Study of Multiple Drug Resistance Pattern and Biofilm Formation of *Candida* Species Isolated from Iraqi Patients

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Abstract

Background: In the genus Candida, there are more than 200 yeast species, although only around 10% of them have been found to be dangerous to people and some other animals. Invasive candidiasis is the most prevalent invasive fungal infection examined, and Candida albicans (C. albicans) is the most common causal pathogen. Objectives: The current investigation is to isolate and identify Candida spp. from various clinical specimens and to calculate multiple drug resistance (MDR) index and the ability of biofilm formation. Materials and Methods: One hundred (100) different clinical samples (ear, mouth, vagina, skin, blood, and urine) were collected from patients who attended Gazi Al Hariri Hospital, Baghdad Teaching Hospital in Medical City and Al-Yarmouk Teaching Hospital. Candida spp. were identified using selective medium. Furthermore, antifungal susceptibility and MDR index were evaluated. Results: The results of isolation and identification for Candida species were revealed that Candida parapsilosis isolated in a high percentage (27.7%) followed by Candida glabrata (22.2%), the lowest percentage was recorded in Candida tropicalis, Candida krusei (5.50%), while the MDR index of isolated Candida spp. toward the antifungal in the current study showed that Candida lusitaniae and Candida krusei and Candida parapsilosis were (100%), the biofilm formation strength was different between the isolates Candida tropicalis and Candida parapsilosis was isolated in high percentage. Candida lusitaniae, Candida krusei, and Candida parapsilosis were the most resistant to the antifungal agents. Candida lusitaniae, Candida glabrata, Candida tropicalis, and Candida rugosa revealed strong biofilm formation.

Keywords: Antifungal, biofilm formation, Candida spp, MDR Candida

INTRODUCTION

Opportunistic yeast infections have been more prevalent over the past few decades, and they are now thought to be the cause of more than 1.5 million fatalities worldwide each year. These infections are more common in intensive care unit patients as well as those with a history of immunosuppressive medications, excessive antibiotic usage, hormonal treatment, or invasive procedures.^[1]

The development of multiple drug resistance (MDR) Candida, although rare compared to antibacterials, is a matter of concern especially in light of the changing epidemiology of Candida infections, showing a shift toward species intrinsically resistant to the most commonly used antifungal drugs. In fact, MDR Candida

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mainly involves acquired resistance in species with intrinsic resistance.^[2] These yeasts can also trigger superficial infections that damage the skin or mucous membranes, in addition to severe infections that can spread and be fatal,^[3] also found in pelvic inflammatory disease^[4] and as air borne microorganism.^[5] According to Pfaller and Diekema,^[6] there are roughly 250,000 new instances of immunosuppressants reported annually, more than 50,000 of which result in death. Other studies have estimated a mortality rate of more than 60%.^[7,8] An invasive candidiasis

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is the most prevalent invasive fungal infection examined, and Candida albicans (C. albicans) is the most common causal pathogen (33.8%–60%) overall, despite an increase in non-albicans Candida species.[9] However, its usage in hospital laboratories has been constrained in part due to a lack of the required specialized infrastructure or because some tests are challenging methodologically. Therefore, it is essential to create novel assays that accurately identify Candida species utilizing methods that are specific, userfriendly, practical, inexpensive, and available to any laboratory. One of the key factors in the evolution of Candida species into significant human diseases is the production of biofilms.[10] Understanding and treating Candida-associated infections are necessary due to the rise in the prevalence of fungal infections, particularly those brought on by C. albicans and other Candida species. The main purpose of the present study was to isolate and identified Candida spp. from different clinical specimens and estimate MDR yeast and their ability to form biofilm.

MATERIAL AND METHODS

Sample collection

One hundred (100) different clinical samples (ear, mouth, vagina, skin, blood, and urine) have been collected from patients who visited the Al-Yarmouk Teaching Hospital, Baghdad Teaching Hospital in Medical City, and Gazi Al Hariri Hospital. The investigation was conducted for the period from December 2021 to February 2022.

