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STUDY OF GENE EXPRESSION OF VIRULENCE FACTORS OF SOME CANDIDA FUNGAL ISOLATED FROM DIFFERENT CLINICAL CASES

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Abstract:

Background: Yeasts of the genus Candida are the main causes of oral candidiasis, and Candida albicans is the most common cause. **Aim of the study:** This study aimed to isolate and diagnosis the yeasts that cause Oral Candida in infected children **Material and methods:** The study included children with oral candidiasis visiting Dhuluiya General Hospital and some private clinics in Dhuluiya during the period from December 2022 to January 2023. During this period, 100 samples were collected from children aged from 1 day to 12 years and of both sexes (males, females). . The diagnosis was made based on microscopic examination, laboratory culture of samples on saprodextrose agar medium, and the use of differentiation medium (Chrom Agar) for isolated Candida to distinguish between the types of the genus Candida spp. And diagnosis using the Vitec device. The genetic effect of silver nanoparticles prepared from the large fungus Schizophyllum Commune on the gene expression of C.albicans yeast was studied on four genes, namely CAT1, CAP1, and LIP3.**Result:** Most of the isolates were C.albicans, with 26 at 59.09%, followed by C.tropicalis, with 17 at 38.64%, and C.parapsilosis, with 1 at 2.27%. The genetic influence of silver nanoparticles from the fungus Schizophyllum Commune on C.albicans yeast gene expression was examined on four genes: CAT1, CAP1, and LIP3. The ALS1 gene encoding C.albicans yeast with code 91 had a stronger effect, with gene expression reaching 0.42. C.albicans with code number 92 was 0.16. **Conclusion:** This study concluded Isolation of three types of Candida spp. and the efficacy of silver nanoparticles made from Schizophyllum against candida albicans.

Keywords: Oral Candida, C.albicans, ALS1, CAT1, CAP1, and LIP3

INTRODUCTION

Oral candidiasis, often known as thrush, is a fungal infection that affects the mouth and is caused by different types of Candida fungi. This illness impacts persons of all age groups, ranging from neonates to the elderly, and is more common among individuals with immunosuppression[1].Candida species' have diversity of virulence factors, such as their production of the protease enzyme and biofilm formation, reduces the efficacy of antibiotics because the protease enzyme breaks down host tissues and damages the affected area. Managing protease synthesis and biofilm formation is crucial to treating candidiasis since biofilms impair the efficacy of conventional antifungals [2]. These fungi also respond to oxidative stress, pH fluctuations, and metabolic flexibility. These fungi express antioxidant genes including CAP1 and CAT1 in response to oxidative stress, which activate free radical and reactive oxygen species pathways. Research shows Previously, antibiotic treatment decreased gene expression of CAP1 and CAT1 in biofilms [3], highlighting their crucial role in yeast biofilm production. After yeast infection, reactive oxygen species are present. CAP1 acts as a transcription factor in yeasts to reduce reactive oxygen species (ROS), which are produced by the host, especially phagocytes, so yeasts express these genes to protect them from free radicals and ROS [4]. Protease enzymes are proteins with a molecular weight ranging between 35-50 kDa, and they have high flexibility towards changes in pH, as SAP1 and SAP3 are well effective at low pH levels, while SAP4 and SAP6 are effective at high pH levels. Candida may adapt to varied environmental conditions in host tissues, making them excellent opportunistic infections[5]. At infection sites, SAP destroys albumin, keratin, and other proteins, which cause invade and pierce host tissue [6]. Therefore, the current study aimed to Detecting the extent to which the expression of genes of some Candida virulence factors is affected

Materials and Methods

Collect fungal samples

A total of 100 clinical samples were gathered from Dhuluiya General Hospital and various private clinics in Salah al-Din city. These samples were collected from patients with oral candidiasis, spanning a wide age range of 1 day to 12 years, and included both males and females. The collection period was from December 9, 2022, to January 2023. Oral swabs were obtained from the samples using Sterile cotton swabs after the infection was diagnosed by specialized doctors, and a questionnaire form was allocated to each patient, which included some information about the patients

Culture Examination

This test was carried out by culturing samples taken from patients on saprodextrose agar medium in plastic Petri dishes and incubating them at a temperature of 37°C for 24-48 hours [7].

