Published Online First: May, 2024
https://doi.org/10.21123/bsj.2024.9415
P-ISSN: 2078-8665 - E-ISSN: 2411-7986
Baghdad Science Journal

Genetic prevalence of antifungal resistance gene in cancer patients with Oropharyngeal Candidiasis from Iraq.

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Received 12/09/2023, Revised 04/11/2023, Accepted 06/11/2023, Published Online First 20/05/2024

Abstract

As the population of immunocompromised patients grew, yeast infections became more common. There has been a significant rate of morbidity and mortality among patients with Candidiasis demonstrating increased resistance to antifungal medications. The main aim of this study was to determine the virulence gene of *Candida albicans*, evaluate antifungals used for Candidiasis, and biofilm formation, which is isolated from the mouth of individuals in the Baghdad Governorate From 1st October 2022 to 28th February 2023. This study included 120 oral swabs collected from people who had oral candidiasis, with an age group between10-65 including both Genders. The results obtained showed that the candidiasis frequency was more prevalent in males between the age group of 11-20 years. These results showed a statistically significant increase in the prevalence of ERG11 virulence gene among the biofilm-forming *C. albicans* fungus compared to other isolates with a significant difference (P = 0.863). Also, the study showed significant resistance of *C. albicans* to the antifungals fluconazole, itraconazole and fluconazole with the ability to form a biofilm. The present study also highlighted an elevated prevalence of the ERG11 gene in *C. albicans* and a strong association between the formation of biofilms and the presence of virulence gene was noted. Candidiasis is one of the fungal infections that are more prevalent among patients, and the virulence factor biofilm has an important role in increasing the pathogenicity of the fungus.

Keywords: Antibiotic susceptibility, Biofilm, *Candida albicans*, ERG11, virulence.

Introduction

The most common fungus pathogen of humans and a common commensal of the mammalian microbiome are both called Candida albicans. They are found primarily on human mucosal surfaces such as the gastrointestinal and urogenital tracts as a commensal and most commonly cause invasive disease as a result of alterations in the normal microbiological flora, breaches in the mucocutaneous barrier, or defects in the host cellular response. In addition to the traditional risk factors for developing IFDs, the presence of central venous catheters, the use of total and parenteral nutrition, the use of indwelling urinary catheters, and the use of broad-spectrum antibiotics increase one's risk of developing invasive candidiasis[1] .Species of the genus *Candida* are part of the common microbiota of humans; however, some of the *Candida* species are known opportunistic pathogens.

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Formation of biofilms, resistance to antifungal drugs, and increase in asymptomatic infections demand more studies on the isolation, identification and characterization of *Candida* from clinical samples [2,3]. Candida infections, whether caused by *C. albicans* or another species, have increased dramatically [4]. Members of the Candida genus, most often *C. albicans* and non-albicans spp., caused the majority of yeast infections. Patients with weakened immune systems, such as those suffering from diabetes, AIDS, malignant tumors, or solid organ transplants, are more vulnerable to candidiasis[5]. The "virulence factors" present in yeast cells, such as biofilm, pigments, and extracellular hydrolytic enzymes, boost cell virulence[6].

Candidiasis is frequently accompanied by the formation of a biofilm (BF), which can occur both in vivo, such as the oropharyngeal surface, and in vitro, such as catheter and indwelling device surfaces.[7] These biofilms are made up of tightly packed micro colonies of cells that form a complex structure that increases drug resistance to drugs such as nystatin, Azole, and amphotericin B[7,8]. These factors increase the pathogen's ability to survive, invade, and disseminate to other organs [5]. Candida infection can vary depending on the site of infection; oral candidiasis is the most frequent kind[9]. Immunocompromised people have more Candida species colonization than healthy people[9]. Cancer patients with oral candidiasis have colonization levels ranging from 30% to 50%[10]. *C. albicans* has been recognized as the primary source of fungal illnesses in cancer patients[11] .

