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Evaluation of antifungal drug resistance among *Candida albicans* isolated from clinical specimens

Kawther Hafez Jabbar Al-Sabti[1*], Raed Ali Hussain Shabaa[1]
[1]*Department of Pathological Analyses, Faculty of Science, University of Kufa, Najaf, Iraq.*

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ABSTRACT

Candida albicans is a dimorphic fungus that causes various diseases in humans and animals. Antifungals mainly used to treat infections caused by this species are azoles, echinocandins, and polyenes; however, these drugs tend not to be successful. The emergence of acquired drug resistance among prevalent fungal pathogens restricts treatment options, which alters patient management. A total of 151 clinical specimens were collected from immunocompromised patients suffering from underlying diseases including cancer, diabetes, and thalassemia. All the collected specimens were cultured on yeast selective media and subjected to a microbiological diagnostic procedure to isolate and identify *Candida albicans* isolates to the species level depending on the morphological characteristics, and the antifungal susceptibility profile was evaluated by Disk Diffusion Method. From the total 151 clinical specimens included in the present study 111 (73.50%) specimens were positive for yeast growth, among them the most predominant species was *C. albicans* at 64 (57.65%). There was a difference in the prevalence of yeast species isolates according to the patient's gender, which was higher in females 76 (68.46%) than in males 35 (31.53%), and according to the age group, the highest prevalence of infection was among the adults than the other age groups. The results of the antifungal susceptibility test showed that from the 64 *C. albicans* isolates, 16 (25%) were multidrug-resistant (MDR) isolates that were non-susceptible to at least one antifungal drug in two different drug classes, while the other isolates varied from non-susceptible to one drug class to susceptible to all the drugs tested in the current study. According to the data obtained by this study, it was concluded that there was a high prevalence of fungal infections caused by the pathogenic yeast species *C. albicans* isolates especially among adult female immunocompromised patients, and there was a concerning multidrug resistance among a concordable percentage of these isolates towards the most common antifungal agents that have been used in the treatment of such infections.

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***Corresponding author Email address*: kawtherh.alsabit@student.uokufa.edu.iq (Kawther Hafez Jabbar Al-Sabti)**

Introduction

Fungal infections, including *Cryptococcus*, *Malassezia*, *Pneumocystis*, *Aspergillus*, and *Candida* species, are the main reason people get sick and die around the world (Shabaa 2020a; AL-Zubaidy & Shabaa 2023). An estimated 2 million life-threatening infections are reported yearly as a result of fungal infections (Gnat et al. 2021). One kind of pathogenic yeast that leads to candidiasis is *Candida*. The genus *Candida* contains over 200 different species; however, only a few of these are dangerous and can cause infections that can be more serious and be either internal or external (Al-Garawi et al. 2022). There are many different organisms in this genus, and over 17 species of *Candida* are known to infect humans. That being said, more than 90% of major infections are caused by *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (Pfaller et al. 2007).

The *Candida* species spreads the most common fungal infection in the oral cavity candidiasis. *Candida albicans* is the most prevalent species in both healthy and immunocompromised, especially cancer patients (Al-Husseini et al. 2020; Talapko et al. 2020; Al-Tameemi et al. 2024; Al-Khalidi & Al-Husseini 2024). *Candida* albicans is a common pathogenic microbe and a part of commensal biota. It is a dimorphic fungus that can infect both healthy and immunocompromised people. It causes a wide range of diseases in both humans and animals. The incidence of lethal invasive infections has increased since the 1980s; these infections are now highly significant, particularly in individuals with impaired immune systems, and they present a serious and challenging public health issue due to growing healthcare expenses and hospital stays (Al-Husseini, 2020; Kumari et al., 2021; Gnat et al., 2021).

Harmful fungi typically cause infections that most commonly respond to azoles, echinocandins, and polyenes (Shabaa 2020). However, these medications usually do not work when the infection involves the C. *albicans* biofilm (Costa-de-Oliveira & Rodrigues 2020; Feng et al. 2021). There are some problems with the antifungals that are currently used to treat candidiasis. These include nephrotoxic polyenes, echinocandins that can only be given through an IV, and some thiazoles that are harmful and have trouble being absorbed (Kullberg et al. 2019).

