IHJPAS. 37 (2) 2024
Ibn Al-Haitham Journal for Pure and Applied Sciences
Journal homepage: jih.uobaghdad.edu.iq
PISSN: 1609-4042, EISSN: 2521-3407

Detection of Some Virulence Factors in *Candida albicans* Obtained from Different Clinical Specimens of Iraqi Patients

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Received: 7 March 2023 Accepted: 3 April 2023 Published: 20 April 202
doi.org/10.30526/37.2.3315

Abstract

Candida albicans is a diploid polymorphic yeast that is found as part of the normal microbial flora in most healthy humans. It's usually a harmless colonizer of mucosal surfaces; it can, however, cause disease in the case where the host suffers from a weakened immune system or is immunocompromised. The present investigation was carried out to detect and investigate the prevalence of some virulence factors, which include coagulase production, biofilm formation, and phospholipase distribution among *C. albicans* isolated from different clinical samples, including oral cavity and respiratory tract, which were collected from May to August 2022 and numbered 280 of different ages and genders of Iraqi patients suspected of respiratory diseases and candidiasis. After detection, among the 102 positive samples, results showed that 58 (56.86%) were from the oral cavity and 44 (43.14%) were from the respiratory tract. *C. albicans* was the most prevalent species among the six types collected of *Candida* species, while *C. glabrata* was the least prevalent. All of the yeast was identified using several conventional methods, such as chlamydospore generation and germ tube production, and grown on HiCrome *Candida* medium, which was confirmed using the VITEK-2 system. Biofilm activity was strong in 34 (48.57%) of *C. albicans* isolates, while 45 (64.29%) strong isolates produced phospholipase and 59 (84.29%) isolates had the ability to produce coagulase.

Keywords: *Candida albicans*, virulence factors, biofilm formation, coagulase, phospholipase.

1. Introduction

Every year, millions of people around the world suffer from morbidity and mortality due to fungus infections with types of *Candida*, *Cryptococcus*, *Pneumocystis*, and *Aspergilla* that are thought to be responsible for two million infections that are potentially fatal in one year [1]. There are currently about 1,500 species of yeast, which are eukaryotic microorganisms classified in the kingdom of fungi [2]. Yeasts' inclusion in the divisions Ascomycota, Basidiomycota, and Deuteromycota is evidence of their phylogenetic variety. *Candida* species belong to the division Ascomycota, class Ascomycetes [2]. *Candida* species are one of the main leading causes of invasive fungal infections in the world-wide [3, 4]. Despite the availability of newer antifungal drugs for the treatment and prevention of Candida spp. infections, invasive fungal infections and resistance to therapy by antifungal drugs are on the rise [5]. *C.albicans* is a common opportunistic pathogen that affects millions of people worldwide and causes a wide range of infections each year, from superficial mucosal to hematogenously disseminated candidiasis.

A variety of virulence factors affect the pathogenicity of *C. albicans* [6]. When the normal oral microbiome balance is out of equilibrium or in individuals with compromised immune defenses, *Candida* spp. will transform from an opportunistic to a pathogenic organism that colonizes, multiplies, stifles other microbes, and causes recurrent infections in the mucous of the respiratory system and the oral cavity and is reliant on numerous virulence factors, including phenotypic switching and dimorphism, sticky qualities, extracellular enzyme synthesis, and biofilm development [7, 8]. Oral candidiasis is considered an opportunistic infection that influences people with cancer, notably those taking chemotherapy. And while this illness typically causes a variety of symptoms, including a burning, painful sensation, a change in taste, trouble swallowing, and decreased saliva output, it can also go unnoticed [9, 10]. Although *C. albicans* is the primary cause of candidiasis and is frequently isolated from the oral cavity and respiratory tract, other non-*Candida albicans* species, such as *Candida glabrata, Candida tropicalis*, and *Candida krusei, Candida parapsilosis*, and *Candida kefyr*, have increased over the past 20 years as a result of various factors, including immune-suppressants and prolonged use of broad-spectrum antifungals [11, 12, 13].