Numerous investigations on the formation of biofilms and extracellular hydrolytic enzymes (proteinase, phospholipase, and lipase) have been undertake[11]. They were discovered to have a strong relationship with yeast pathogenicity by enhancing adhesion, penetration, host defense modulation, or colonization[12]. Yeast cells, on the other hand, aggregate form a thin covering known as a biofilm that adheres to solid surfaces[13]. This layer, however, has a thin, strong appearance and characteristics associated with pathogenicity and antifungal medication resistance[14]. Azoles are the first-line antifungals utilized in therapy. By encoding the gene ERG11 (Erg11p), the azoles work by inhibiting lanosterol 14-demethylase, an enzyme that aims to limit the biosynthesis of ergosterol, which is required for the synthesis of the cell membrane of fungi resistant to azoles. Lanosterol 14-demethylated azoles are produced by mutagenesis and gene expression of the ERG11 gene[15].

Candida frequently sticks to biomedical equipment and develops into a hardy biofilm that may survive extremely high antifungal doses. The central venous catheter (CVC), which is used to infuse fluids, nutrition, and/or cytotoxic medications, is the medical device that is most frequently contaminated by Candida biofilms. The distal tip of the catheter can be contaminated at the time of insertion or, alternatively, organisms can migrate down the catheter wound, but more frequently, Candida can have its origin on the patient's skin or the hands of nursing staff[16].

The ability of each species to create extracellular polymeric substances (EPS) and exhibit dimorphic growth, as well as the substratum of the biofilm, the accessibility of carbon sources, and other factors, all affect the biofilm's features. Additionally, pathogenic yeasts of the Candida genus exhibit a high degree of complexity and diversity in the transcriptional regulation of activities like adhesion, biofilm formation, filamentation, and EPS generation. The antifungal resistance that is typically present in Candida biofilm cells, potentiated by EPS, which acts as a barrier to drug diffusion, and by the overexpression of drug resistance transporters, has implications for both the persistence of colonization and infections[17].

In the plasma membrane of Candida cells, ergosterol predominates over all other sterols. Additionally, antifungal medications (such as azoles and amphotericin B) function as ergosterol synthesis inhibitors by attaching to the particular enzyme lanosterol demethylase involved in the manufacture of this sterol. The discovery that Candida mutants with altered ergosterol production exhibit increased resistance to azoles and amphotericin B prompted the researchers to wonder whether Candida biofilm cells might use comparable mechanisms of resistance[18].

Numerous investigations revealed that when comparing the membranes of biofilm cells to those

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of planktonic cells, the latter exhibited a lower content of ergosterol, particularly during the latter stages of biofilm development. This study shows that ergosterol is not as important for maintaining membrane fluidity in cells from established biofilms, which may restrict the effectiveness of medications that target ergosterol. In fact, numerous investigations have shown that different Candida species have altered sterol pathway genes' transcriptional profiles. When compared to their planktonic counterparts, ERG25 and ERG11 in vitro biofilm development increased according to a *C. albicans* microarray investigation [17].

In Iraq, there is not much research or scarcity of research and information regarding the drug resistance of fungi and its molecular mechanisms, especially in *C. albicans*. Therefore, this research was designed to find out the prevalence of disease causing *C. albicans* and its resistance to antifungals. We also aimed to find the prevalence of ERG11 responsible for antibiotic resistance among the isolates isolated from the mouth and pharynx of subjects[16].

Materials and Methods

Patients and Samples collection: Between October 1st, 2022 and 28th February 2023, 120 male and female patients with cancer were admitted to Medical City, Specialist Children's Hospital, Baghdad Hospital, and Central Teaching Hospital of Pediatrics. To complete this project, Middle Technical University/College of Health and Medical Techniques/Department of Medical Laboratory Techniques inked agreements. A sterile pre-moistened swab was used to collect duplicate samples of oral thrush. The clinical mycology laboratory received all samples. The process of collecting samples was done by rinsing the mouth with 10 ml of sterile water for half a minute, and the rinse components were settled by centrifugation for 15 minutes at 2000 rpm. In ideal conditions, 37 °C, *C. albicans* were dispersed by germ tube formation experiment (2 hours in serum at 37 °C), after which microscopic examination was carried out to reveal the germ tube[19] . Chlamydospore production on Corn Meal Agar (CMA), colony color on CHROMagar Candida medium a (CHROMagar, Paris, France),

Conventional Identification

Plate-Base Method: After preparing the samples, the type of Candida was diagnosed using a chromogenic candida luminescent agar (BCA)medium, according to the manufacturer's instructions,[12] Sub-cultured isolates were then incubated for 2 days at 37°C. The yeast colony color on the plate was used for identification and growth was evaluated every day for 3 days.