Microscopic Features

This test was performed to observe yeast cells. If a portion of the colony growing on saprodextrose agar medium was taken using a loop carrier, then mixed with a drop of normal saline solution and placed on a glass slide, left to dry by passing it over a flame, then stained with lactophenol cotton blue dye, covered with a slide cover, and examined with a microscope at 100 x power, then transferred. For the force 400 x [8]

Hicrome™ Candida Differential Agar Base

This test was performed with chromium agar differential medium. The medium is characterized by being a selective differentiation medium for isolating and developing yeast, identifying colonies belonging to Candida species, and differentiating them according to the colony's colors and external appearance. A portion of the activated colony at 24 hours old is taken and planted on chromium agar medium at a temperature of 37°C for 48 hours [8].

Identification by Vitek

The diagnosis was performed using the **Vitek** device with the aim of final and conclusive confirmation of the diagnostic results obtained from previous biochemical tests for oral isolates using the YST Card, according to the instructions of the French processing company Biomr ieux.

Detection of Virulence Factors

Congo Red Agar (CRA)

This test was conducted to detect the ability of yeasts to form a biofilm. Yeast colonies were streaked on Concho red medium, and then the dishes were incubated at a temperature of 37°C for 48 hours. The results were measured by recording the color and shape of the colony, as the appearance of a black color or crystalline texture is evidence of the formation of Biofilm: If the color of the medium remains pink, this indicates that the biofilm has not been formed[9].

Protease Test

The test was conducted to detect the ability of Candida species to produce the protease enzyme using milk agar medium. The medium was inoculated by transferring the 24-hour-old colony in the form of a straight line on the surface of the medium, and then incubated at a temperature of 37°C for a period of 24 to 48 hours. The formation of transparent halos around the inoculation area in the agricultural medium is evidence of the test being positive [10]

Macrufungi Sample

A sample of the common Shizophyllum mushroom was obtained from the Mycology Laboratory/Department of Life Sciences/Tikrit University. The fungus was previously identified according to traditional and molecular methods by a M.D. Sarah Qahtan Suleiman**.**

Preparation of S.commune extraction

10 tablets were taken from the S.commune fungal colony and immersed in a glass beaker containing 500 ml of sterile liquid dextrose potato medium with an antibiotic added to it to prevent bacterial growth according to the method of [11].

Biopreparation of silver nanoparticles from S.commune mushroom sample

Silver nanoparticles were prepared from the aqueous extract of **S.commune** by adding the prepared extract in the form of drops to a solution of silver nitrate (prepared in advance at a concentration of 5 mM), then leaving the solution to mix for 30 minutes without heat, while monitoring the color change according to [11].

100 microliters of suspension of the isolates tested for study were taken individually and spread on the surface of Petri dishes containing Muller Hintone agar culture medium, then the dishes were left for a quarter of an hour. After that, holes with a diameter of up to 5 mm were made in the culture medium using a sterile cork drill. Then the holes were filled with about 60 microliters of a solution of silver nanoparticles prepared at different concentrations (25%, 50%, and 100%). Ionic water and mushroom extract were used as negative control agents, while the two antibiotics, Nystatin and the antibiotic Levofloxacin, with candida and bacteria, respectively, were used as positive control agents. The experiment was carried out with 3 replicates for each treatment. The plates were incubated at 37°C for 24 hours. After the end of the growth phase, the diameters of the inhibition zones were measured for the concentrations used[12].

