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Detection of some virulence factors among *Candida albicans* isolated from patients and prevalence of candidalysin gene *CEEc1*

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Abstract

Candida albicans is a common cause of respiratory infection and oral candidiasis in people; it is an opportunistic yeast pathogen and a major cause of morbidity and mortality in the immunocompromised persons and causes superficial infections of mucosal surfaces which affect millions of people throughout world. The main goal of this study was investigating the prevalence of some virulence factors which has the ability to configure biofilm formation, proteinase, hemolysin production among *C.albicans* isolates that include prevalence of candidalysin gene *Eec1*. Samples were collected during the period May and August of 2022 from 280 samples (swabs) of different ages and sexes of non-duplicated Iraqi patients suffering from oral candidiasis and respiratory diseases. The results showed that 102 were positive samples, 58(56.86%) from oral cavity and 44 (43.14%) from respiratory tract, while 178 of them were negative. *Candida* isolates were identified using conventional methods by grown on HiCrome *Candida* medium, germ tube production, chlamydospore formation and confirmed using VITEK-2 system, susceptibility of *Candida* isolates to antifungal drugs was examined by disk diffusion method, performed as recommended by (CLSI) M44-A document. The isolates showed a high level of susceptibility to Amphotericin-B (93.20%), Nystatin (90.20%), and Clotrimazole (85.92%). Prevalence of Candidalysin gene *Eec1* among 70 isolates of *C. albicans* was investigated using polymerase chain reaction (PCR) technique, the results revealed that 41(58.57%) were harboring *Ece*1 gene for the oral cavity and respiratory tract. Only 34 (48.57%) *C. albicans* isolates were strong producer of biofilm, while 30 (42.86%) isolates produced proteinase, 20 (28.57%) of isolates had the ability to hemolyze the blood.

Keywords: Biofilm formation, *Candida albicans*, Candidalysin, Hemolysin, Virulence factors.

Introduction

Fungi make up approximately 7% of all eukaryotic organisms found on earth[1]. Fungal infections are a major cause of morbidity and mortality in the global population with species including *Candida, Cryptococcus*, *Pneumocystis*, and *Aspergillus* and contributing to an estimated 2 million life-threatening infections reported each year [2]. It is critical to understand the molecular mechanisms that support fungal pathogenesis and host immunity

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better and use this understanding to create of new diagnostics, vaccines, and, immunotherapies [3].

Yeasts are eukaryotic microorganisms classified in the kingdom of fungi, with about 1,500 species. The phylogenetic diversity of yeasts is shown by their placement in the divisions Ascomycota, Basidiomycota, and Deuteromycota. The *Candida* spp. belong to Ascomycota commonly known as *ascomycetes* [4].

Candida albicans is one of the most dangerous fungi to human health. This yeast despite being a normal component of the commensal flora can infect the skin, mouth, vagina, and gut in both healthy and immunosuppressed people. Furthermore, *C. albicans* is responsible for invasive candidiasis, an infection of the blood, heart, and other organs in hospitalized patients. Even in otherwise healthy patients, invasive candidiasis has high mortality rates about 50% [5,6].

Hyphae constitute an important stage in the illness progression because is the most invasive morphology of yeast, as it is necessary for diffusion into the bloodstream during systemic infections. In addition, the hyphal formation is usually accompanied by the development of various additional virulence factors, such as adhesions, invasions, metal acquisition factors, hydrolytic and detoxifying enzymes, all of them are playing a role in the pathogenesis of *C. albicans* [7,8]. Dimorphic fungal phases are formed due to immunodeficiency, stress, and other external factors, the morphological change of *Candida* spp. increases the yeast overgrow and virulence in their hosts. The host recognition biomolecules (adhesions), phospholipases, secreted aspartyl proteases, and hemolysins are connected to the active invasion of host tissues, they are among the fungus's virulence factors. Thus, *C. albicans* can produce a variety of diseases, such as vulvovaginitis and oropharyngeal candidiasis, as well as hematogenously disseminated systemic candidiasis [9].

Due to their immunocompromised state and the side effects of chemotherapy, cancer patients are at significant risk for fungal infection, especially by *Candida* species. Patients with cancer are more at risk for developing oral candidiasis when they are receiving chemotherapy, this infection typically comes with several symptoms such as burning, pain, taste changes, decreased saliva secretion, and difficulty swallowing, but it can also remain unrecognized [10]. Candidalysin is a virulence factor of the *C. albicans* genus encoded by the *ECE1* gene where it is secreted in people whose immunity is weak or who are immunosuppressed, which leads to *C. albicans* being a dangerous pathogen causing many diseases such as cancer and dermatitis in addition to its infiltration to the body organs and the possibility of it even reaching the brain.

In Iraqi patients, *C. albicans* is widespread among people particularly children and young as well as adults who suffer from different diseases. For this reason, the main aims of the current study are the detection of some virulence factors among *C. albicans* isolates collected from Iraqi patients and to study the prevalence of Candidalysin gene *ECE1*, as well as investigating antifungal activity of some antifungal drugs against the tested isolates.

Materials and Methods

Candida spp. isolates

One hundred and two clinical isolates of *Candida* spp. were included in this study. These isolates were collected from 280 clinical samples of Iraqi patients suffering from infected respiratory tract and oral cavity, during routine work at the Medical City Hospitals and Al-Yarmouk Teaching Hospital, AL-Imamein AL- Kadhimaein Medical City Educational, Baghdad, Iraq. The isolates had previously had been identified as *Candida* spp. based on routine presumptive tests.

Re- Identification of *Candida* spp. Isolates

Various tests were used to confirm the identification of obtained *Candida* isolates. In brief, all the isolates were subjected to germ tube formation test[11], and chlamydospore formation test[10]. Subsequently, each isolate was streaked on HiCrome *Candida* agar (HImedia, India) and the plates were incubated at 37

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°C for 48 h. Afterwards, the characteristic colors of the developed colonies were observed. Finally, the identification of *Candida* spp. isolates was confirmed using VITEK-2 system (BioMerieux, French).

