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Antifungal Susceptibility Profile Of Clinical Isolates Of Candida Species And Their Resistant To Azole Compounds

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ABSTRACT

Background: *Candida infections are a major health problem where the most important predisposing factors for the infections are the use of antibiotics, indwelling catheters, immunosuppression, chemotherapy and radiotherapy.*

Objective:

To investigate antifungal susceptibility profile of Candida isolates toward some antifungal drugs.

Methods:

Different clinical specimens from patients suffering from candidiasis were collected. Isolation and identification of Candida spp. were done according to the laboratory standard methods. The antifungal susceptibility test for C. albicans isolate using discs diffusion method and determining the minimal inhibition concentration (MICs) were conducted to determine the values of drug resistance and detection of some genes of resistant using specific primers and PCR method.

Results:

The isolation and identification results showed that causative agents of candidiasis were C. albicans C. krusei C. Tropicalis C. glabrata The results of disc diffusion methods showed that the amphotericin B is the most effective drug followed by ketoconazole, fluconazole then nystatin ,while the results of MICs showed that amphotericin B inhibited (97%),followed by ketoconazole (50%), fluconazole (31%) and nystatin (26%). The PCR assay showed the amplification of two genes related with antifungal resistant when amplify with DNA extracted from Candida isolates.

Conclusion: *The study proved the emergence of drug resistant phenomena against azole agents in Candida spp.*

KEYWORDS: *Candida spp; Antifungals susceptibility; Disk diffusion; MICs.*

INTRODUCTION

Candida species is a member of the normal human microbiome, usually being commensal and harmless, but under certain circumstances, it becomes an opportunistic pathogen and produces candidiasis if the defense mechanisms of the host are damaged [1]. Antifungal therapy is a critical component of the patient management for acute and chronic diseases [2]. Resistance has been defined as persistence or progression of an infection despite appropriate antimicrobial therapy [3].Both primary and secondary resistances to antifungal agents have been observed [4]. As described previousely, clinical isolates that are resistant to one azole are frequently cross-resistant to other azole drugs and can be cross-resistant to polyenes as well. Similarly, there has been insufficient analysis to determine if alterations in other enzymes in the ergosterol pathway will result in cross-resistance [2].

On the other hand, the relative safety of azoles agents encourages the wide use of these agents that may play a role in the appearance of resistance strain toward these agents [5]. The study aimed at investigating the antifungal susceptibility profile of *Candida* isolates toward selected antifungal agents and detection of cross resistance of these isolates toward azole agent.

MATERIALS AND METHODS

A total of 100 clinical specimens (50 oral swabs from oral thrush; 50 vaginal swabs from vaginal candidiasis) were obtained from patients included males and females of different age groups whom attended Hospitals during the period from march 2021to September 2021, and clinically diagnosed by specialist physician. Specimens were taken using sterile swabs, and then transported to the laboratory for diagnosis. The control group includes 50 apparently healthy subjects with no symptoms of candidiasis were enrolled in this study. All swabs were subjected to culture on (SDA) for detection of Candida species. A Portion of colonies removed from the culture and placed on a slide with lacto phenol cotton blue or Gram stain, teased apart and covered with a cover slip and tested under the light microscope [6]. Germ Tube Production ;Dalmau Plate Technique ;Resistance to Cycloheximide (Actidione) Test ;Urease Test ;Growth ;Sugar Fermentation Test; Sugar Assimilation and Tobacco Test were tested for biochemical and physiological confirmation of Candida isolates [7].