Identification and conformation of Candida spp

Chromagar media (*Candida* agar) is a medium used to identify organisms of the *Candida* species based on the color of the growing colonies. This medium was prepared in accordance with the manufacturer's instructions, which stated that 47 g of powdered medium should be dissolved in 100 mL of distilled water, brought to a boil in a water bath, and then poured into Petri dishes to be stored until use. This medium was used to distinguish between types of *Candida*. A confirmation diagnostic utilizing the Vitek-2 YST System was performed. VITEK®2 ID cards are disposable, self-contained cards designed for use with the VITEK®2 system. They are practical and secure.

Antimicrobial susceptibility and multiple drug resistance (MDR) Candida

Antibiotic susceptibility analysis will be performed utilizing antibiotic discs that are easily accessible on the market and a modified version of Kirby Bauer's Disk diffusion technique. According to the manufacturer's recommendations, the diameter of the inhibitory zone was used to classify stains as susceptible, moderately resistant, or resistant, which matched the interpretive criteria advised by Clinical and Laboratory Standards Institute (CLSI).^[12] The list of antibiotics employed includes fluconazole, voriconazole, ketoconazole, clotrimazole, nystatin, and

amphotericin B. The amount of antibiotics to which an isolate is resistant is divided by the total amount of antibiotics to which the organism has been exposed to produce the MDR index.

Quantitative biofilm formation assay^[13]

The potential of (Candida spp) isolates to form biofilms was assessed using a 96-well microtiter plate test based on the crystal violet staining technique. Briefly, 20 L of suspended yeast from strains 0.5–0.7 McFarland (1.108 cfu/ mL) were added to each well of a 96-well flat-bottomed sterile polystyrene microplate, which was previously filled with 199 L of Mueller-Hinton broth supplemented with 1% glucose. At 37°C, microplates are incubated for 48 h. The adhering cells were rinsed twice with phosphatebuffered saline (PBS), and wells were dried at 60°C for 1 h or less. The liquid media was then discarded. After that, it was stained for 15 min with 150 L of 2% crystal violet. The microplate wells treated with crystal violet were then rinsed twice with PBS to remove the stain. Following the air drying of the microplate's wells, 150 L of 95% ethanol were used to re-solubilize the dye from the biofilms that lined the plate's walls. A microplate reader measures the microplate spectrophotometrically at 570nm after 5-10 min. At least three new samples were used each time the experiment was performed.

Ethical approval

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients, verbal approval before samples were taken. The study protocol and the subject information and consent form were reviewed and approved by a local ethics committee at University of Baghdad.

RESULTS

Isolation and identification of *Candida* spp

The findings of isolation and identification of *Candida* spp. in studied samples were reported. The results showed that *Candida parapsilosis* was isolated in high percentage 27.70%, followed by *Candida glabrata* 22.22% and *Candida lusitaniae* (16.60%), while both of *Candida albicans, Candida rugose* were (11.11%). Finally, the lowest percentage recorded in *Candida tropicalis*, *Candida krusei* (5.50%) [Figure 1].

All *Candida* spp. were identified using the CHROMagar *Candida* culture medium, which selective media identified some of the species involved. Figure 2 when compared to the CLSI technique (Clinical and Laboratory Standards Institute), the ViteK-2system, a system with great reproducibility and accuracy.^[13] Due to their high repeatability and quick diagnostic tests with *Candida* spp, then followed by screening with the automated ViteK-2®