Molecular Study

RNA extraction

The Transzol Up Plus RNA Kit prepared by TRANS was used

Table (1): Primers used in rt-qPCR

No.	Gene Name	Sequence		Band size	No. of bases
1	ALS1	F	CATCATTGACTCAGTTGT	117	18
		R	CAGTGGAAGTAGATTGTG		18
2	CAP1	F	AGTCAATTCAATGTTCAAG	87	19
		R	AATGGTAATGTCCTCAAG		18
3	CAT1	F	GACTGCTTACATTCAAAC	117	18
		R	AACTTACCAAATCTTCTCA		19
4	LIP3	F	TCTCACCGAGATTGTTGTTGGA	68	22
		R	GTTGGCCATCAAATCTTGCA		20
	Total	-	-		152

cDNA Synthesis

The method of measuring gene expression using rt-qPCR technology requires converting single strands in RNA into complementary strands of DNA. In order to accomplish this, the Easy Script First Strand DNA Synthesis kit, prepared by TRANS,

RESULT

Morphological Identification of Isolated Fungi

The yeast colonies cultivated on saprodextrose agar medium at a temperature of 37°C and incubated for 48 days exhibited a white to cream colour. They had a smooth texture and a convex surface, as depicted in Figure 1.

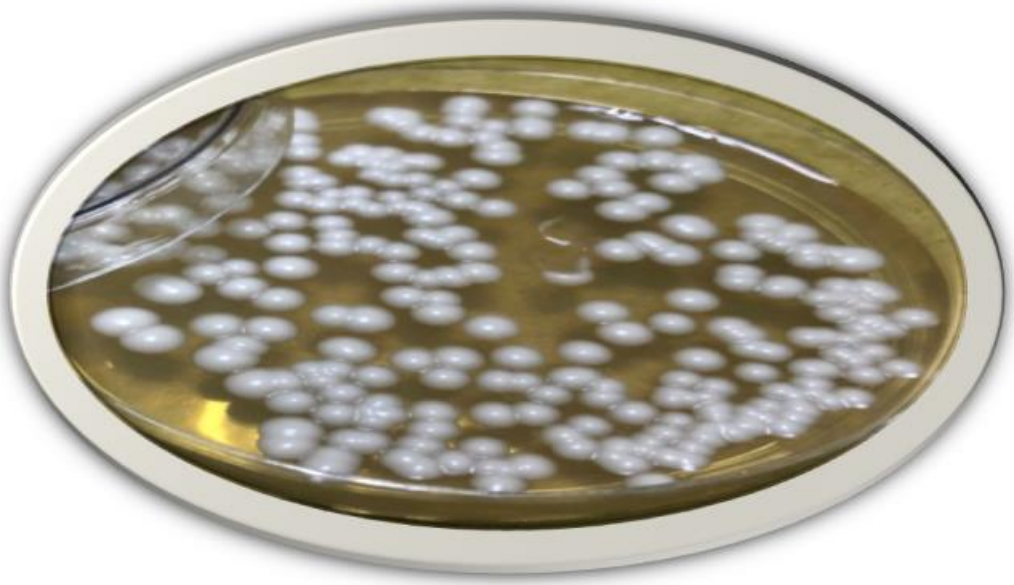

Figure (1): Growth of e C.albicans on saparoid dextrose agar medium at a temperature of 37°C for 48 hours

Microscopical Characteristics

The present study observed that the presence of spherical to oval-shaped or longitudinal and budded cells of varying sizes for some Candida isolates after staining with lactophenol cotton blue dye (Figure 2).