Germ Tube Test: *C. albicans* germ tube assay was performed for all yeast isolates and other related isolates by adding each isolate to 0.5 ml of human blood serum in a test tube. After incubation for 3 hours at 370 °C, 1–2 drops of the suspension were added to 10% KOH onto the slide and examined under a light microscope to examine the germ tubules.[13,15]

Detection of Virulence Factor

Biofilm formation: The biofilm formation of the isolated yeasts was investigated were evaluated by the method described by Coffey BM et al[20] . Fungal cultures grown overnight in Saubroud dextrose broth were diluted in sterile saline to 0.5McFarland ($1.5x10^8$ CFU/mL). About 15µl of this fungal suspension was added to 96-well microplates containing 150µl of Saubroud broth. The plates were then incubated at 37^0C for 24- 48 hr to facilitate biofilm growth. Following incubation, the wells were washed with PBS and stained with crystal violet (0.1%) for 15min. The stained cells were then decolourized with 33% acetic acid and the absorbance was recorded at 492nm was measured in Genetix microplate reader. The biomass adhered to is estimated to be proportional to the absorbance. The intensity of the biofilm growth was assessed using OD cut-off values[15.]

Antifungal susceptibility testing: The antifungal sensitivity test was done using the Kirby-Bauer disk diffusion method [12]. In brief, the inoculum was adjusted to 0.5McFarland with saline. Standard

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antifungals (Liofilchem R srl, Italy) like Fluconazole FLU (100mg), Itraconazole ITR (50mg), Nystatin NY and Amphotericin B were added to their respective wells and incubated at 37°C for 24-48hr. Following incubation, inhibition zones were measured in millimeters and the findings were interpreted using interpretative breakpoints established by CLSI/EUCAST for in vitro sensitivity testing [21].

DNA extraction and PCR amplification: The extraction of DNA was done, based on bead beating tube and spin columns as described by gene aid[22]. Primer for ERG11 gene (F: 5′-GCAGCA GCA GTA TCCCATCT-3″, R: CTCATGGGGTTGCCAATGTT-3′[23].Was designed using primer 3 software and procured from Sigma Aldrich. ERG11 gene was found to be responsible for the resistance against azole exposure in *C. albicans*. Amplitaq Gold master mix was used in the amplification using the System PCR 9700 of GeneAmp Thermocycler (Applied Biosystems, CA, USA). DNA polymerase (5U/L), 125mM $MgCl_2$; and 10 mM dNTP mixture. The PCR was performed in a total reaction volume of 10.5µl. 2µl of each primer was added to the master mixture. Then the following amplification protocol: 95°C for 5min for Initial denaturation, then 35 cycles of denaturation at 95°C for 1min, Annealing at 53°C for 30sec, and the elongation step was at 72°C for 2min. The amplicons were separated on 1.5% agarose gel using TBE buffer. PCR product to be expected at 751bp.

Table 1. Table showing the primer sequence used for the molecular study of the isolated *C. albicans.*

Gene		Sequence of Primers	Annealing Temp.	Size of Amplicon (bp.)
CALBI	**FW**	GCAGCACAGCAGTATCCCATC	59.8	751bp
	RV	CTCATGGGGTTGCCAATGTT		

Statistical analysis: The statistical analyses were performed using SPSS (Statistical Package, Version 20.0). The variables were subjected to student's t-test and were tested for significance at $p<0.05$. The SD, which was calculated using Microsoft Excel 2010, was used to quantify the dispersion.