The *C. albicans* is widespread among people, particularly children and young, as well as adults who are suffering from different diseases such as immune disease, for these reasons, the main aims of the study were to identify *C. albicans* and their epidemiology in the oral cavity and respiratory tract of Iraqi patients and detect the prevalence of some virulence factors among *C. albicans* isolates by using phenotypic methods.

2. Materials and Methods

2.1. Sample collection

All the *Candida* spp. isolates were collected from different ages and genders of non-duplicated Iraqi patients suspected of respiratory diseases and candidiasis during the period between May and August of 2022 from Baghdad hospitals (Medical City Hospitals, Al-Yarmouk Teaching Hospital, and AL-Imamein AL-Kadhimaein Medical City Educational). During the present study, a total of 280 clinical samples (Swabs) were collected and cultured on Sabouraud Dextrose Agar (SDA), supplemented with chloramphenicol at a concentration of 10 mg/mL as an antibacterial, and incubated at 37 °C for 24-48 h.

2.2. Identification of *Candida* spp.

2.2.1. Microscopic examination

A single colony was transferred from SDA medium (Direvo, Germany) to a glass slide using a sterilized loop and mixed with a drop of distilled water. After drying, the prepared sample was spread out, fixed on a flaming fire, and stained with lactophenol cotton blue or Gram stain according to the instructions of the manufacturer. It was then investigated under a light microscope at 100X to detect yeast shape, budding form, and arrangement [14, 15].

2.2.2. Germ tube formation

This test was performed according to [16, 17], by growing *C. albicans* isolates on human serum after being incubated for 2–3 hours at 37 °C, the ability of the yeast isolates to form the germ tube was tested under a light microscope at 100 x.

2.2.3. Chlamydospores formation

Chlamydospore formation by *C. albicans* isolates was investigated on corn meal agar medium with tween-80 (Bangalore, India), which promotes the growth of chlamydospores. The streak method was used to culture and develop the isolated yeast on this medium, which was incubated for 2–5 days at 28 °C. After that, a slide sample from the growing colonies was prepared and

tested, viewed under a light microscope to identify the presence of thick-walled chlamydospores [16, 18].

2.2.4. Culturing on *Candida* HiCrome medium

Colonies of isolated *Candida* spp. were cultured on SDA and transferred to HiCrome *Candida* agar (HI-media, India). This medium, which is thought to be selective and differential, makes it easier to isolate and identify *Candida* spp. When the enzymes made by *Candida* spp. react with the chromogenic substrate in HiCrome medium, colonies of different colors form. The culture of yeasts on medium was incubated at 37 °C for 48 h. During this time, changes in the color of the yeast colonies were observed. This medium was prepared and autoclaved in accordance with the directions of the HiCrome Agar Company [19].

2.2.5. Identification of *Candida* spp. isolates using VITEK-2 system:

For more conformation and identification of *Candida* spp. isolates, all the collected isolates were subjected to identification in the AL-Imamein AL-Kadhimaein Medical City Educational Lab Division, Baghdad, Al-Kadhimiya, using the Vitek2 compact device equipped by the French company BioMerieux, Marcy, which is an automated system approved for the diagnosis of yeasts and bacteria, which is mainly based on a set of biochemical reactions and gives results within hours [20].