Multidrug resistance (MDR) can happen for many reasons, including biofilms that make it harder for the antifungal to reach the target; spontaneous mutations that make the target more or less susceptible; chromosome changes; overexpression of multidrug efflux pumps; and being able to get past the host's immune system (Costa-de-Oliveira and Rodrigues, 2020; Al-Khfaji et al., 2023; Hassan and Motaweq, 2024). The growth of acquired drug resistance among common fungal infections limits treatment options and modifies patient care. To improve therapies, diagnostics, and intervention techniques that may overcome and prevent resistance, a deeper comprehension of mechanism-specific resistance and the biological mechanisms that contribute to resistance formation is essential (Bonhomme & d'Enfert. 2013; Abdulla et al. 2024).

Materials and Methods

Sampling

A total of the 151 clinical specimens included 111 (73.5%) specimens from patients with cancer, 32 (21.1%) specimens from patients with diabetes, and 8 (5.2%) from thalassemia patients who attended the Euphrates Cancer Hospital, AL-Sadder Medical City, and AL-Zahraa Educational Hospital in Najaf Province, which comprised 45 (29.8%) males and 106 (70.1%) females.

Phenotypic and biochemical identification of yeast

For identification of taxa, we used the phenotypic and biochemical criteria listed in the most relevant identification keys, such as those by Collee (1996), De Hoog et al. (2005), and Burns et al. (2008).

The yeast colonies grew bigger than the molds on Sabouraud dextrose agar that had antibiotics (amoxicillin, tetracycline, and gentamicin) added to it. The Petri dishes were then incubated at 37°C and 25°C separately. The data on growth at both temperatures were thought to belong to the abnormal categories. After 24 to 48 hours, colonies can be identified by their differences in size, color, and shine (Ellis 1994). The yeasts were then examined microscopically.

For microscopic examination of the recovered taxa, lactophenol cotton blue as mounting medium was used according to Morello *et al*. (2003) and Kayser *et al*. (2005).

For the identification of Candida based on their colonies color, HiCrome™ *Candida* differential agar was used. Twenty four hours old *Candida* isolates established on SDA were inoculated on HiCrome™ *Candida* differential agar and incubated at 37°C for 24 to 48 hours. According to the manufacturer's instructions, the assay was used to presumptively identify *Candida* spp. by color of the resulting growth where, *C. albicans / C. dubliensis* = green, *C. tropicalis* = blue, *C. parapsilosis* = white to purple, and *C. glabrata* = cream to white (Baradkar *et al*., 2010). However, pale pink colonies are produced by species of the *C. haemulonii* complex, such as *C. auris* and others like *C. krusie* (Kathuria *et al*. 2015).

Antifungal susceptibility test

We used the disk diffusion method for this study and seven different kinds of antifungal drug discs: amphotericin-B (AMB 100 µg), fluconazole (FLC 10 µg), itraconazole (IT 10 µg), miconazole (MIC 30 µg), and nystatin (NS 50 IU). The disk diffusion test was carried out according to CLSI (2018). A 0.5 McFarland density criterion was met by turbidity-adjusted yeast inoculum suspensions, yielding an inoculum with 5×10^6 yeast cells/ml. In the disc diffusion approach, agar plates were directly inoculated with this liquid. In 90-mm diameter Petri dishes, Mueller-Hinton agar enriched with 2% glucose and 0.5 mg/L of methylene blue (20 ml medium/plate) was distributed.

A cotton swab dipped in a cell suspension and adjusted to the turbidity of a 0.5 McFarland standard was used to inoculate the agar surface. The swab was rotated multiple times to eliminate any extra fluid, and the excess fluid was removed by pressing strongly against the inside wall above the fluid level. The swap ensured a uniform dispersion of the inoculums by streaking the dried surface of the agar using the normal procedure. The plates were kept open for 15-20 minutes to allow any surplus liquid to seep in. Using sterile forceps, the antibiotic disks were positioned on the plate surfaces. The plates were then incubated at 35 to 37C° and read 24 hours later.

The diameter of the inhibition zone for each antifungal medication was converted into sensitive and resistant groups using an interpretation chart from CLSI document M44, 3rd ed. (2018). At the 24-hour growth-decreasing changeover point, the widths of the inhibition zones were determined in millimeters. The inhibitory zone for azoles was measured up to colonies of typical sizes. Additionally, the clean zone devoid of evident development for Polyenes was measured.

Results and Discussion

Out of the total 151 clinical specimens included in the present study, 111 (73.50%) specimens were positive for yeast growth. The morphological characteristics and the biochemical tests results shows differences in the distribution of isolates among clinical specimens, the greatest number of yeast isolates were in the urine specimens which were 29 out of 39 specimens (74.35%) followed by oral swap specimens 82 out of 112 specimens (72.21%) (Figure 1).

