the blood is sterile, but when the immunity is suppressed or weakened as a result of a specific symptom or disease, the whole body and the blood are also vulnerable to any opportunistic microbe that grows and multiplies with it. So the presence of microbes in the blood is a strong indicator of weak immunity in cancer patients to the point of penetration of Candida into the blood. We concluded from our current study that some pathogenic microbes can be isolated from the sera of cancer patients, as some types of Candida were isolated, which include *C.albicans, C. tropicalis, ,and C.guillermondii*.Our results of this study indicated the capacity of some of *Candidia spp.* to produced phospholipase enzyme. Not all *C. albicans* isolates was positive for phospholipase production, we also conclude that some genes of virulence factors don't necessary to express and *Candida* can be pathogenic without it.

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