Antifungal susceptibility testing of *Candida* spp. isolates

The antifungal susceptibility tests for all clinical *Candida* isolates were performed using disk diffusion method in accordance with CLSI standards[12]. A suspension of *Candida* isolates was made by selecting 5–6 colonies from an overnight culture on an SDA plate. It was suspended in 5 ml of sterile normal saline; the turbidity was adjusted to 0.5 McFarland standards. A sterile cotton swab was moistened in inoculum suspension and streaked on Mueller-Hinton agar medium (MH-GMB), it was prepared and autoclaved according to the instructions of the manufacturer and supplied by 2% dextrose and 0.5 g/mL methylene blue. All plates were left for 30 mints at room temperature; antifungal disks were placed on the surface of (MH-GMB) medium. The plates were incubated at 37°C for 24 h. The inhibition zone formed around the antifungal disks was measured in inhibition and calculated in (mm), and interpreted as described by CLSI (Sensitive S, resistant R, susceptible dose dependent (SDD)[13].

Assessment of some virulence factors

Production of Hemolysin

The hemolytic activity among *C. albicans* isolates was determined, 70 isolates of *C. albicans*, were subjected to this test performed on SDA supplemented with 5% sheep blood and 3% glucose. Then, plates were incubated for 24- 48 h at 37°C in 5% CO_2, hemolysin production was evaluated by the formation of a zone completely clear of blood around the yeast colonies as detected by transmitted light. A hemolytic index was created by dividing the colony's diameter by the combined diameter of the colony and its translucent halo. (Hz value) representing the intensity of the hemolysin production by different *C. albicans* isolates (when Hz = 1, there is no hemolysin activity (negative); Hz = 0.7− 0.99, is weak positive; Hz = 0.5-0.69, is moderately positive; Hz < 0.5, strong positive) [14].

Determination of proteinase activity

Extracellular proteinase activity of clinical *C. albicans* isolates confirmed during this study was analyzed according to Staib *et al.*[15], with few modifications, using a medium composed of (Dextrose 2%, KH_2PO_4 0.1%, $MgSO_4$ 0.05% and supplemented with agar 2%), Autoclaved at 115 °C for 15 min, mixed well after cooling to 50°C and supplemented with 1% Bovine serum albumin (BSA) solution, yeast suspension of 1×10^8 cells/ mL was prepared, and 10 µL of yeast suspension was inoculated onto the surface of the prepared medium. The Petri dishes were incubated for 24-48 h at 37 °C. After that, the Petri dishes were fixed with 20% Trichloroacetic acid (TCA) and stained with 1.25% amidoblack, and Acetic acid 15% was used for decolorization. The proteinase activity was seen as opaqueness of the petri dishes agar, corresponding to a zone of proteolysis around the yeast colony not stained with amidoblack. The test was done on three different occasions for each *C. albicans* isolate tested. The proteinase activity (Prz) of 70 *C. albicans* isolates was tested by a halo zone formation around the inoculation area on BSA medium. Less than one (Prz1) indicates proteinase activity, whereas a Prz value of 1 indicates no activity [16].

Assessment of Biofilm formation by Clinical *C. albicans* isolates

To assess the ability of all *C. albicans* isolates for biofilm development, the yeast isolates were grown on SDA at 37 °C for 24-48 h, before being suspended in yeast extract peptone dextrose medium (YPD), the pH was adjusted to 7.2, culture of the yeast was adjusted to a 0.5 McFarland standard ($1.5\ x10^6$ cells/mL) as a yeasts suspension, In this experiment Crystal violet staining was used according to Jin *et al*, [17]. Briefly, 200 µL of yeast suspension was seeded into a well of the sterile 96-wellmicrotiter polyester plate. Then, the plates were sealed and incubated for 24 h at 37°C, Thereafter, the wells containing medium and yeast planktonic cells were washed using 200 µl of PBS for three time. Then, 110 µL of a crystal violet 0.4% solution was added to each well plate. After 45 minutes of incubation at room temperature in the dark, it was washed thoroughly several times with water. Crystal violet was used to uniformly label adherent cells, which often develop

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biofilm on all side wells. The resulting biofilms produced by *C. albicans* isolates were fixed with 200 µl of 95% methanol, were used to solubilize crystal violet stained biofilm. Of that, 100 µl were transferred to a fresh plate that had already been read[6]. Following the measurement of the optical density (OD) at 595 nm, the results were read as follows: Based on the established OD cut-off values (ODc) and biofilm density, biofilm production was divided into four categories[18] Table 1.

Table 1. Classification of biofilm formation according to biofilm density.

Optical Density Values (OD)	Interpretation of biofilm production
OD ≤ ODc	No biofilm production
ODc < OD ≤ 2 × ODc	Weak biofilm production
2 × ODc < OD ≤ 4 × ODc	Moderate biofilm production
4 × ODc < OD	Strong biofilm production

Detection of candidalysin *ECE1* gene among *C. albicans* isolates

DNA Extraction:

For the detection of *Ece1* gene existence among *C. albicans* isolates, the whole genomic DNA of *C. albicans* isolates were extracted and purified using yeast genomic DNA extraction kit (TransGen, Biotech/China) and the steps of extraction were done based on the manufacturer's instructions. Firstly, colonies of *C. albicans* were grown on SDA as a pure culture for 18-24 hours at 37°C before being processed for DNA extraction and PCR analysis, after taking a pure yeast colony. The extracted DNA samples were measured for concentration and purity using Nano drop UV spectrophotometer at OD ranging between 260-280 nm, samples of DNA were preserved in deep freeze at -20 C until used in PCR.

For amplification of *ECE1* gene specific primers F-AGCTGTTGACACAGCCATGA (Tm, 60.7) and R-TCTGAAACAATTTGAGCAGCA (Tm, 56.3) which used for this purpose. PCR was done on 25 µL of reaction mixture (Taq PCR PreMix 12.5 µL, forward and reverse primers 2 µL for each one, DNA 4 µL and 6.5 µL from nuclease free water). PCR program was initiated by an initial denaturation step 1 (at 94°C 5, min 1), then denaturation step 2 (at 94°C, for 30 sec), followed by 35 cycles, Annealing step (at 58 °C for 40 sec), in the final extension (at 72 °C for 5 min). PCR products of *ECE1* gene were visualized on agarose gel, this gel was prepared in 2%, by dissolving 2 g of agarose in 100 mL, electrophoresis was performed for 60 min at 70 volts, the bands that appeared on the gel were detected under UV trans-illuminator at 302 nm, compared and photographed with Ladder size. Four isolates of *C. albicans* from the positive of *ECE1* gene were selected for sequencing using Sanger method in (Macrogen Company, South Korea. Dna.macrogen.com).The obtained sequences deposited in the NCBI under accessions numbers OQ343341, OQ343342, OQ343343 and OQ343345.