There are several causes for the increase of candidiasis infections among immunocompromised patients such cancer and renal failure patients, including chemotherapy and radiation therapy which itself can impair cell-mediated host immunity, which is crucial for preventing fungal infections. Chemotherapy and radiation therapy have the same potential to cause mucositis, or inflammation and injury to the oral mucosa, which can lead to xerostomia and hyposalivation, that in turn, increases the growth, colonization, and infection of oral yeast. When oral candidiasis manifests it causes pain or burning sensation in the mouth and lead to poor nutrition or even invasive infections like candidemia or esophagitis, this can significantly increase morbidity (Jasiem & Al-Husseini 2023; AL-Tameme *et al*.2023; Al-Msaid *et al*. 2024). Other risk factors that have been linked to the development of this oral disease include corticosteroids, wide spectrum antibiotics, and diabetes (Bergmann, 1991; Samonis *et al*. 1998; Chitapanarux *et al*. 2021).

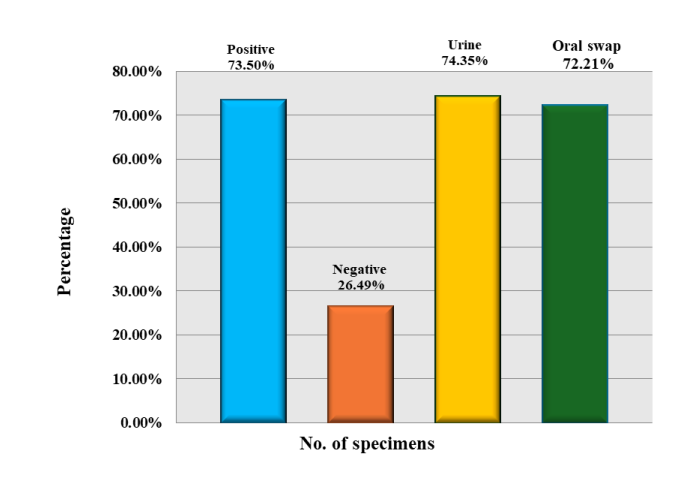

Fig 1. The prevalence of *Candida* isolates according to specimens.

Prevalence of yeast isolates according to gender

The results revealed that the prevalence of yeast species isolates was higher in females 76 (68.46%) than in males 35 (31.53%) (Figure 2).

The primary cause of the greater prevalence of candidiasis in females over males is the anatomical and physiological variations between the sexes. *Candida* spp. are found in small amounts in the skin, mouth, and genital tract than in other parts of body. Particularly, the female vaginal surface is larger than those in males, thus providing more area for yeast to colonize and grow. This creates the perfect warm and moist environment for yeast overgrowth, especially when hormonal changes occur (during the menstrual cycle, during pregnancy, or menopause), or when certain medications like birth control pills or antibiotics are taken. Additionally, changes in hormone levels, especially those of estrogen, can impact the ecology within the vagina. Excessive estrogen may promote yeast development. Female candidiasis risk can be elevated by hormonal changes throughout the menstrual cycle,

pregnancy, or hormone therapy (Thomas-White *et al.* 2018; Komesu *et al.* 2020; Jasiem & Al-Husseini, 2024).

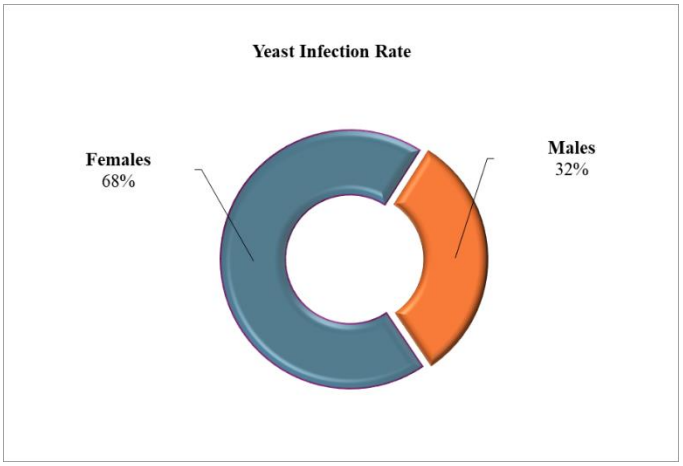

Fig 2. The prevalence of yeast isolates according to gender.