Results and Discussion

Study population: The present study comprised 120 male and female patients diagnosed with cancer and were hospitalized at Medical City Specialist Children's Hospital, Baghdad Hospital, and Central Teaching Hospital of Pediatrics. The observed results were about 45.83% (55/120) of the subjects was females and the rest 54.16% (65/120) were male. A significant observation was funded, where a larger proportion of females (27.27%) to be in the age group of less than 10 years old (15/55). And a higher percentage of males (30.77%) are in the age group of 11-20 years old (20/65). Further, also had confirmed data, where the majority of patients had inflammatory symptoms of oral candidiasis, like severe pseudomembranous candidiasis, oropharyngeal candidiasis, and angular cheilitis.

After incubation, 90% (100 of the samples) of the positive sample swabs showed development on the SDA plates, whereas the rest samples did not. The morphological identification of the entire positive (n=90) samples was investigated, for the development of colonies on Brilliant green and the formation of germ tubes. Among the cultured isolates about 92 and 83% showed positive growth on SDA and CHROM agar respectively. On the other hand, about 83% showed the development of germ tubes (Table 2, Fig. 1)

Table 2. C. albicans growth on Saubrouds Dextrose Agar (SDA), Germ tube and CHROM agar culture)

	SDA	Germ tube	CHROM agar
Number positive	110	100	100
Percentage	92%	83%	83%

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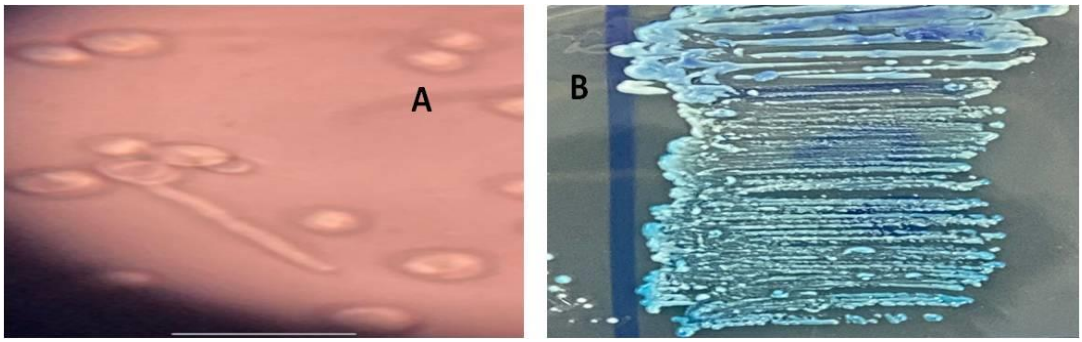

Figure 1. Photographic images showing A: germ tube formation in *candida albicans;* B: *candida albicans* on Brilliance candida agar (BCA).

Table 3. Table showing the antibiotic susceptibility in *C. albicans* isolated from the patients. (n=90). All the values are average of triplicates.

Antifungal	Sensitive (%)	Resistance (%)	Intermediate (%)	Total no.
Flucanozole	16.60	72.20	11.11	
Itrconozole	33.30	61.11	5.55	90
Nystatin	88.88	1.12	10	
Amphotericin B	94.44	0	5.56	

According to the Clinical and Laboratory Standards Institute criteria, the antifungal susceptibility test revealed that resistance impacted all of the families evaluated in this investigation. The isolated strains showed less susceptibility to Flucanozole (72.20%) followed by Itraconazole (61.11%). Among the 90 isolates tested, almost all of them were found to be susceptible to Amphotericin B. There were no resistant isolates observed for Amphotericin B. Among the sensitive isolates 94.44% were found to be highly sensitive and 5.56% were intermediate. (Table 3) **Antifungal susceptibility testing:** With the ability of *C. albicans* to form a biofilm, its resistance to antifungal drugs increased, and the increase was as follows for the biofilm-forming isolates: flunazole, itraconazole, amphotericin B, and nystatin (83.1%, 67.5%, 5.2%, and 11.7%, respectively) compared to isolates that did not form biofilm (84.6%, 61.5%, 7.7% and 7.7%, respectively). There was a significant increase in the prevalence of ERG11 virulence gene (96.6%) among biofilm-forming isolates compared to non-biofilm-forming isolates (11.9%).

