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RESEARCH PAPER

Isolation, Identification and Antifungal Susceptibility Testing of *Candida* Species from Dermatologic Specimens in Duhok Province

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A B S T R A C T:
Candida species. is a member of the mucous membrane of the normal flora, gastrointestinal tract and skin. They are endogenous opportunistic pathogens that cause secondary infections with underlying immunocompromised condition. Candidiasis is a common fungal infection in human. This study was aimed to isolation and identification of *Candida* using germ tube, chlamydospore formation, and chromogenic agar testes and to asses antifungal susceptibility to some isolates. A total of 180 samples of skin swabs and skin particles, nail and hair were collected from patients in Duhok Province Hospitals, outpatient and a number of private clinic dermatologists in Duhok city. A Total of 63 *Candida* isolates was detected in this study. These isolates were subjected to germ tube test, chlamydospores formation and inoculation on commercially available CHROMagar. *Candida* albicans was the main yeast species isolated followed by *C. krusei, C. tropicalis* and *C. glabrata* CHROMO agar is a convenient and very fast method of identifications of *Candida* species even in resources poor settings. The result of antifungal sensitivity test revealed that itraconazole is the main active antifungal against all *Candida* species with the minimum inhibition concentration (MIC) of 25 µg /ml. followed by terbinafine, fluconazole and griseofulvin.

KEY WORDS: *Candida*, Non- albicans *Candida*, CHROM agar, Duhok province.
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1. INTRODUCTION:

Candidiasis refers to fungal infection caused by several species related to genus *Candida*. The Incidence of Candidiasis is increasing worldwide. The infections by *Candida* increased dramatically in their prevalence during the past 20 years (Kim et al., 2016). *Candida* species are the main common causes of the fungal infections. The species of *Candida* produce infections ranges from superficial mucosal infections, cutaneous to invasive and non–life-threatening mucocutaneous infections that may involve substantially any organ that caused by alteration of immune defences (Dabas, 2013).

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Recently, *Candida albicans* is the most common opportunistic invaders in humans and is associated with nearly (60-80) % of the nosocomial fungal infection), that can increase mortality and morbidity in hospitalized patients (Pappas et al., 2004; Kim and Sudbery, 2011).

There are many conditions in which the normal balance between the host and *Candida* is change and leads to pathologic situation: elders, diabetes, pregnancy, malignancy, steroid therapy, extensive administration of antibiotics, and AIDS. *Candida* infections constitutes the main widely recognized fungal infection in AIDS patients. (Hasan et al., (2009); Fidel, (2006). During last decades an increase of prevalence of non-albicans *Candida* (NAC) species have been noticed (Mokaddas et al.,2007, Srinivasan et al.,2006)

Together with the *C. albicans* there has been a greater concern of the importance of the non-

albicans *Candida* species (NAC) in human disease mainly *C. glabrata* and *C. krusei*. These species have received awareness because it promotes resistance to different antifungal agents (Rajeswari et al., 2018).

The traditional methods for identification of *Candida* isolates to species level has done using germ tube test, fermentation tests, sugar assimilations and chlamydospore formation. Recently newer methods include CHROM agar, Viteks 2 / ID system, API (Analytical Profile Index) system, and molecular techniques have been widely employed (Jain et al., 2012). Since Vitek 2ID system, API system, and molecular techniques are expensive and time consuming, use of CHROM Agar for species identification would be of benefit and useful for rapid and easy differentiation (Abdel et al.,2007).

The CHROMagar contain chromogenic substances that reacts with *Candida* secreted enzymes and produces different pigmentation of colonies. These Enzymes are species specific, allowing fungus to be identified to a species level by their colony characteristics and color (Mehta and Anupama, 2016).

The identification of non- albicans *Candida* species is very important. CHROM agar as a selective medium is used to identify a clinically *Candida* species. according to growth color and other characteristics (Lymn et al., 2003). It is useful to detect mixed cultures of *candida* in clinical samples.