2.3. Study of some virulence factors for *C. albicans.*

2.3.1. Determination of phospholipase activity.

By evaluating the extent of the zone of precipitation after the growth on egg yolk agar, the phospholipase activity of *C. albicans* was discovered. [21] The egg yolk medium contained 10% sterile egg yolk, 11.7 g of sodium chloride, 0.11 g of calcium chloride (BDH, England), and 13.0 g of SDA (they are all placed in 184 ml of distilled water) [22, 23]. All of the substances were combined and sterilized, with the exception of the egg yolk, which was then centrifuged at 500 g for 10 minutes at room temperature. Twenty ml of the supernatant were then added to the sterilized medium that had already been prepared. [22]. Inoculating 10 µl aliquots of yeast from the yeast suspension (about 10^8 yeast cells/ml) were deposited onto the surface of egg yolk agar medium and left to dry at room temperature [22] and incubated at 37 °C for 48 h. When a precipitation zone could be seen surrounding the yeast colony on the plate, the phospholipase activity of 70 isolates of *C. albicans* was regarded as positive. The ratio of the diameter of the yeast colony to the overall diameter of the colony plus the precipitation zone was used to calculate the amount of phospholipase activity (Pz). Phospholipase activity is indicated by (Pz) values less than one, while (Pz) values greater than one indicate no activity [24].

2.3.2. Determination of Coagulase Activity

Candida isolates were grown on Sabouraud dextrose agar (Direvo-German) with 1% chloramphenicol added after the purity of the cultures was checked. Next, the plate was kept at 37°C for 24-48 hours, and the yeast growth colonies were carefully transferred into a glass tube with 5 ml of EDTA-rabbit plasma and incubated there for 4-6 hours at 35°C. The tube was re-incubated for 24 hours in the absence of a clot, then re-examined [25]. For the coagulase test's positive and negative controls, the strains *Staphylococcus aureus* and *Staphylococcus epidermidis* were employed. The presence of a clot served to measure coagulase production [25].

3.3. Determination of Biofilm formation

Using Christensen's tube adherence method, biofilm formation for 70 isolates of *C. albicans* was determined [26]. Once the tryptone soya broth (TSB) medium (HI-Media, India) was sterilized and supplemented with glucose (final concentration of 8%), a loopful of the yeast from the SDA plate was injected into the tube. after that, incubated at 37°C for 24 hours. The tube

contents were aspirated out and washed three times with phosphate buffered saline (PBS), and the pH was adjusted to (7.2). The walls of the tubes were stained with 1% crystal violet for 3 hours. The stain was removed from the tubes, then inverted, left to dry, and fixed with 200 µl of 95% methanol. The result is considered "positive" by the presence of colored layers in the inner wall of the tubes. A negative (0+), weak positive (1+), moderate positive (2+), and strong positive (3+) rating were given for the test of biofilm formation. Each isolate was tested at least three times and read independently by two different observers.

3.4. Statistical Analysis

The data for the current study were analyzed using Microsoft Excel and IBM SPSS V26. A chi-square test was used for categorical data [27].

3. Results and Discussion

3.1. Isolation and identification of *Candida* spp.

During the 3-months study period, and out of 280 clinical samples 102(36.43) yeast isolates were collected from of different ages and genders of non-duplicated Iraqi patients who suspected of respiratory diseases and candidiasis from some hospitals in the capital of Baghdad, While 178 (63.57%) of clinical sample were negative as **Figure (1)**. The isolates were identified as *Candida* species. Most *Candida* spp. is easily identified using classical methods such as cultural and morphological characteristics [28].

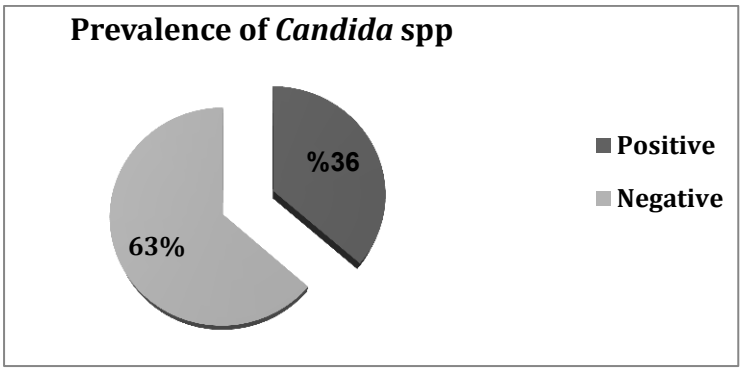

Figure1. The percentage of positive and negative culture of collected samples from patients cultured on SDA medium.