Table 4. Comparison between biofilm forming *C. albicans* and non-biofilm forming *C. albicans*.

n = 90	Biofilm forming 85.6%	Non-biofilm forming 14.4%	P value
Nystatin	11.70%	7.70%	P=0.672
Fluconazole resistance	83.1%	84.60%	P=0.893
Itraconazole resistance	67.50%	61.50%	P=0.672
Amphotericin B resistance	5.20%	7.70%	P=0716

The result after chi square performed was non-significant >0.05 so there we deduced no significant relation between biofilm formation and antifungal resistance.

Antifungal susceptibility tests in *C. albicans*

The *invitro* An antifungal susceptibility study was conducted on five antifungals used for *C. albicans* showed that all *C. albicans* isolates (100%) were susceptible to AMB, ITC, VRC and CAS with MICs range from ≤ 17 mm The result was resistance into fluconazole and itraconozole (Fig. 2) and this resistance help to survive and increase pathogenicity. According to the Clinical and Laboratory Standards Institute criteria, the antifungal susceptibility test

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revealed that resistance impacted all of the families evaluated in this investigation.

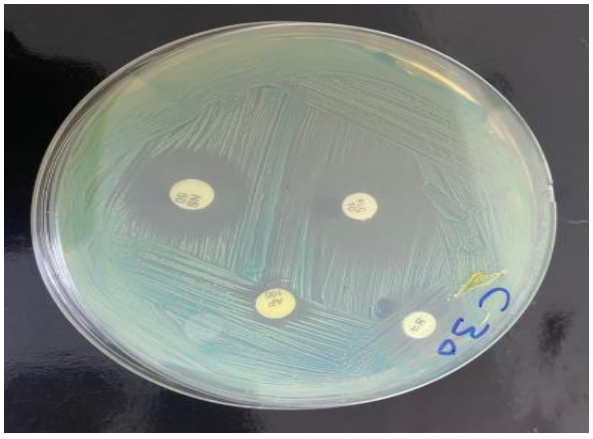

Figure 2. Plate showing the inhibition zones with different antifungals against *C. albicans.*

Evaluation of Virulence Factor: In the table was presented, the results of the biomass of the biofilms (Bm) of the candida. In the current study, the results showed the ability of *C. albicans* to produce biomass. After 72hr of incubation high, low and moderate biofilm producers were found to be 81.11, 5.56 and 13.33 respectively.

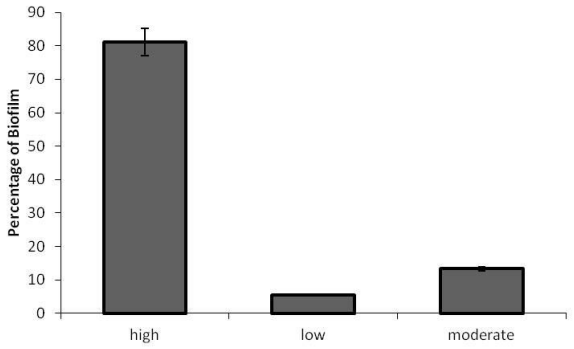

Figure 3. Graph showing the percent of biofilm producers. All the values are average of triplicates. High, low and intermediate percentage can be seen in the graph.

Virulence Factor Evaluation.

This study looked at the virulence factor, as well as the isolate's capacity to biofilm. Biofilm formation may contribute aggressively to both systemic and superficial candidiasis by increasing antifungal resistance. In the current study, the potential for producing thin biofilms was classified into three categories: high, medium, and poor, and then the results were evaluated by measuring the number of adhering cells from *C. albicans* at the bottom of the well using cut-off values OD and under microscopic magnification 40 and for three different times after three days (72) hours. The outcome was 65% strong, 21% low, and 14% intermediate (Table 3).