The present study aimed to isolation and identification of *Candida* species in Duhok hospitals using germ tube test, chlamydospores formation and CHROMagar to asses antifungal susceptibility of some isolates against various antifungal.

2. MATERIALS AND METHODS

2.1 Samples Collection

From Azadi Teaching Hospital, outpatient and a number of private dermatophyte clinics dermatologists in Duhok city, 180 samples including hair, nail, skin scraping and skin swabs were collected also from patients with suspected candidiasis in these tissues and ages from 1 to 61 and above years, samples were obtained during February 5th to June 5th/2018 by using sterilized fine scissors, forceps, nail clipper, scalpel blades, and 70% ethanol.

Appropriate material including skin scrapings, hair or nail clippings were taken from the infected tissues, which were treated with 70% ethanol prior of sample collection. Out of the material collected, part of it was used for direct potassium hydroxide (KOH) examination and remaining part was used to inoculate SDA, Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) medium for culture and isolation causative fungi.

2.2 Direct Examination

Direct microscopic examination was carried out for samples (skin scraping and nails) were placed on a clean glass slide; then a drop of 10% KOH was added to them. A clean cover slip was placed on the sample and with gentle pressure to prevent the air bubbles formation. The slide was examined by using light microscope. This allows complete visualization of pseudohyphae as well as the budding oval yeasts cells, (Dismukes et al., 2003; Shamim et al., 2005; Singh and Beena, 2003) which corresponds to various *Candida* species.

2.3 Cultures Media

All the collected samples of hair, nail and skin were inoculated directly, on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Sabouraud Dextrose Agar (SDA) containing chloramphenicol 0.5 g/ml was added to prevent bacterial contamination. To prevent the growth of saprophytic fungi, cycloheximide (Actidione) 0.05 g/l was added to SDA (Campbell et al., 2013). The inoculated plates were incubated for 48hrs. The three media (SDA (CONDA, Spain)), PDA (LAB-M, UK) and MEA (LAB-M, UK)) were prepared as per instructions provided by manufacturers.

2.4 Germ Tube test (GTT)

This test is a rapid method for differentiation between *Candida dubliniensis* and *Candida albicans* by its efficiency to produce short, tube, delicate like structure known as germ tubes when it is incubated in serum of human blood at 37 ℃ for two hours. Germ tubes differs from pseudo-hyphae because it is elongations of daughter cells from the mother cell without shrinkages at the origins (Aryal, 2015; Deorukhkar and Saini, 2014).

2.5 Chlamydospores formation

An isolated colony from the primary culture medium was obtained. The plate of cornmeal agar was inoculated by making three parallel streaks about half inch apart at a 45 angle to the culture medium. Formation of large, highly retractile, thick walled, terminal spore was called chlamydospore. The test was used for the identification of *C. albicans* (Yadav et al., 2018).

2.6 CHROM agar *Candida* / culture media

All colonies of *Candida* isolates on SDA, MEA and PDA were sub cultured by streaking a loop full of culture on to CHROMagar *Candida* medium and incubate at 37 ℃ for 48 hours. This is a selective and differential medium which assist rapid isolation and presumptive identifications of many clinically important *Candida* species on the bases of colonies color and characteristics types.

As per manufacturer's instructions (ACUMEDIA –LAB, NEOGEN –UK). (Table 1). CHROMagar medium contain chromogenic substrate which react with the secreted enzymes by *Candida* species to yield colonies of different pigmentation, which allow to species identification as described by Odds and Bernarets,1994; Mathavi et al., 2016).

2.7 Antifungal susceptibility

Antifungal susceptibility test is a method used to detect antifungal resistance and it used to detect the preferable treatment for a specific fungus. In vitro susceptibility tests are available for patient care determination, for drug discovery and development or used in epidemiological studies. Clinical microbiology depends on these methods to choose the agent for a fungal infection, and also to recognize the global and the local epidemiology of antifungal resistance.