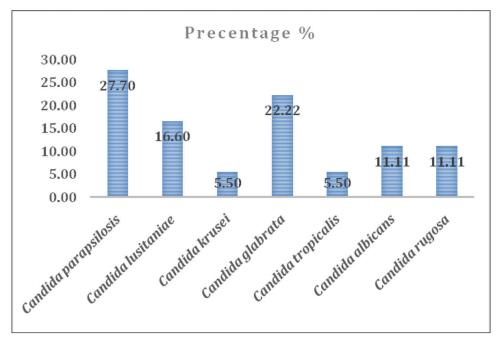


Figure 1: Percentage of Candida spp in samples

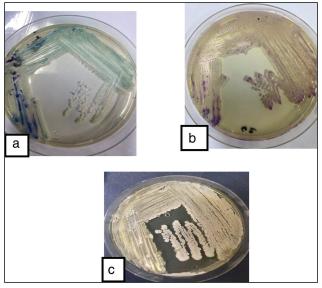


Figure 2: Candida spp. on CHROMagar. (a) Mixed sample. blue to purple: candida tropicals, Green: Candida albicans, white: candida glabrata. (b) Candida lusitaniae. (c) Candid aparapsilosis

and Etest® systems. Both methods are more complicated and time-consuming than the CLSI standardized broth microdilution method, which serves as a reference for antifungal susceptibility testing even after being challenging and labor-intensive to use on a regular basis. However, both methods have advantages over the CLSI method.^[14]

Antifungal and multiple drug resistance index

From the other side of the current study MDR index of isolated *Candida* spp. toward the antifungal reported that in

Candida lusitaniae, Candida krusei and Candida parapsilosis were 100%, followed by Candida tropicalis (66.6%) and Candida albicans, Candida rugose, and Candida glabrata (33.3%). However, some isolates of Candida albicans (50%) and Candida parapsilosis (83.3%) and Candida glabrata (16.6%) are shown in Figures 3 and 4.

Determination of biofilm formation

Moreover, the biofilm formation of isolated *Candida* spp. Figure 5, revealed various biofilm formation strengths as some of *Candida parapsilosis* were not able to form biofilm and some formed weak and moderate biofilm, while *Candida albicans* showed isolates not able to form biofilm and some formed a weak biofilm same in *Candida krusei* revealed a weak biofilm formation strength, *Candida lusitaniae* and *Candida glabrata* revealed weak to strong biofilm formation strength, *Candida tropicalis* and *Candida rugosa* showed strong biofilm formation strength, and *Candida albicans* with significant differences between isolates as shown in Table 1 and Figure 6.

DISCUSSION

The isolation results were consistent with those reported by Montes *et al.*,^[15] who revealed during culture from different clinical samples. Our findings showed that *C. parapsilosis*, not *C. albicans*, was the most common species isolated from blood (41.7%). This finding is intriguing since *C. parapsilosis* is complex and has become a more prevalent cause of fungemia^[16] because of its ability to colonize skin and spread to patients when medical professionals manipulate intravascular catheters. Another investigation using 11 different clinical specimens taken

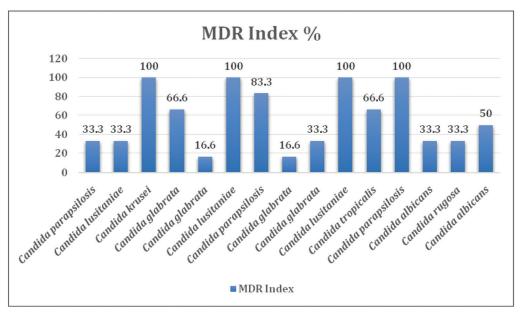


Figure 3: Multiple drug resistance index of isolated *Candida* spp. Antifungls: fluconazole, voriconazole, ketoconazole, clotrimazole, nystatin, and amphotericin B

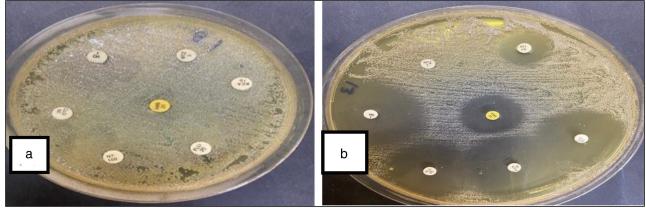


Figure 4: Antimicrobial susceptibility of isolated Candida spp. (a) Candida parapsilosis, (b) Candida glabrata