Antifungal resistance: The result was resistance to fluconazole and itraconozole (Fig. 3) and these resistances help to survive and increase pathogenicity. Detection of antifungal susceptibility test results, all fungal strains that were evaluated according to the criteria of the Clinical and Laboratory Standards Institute were investigated in this study.

Table 5. Table showing the percent of biofilm producers and antifungal resistance developed within the strain after 72hr incubation.

Results After 72hr	Biofilm	Antifungal
Strong	65%	60% resistance
Intermediate	21%	12% resistance
Low	14%	1% resistance

The relation between biofilm and antifungal: Stronger the biofilm formation, more is the resistance to antifungals is seen after 72hr of incubation. Stronger (65%) biofilms were found to be 60% resistance, while intermediate (21%) were found to be 12% resistance.

Determination of mutated *ERG11* gene: Only *C. albicans* strains with one of azole resistance genes were chosen for the identification of the resistance gene (ERG11). In total. The DNAs of 30 isolates (*C. albicans)* were tested. 8 of the isolates exhibited the *ERG11* resistance gene. (Fig. 4) shows the depiction of bands acquired via UV visualization following agarose gel electrophoresis.

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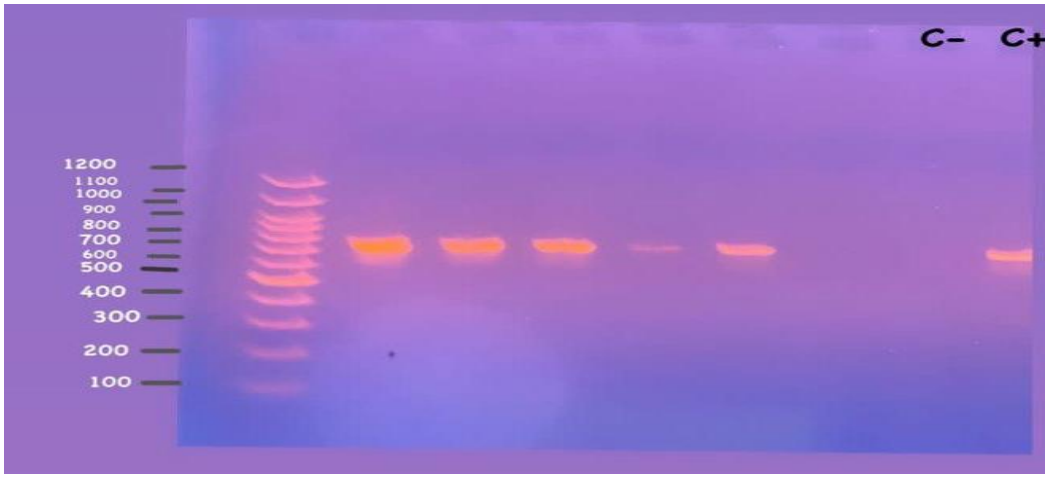

Figure 4. Gel electrophoresis of simleplex PCR products of ERG11 gene of *C.albicans* on 1% agarose gel at 75 Volt /cm for 1 hour. Lane 1: 1000bp DNA ladder.

Discussion

Candida is a fungus that belongs to the normal flora of the oral cavity which is pathogenic under certain conditions. It can be a cause of opportunistic infections when the host immune system is impaired[8]. *C. albicans* is known to be carried out in the oral cavity of 50 % of the world's population as a part of normal flora. There is a higher prevalence of *Candida* carriage in the oral cavity of immunocompromised patients when compared with a healthy population[24] . Invasive infections in the mucosa lining the oral cavity of immunocompromised patients is the results of the colonization of *C. albicans*. The prevalence of *C. albicans* in the oral mucosa of cancer patients in general (100%) and in our current study the antifungal susceptibility profile showed that all tested isolates were sensitive to FLC, NYS, AMB, VRC.

The results of the study of the activity of the thin biofilms of *C. albicans*, measured using violet crystal, were the highest activity of *C. albicans* with high levels, followed by medium and then low [25] .

In our study, antifungal resistance as a virulence factor is an important step in fungal pathogenicity. *C. albicans* had the highest frequency of ERG11 genes. This result was similar to previous studies in Iraq and Iran[26]. That showed that expression of ERG11 in *C. albicans* plays an important role in the spread of Candida infection.