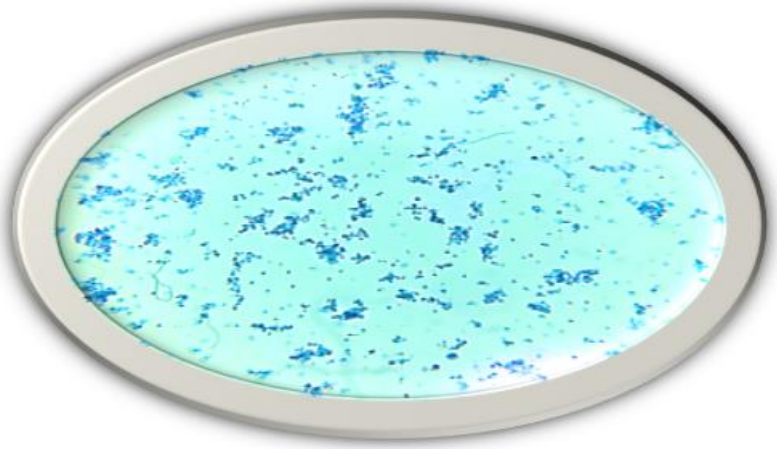

Figure (2): C.albicans stained with cotton-lactophenol blue dye (400x) after growth on saprodextrose agar medium at 37°C for 48 hours.

Differential diagnosis using Chrome agar

Figure (3) show that all isolates are able to grow on the chromium agar medium, as the colonies appeared in different colors that matched the instructions of the company that prepared the chromium agar medium. The C.parapsilosis yeast colony appeared in white, the C.tropicalis yeast colony appeared in dark blue, while the C.albicans yeast colony appeared in light green.

c= C. parapsiolosis.

Figure (3): Growing colonies of Candida species isolated on chromium agar medium, a=C. albicans, b=C. tropicalis, c= C. parapsiolosis.

The effectiveness of biofilm production for Candida spp.

The results showed a significant difference in the number of isolates that produced and did not produce biofilms. Out of 26 isolates, only 8 isolates of the C. albicans formed biofilms, with a percentage of 30.77%. Of these, 5 isolates formed black colonies and 3 isolates appeared in crystal form. The results also showed that 47.06% of the C. tropicalis isolates formed a biofilm, with only 8 isolates out of 17 isolates, 6 of which appeared in black color and 2 of which appeared in crystal form. The C. parapsilosis isolate also formed a biofilm represented by the formation of a black-colored colony. As shown in Table (4).

Table 4: Types and number of Candida isolates producing biofilm on Congo red agar medium.

Candida Spp	Total	No. isolates produce black colony	%	isolates not produce black colony	%	No. isolates produce crystal	%	No. isolates not produce crystal	%	Percentage of isolates produced %
C.albicans	26	5	19.23	21	80.76	3	11.53	23	88.46	30.77
C.tropicalis	17	6	37.5	10	62.5	2	12.5	14	47.06	47.06

C.parapsilosis	1	0	0	1	100	1	100	0	0	100
Total	44	11		32		6		37		
* Chi-Square = 8.410 P-Value = 0.050										

Ability of isolated Candida species to produce protease enzyme

The chi-square test showed that there were no significant differences in the ability of the Candida isolates isolated in the current study from cases of oral candidiasis to produce the protease enzyme. Despite this, the results shown in Table (5) showed that the C. tropicalis yeast isolates are The highest producer of the proteinase enzyme was 76.47%, with a rate of 13 out of 17 isolates, and the yeast C. albicans was ranked second highest among the proteinase-producing and proteolytic isolates, which appeared in the form of a transparent halo around the colonies growing on the medium, with a rate of 69.23%, with 18 isolates out of 26 isolates, while the only isolate of C. parapsilosis yeast did not show the ability to produce the proteinase enzyme.

Table (5): Proteinase production by Candida species.

Candida Spp	Total	No. isolates that produce protease	%	No. isolates that not produce protease	%
C.albicans	26	18	69.23	8	30.76
C. *tropicalis*	17	13	76.47	4	23.52
C.parapsilosis	1	1	100	0	0
Total	44	32		12	
P-value	0.62				

The effect of silver nanoparticles produced from the fungus S. commune on the gene expression of some virulence genes of Candida yeast.