However, microdilution methods are the reference techniques or gold standard. Two organizations, the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antibiotic Susceptibility Test (EUCAST) have standardized methods to proceed antifungal susceptibility testing (Alastruey-Izquierdo et al., 2015).

2.8 Agar well diffusion method

Agar well diffusion method was used to estimate the activity of antimicrobial agent. Which similar to disk-diffusion method procedure, this test is performed using Casitone Agar. The inoculation of agar plate surface is performed by spreading 0.1 ml of the inoculum of microbial over the entire agar surface. Consequently, a hole with a diameter of 6 millimetres is punched aseptically by an antiseptic cork borer, and then a volume of 100 μL of the antimicrobial agent is subjected to the well. Furthermore, under suitable conditions agar plates are incubated, bases on the tested microorganism. The antimicrobial agent distributes in the medium and prevent the growth of the tested isolate (Balouiri et al., 2016).

3. RESULTS AND DISCUSSION

A total of 63 *Candida* isolates was obtained from diverse dermatological specimens including nail, skin particles and skin swabs. The majority of the isolates were from skin particles 33 (52.4%) followed by nail samples 15 (23.8%) and skin swabs 15 (23.8%) shown in table 2. All the samples were directly cultured on Sabouraud Dextrose Agar (SDA) containing antibacterial antibiotics chloramphenicol and cycloheximide the identification of the *Candida* species was performed depending on growth characteristics, chlamydospores formation and germ tube formation as well as CHROMO agar.

On Sabouraud Dextrose Agar (SDA) media, the colonies of *Candida* species were slimy, white to creamy, soft, round, and wrinkled to smooth, with a characteristic yeast (figure 1) grow rapidly during 2-3 days. Lactophenol cottons blue stain examinations of *Candida* isolates showed oval to spherical, with a presence of some budding (figure 2).

The germ tube test (GTT) formed within two hour of incubation and this is a differential *Candida* dubliniensis and *Candida albicans* (figure 3) .The results of this study revealed all *Candida albicans* were positive for germ tube test and in agreement with that found by Sheppard et al., (2008), they mentioned all *C. albicans* isolates were positive for germ tubes test when tested directly from the colony, while all non- species of albicans were negative to germ tube test when tested directly.

Distribution of samples of *Candida* isolates in Table 3. *Candida albicans* 33 (52.4%) was the most common isolated species. Among the non-albicans *Candida*, *C. krusei* 13 (20.6%), *C. tropicales* 9 (14.3%) and *C. glabarata* 8 (12.7%). As shown in the present study *Candida albicans* is predominant among all isolated species, same prominence was seen also by Srinivasan, (2006) however, higher incidence of non-albicans *Candida* had been recorded in many studies. *Candida krusei* was reported to be as the most identified species followed by *C. tropicalies*. The lowest incidence rate was *C. glabrata*. Similar results had been seen by Yucesoy and Marol, 2003.

Non-albicans *Candida* species are on the rises because of the increasing immunocompromised conditions, patient receiving prolonged antibacterial and offensive cancer chemotherapy, organ transplantation or undergoing invasive surgical procedure. (Mohandas and Ballal, 2011). Several studies have shown a considerable increase of the non-albicans *Candida* infections.

Different species of *Candida* previously known to causes an epidemic disease of animals in laboratory and in humans onychomycosis, has emerged as an opportunistic fungal pathogen capable of causing outbreaks of fungemia (Jaya and Harita, 2013).

Conventional speciation of *Candida* isolates was performed by germ tube test and chlamydospore formation which shown in Figure 4. These tests are laborious and time consuming. CHROM agar is a fast method to spectate the various *Candida* species. It facilitates the detection and identification of *Candida* species from mixed culture and provides results within 24- 48 hours.

All *Candida* isolates were positive for the germ tube test and they all showed distinguished growth on both 37°C and 45°C, while Non-albicans *Candida* are all negative for the previous test therefore, the CHROM agar was used to set up their identification.