Statistical Analysis:

The data obtained during this study were analyzed using the following software, Microsoft excel, IBM SPSS V26, and Minitab v.18. The results reported in this study were expressed as N (%). Z-test was used to compare two proportions. One-way analysis of variance was used for biofilm analysis. The chi-square test of association and chi square goodness of fit was used for categorical data. $P \leq$ l 0.05 and 0.01 were considered significantly and highly significantly different [19].

Results and Discussion

During the present study, A total of 280 clinical samples (swabs) were collected from Iraqi patients suffering from an infection by *Candida* spp., the samples included oral cavity and respiratory tract swabs, these clinical swabs were collected between May and August in 2022 from a number of hospitals in Bagdad city, the swabs were cultured on SDA medium at optimum conditions. The results showed that there were 102 (36.43%) positive samples for *Candida* spp. growth, while 178 (63.57%) were negative cultures as shown in Fig. 1.

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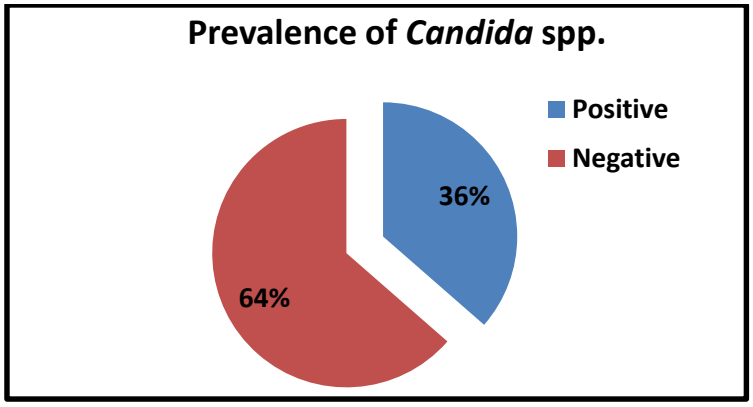

Figure 1. The percentage of positive and negative cultures of collected swab samples from patients on SDA medium.

In the current study, less than half 36.43% of the visited patients to targeted hospitals were positive for growth of *Candida* species. Previous reports and studies reported that nearly 10% of the common species in the oral cavity behave as opportunistic yeast pathogens, and cause infections and diseases like oral candidiasis[20,21]. The opportunistic infections of the oral cavity are common among the immunocompromised patients such as AIDS and HIV [22]. *Candida* spp. colonize the oral cavity in varied degrees according to the age, in newborns it ranges between 42-45%, in healthy children about 50-64%, in healthy adults it ranges between 30-45%, in wearers of denture 55-65%. In peoples infected with oral microbes 65-85%, while in immunocompromised individuals like those infected with HIV and/or undergoing chemotherapy treatment like patients with acute leukemia, it is ranges between 90-95% [23, 24].

Distribution of Candida isolates according gender

Candida spp. isolates were collected from clinical sources (oral cavity and respiratory tract), distributed according to patient's gender.

Isolates of *C. albicans* and *C. tropicalis* were mainly isolated from patients of each sex and no significant differences appeared between the isolates based on gender only in *C. kruzei*. The frequency of collected *Candida* spp. isolates based on gender is illustrated in Table 2.

Table 2. Distribution of clinical *Candida* spp. isolated from different specimens according to gender.

Types of *Candida* spp.	Male	Female	Total	*P*-value¥
	N (%)	**N (%)**	**N (%)**	
C. albicans	30 (42.9)	40 (57.3)	70 (68.6)	0.088 N.S
C. tropicalis	7 (63.6)	4 (36.6)	11 (10.8)	0.184N.S
C. kruzei	1(16.7)	5 (83.3)	6 (5.9)	0.002**
C. kefyr	2(33.3)	4 (66.7)	6 (5.9)	0.221 N.S
C. parapsilosis	2(40.0)	3 (60.0)	5 (4.9)	0.519 N.S
C. glabrata	1 (25.0)	3 (75.0)	4 (3.9)	0.102 N.S
Total	43(42.16)	59 (57.84)	102 (100)	

Data presented as N (%), ¥: Z-test was used to test two proportions. N.S: Not significant (P>0.05)*, ** Significant and highly significant ($P \leq 0.05$) and ($P \leq 0.01$) respectively

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Identification of *Candida* spp.

All isolates of yeasts obtained during this study were subjected to identification using some phenotypical characters (Morphological Characteristics of colonies on SDA medium, Chlamydospore spores, Germ tube production, and color of colonies on HiCrome *Candida* agar). The results of the identification were confirmed using automated method VITEK-2 system. The number of *Candida* isolates, according to species is listed in Table 3.

Table 3. Identification of Candida isolates spp. collected from clinical sources using various techniques.

Candida isolate	Species Identification method						Number and percentage N (%)
	Germ tube		**Chlamydospores**		**HiCrome agar**	**Vitek 2 system**	
	Positive	**Negative**	**Positive**	**Negative**			
C. albicans	70	0	70	0	Green	70	70
C. tropicalis	0	11	0	11	Blue	11	11
C. krusei	0	6	0	6	Pink	6	6
C. kefyr	0	6	0	6	Cream with center pale pink	6	6
C.parapsilosis	0	5	0	5	White to cream	5	5
C. glabrata	0	4	0	4	Light pink	4	4

Six species of *Candida* were identified during this study as shown in the Table 5, our finding indicates that *C. albicans* was the most frequently isolated in 70 (68.63%) of the positive sample, while *C. glabrata* 4(3.90%) was the lowest frequently isolated *Candida* collected during the isolation.

The Morphological features of *Candida* spp. growing on SDA medium, show the morphology of yeast colonies, they are white to creamy, curved, smooth to wrinkled, soft and round, and also emits a yeast odor. So, morphological appearances of *C. albicans* colonies on SDA are white to cream-colored smooth, glabrous.

Candida spp. was examined under light microscope after staining with Lacto phenol cotton and crystal violate, the results of the examination showed the oval shape with budding cells of *Candida* yeast, the features of yeast cells with budding as shown in the Fig. 2.