The effect of silver nanoparticles manufactured from the fungus S. commune was evaluated on four isolates selected for the study (C.albicans 91 and C.albicans 92) after treating them with the nanosolution at a concentration of 100% (the concentration that achieved the highest rates of inhibition in the effectiveness experiments). The test was done using quantitative polymerase chain reaction technology by calculating Folding Change (FC) values. FC values below 1 were considered the Threshold limit for the occurrence of decreased expression of down-regulated virulence genes selected for the study (ALS1, CAP1, CAT1, and LIP3).

The results showed a clear down-regulated decrease in the expression of the ALS1 gene for all isolates studied, and this effect was higher for the two C. albicans isolates, as the FC value for the two isolates (91 and 92) reached 0.42 and 0.16, respectively.

Isolate C. albicans 91 also showed an effect in the expression of the CAP1 gene, as the FC value reached about 0.03, while the expression level of the up-regulated gene increased for isolate C. albicans 92, with an FC value of 2.55. It appears that a decrease in the level of expression of the CAT1 gene occurred for isolates C.albicans 91 and C.albicans 92 only, as the FC values for the two isolates reached 0.74 and 0.43, respectively.

While the level of genetic aroma increased for the two isolates, C.albicans 91 and C.albicans 92, as the FC values reached about 7.54 and 3.17, respectively.

Table (6): The effect of silver nanoparticles on the gene expression of the virulence genes ALS1, CAP1, CAT1, and LIP3 of the yeast species studied.

Type isolation	LIP3	CAT1	CAP1	ALS1
Candida albicans 91	7.547 a A	0.743 a A	0.029 c B	0.419 b A
Candida albicans 92	3.166 a B	0.434 c B	2.550 b A	0.163 d B

Discussion

The process of diagnosing fungi is very important, as fungal isolates differ in their ability to cause infection [13], and in their resistance to antifungals [14]. Yeast colonies growing on Sabroid Dextrose Agar medium at a temperature of 37°C and after 48 days of incubation showed a white to cream color with a smooth and flat texture. SDA medium was used because it is characterized as a medium that promotes the growth of Candida and is a low pH medium that prevents the growth of bacteria present in the mouth, in addition to adding an antibacterial to the growth medium to ensure obtaining pure fungal colonies [15]. Lactophenol pigment is characterized by its ability to give the blue color or dye present in the walls of fungal cells, spores, and other fungal structures such as fungal hyphae, and this result is consistent with what was indicated by [16]. The biofilm is one of the most important factors of Candida virulence, which plays a major role in the infection of candidiasis, as these biofilms have a vital role in resisting the host's immune system mechanisms and resisting the effect of antifungals [17].

The results of the current study are consistent with the study of [18], It indicated that most Candida species from disease and body sites can produce biofilms.The study done by[19], showed that the Candida species C. albicans and C. tropicalis is the most biofilm producing species. It is also consistent with the results of the study by [20], which showed that the yeast C. tropicalis is the species most capable of forming biofilms after the yeast C. albicans. The current study's results may differ from others due to sample size or other factors. Use of alternative methods and Candida's ability to create biofilms vary by species, strain, and infection site[21]. Extracellular lytic enzymes help invade and colonise host tissues and evade the immune system by destroying immunoglobulins and complement proteins, and cytokines [22], hence this study examined oral candidiasis Candida species' protease enzyme production.

The results of the current study are consistent with the results of the study of [22], which showed that the yeast isolate C. tropicalis was the most proteinase-producing isolate compared to other types of Candida isolated from the oral cavity of children with oral candidiasis. This percentage was lower than what was reported in the aforementioned study, which amounted to (96.8)%, and was higher compared to the study of [23], which amounted to 30.7%. This discrepancy in the results may be attributed to the difference in sample sizes between the current study and other studies.