Morphological appearances of colonies of *C. albicans* on SDA were white to cream-colored, smooth, glabrous, and yeast-like. Under microscopic examination, the shape of *Candida* yeast cells was spherical to oval, with the presence of budding, and much larger than bacterial cells. Previous studies indicated that the development of germ tubes is typically used to identify *C. albicans* strains, *C. dubliniensis* strains, and some *C. tropicalis* strains when growing under certain non-optimal and unfavorable conditions [29]. The results of the examinations were shown in this study. All C. *albicans* isolates produced germ tubes and chlamydospores, while the other species did not produce any of them. The Vitek is more sensitive and specific for the diagnosis of *C. albicanis* than other methods (culture and germ tube tests). The Vitek is a very important test for the diagnosis of *C. albicanis,* and it has sensitivity and specificity to differentiation for *Candida* spp.

Hichrome *Candida* agar medium was used in the differentiation and identification of collected yeast isolates [30], which were identified according to the colony color on Hichrome *Candida* agar as shown in **Table 1.** Distinctive and special diagnoses for each *Candida* type are based on

the basic substance of those enzymes in the medium [30, 31]. This test is accurate and high in diagnosing the different species of *Candida.* This is because it contains a substrate called the chromogenic mixture, where the enzymes of each type of *Candida* yeast interact with its substrate in this medium. Such as type *C. albicans produces a* specific enzyme (BN-Acetylgalactosaminidase) that works on its substrate in the medium **Table 1.** [32, 33].

Table 1. Morphology characteristics and Color of *Candida* spp. on Hichrome *Candida* agar medium

Candida Species	HiChrome *candida* agar color and morphology
C. albicans	Light green-smooth-brilliant
C. tropicalis	Blue-dark blue with dark halo in agar-smooth-brilliant
C. kruzei	Pale pink-smooth and brilliant
C. kyfer	Pink -purple
C. parapsilosis	Light pink-smooth and brilliant
C. glabrata	Cream- smooth- brilliant

In the present study, according to cultural and morphological characteristics, 102 isolates were identified. Six isolates of *Candida* spp. were identified, including *C. albicans* 70 (68.63%), *C. tropicalis* 11 (10.78%), *C. krusei* 6 (5.88%), *C. kyfer* 6 (5.88%), C. *parapsilosis* 5 (4.90%), and *C. glabrata* 4 (3.90%), as shown in **Table 2.** The most frequently isolated *Candida* spp. from clinical manifestations are *C. albicans* (predominant worldwide), followed by C. *tropicalis,* while the less frequently and less commonly isolated were *C. glabrata* from clinical manifestations, as also described in some other studies.

Table 2. Number and percentage of *Candida* spp. isolated from different clinical sources

No	Types of *Candida* spp.	No. of Isolates	Percentage %
1	*C. albicans*	70	68.63
2	*C. tropicalis*	11	10.78
3	*C. krusei*	6	5.88
4	*C. kyfer*	6	5.88
5	*C. parapsilosis*	5	4.90
6	*C. glabrata*	4	3.90
	Total	102	100
	Chi square test	*P*-value	0.001**

The current study proved that females 59 (57.84) are more susceptible to infection with candidiasis than males 43 (42.16) (**Figure 2)**, especially pregnant women and people with immune system disorders.