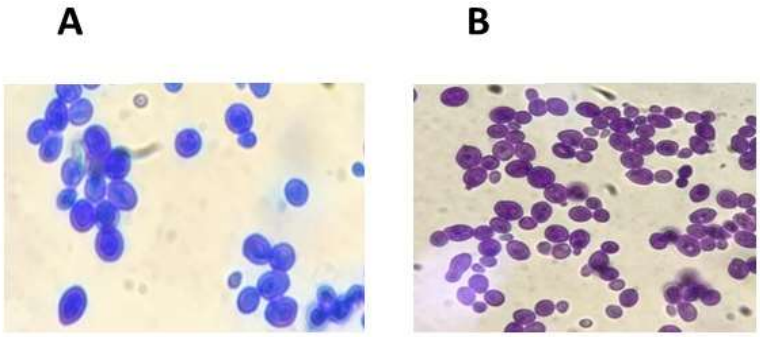

Figure 2. Microscopic features of *C. albicans* cells stained with A- Lacto phenol cotton blue B- crystal violate dyes, examined under light microscope (100X).

HiChrome Candida agar medium was used in the differentiation and identification of collected isolates, *Candida* species were identified according to the colonies color on HiChrome *Candida* agar as shown in the Fig. 3. Chromogenic agar medium was used for the identification of *Candida* as an

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alternative technique in resource limited settings because of its ease of use and lower costs; this allowed a fast presumptive differentiation and identification of the common clinical species of *Candida* [25]. The different resulted colors may depend on the reaction between the enzyme released from the yeast and this chromogenic mix, the reactions produced during incubation were revealed by spontaneous color changes in the organism[26].

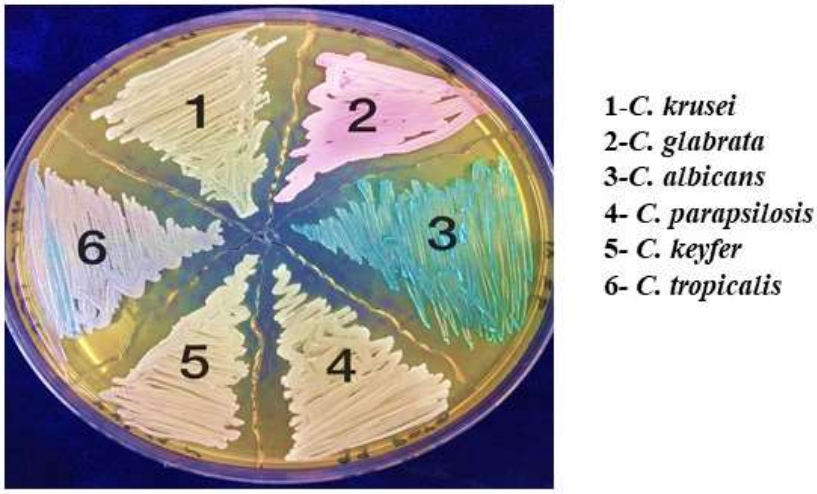

Figure 3. Differentiation of *Candida* isolates according to colony color grown on HiChrome *Candida* Agar medium for 24h at 37°C.

As clear in Fig. 4, most *Candida* spp. are easily identified using classical methods such as microscopic and cultural features, and identification according to individual species can be differentiated using some biochemical tests and physiological characteristics [27]. Previous studies and reports indicated that the identification of *C. albicans* strains and some strains of *C. dubliniensis* and *C. tropical* is generally done by production of germ tube under unfavorable condition [28]. For example, *C. albicans* when grown under certain non-optimal conditions, can produce chlamydospores, these spores are round with thick cell walls[29].

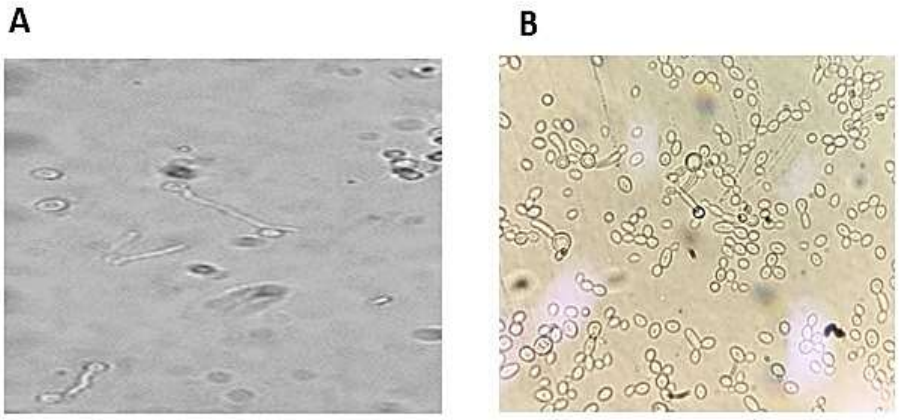

Figure 4. *C. albicans* isolate produced A-germ tube when grown on human serum after 3 h incubation at 37 °C. (40 X), B-Chlamydospore formation on corn meal broth supplemented with 10% tween 80after 24-48 h incubation at 37 °C.

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Antifungal susceptibility testing:

Antifungal susceptibility patterns of all clinical *Candida* isolates obtained from Oral cavity and respiratory tract samples to nine antifungal drugs was evaluated *in vitro* using the disk diffusion method according to CLSI guidelines (CLSI document M44-2). The results are expressed as resistant (R), Susceptible does-dependent (SDD) and sensitive (S), according to the values of inhibition zone diameter and they are summarized in Table 4. Among the used antifungals drugs, amphotericin-B was highly active towards more *Candida* isolates, the number and the percentage of susceptible isolates were 94.14%. The least susceptibility was recorded towards Metronidazole 100%, while the susceptibility values of other antifungal drugs against *Candida* isolates were recorded as, 90.2, 85.29, 70.59, 51.96, 49, 47, and 47% for clotirmazole, nystatin, fluconazole, voriconazole, miconazole, itraconazole and ketoconazole respectively. *C. albicans* isolates showed the highest antifungal resistant for metronidazole 100% and the lowest resistant was recorded against the antifungal drug amphotericin-B 2.94%. On the other hand, the non-*C. albicans* isolates also showed high susceptibility towards amphotericin-B, clotrimazole and itraconazole and the percentages of sensitivity were

Table 4.Antifungal susceptibility of *Candida spp.* isolated from the oral cavity and respiratory tract of patients.