Explanation of the reasons for the decrease in the expression of the studied genes. The study by [24] showed that a silver nano solution stimulates the accumulation of hydroxyl radicals and reactive oxygen species, causing apoptosis by causing a defect in mitochondrial functions. It is also noted that there is an increase in gene expression in some treatments, as the gene expression of the LIP3 gene increased for both isolates of the C. albicans type, and the gene expression of the CAP1 gene increased in the C. albicans 92 isolate. This is consistent with the results of several studies, such as the study by [25], which showed an increase in the level of expression. Genetic analysis of the SAP7 gene when four different isolates of C.albicans yeast were exposed after being treated with zinc oxide nanoparticles (ZnONPs), in addition to other treatments with various other nanosolutions. This increase is explained by several mechanisms, including the activation of the stress response in yeast C. albicans when exposed to silver nanoparticles, followed by stress factors that lead to the activation of stress response pathways. These pathways may lead to an increase in gene expression as a form of adaptation for the fungus to maintain its survival [26].

CONCLUSION

1. Isolation of three types of Candida spp. The species C.albicans ranked first among the other isolated species.
2. The effectiveness of silver nanoparticles prepared from the large fungus Schizophyllum Commune against candida albicans
3. Silver nanoparticles prepared from the large fungus Schizophyllum Commune showed an inhibitory effect.

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دراسة التعبير الجيني لعوامل ضراوة بعض عزلات فطرية لجنس المبيضات Candida من حالات سريرية مختلفة

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كلية العلوم, جامعة تكريت, تكريت , العراق

الخلاصة

تمهيد: تعد الخمائر من جنس المبيضات من المسببات الرئيسة لداء المبيضات الفموي، وتعد المبيضات من النوع albicans المسبب الاكثر شيوعًا. **الهدف من الدراسة:** هدفت هذه الدراسة الى عزل وتشخيص الخمائر المسببة لداء المبيضات الفموي Oral Candida عند الأطفال. **المواد وطرق العمل:** شملت الدراسة الاطفال المصابين بداء المبيضات الفموي المراجعين لمستشفى الضلوعية العام وبعض العيادات الخاصة في الضلوعية خلال المدة من كانون الأول ٢٠٢٢ ولغاية كانون الثاني ٢٠٢٣ وخلال هذه المدة جمعت ١٠٠ عينة من أطفال تتراوح أعمارهم من ١ يوم – ١٢ سنة ومن كلا الجنسين (ذكور ، اناث). تم التشخيص اعتمادا على الفحص المجهري , الزرع المختبري للعينات على وسط السابرويد دكستروز اكار, استخدام الوسط التفريقي (Chrom Agar) للمبيضات المعزولة للتمييز بين أنواع الجنس Candida spp. و والتشخيص باستخدام جهاز الفايتك. كما و تمت دراسة التاثير الجيني لجسيمات الفضة النانوية المحضرة من الفطر الكبير Schizophyllum Commune على التعبير الجيني لخمائر C.albicans على اربع جينات وهم CAT1, CAP1, LIP3 . **النتائج:** اظهرت النتائج ان اكثر الأنواع المعزولة تعود الى النوع C.albicans اذ تبلغ عدد العزلات ٢٦ عزلة وبنسبة 59.09 % تلاه نوع C.tropicalis اذ بلغ عدد العزلات 17 عزلة وبنسبة 38.64 % تلاه النوع C.parapsilosis وكانت بواقع عزلة 1 وبنسبة 2.27 %. كما أظهرت النتائج تاثير المستخلصات النانوية على الجين ALS1 المشفر لخميرة C.albicans ذات الشفرة رقم 91 اذ بلغ التعبير الجيني 0.42 و ذات الشفرة رقم 92 بلغت قيمته 0.16. **الاستنتاج:** خلصت هذه الدراسة إلى عزل ثلاثة أنواع من المبيضات Candida spp و فاعلية جسيمات الفضة النانوية المحضرة من الفطر الكبير Schizophyllum Commune المضادة للفطريات.