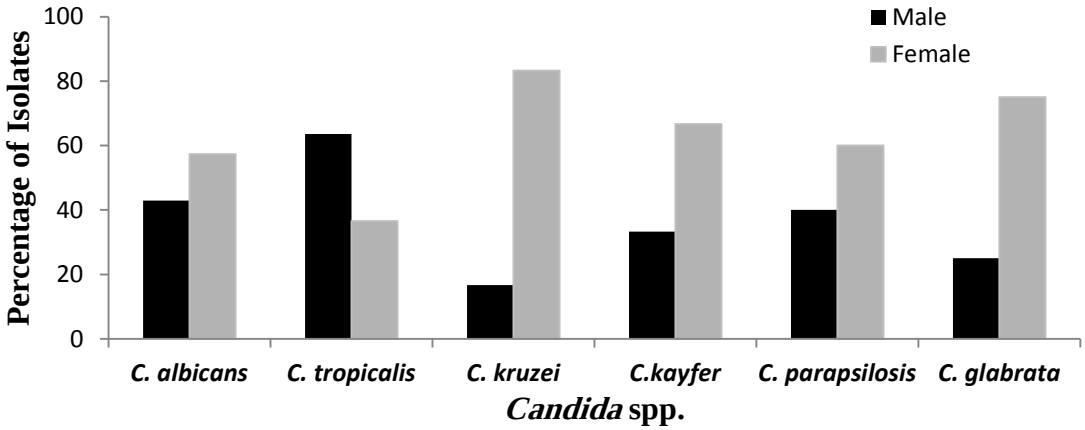

Figure 2. Distribution of clinical *Candida* spp. isolated from different specimens according to gender.

As well as patients undergoing chemotherapy for cancer, those taking immunosuppression therapies to preserve transplanted organs, and AIDS patients.

3.2. Phospholipase

Phospholipase production was seen in 63 (90%) of 70 cases, while 7 (10%) did not show any activity to produce phospholipase in *C. albicans* isolates, as shown in **Table 3**.

Table 3. Phospholipase activity production from 70 isolate of *C. albicans*.

No. of Candida Isolates 70 (%)			
Negative	Weak	Moderate	Strong
7(10)	5(7.14)	13(18.57)	45(64.29)

The extracellular phospholipase enzyme lyses host cells to facilitate adhesion and penetration [34]. The phospholipase enzyme breaks down the phospholipids of the cell membrane, causing cell lysis or host cell damage. This is a major mechanism contributing to microbial virulence [34, 35]. According to several earlier investigations, 30 to 100% of *Candida* spp. from various patient groups and locations indicated phospholipase activity [36, 37]. Also, it may depend on the site; as reported by a previous study, phospholipase activity was discovered in 55, 50, and 30%, respectively, of the *Candida* spp. isolated from blood and wound infections and urine [36] or affected by growth conditions [38]. For example, the presence of a high concentration of salivary glucose combined with a reduced salivary secretion rate enhances the growth of yeasts and their adherence to epithelial oral cells [39].

3.3. Coagulase

The coagulase tube test was used to assess the production of *Candida* coagulase, and the majority of the *C. albicans* 59 (84.29%) strains were successful in causing clot formation from EDTA-rabbit plasma after 24 h of incubation. lower percentages of isolates tested (15.71) were negative for producing coagulase, as shown in **Figure 3.**