Species (No) / Antifungal		*C. albicans* No(70)	*C. tropicalis* No(11)	*C. kruzei* No(6)	*C. kefyr* No(6)	*C. parapsilosis* No(5)	*C. glabrata* No (4)	Total (102)
Fluconazole	S (%)	50(71.43)	7(63.64)	4(66.67)	5(83.33)	3(60)	3(75)	72(70.59)
	SDD (%)	5(7.14)	1(9.09)	0(0)	0 (0)	0(0)	0(0)	6(5.88)
	R (%)	15(21.43)	3(27.27)	2(33.33)	1(16.67)	2(40)	1(25)	24(23.53)
Clotrimazole	S (%)	65(92.86)	9(83.33)	4(66.67)	6(100)	4(80)	4(100)	92(90.20)
	SDD (%)	0(0)	1(9.09)	0(0)	0(0)	0(0)	0(0)	1(0.98)
	R (%)	5(7.14)	1(9.09)	2(33.33)	0(0)	1(20)	0(0)	9(8.82)
Ketoconazole	S (%)	30(42.86)	6(54.55)	3(50)	2(33.33)	4(80)	3(75)	48(47.06)
	SDD (%)	10(14.29)	1(9.09)	1(16.67)	1(16.67)	0(0)	1(25)	14(13.73)
	R (%)	30(42.86)	4(33.33)	2(33.33)	3(50)	1(20)	0(0)	40(39.22)
Voriconazole	S (%)	35(50)	6(54.55)	3(50)	3(50)	2(40)	4(100)	53(51.96)
	SDD (%)	5(7.14)	1(9.09)	0(0)	2(33.33)	0(0)	0(0)	8(7.84)
	R (%)	30(42.86)	4(33.33)	3(50)	1(16.67)	3(60)	0(0)	41(40.19)
Micaconazole	S (%)	30(42.86)	8(72.73)	4(66.67)	2(33.33)	2(40)	4(100)	50(49.02)
	SDD (%)	20(28.57)	2(18.18)	0(0)	2(33.33)	1(20)	0(0)	25(24.51)
	R (%)	20(28.57)	1(9.09)	2(33.33)	2(33.33)	2(40)	0(0)	27(26.47)
Nystatin	S (%)	65(92.86)	8(72.73)	4(66.67)	4(66.67)	3(60)	3(75)	87(85.29)
	SDD (%)	3(4.29)	2(18.18)	1(16.67)	2(33.33)	1(20)	0(0)	9(8.82)
	R (%)	2(2.86)	1(9.09)	1(16.67)	0(0)	1(20)	1(25)	6(5.88)

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Amphotericin-B	S (%)	68(97.14)	9(83.33)	5(83.33)	5(83.33)	4(80)	4(100)	95(93.14)
	SDD (%)	2(2.86)	1(9.09)	0(0)	1(16.67)	0(0)	0(0)	4(3.92)
	R (%)	0(0)	1(9.09)	1(16.67)	0(0)	1(20)	0(0)	3(2.94)
Itraconazole	S (%)	30(42.86)	8(72.73)	2(33.33)	2(33.33)	2(40)	4(100)	48(47.06)
	SDD (%)	15(21.43)	1 (16.67)	2(33.33)	3(50)	1(20)	0(0)	22(21.57)
	R (%)	25(35.71)	3 (27.27)	2(33.33)	1(16.67)	2(40)	0(0)	32(32.35)
Chi square test *P*-value		**0.001****	**0.006****	**0.121**$^{N.S}$	**0.015***	**0.369** $_{N.S}$	**0.007****	

Our results showed that the *Candida* isolates obtained from oral cavity and respiratory tracts had the highest susceptibility in vitro to Amphotericin-B. Amphotericin-B has a high molecular weight, and is almost completely insoluble in H_2O. These traits resulted in a low permeability by human stomach and gastrointestinal [30]. This antifungal has a broad spectrum of activity towards *Candida* spp., a few other non-albicans *Candida* may be less susceptible [31]. The current results revealed that the collected clinical *Candida* spp. isolates showed high susceptibility 87% to nystatin, this finding is consistent with previous studies which reported that nystatin showed low resistant against all tested *Candida* spp. [32]. Nystatin treatment of *Candida* plays a significant role in its activity through the interaction with the ergosterol found in cell membrane of yeasts, making it porous and lead to the lysis of the cell membrane, thus exerting its antifungal effect, this action is considered a mechanism to change the composition and main function of cell membrane [33]. *Candida* isolates were susceptible to Clotrimazole in 92% of the total tested isolates. It was found to be the most useful antifungal drug against *C. albicans* and non-*albicans candida* isolates. One of the most commonly used antifungal drugs for *Candida* spp infection is fluconazole, among *Candida* spp. 70% were susceptible to fluconazole. This percentage corresponds to 1qq [34]. The *Candida* spp. isolated during this study showed varied levels of susceptibility against different antifungals, Fluconazole 70%, voriconazole 51%, miconazole 49%, Itraconazole 47, respectively, which was in accordance with other reports[34, 35]. Groups of Azoles are five-membered heterocyclic component with antifungal properties. They are classified into 2 groups' imidazole and triazole. Triazoles consist of 3 nitrogens in the azole ring and they include (fluconazole, itraconazole, voriconazole, isavuconazole, and posaconazole). Fluconazole is considered the most common azole used during therapy. The other common triazoles include voriconazole and posaconazole, and isavuconazole are more potent against resistant fungal pathogens.

Imidazoles contain 2 nitrogens in the azole ring, include (clotrimazole, econazole, ketoconazole, miconazole, and tioconazole) [36, 37]. Azoles are considered the most common antifungal drug class used in the treatment and prevention of *Candida* spp. infections. Azoles target the enzyme 14α–demethylase (Erg11p), a very important enzyme in ergosterol biosynthesis which Azoles bind to Erg11p, thereby lowering the ergosterol levels of the cell [38]. In this study, clotrimazole showed high potency 92% against the tested isolates of *Candida* spp.

Detection of some virulence factors among *C. albicans* isolates

Biofilm formation assessment

The biofilm formation among *C. albicans* isolates was evaluated using a quantitative crystal violet assay by 96 microtiterplates. In our study, among the clinical *C. albicans* isolates tested for the biofilm formation, 67 (95.7%) had the ability to develop and produce biofilm *in vitro* in broth medium at absorbance value more than 0.061. While there were 3 isolates 4.29% that did not develop biofilm in broth medium and their absorbance value was lower than 0.12 as shown in Fig. 6. According to the values of

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biofilm production in the culture medium, we classified the *C. albicans* isolates into, strong producers 34 (48.57%), moderate producers 28 (40%), and weak producers 5 (7.14%). Whereas, among the 70 isolates, there were three isolates 4.29% that did not develop a biofilm as reported in Fig. 5.