Prevalence of pathogenic yeast isolates according to age group

The study involved patients aged 3-85 years, who were categorized into four age groups: Child (3–12 years), Adolescence (13–18 years), Adult (19–59 years), and Senior Adult (60 years and above) (Nithyashri & Kulanthaivel 2012).

Compared to the other age groups in this investigation, the adult age group had the greatest infection rate of *Candida* spp. (62.16%), as indicated by the data in table 1. This age group's high incidence of candidiasis is linked to rising cancer and diabetes risks, as well as an aging population that takes more drugs, which raises the risk of adverse effects. The increased prevalence of infection in this age group may also be related to the growing number of patients with underlying illnesses and their longer longevity as a result of improvements in medical treatment (Mbakwem-Aniebo *et al*. 2020). The infection rate of the Adult age group was followed by Senior Adult which was 31(27.92%), Child 8(7.20%), and Adolescence 3(2.70%).

Table 1 Prevalence of yeast species isolates according to age group.

Age range (years)	No. of positive cases	%
Child (3-12 years)	8	7.20%
Adolescence (13-18 years)	3	2.70%
Adult (19-59 years)	69	62.16%
Senior Adult (60 years and above)	31	27.92%
Total	111	100%

Cultural characteristics

Cultural characteristics were used as a starting point for *C. albicans* identification. Sabouraud dextrose agar plates (SDA), which are ideal for the cultivation of opportunistic and pathogenic fungi, were used to cultivate all specimens. The colonies of *Candida* spp. grew fast in 24-48 hours as white, cream-colored to yellowish, smooth, glistening with a dry texture. Certain isolates had formation-footed colonies, while *Cryptococcus* species colonies ranged in color from bright white to tan or were translucent and had a mucous consistency (Jatta *et al*., 2009; Bhavan *et al*., 2010 and Singh *et al.,* 2013).

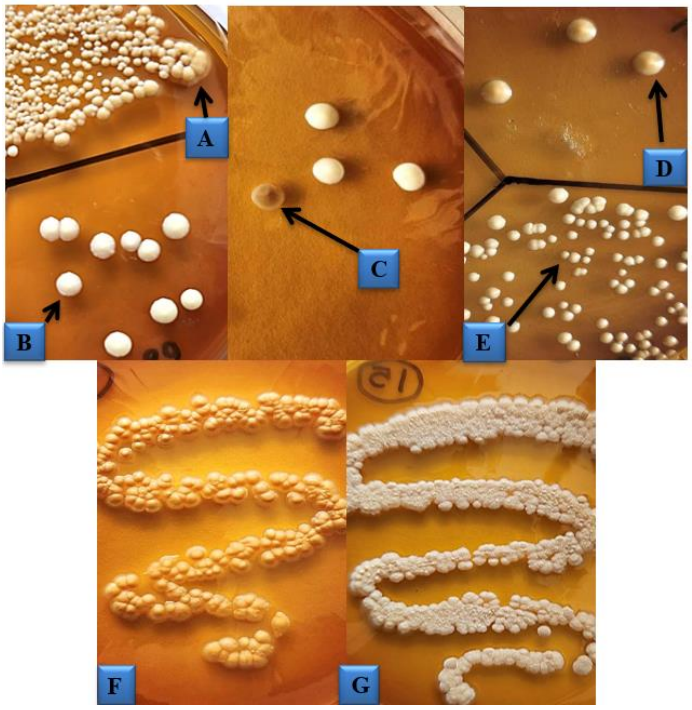

Fig 3. Growth of *Candida* spp. on SDA at 37°C for 48 hr, A: Footed colonies, B: White colonies, C: Tan colonies with a mucous consistency, D: Yellowish colonies, E: Small Cream colonies, F: Glistening with dry texture colonies, G: Dry colonies.

Microscopic criteria

There are several advantages of microscopic examination including its low cost and quick diagnostic turnaround, which make it ideal for determining whether a colony is a yeast isolate based on characteristics like budding formation (Deorukhkar and Saini, 2014). The lactophenol cotton blue microscopic preparations of *Candida* spp. isolates (Figure 4) showed round, oval to elongated budding yeast cells (Boon *et al*., 2013). Round-to-ovoid budding with elongated, pseudohyphal-like structures was observed in several isolates of *Candida* spp. This observation may suggest that the isolates are related to the infamous *C. auris* (Borman *et al*., 2016).