This medium was originally developed for presumptive identification and selective isolation for many important clinically yeasts species for example *C. tropicales, C. glabrata, C. albicans* and *C. krusei* depending on the basis of differences in surface of colonies and color as shown in Figure 5 (Tornai-Lehoczki and Dlauchy, 2003).

The recent study confirmed the previous investigations concerning the high accuracy of CHROMagar medium. We found assumed identification of 100% of *C. albicans* isolates, *C. tropicalies, C. glabrata* and *C. krusei* isolates. None of the remaining species was misidentified as one of the four species. The results of this study confirmed those of Odds and Bernarets, (1994).

To differentiate between *Candida* with no requirement for germ tube confirmation test the most successful detector is CHROMagar-*Candida* test which has been reported by Odd et al., 1994.

The result of our study is in agreement with many previous studies. Mathavi and Priyadarsini, 2016 have reported that although the CHROMagar *Candida* is not only a simple, cost/ effective and reliable method for the identification of chlamydospore-negative atypical *C. albicanes*, but it is also used to differentiate different groups of chlamydospores negative *Candida* species.

3.1 Antifungal Susceptibility Test

Antifungal susceptibility testing was performed using four common antifungal drugs in the current study (Terbinafine, Itraconazole, Fluconazole, and griseofulvin) by using well diffusion method to determine minimum inhibition concentration (MIC) for each antifungal

Four pathogenic isolates of yeasts include *C. albicans, C. krusei, C. tropicalis, C. glabrata* were tested for their in-vitro sensitivity to selected antifungal drugs.

3.1.1 Terbinafine

The results in table 4, shows that *Candida krusei* (Figure 6 A) and *Candida tropicalis* had MIC at (25µg /ml) as well as *Candida albicans* and *Candida glabrata* had MIC at (50 µg /ml) and both had resistance at 25 µg /ml. The results were compatible with the study of Abdullah and Saadullah, (2017) in Duhok who found that *C. glabrata* had MIC of terbinafine at 100 µg /ml.

Terbinafine is the major agents of allylamines. It inhibits the synthesis of ergosterol, a key sterol component in the plasma membrane of the fungal cell. Terbinafine inhibits its squalene epoxidase, the enzyme which catalyses the conversion of squalene to squalene-2,3 epoxide, a precursor of lanosterol, which in turn is a direct

precursor of ergosterol. It can be found topical and oral (Dismukes et al., 2003).

3.1.2 Itraconazole

The results of itraconazole against *Candida* species were shown in table 4. That all isolates had MIC at (25 µg /ml) successfully (figure 6 B). Our findings are in agreement with sabaly, (2014) in Duhok which found MIC of itraconazole at different concentration.

Itraconazole is a member of Azole family, are active against a wide spectrum of pathogenic fungi, encompassing *Candida* species and dermatophytes.

Whole azole antifungal agents share same primary, common mechanism of action, which inhibits cytochrome P450-dependent enzyme lanosterol 14 alpha-demethylase. This is essential enzyme to convert lanosterol to ergosterol, avital component of the fungl membrane, which lead to cell membrane disruption by rising in permeability, that result in cell lysis and death (Wormser et al., 2010).

3.1.3 Fluconazole

So, *Candida albicans* (figure 6 C), *Candida tropicalis* and *Candida glabrata* had showed MIC at (25 µg /ml). on the other hand, *Candida krusei* had MIC at (75 µg /ml) concentration only that is shown in Table 4. However, the results were contradictory to a study by Ali et al., (2018) in Baghdad which found fluconazole is ineffective against *Candida* species.

The antifungal spectrum of fluconazole is less than that of other azoles. It has excellent activity against most *Candida* species (Schaechter et al., 2007). Fluconazole is used for candidiasis, urinary tract infection, onychomycosis and peritonitis (Sheppard and Lampiris, 1998).