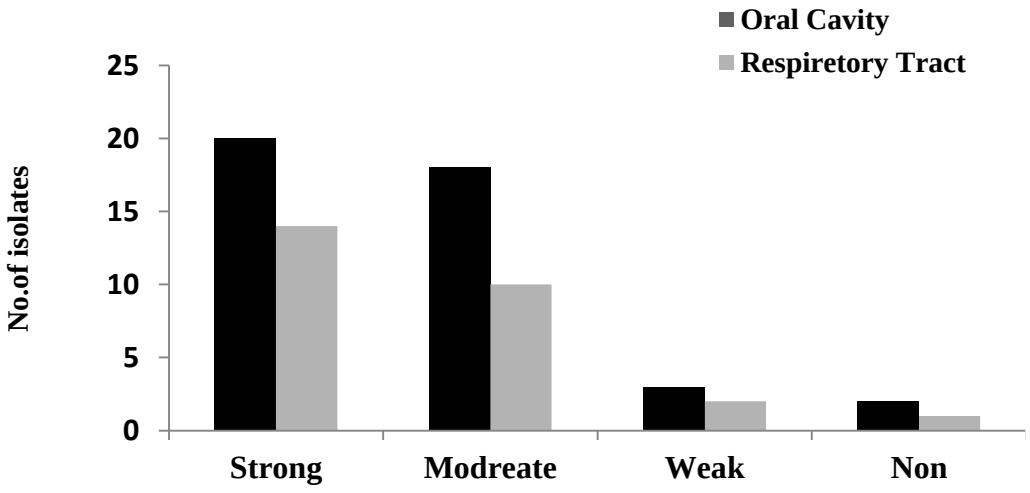

Figure 5. The biofilm capacity of 70 *C. albicans* isolated from oral cavity and respiratory tract.

As mentioned, *Candida* is the most common colonizer of the human oral cavity and plays an essential role in wide oral infections and diseases. However, some scientists reported that there is a possible link between oral cavity and colonization of the lungs by *Candida*, which may lead to respiratory infection. Studies and reports have disrobed respiratory microbial pathogens colonizing the oral cavity, also the oral pathogens inhabits and colonizes the lungs[39, 40]. *Candida* spp. is the commonest fungus colonizing the oral cavity of humans, several studies indicated that *C. albicans* is considered to be the strongest producer of biofilm among *Candida* spp. isolated from different sources, through their ability of biofilms formation and morphology of the hyphae shift, displaying a biofilm prevalence nearly 100% and so becoming an important menace in hospital-acquired infections[41, 42]. Recently, many reports and studies demonstrated that the majority of diseases and acute clinical implications caused by *Candida* spp. is related to its ability of biofilm production on attached surfaces[42, 43]. Biofilm development by *C. albicans* is initiated to adhere on both abiotic and biotic surfaces and it is considered as a significant contributing factor to the beginning of the infection and pathogenicity. *Candidal* yeasts vary in their ability to produce a biofilm depending on the species. Most studies reported that the pathogenic effects are caused by *C. albicans* and to a lesser extent by other species of *Candida* commonly associated with biofilm production that can be produced both on plastic surfaces of clinical devices and mucosal surfaces. The chemical structure of the biofilm is composed of matrix materials of enclosed small colonies of yeast, pseudo-hyphae and hyphae ordered in a complex structure [44, 45].

Proteinase activity

Proteolytic activity was evaluated in 70 *C. albicans* isolates collected during this study; the proteinase activity (Prz) of all *C. albicans* isolates was determined by the formation of proteolytic zone around the colonies of yeasts growing on prepared medium after growing for 48 h. The results of this study showed strong proteinase activity in 18 and 12 (42.85%) isolates collected from oral cavity and respiratory tract respectively, 33 isolates 47.14% exhibited moderate proteinase activity, while 5 isolates 7.14% exhibited weak activity Fig. 6.

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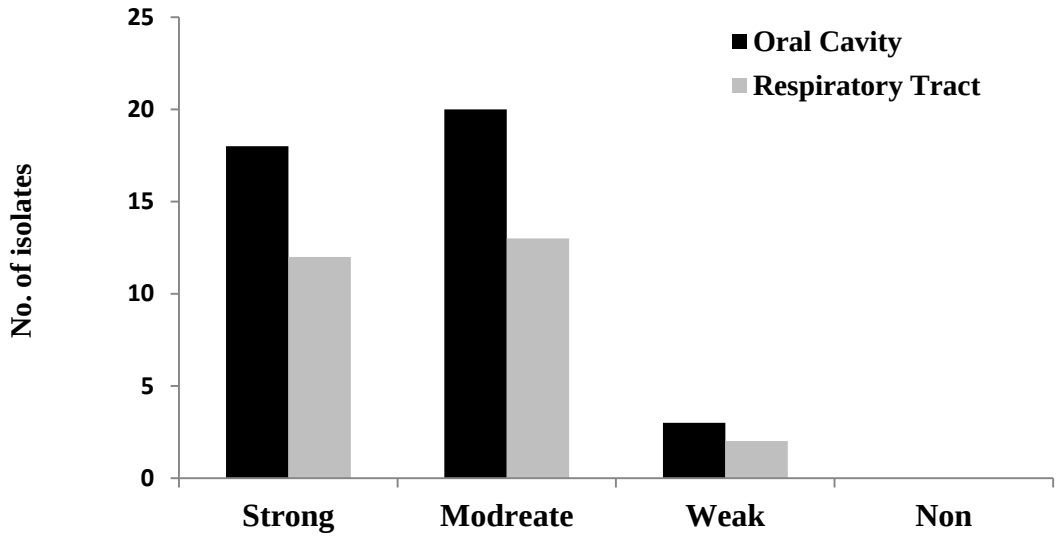

Figure 6.The proteolytic activity of isolates of 70 C. *albicans* on BSA agar medium at 37 ℃ for 24 h.