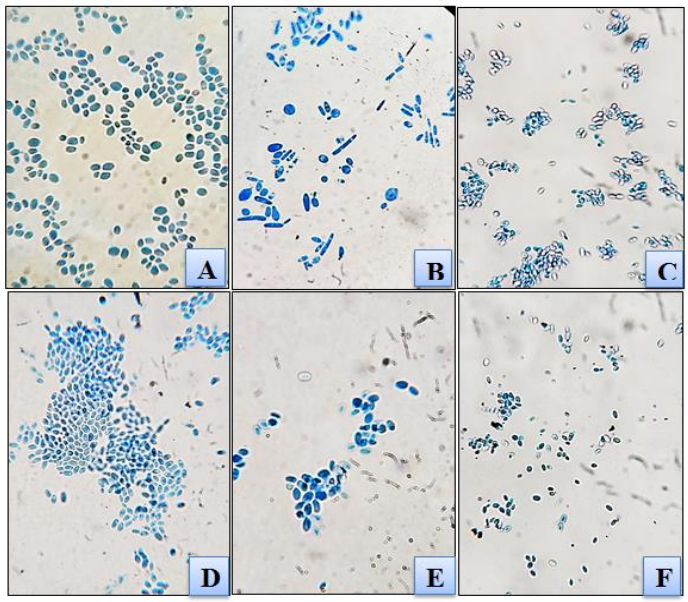

Fig 4. Yeast species mounted in lactophenol, A, C, D, E, and F: spherical, oval to elongated budding yeast cells; B: round-to-ovoid budding with elongated, and pseudohyphal-like forms.

Biochemical identification of Candida species

After up to 48 hours of incubation on HiChromeTM Candida Differential Agar, colonies with varying colors were observed, indicating that the isolates of *Candida* spp. were recognized down to the species level (Mulet *et al*., 2022). Isolates with pale green colonies are considered *C. albicans*, while the isolates that produced slightly bluish-dark green colonies are considered *C. dubliniensis*. Because *C. dubliniensis* and *C. albicans* are similar in forming germ tubes and producing colonies that are nearly identical in color on chromogenic medium, the tiny color variation between the colonies on Hicrome Candida Differential Agar helped distinguish the two species (Otero-Silva *et al*.,2004; Murray *et al*., 2005).

The metallic blue colonies are identified as *C. tropicalis*, the white to purple as *C. parapsilosis*, and the cream to white colonies as *C. glabrata* (Baradkar *et al*., 2010). However, the isolates that formed light pink colonies with white borders are thought to belong to the *C. haemulonii* complex species, including *C. auris* and *Teunomyces krusei* (formerly known as *C. krusie*) (Sayyada *et al*., 2010; Kathuria *et al., 2015 and* Noster *et al*., 2022).

Distribution of clinical isolates according to candida species

According to the findings of the morphological and biochemical identification tests carried out during this investigation, *C. albicans* was the most common species in about 64 out of 111 clinical isolates (57.65%), followed by *C. glabrata* 17 (15.31%), as shown in table 2.

Fig 5. Growth of yeast isolates on HiCrome™ *Candida* differential agar shows different colony colors: *C. albicans* = pale green, *C. dubliniensis* = slightly bluish-dark green, *C. tropicalis* = metallic blue, *C. parapsilosis* = white to purple, and *C. glabrata* = cream to white, *C. haemulonii* complex including *C. auris* and *Teunomyces krusei* = light pink.

Table 2 Distribution of clinical isolate according to *Candida* species.

Yeasts specie	Number of isolates	Percentage
C. albicans	64	57.65%
C. glabrata	17	15.31%
C. dubliensis	9	8.1%
C. parapsilosis	3	2.7%
C. tropicalis	9	8.1%
C. auris	5	4.5%
Teunomyces krusei	4	3.6%
Cryptococcus spp.	1	0.90%
Total	111	100%

The Antifungal Susceptibility Test for C. albicans Isolates

The disk diffusion method was used in this investigation, and the data were interpreted using the standard approach described in CLSI document M44, 3rd ed. (2018). The results showed that each drug had three categories of susceptibility: susceptible, intermediate, and resistant (Figure 6). The susceptibility pattern of *C. albicans* isolates for Miconazole was 19 (29.68%) resistant and 37 (57.81%) susceptible with 8 (12.5%) intermediate isolates while the susceptibility pattern of Itraconazole was 11 (17.18%) resistant, 39 (60.93%) intermediate and 14 (21.87%) susceptible. The susceptibility pattern for

Amphotericin-B was 10 (15.62%) intermediate and 54 (84.37%) susceptible with no resistant isolates, which differed slightly from that of Nystatin which was 8 (12.5%) intermediate and 56 (87.5%) susceptible with no resistant isolates while most isolates were susceptible to Fluconazole 61 (95.31%) with only 2 (3.12%) intermediate and 1 (1.56%) resistant.