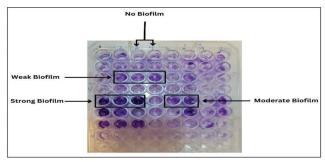


Figure 5: Microtiter plate assay of biofilm formation

from hospitalized persons in Mexico City with probable fungal infections revealed that *C. albicans* was more common, followed by *C. tropicalis* and *C. glabrata*.^[17] One hundred and five urine samples from patients with renal failure were collected by Othman *et al*.^[18] using a sterile urinary cap. Various diagnostic methods were employed to

characterize the isolated *Candida spp.*, including *C. albicans* (20%), *C. parapsilosis* (20%), *C. glabrata* (32.72%), and *C. krusei* (27.27%). However, a study was conducted by Arya and Naimshree. ^[19] revealed variations in the percentage of *Candida* spp. identified by CHROM agar from different clinical samples, with *C. albicans* being isolated in 48.4% of species. 248 samples were collected, where urine sample was the most common sample (68.1%) followed by pus (13.3%) and blood, which the species were isolated *C. glabrata*, *C. krusei*, and *C. tropicalis*.

Moreover, the results were compatible with El-Ganiny *et al.*^[20] regarding antifungal susceptibility. In this study, the highest level of resistance was observed against fluconazole. The *candida* spp. showed that fluconazole (FLU) resistance was *C. glabrata* (66.6%), *C. krusei* (16%), *C. tropicalis* (12.5%), and *C. albicans* (10%). Furthermore, the results showed an agreement with Terças *et al.*^[21] who revealed that results showed that all

strains of *C. guilliermondii*, *C. parapsilosis*, *C. albicans*, and *C. tropicalis* displayed as sensitivity to all antifungals drugs tested. Four of the six *C. krusei* isolates exhibited intermediate susceptibility to flucytosine, four of them showed resistance to fluconazole, and all of them were susceptible to amphotericin and voriconazole. Three different *C. glabrata* isolates were positive for resistance to fluconazole, amphotericin B and voriconazole. Furthermore, *C. krusei* naturally resists FLU.^[22] The observed fluconazole resistance in *C. tropicalis* ranges from 0% to 83% and from 4% to 9% in the USA, according to several perspective studies.^[23,24] Fluconazole is the antifungal, that is, prescribed most frequently, and due to

Table 1: The biofilm formation values of <i>Candida</i> spp		
Isolates	Mean	SE
Candida parapsilosis	0.24	0.010
Candida lusitaniae	0.36	0.090
Candida krusei	0.35	0.020
Candida glabrat	0.41	0.131
Candida glabrata	0.31	0.043
Candida lusitaniae	0.35	0.039
Candida parapsilosis	0.30	0.029
Candida glabrata	0.36	0.035
Candida glabrata	3.89	0.161
Candida lusitaniae	2.69	0.322
Candida tropicalis	3.15	0.694
Candida parapsilosis	0.23	0.029
Candida albicans	0.29	0.084
Candida rugosa	1.78	0.095
Candida albicans	0.20	0.041
Candida parapsilosis	0.13	0.000
Candida parapsilosis	0.64	0.098
* P value = 0.00		

*P value: highly significant

its extensive use in treating *Candidiasis*, all *Candida* spp. have developed a resistance to the antibiotic.^[24]