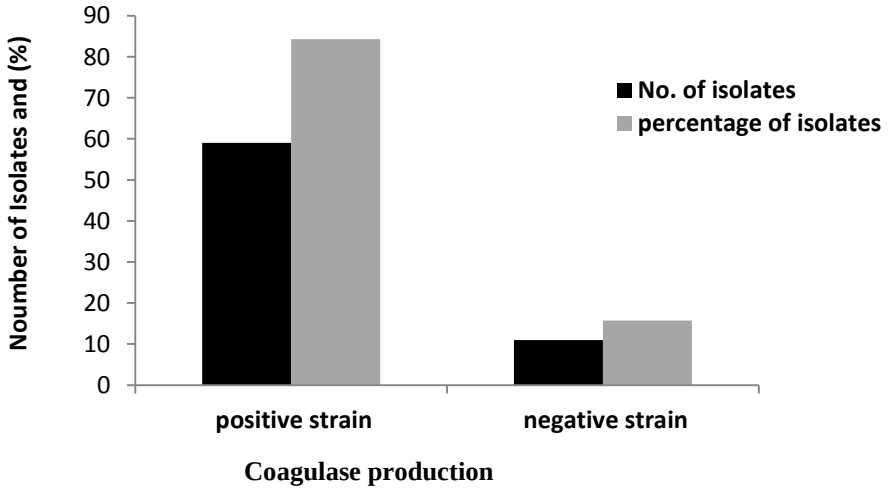

Figure 3. Coagulase activity of 70 isolates of *C. albicans*.

Plasma coagulase is an enzyme that binds plasma fibrinogen, starts a chain of events that causes plasma to clot, and aids in the detection of *S. aureus* [40, 41]. Due to their increased use in clinical microbiology laboratories and the recent replacement of the outdated coagulase test with more accurate and speedy diagnostic tools like Pastorex Staph-Plus, a variety of diagnostic kits are now being employed. The body of an organism can be damaged by hydrolytic enzymes that

are expressed on the surface of microbial pathogens; however, the pathogenic effects of these enzymes are still unclear. The first reported coagulase production from *Candida* species was by [25], who detected high coagulase activity in *C. albicans* (88.5%), and this percentage is consistent with our current study, where we found that 84% of *C. albicans* isolates were producers of this enzyme. Other studies [42] found that 68.9% and 64.7% of *Candida* isolates produced coagulase enzyme.

3.4. Biofilm Formation

The biofilm formation was tested using tube adherence methods. The Biofilm formation of 70 isolates of *C. albicans* was evaluated. The biofilm could be formed by 67 (95.7%) of the isolates, of which 34 (48.57%), 28 (40%), 5 (7.14%) developed strong, intermediate, and weak biofilms, respectively. Hence, among all the isolates, the growth of strong biofilms was greater than that of intermediate and weak biofilms. Also, among the 70 isolates, there were three isolates (4.29%) that did not produce a biofilm, as shown in **Table 4.**

Table 4. *C. albicans* and biofilm formation capacity.

***C. albicans* (N=70)**			
No production	weak	Moderate	Strong
3	5	28	34
4.29%	7.14%	40%	48.57%

Biofilms are collections of unicellular microorganisms that attach to surfaces and build multicellular structures [43]. Chemical structure composed of matrix materials enclosed by small colonies of yeast and pseudo-hyphae and hyphae arranged in a complex structure. Most studies reported that the pathogenic effects and oral infections are caused by *C. albicans* associated with biofilm production, which can be produced both on the plastic surfaces of clinical devices such as prosthetic heart valves, joint replacements and on mucosal surfaces [44, 45, 46]. *Candida* is the most common colonizer of the human respiratory tract and oral cavity, which plays an essential role in wide oral infections and respiratory diseases [46, 47].

4. Conclusion

During this work, we collected and identified 102 *Candida* isolates from clinical sources which include oral cavity and respiratory tract infection of Iraqi patients. We found 70 isolates were identified as *C. albicans* from the clinical sources have different types of virulence factors responsible for the pathogenicity of this yeast. . The current results revealed that 48.57% from *C. albicans* isolates were strong producer for biofilm, and 40% were moderately biofilm producer, while 4.29% non-producer of biofilm and *C. albicans* isolates showed varied phospholipase activity, 64.29% was strong activity and 7.14% of isolate have weak activity. The percentage of positive coagulase production among *C. albicans* isolates was 84.29% while 15.71% was negative in production.

Acknowledgment

Many thanks to the Department of Biology, College of Science for women, University of Baghdad for facilitating the work of the practice parts in this article.

Conflict of Interest

There is no conflict of interest.

Funding

There is no funding for the article.

Ethical Clearance

The samples were gained according to Local Research Ethics Committee approval in Iraqi Ministry of Health No. 3616 on 24/1/2022.

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