Table 3 The antifungal drugs susceptibility patterns of *C. albicans* isolates.

Antifungal disc	Sensitive (%)	Intermediate (%)	Resistance (%)
Amphotericin-B (100 µg)	**54** (84.37%)	**10** (15.62%)	**0** (0%)
Fluconazole (10 µg)	**61** (95.31%)	**2** (3.12%)	**1** (1.56%)
Nystatin (50 IU)	**56** (87.5%)	**8** (12.5%)	**0** (0%)
Miconazole (30 µg)	**37** (57.81%)	**8** (12.5%)	**19** (29.68%)
Itraconazole (10 µg)	**14** (21.87%)	**39** (60.93%)	**11** (17.18%)

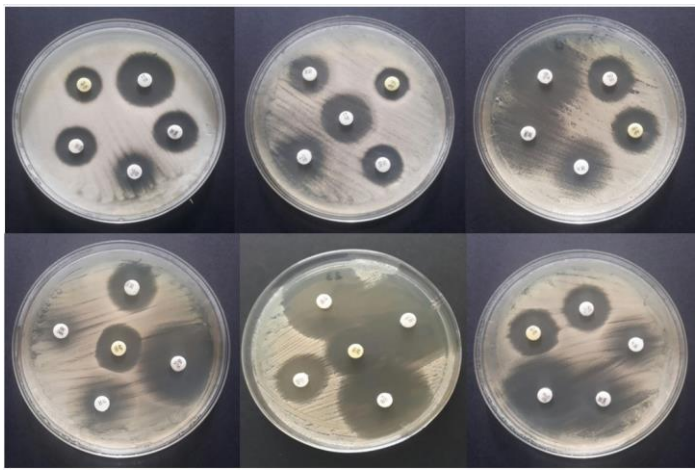

Fig 6. Antifungal sensitivity test by disk diffusion method on Mueller-Hinton agar supplemented with 2% glucose, showing the difference of growth inhibition zone among the isolates.

Distribution of multidrug-resistant C. albicans isolates

Acquired non-susceptibility to at least one agent in three or more antimicrobial groups was the definition of multidrug resistance, or MDR (Magiorakos *et al*. 2012). The only four drug classes currently available for the systemic treatment of Candidiasis are the Azoles (fluconazole, itraconazole, isavuconazole, posaconazole, and voriconazole), Polyenes (amphotericin B), Echinocandins (anidulafungin, Caspofungin, micafungin), and Pyrimidine analogue (flucytosine). As a result, this definition cannot be directly accepted for resistance in *Candida* spp. Only the first three of these drug classes—Amphotericin B, fluconazole and echinocandins—are permitted to be used alone to treat candidiasis; they are only advised as first-line treatments for invasive candidiasis. It is therefore fair to define MDR *Candida* as an isolate resistant to at least one compound in at least two distinct medication classes (Arendrup and Patterson, 2017).

According to that and depending on the results of the antifungal susceptibility test of the current study, from the total 64 *C. albicans* isolates, 17 (26.56%) were MDR isolates that were non-susceptible to at least one antifungal drug in two different drug classes while the other isolates varied from non-susceptible to one drug class to susceptible to all the drugs tested in the current study (Table 4- Supplementary materials). Formation of biofilms, which diminish the accessibility of the antifungal, selection of spontaneous mutations that increase expression or decreased susceptibility of the target, altered chromosome abnormalities, overexpression of multidrug efflux pumps and the ability to escape host immune defenses are some of the factors that can contribute to antifungal tolerance and resistance (Costa-de-Oliveira and Rodrigues, 2020).

Conclusions

The most prevalent *Candida* species is *C. albicans* it is responsible for more than 50% of the candidiasis infections among patients especially among adult female immunocompromised patients. From the 64 isolates tested for drug susceptibility during this study, 17 (26%) isolates were considered multidrug resistance (MDR) isolates because they were non- susceptibility for at least one agent in more than one Antifungal drug, this is a concerning result indicates that *C. albicans* isolates are developing resistance for the common Antifungal drugs that currently used to treat infections cause by this species.

Ethical approval of sampling

All the participants provided informed consent for inclusion in the study and were assured that all the information provided would be used solely for this study and treated confidentially.

Conflict of interest

The authors declare that they have no conflict of interest.

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