Proteinase is hydrolytic enzyme that plays an important role in the infection and pathogenicity of opportunistic *Candida* spp. in humans[46-48]. The proteolytic activity on BSA agar method has been demonstrated in the current study and other reports and studies among clinical strains of *C. albicans* and non-*C. albicans* spp.,[49-51]. A previous study by Kantarcioglu and Yuce[52] revealed that the positive level of Protease activity among clinical *Candida* isolates collected from different clinical sources was 78.9%. Also, the protease and phospholipase activities were investigated in 122 isolates of *Candida* spp. collected from several anatomically distinct sites of healthy adults, the results of this study reported that the *C. albicans* was positive to protease particularly those isolated from skin, urogenital and oral cavity[53]. Production of hemolysin is an essential virulence factor for *C. albicans* and non-*C. albicans* to obtain iron from the lysed red blood cells which permits growth in the host and supports the initiation of the infection in blood and mucosal tissues[54]. In our study, all of the collected *C. albicans* isolates were positive of hemolysin and more than 80% showed strongly and moderately positive results, these results are consistent with Luo *et al* [3], who reported that *C. albicans* and other *Candida* isolates displayed alpha and beta hemolytic activities after examining 70 *Candida* isolates on blood agar media. While Nouraei *et al*., revealed that all of the *C. albicans* isolates collected from stock in Iran exhibited strongly positive results of the production of hemolysin [55].

Hemolysin activity

In our findings, all of the 70 *C. albicans* isolates produced hemolysin, Strong hemolytic activity was observed in 20 isolates 28.57%, whereas the higher number of isolates, 36 (51.43%) showed moderate hemolysin activity and 14 isolates 20%, showed weak hemolysin activity Fig. 7.

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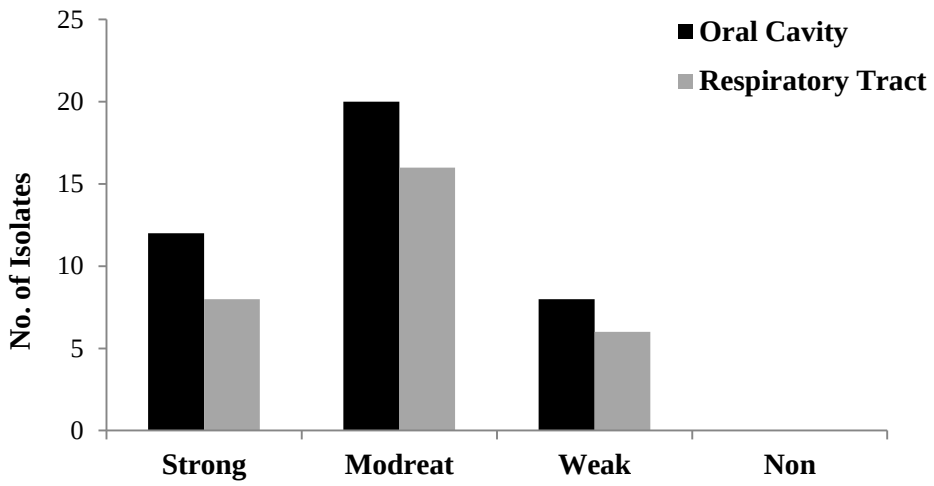

Figure 7. Distribution of hemolytic activity among *C. albicans* isolates on blood agar medium.

The hemolytic activity among *C. albicans* was firstly reported in 1951, and since then many reports and studies have demonstrated this activity [56], but the factors of *C. albiacns* responsible for hemolytic activity was not detected or understood well. In 2016 Moyes *et al*. and Manns *et al.* in 1994 discovered the new type of fungal toxin called candidalysin, this cytolytic toxin is the first fungal peptide toxin detected in a human fungal pathogen[57, 58]. Candidalysin produced by strains of *C. albicans* and it is very important during invasion of mucosa and other human tissues[59]. In previous studies and reports to elucidate the activity of candidalysin in the lysis of red blood cells, the wild type of *C. albicans* was incubated parallel to another strain harboring *Ece1* gene (mutant strain) on blood agar medium, the obtained data showed both the mutant and wild strains had the ability to produce beta-hemolysis[59, 60]. This may indicate that there are other factors causing the hemolysis in RBCs and produce the hemolytic halo zone around the colonies of yeast strains, possibly aspartic protease, that is responsible for the hemolysis in RBCs found in blood agar medium[61]. However, some studies revealed that the strains of *C. albicans* have the ability to synthetic and secret candidalysin and its direct precursor P3 which are considered as strong hemolytic peptides[60]. The strains of *C. albicans* harboring *Ece1* gene play an important role in the lysis of RBCs present in the medium of blood agar, this is further supported by the data that revealed that ECE1 expression is induced in the existence of hemoglobin[60,62]. *C. albicans* has different virulence factors like the morphological transition from unicellular yeast to hyphae and the production of lysis enzymes. The pathogenicity of *C. albicans* is initiated when it comes in contact with host cells[63,64]. Several researchers and scientists have demonstrated that candidalysin is produced by *C. albicans* strains only when it grows in the hyphal form and causes damage to host cells particularly the epithelial cells during mucosal infection[65]. Indeed, there are clear differences in the *Ece1* gene expression levels noticed and reported in *C. albicans*, and some strains of *C. dubliniences* and *C. tropical* when grown in the presence of oral epithelial cells *in vitro*, independent of the hypha formation by the strains of yeast[66, 67].

In this study, to detect the prevalence of candidalysin *Ece1*gene among clinical *C. albicans* isolates, the specific primers of the tested gene were designed according to the complete sequence deposited in the gene bank. The genomic DNA of all *C. albicans* isolates was extracted and subjected to PCR using designed primers, after electrophoresis the PCR product was visualized. As predictable, the bands with molecular size 435 bp were amplified from analyzed *C. albicans* isolates, Fig. 8. The results revealed that there are 15 (60%) positive isolates

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collected from the oral cavity of males for *Ece*1 gene and 6 (37.5%) from the respiratory tract, while 10 (40%) of isolates collected from the oral cavity of females were positive for the targeted gene. Also, 10 (62.5%) from respiratory tracts of females were harboring this gene as shown in Table 5. The obtained sequences of *Ece1* gene for selected four isolates of *C. albicans* were deposited in gene bank under accession numbers and the data of alignment showed 100% percentage with the sequences of the same gene of the others isolates of *C. albicans*.