3.1.4 Griseofulvin

The table 4 shows the results of antifungal griseofulvin against *Candida* species isolates, which reveal all resistance to griseofulvin at different concentration (Figure 6 D). Nowadays, the use of griseofulvin has largely been supplanted by terbinafine and itraconazole (Schaechter et al., 2007).

Griseofulvin is an oral agent used only for treatment of superficial dermatophyte infections. And is not active in vitro against *Candida* species. Griseofulvin's mechanism of action prevent cellular mitotic division by effecting on microtubules, but it is deposited in newly forming skin where it binds to keratin, protecting the skin from new infection. Because its action is to prevent infection of these new skin structures, griseofulvin must be administered for 2–6 weeks for skin and hair infections to allow the replacement of infected keratin by the resistant structures (Schaechter et al., 2007, Katzung et al., 2015, Dismukes et al., 2003).

4. CONCLUSIONS

The present study reveals that the most frequently isolated *candida* species from dermatologic specimens was *C. albicans* followed by nonalbicans *Candida* these are confirmed using CHROMagar *Candida* medium. It is important to increase the awareness of the public concerning the infection by *Candida* and this can be achieved carried out by predominantly designed educational program.

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Conflict of Interest (1)

Table (1) Color of different *Candida* species. on CHROM agar for identifications

Name	Colour on CHROMO agar
Candida albicans	Green
Candida tropicales	Blue
Candida krusei	Purple-Pink
***Candida* glabrata**	White-Purple

Table (2) Distribution of *Candida* isolates in different dermatologic samples

Samples	Number of *Candida* isolate
Skin	33
Nail	15
Skin swap	15
Hair	None
Total	63

Table (3) Distributions of *Candida* isolates in different dermatologic sample

Samples (source)	*C. albicanes*	*C. glabrata*	*C. tropicales*	*C. Kruseei*
Skin	19 (57.6%)	5 (62.5%)	1 (11.1%)	7 (53.8%)
Nail	5 (15.2%)	2 (25%)	5 (55.6%)	4 (30.8%)
Skin swap	9 (27.3%)	1 (12.5%)	3 (33.3%)	2 (15.4%)
Hair	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	33 (52.4%)	8 (12.7%)	9 (14.3%)	13 (20.6%)

Table (4) Total phenolic content of two medicinal bulb extracts in different solvents

Fungal isolates	MIC of terbinafine (µg /ml)	MIC of Itraconazole (µg /ml)	MIC of Fluconazole (µg /ml)	MIC of Griseofulvin (µg /ml)
C. krusei	25	25	75	-
C. albicans	50	25	25	-
C. glabrata	50	25	25	-
C. tropicalis	25	25	25	-

Figure 1: Creamed colored colonies on SDA.