In accordance with the previous research, the current investigation found C. lusitaniae to be resistant.[25] Echinocandins usually have success against C. lusitaniae. The FKS genes encode beta-1,3-glucan synthase, which is the target of echinocandins. A few missense mutations have been discovered in the C. lusitaniae FKS1 hot spot 1. (HS1). Using amphotericin B (AMB), caspofungin (CAS) and azoles to treat chronic candidemia in a child with immunosuppressed enterocolitis and visceral adenoviral disease, the rapid emergence of antifungal resistance in C. lusitaniae. FCZ resistance can be built up in C. glabrata after first coming into contact, whereas it is already present in C. krusei. The findings of this study are consistent with several investigations that showed that C. krusei has innate resistance to FCZ and that C. glabrata and C. famata had greater resistance. The findings of this study are in agreement with numerous investigations that showed that C. krusei has intrinsic resistance to FCZ and that C. glabrata and C. famata species had greater resistance to this antifungal medication.[26]

In addition, the findings of biofilm formation were corroborated by the study conducted by Marak and Dhanashree, [27] who reported that *C. albicans* (45.5%) was found to be the most prevalent species among the 90 *Candida* species that were isolated, followed by *C. parapsilosis* (2.22%), *C. glabrata* (3.33%), *C. krusei* (20%), *C. tropicalis* (28.88%), and *Candida* spp. were found in the following samples: pus, bile aspirate, deep tissue, high vaginal swabs, suction tips, blood, wound swabs, and urine, the age range of 51–60 years was more prone to candidiasis, and more women than men were affected. The most isolates were *C. albicans* then *C. parapsilosis*, *C. tropicalis*, and *C. krusei* isolates which generated biofilm while *C. glabrata*, did not exhibit any biofilm generation.

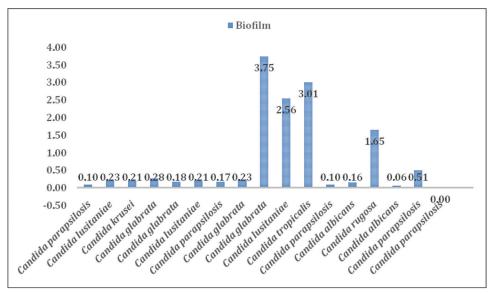


Figure 6: Biofilm formation of *Candida* spp

However, Kuhn *et al.*^[28] found that *C. parapsilosis* produced biofilms at a rate of 100%, followed by *C. krusei* and *C. tropicalis* among the *non-Candida albicans* species. However, demonstrated that compared to other *Candida* species, *C. albicans* forms quantitatively more biofilms.

According to Tulasidas *et al.*,^[29] a total of 176 clinical isolates were tested for biofilm development; of the 74 blood culture isolates, 55 (74%) produced biofilms; *C. haemulonii* (100%), *C. tropicalis* (22%), and *C. krusei* (21%), equation 1, displayed the strongest adherence. 45 (44.11%) of the 102 cervical swab isolates produced biofilms, while *C. tropicalis* (43%) showed high adhesion. Sahal and Bilkay^[30] noted a high biofilm development rate. *Candida* species including *C. orthopsilosis C. tropicalis*, *C. glabrata*, and *C. parapsilosis* were discovered to be the most prevalent species, and isolates of *Candida tropicalis* with a high ability for biofilm formation were shown to have higher rates of fluconazole resistance.^[31]

Alikhani *et al.*^[32] found that biofilm manufacturing was applied to all 50 clinical isolates. 19 (48.7%) of the 39 *C. albicans* isolates were shown to produce biofilms. Of the 11 *C. glabrata* isolates (54.5%) produced biofilms. Biofilms are ubiquitous, intricate, interdependent groups of surface-associated microbes that can grow on any surface, including medical equipment.^[33] A crucial predictor of virulence during candidiasis, the pathogenicity of *Candida species* is linked to their capacity to create biofilms.^[34-37]

Conclusion

The findings of isolation and identification of *Candida* spp. in studied samples were reported. *C. parapsilosis* was isolated in a high percentage, in same time, *C. lusitaniae*, *C. krusei*, and *C. parapsilosis* were the most resistance to the antifungal. Finally, *C. lusitaniae*, *C. glabrata*, *C. tropicalis*, and *C. rugosa* revealed a strong biofilm formation strength.

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Conflicts of interest

There are no conflicts of interest.

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