Conclusion

The awareness of oral candidiasis, and the reduction of risk factors associated with oral candidiasis such as the use of dental prosthesis in patients with CA are essential. Furthermore, it is necessary to identify *C. albicans*, in understanding and defining the pathogenesis and antifungal susceptibility of candidiasis. The present study highlights the prevalence of resistance to antifungal drugs among *C. albicans* which are not uncommon. Moreover, there was a high prevalence of ERG11 gene in *C. albicans*. The resistance to antifungal drugs was common among isolates with the capacity to form the biofilm. There was an association between biofilm formation and virulence genes. Candidiasis is one of the fungal infections that are more prevalent among patients, and the virulence factor biofilm has an important role in increasing the pathogenicity of the fungus.

Acknowledgment

We would express our thanks to the University Middle technical university/college of health and

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medical techniques/ department of Medical Laboratory Techniques and supporting this work. The project was funded by authors.

Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- No animal studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Baghdad.

Authors' Contribution Statement

This work was carried out in collaboration between all authors "Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing and writing original draft preparation .review and editing, visualization, supervision and project administration.

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Published Online First: May, 2024
https://doi.org/10.21123/bsj.2024.9415
P-ISSN: 2078-8665 - E-ISSN: 2411-7986
Baghdad Science Journal

السمه الوراثيه لجين مقاومه المضادات الفطريه لدى المرضى العراقيين

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الخلاصة

مع تقدم الطب وتزايد عدد المرضى الذين يعانون من ضعف المناعة، أصبحت عدوى الخميرة أكثر شيوعًا .كان هناك معدل كبير من الامراضية والوفيات بين المرضى الذين يعانون من داء المبيضات مما يدل على زيادة المقاومة للأدوية المضادة للفطريات .الهدف الرئيسي من هذه الدراسة هو تحديد جين الضراوة لفطر المبيضات البيضاء وتقييم مضادات الفطريات المستخدمة لداء المبيضات وقابلية تكوين الأغشية الحيوية التي يتم عزلها من أنسجة المبطن للفم عند الأفراد في محافظة بغداد خلال الفترة من 1 تشرين الأول إلى 28 شباط 2023. وتضمنت هذه الدراسة 120مسحة فموية من أشخاص مصابين بداء المبيضات الفموي، تتراوح أعمارهم بين 10-65 سنة من كلا الجنسين .أظهرت النتائج التي تم الحصول عليها أن انتشار داء المبيضات كان أكثر انتشارا في الذكور الذين تتراوح أعمارهم بين 11-20 سنة، بينما كان أكثر انتشارا بين الإناث بين المرضى أقل من 10 سنوات .أظهرت هذه النتائج زيادة ذات دلالة إحصائية في انتشار جين الفوعة ERG11 بين فطر المبيضات البيض المكون للأغشية الحيوية مقارنة بالعزلات الأخرى مع وجود فرق معنوي (P =0.863).كما أظهرت الدراسة دلالة معنوية إحصائياً مقاومة فطر المبيضات البيض لمضادات الفطريات فلوكونازول وإيتراكونازول وفلوكونازول مع قدرته على تكوين الغشاء الحيوي .سلطت الدراسة الحالية الضوء على معدل انتشار أعلى بكثير لمقاومة مضادات الفطريات بين المبيضة البيضاء وارتفاع معدل انتشار جين ERG11 في المبيضة البيضاء .وكذلك وجود علاقة قوية بين تكوين الأغشية الحيوية ووجود جين المضاد للعلاجات الفطرية .
إن داء المبيضات هو أحد الالتهابات الفطرية الأكثر انتشارًا بين المرضى، كما أن عامل ضراوة الغشاء الحيوي (البيوفيلم) له دور مهم في زيادة القدرة المرضية للفطر.

الكلمات المفتاحيه: حساسيه للمضادات الحيويه ،الغشاء الحيوي، المبيضات البيض، ERG1 ، الضراوه.