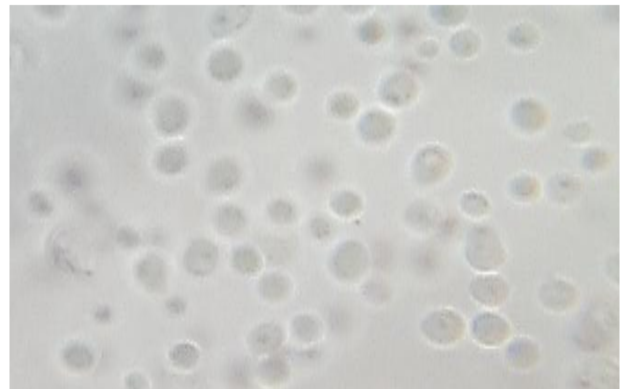

Figure 2: *Candida* Under Microscope

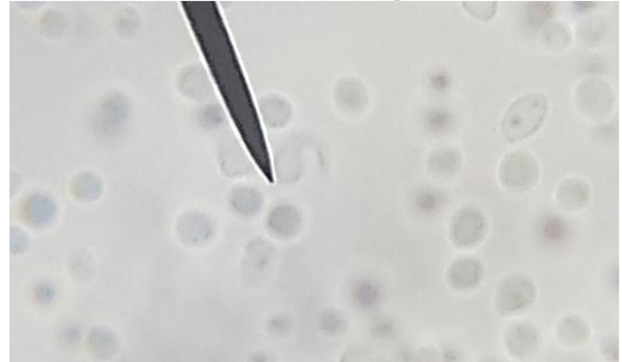

Figure 3: Germ tube test for *Candida*

Figure 4: Chlamydospore formation under 40X light mictoscopy

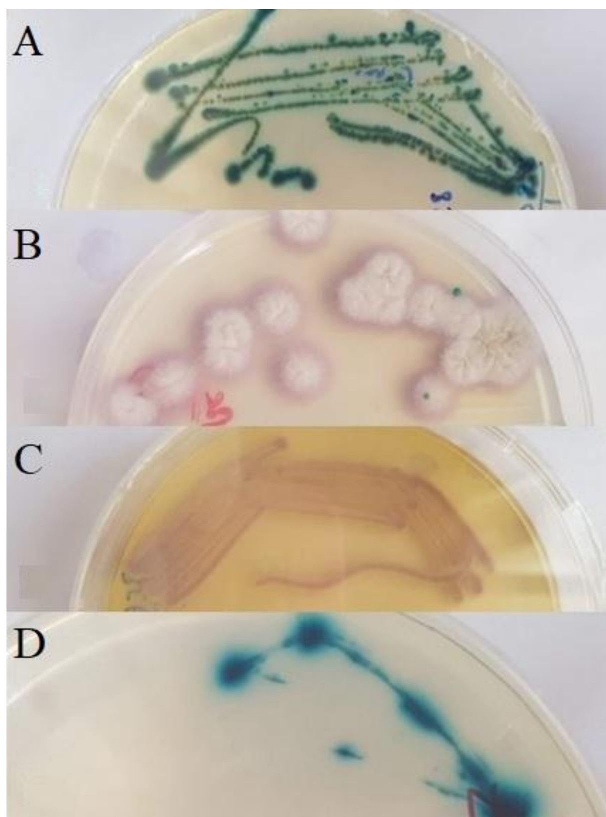

Figure 5: (A) C. albicans on CHROM agar (B) C. glabrata on CHROMagar (C) C. krusie on CHROMagar (D) C. tropicalis on CHROM agar

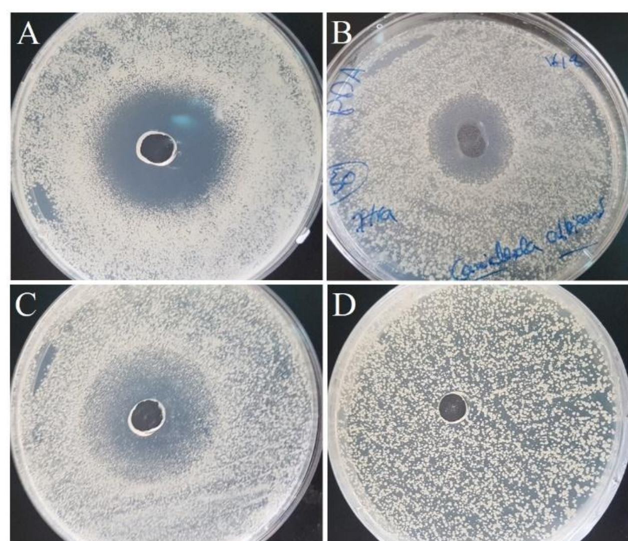

Figure 6: Susceptibility test results of (A) *Candida* krusei to antifungal Terbinafine at 100 µg /ml (B) *Candida* albican to antifungal Itraconazole at 50 µg /ml (C) *Candida* albicans to antifungal Fluconazole at 75 µg /ml (D) *Candida* krusei to antifungal Griseofulvin at 25 µg /ml

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