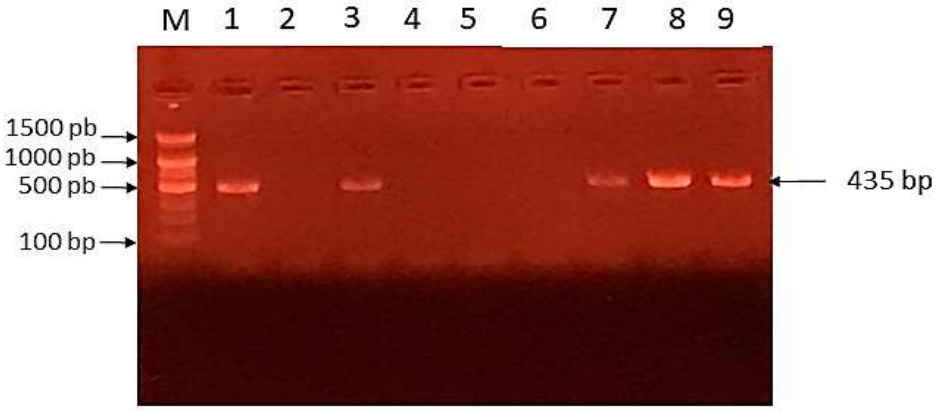

Figure 8. Electrophoresis of the PCR product of the *Ece1* gene of *C. albicans* isolates using agarose gel at a concentration of 2% for 45 minutes under a voltage of 70 volts. After staining with ethidium bromide. M. Gene ruler 1500 kb, Lane 1-9; PCR products of *C. albicans* isolates.

Table 5. Distribution of the candidalysin (*Ece1*) gene among *C. albicans* isolates according to source of isolation and gender.

Gender	Oral cavity		Respiratory tract		Total	*P*-value	*P*-value
	Positive	**Negative**	**positive**	**Negative**		+ve *vs*+ve	-ve *vs* -ve
	N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**		
Male	15(60.0)	5(29.4)	6(37.5)	4(33.3)	30(42.8)	0.002**	**0.638** N.S
Female	10(40.0)	12(70.6)	10(62.5)	8(66.7)	40(57.2)	1.00 N.S	**0.197** N.S
P-value	0.149 N.S	0.008**	0.144 N.S	0.083 N.S			

Data presented as N (%), ¥: Z-test was used to test two proportions. N.S: Not significant*, ** Significant and highly significant and respectively.

Conclusion

The results obtained in the present study demonstrated that the isolates of *C. albicans* collected from oral cavity and respiratory tract infections of Iraqi patients have different types of virulence factors responsible for the pathogenicity of this yeast. Moreover, in our study the distribution of Ece1 gene among the collected *C. albicans* isolates showed that there are 41 isolates obtained from oral cavity and respiratory tracts harboring the tested gene in each gender with hemolytic activity in all *C. albicans* isolates.

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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.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Authors' Contribution Statement

This work was carried out in collaboration between all authors. S. A. S. and S. R. M. Contributed to the design and implementation of the research, to the analysis of the results and the writing of the manuscript.

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الكشف عن بعض عوامل الضراوة بين المبيضات المعزولة من المرضى وانتشار جين candidalysin *ECE1*

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الخلاصة

المبيضات البيضاء هي مسبب شائع لعدوى الجهاز التنفسي وداء المبيضات الفموي لدى الناس ، ومسببات أمراض الخميرة الانتهازية والأسباب الرئيسية للمرض والوفيات لدى الأشخاص الذين يعانون من نقص المناعة وتسبب التهابات سطحية على الأسطح المخاطية التي تصيب ملايين الأشخاص في جميع أنحاء العالم. كان الهدف الرئيسي من هذه الدراسة هو التحقق من انتشار بعض عوامل الضراوة التي لها القدرة على تكوين الأغشية الحيوية(Biofilm) ، والبروتينيز(Proteinase) ، وإنتاج الهيمولايسين(Hymolysine) بين عزلات *C. albicans* التي تشمل انتشار جين*(ECE1)* Candidalysin . تم جمع العينات خلال الفترة من ايار 2022 الى اب 2022 من 280 عينة (مسحة) لمرضى عراقيين من مختلف الاعمار والاجناس غير مكررين كانوا يعانون من داء المبيضات الفموي وامراض الجهاز التنفسي في مستشفيات بغداد (مستشفى مدينه الطب و مستشفى اليرموك التعليمي ومستشفى مدينه الامامين الطبية التعليمية). تم زراعة العينات التي تم جمعها على وسط اكار السابروويد دكستروز (SDA) مضاف اليه كلورامفينيكول كمضاد للبكتيريا بتركيز 10 ميكجم / مل ، وتم حضن الاطباق الملقحة على وسط SDA عند 37 درجة مئوية لمدة تتراوح بين 24-48 ساعة.أظهرت نتائج الاستنبات أن 102 عينة كانت موجبه، منها58 (56.86%) من تجويف الفم و 44 (43.14%) من الجهاز التنفسي، بينما 178 منها كانت سالبة , تم الكشف عن عزلات المبيضات باستخدام الطرق التقليدية والتي تشمل (النمو على وسط HiCrome Candida ، إنتاج الأنبوب الجرثومي Germ tube ، تكوين الابواغ الكلاميديه Clamydospore وتم تأكيد نتئج التعريف باستخدام نضام VITEK-2 بينت النتائج ان من بين 102 عزلة *C. albicans* هي الاكثر شيوعا بين الانواع بنسبة 68.63 % ثم تلتها *C. tropicalis*بنسبة 10.78% ثم *C. kruzei* 5.88% و *C. parapsilosis* 4.9 % بينما كانت *C. kefyr* 5.88% *C. glabrata* 3.9 % وتمثل النسبة الاقل بين الانواع . تم فحص حساسية عزلات المبيضات للأدوية المضادة للفطريات بطريقة انتشار القرص ، والتي أجريت على النحو الموصى به بواسطةM44-A (CLSI) -وثيقة . أظهرت العزلات درجة عالية من الحساسية للأمفوتريسين-ب 93.14% وكلوتريمازول 90.20% ونسياتين 85.92% ،تم التحقق من انتشار جين(*ECE1*) Candidalysin بين 70 عزلة من *C. albicans* باستخدام تقنية تفاعل البلمرة المتسلسل PCR ، وأظهرت النتائج أن 41 58.57% تحتوي على جين *Ece*1 في تجويف الفم والجهاز التنفسي. و 34 عزلة 48.57% من *C. albicans* كانت منتجة قوية للغشاء الحيوي, بينما 30 عزلة 42.86% عزلة انتجت بروتيناز و 20 عزلة 28.57% عزلة لديها القدرة على تحلل الدم.

الكلمات المفتاحية: خميرة المبيضات ، كانندالايسين، عوامل ضراوه، الهيمولايسين، تكوين الفلم الحيوي.