ANTIFUNGAL SUSCEPTIBILITY TEST

Twenty isolates belong to the species of *Candida i*solates were tested for antifungal susceptibility. Tube containing 5 ml of sabouraud's dextrose broth was inoculated with two loopfuls of a yeast colony to be tested from (1-2 days SDA) old cultures. Tubes were incubated overnight at 35 C°, then diluted by 1:100 with sterilized distilled water then shacked vigorously. The suspension was adjusted into 10^5 cell/ml compared with MacFarland standard solution (0.5) [8]. The quality control for antifungal susceptibility test testing was performed by using *Candida albicans* ATCC 10231as reference strain. Emmons modification sabouraud's agar (ESDA) (2% dextrose) was used for *in vitro* susceptibility test. The pH of the medium was adjusted to 6.8-7.0. Amphotericin B, nystatin, fluconazole and ketoconazole were prepared in an initial concentration of 10000µg/ml by dissolving 50mg of the agent in 5ml of dimethyl sulphoxide (DMSO) in a clean sterile screw-capped glass vials. The stock solution was kept at -20C°for further use [9]. Batches of small discs number 100 of absorbent paper (6.5 mm diameter) were dispensed in screw-capped vials, sterilized by autoclaving, and left in the electric oven for 30 minutes at 40 °C to be dried. Final concentration of the (1000 µg/ml) was prepared from the original stock solutions of each antifungal. One ml of final concentration was added to each vial containing 100 discs to obtain a concentration of 10 µg /disc. These discs were stored in wet condition in screw-capped vials tightly screwed and kept at -20 °C until used [10]. Disc Diffusion Method was carried out according to McGinnis [9]. The antifungal discs (10µg /disc) of Amphotericin B ;Nystatin; Fluconazole and Ketoconazole were placed on the surface of the medium and left in the refrigerator for 1-3 hours pre diffusion. At the same time, growth controls without antifungal discs of all strains on ESDA were prepared. The inoculated plates incubated at 30°C and the assay was recorded after 24-48 hours of incubation. The inhibition zones of were expressed as diameter of the clear zones around the antifungal discs measured in millimeters. Duplicate plates were used. The diameter of inhibition zone for individual antifungal agent were compared with standard values of CLSIs, 2021. Three ml of (SDB) was added to each of two vials containing 27ml of (ESDA) maintained at 50-52°C in water bath the vials were mixed well, a liquid of 3ml volume was poured in to separate sterilized Petri dish and then allowed to harden at room temperature [9].

DETERMINATION OF MINIMAL INHIBITORY CONCENTRATIONS (MICS)

The minimal inhibition concentrations (MICs) of the test agents toward 20 *Candida* isolates were established

using agar dilution method [11]. An inoculum of 0.05 ml was poured on the surface of each Petri dish prepared previously. The inoculated plates were left undisturbed to permit the inocula to be absorbed in to the medium surface.The inoculated plates were incubated at 37 C° until the macroscopic growth appeared on the control plates . The MIC polyene were recorded as the lowest consternation of drug showing no growth while the MIC for azoles drug was recorded as the lowest consternation that reduced (80%) of Candida growth .Standard values of MICs of antifungal used in this study based on CLSIs,2021 are used for confirm the results.

DETECTION OF RESISTANT GENES FOR AZOLE COMPOUNDS

Two set of oligonucleotide primers were used to detect the genes encode to azoles resistant by *Candida* spp Table 1. The bioCorp DNA primers were prepared depending on manufacturer's instructions by dissolving the lyophilized primers with TE buffer to form stock solution with concentration of 100 pmol/µl, after spinning down and stay over night at 4C°, primers working solution were prepared by diluted the stock solution with TE buffer, using the equation C1V1=C2V2 to get final working solution 20 pmol/µl for all primers.

Table 1 . Oligonucleotide Primers For Resistant Azole Compounds

Primer	Sequence (5'-3')		Product Size
Sterol esterase gene	F	AGCGGTTCTCATGAATCAGC	415bp
	R	CAGAAGAAACAGCAGGTGATGG	
Proteinase SAP1 gene	F	GTTGGTTTTGGTGGTGCTTC	224bp
	R	TTGTTCACGTTGAGCCATGG	

PCR is performed as follows: the reaction solution consisted of 22.5 µl of each tube PCR consists of: 14.3 µl of PCR grade water, 5.0 µl of 5x kapa2G buffer B with MgCl2, 0.50 µl of 0.2mM dNTP, 1.25 µl of 0.5µM of each primer and 0.2 µl of kappa 2G robust hot start(5 unit/µl), then added 2.5 µl of DNA sample .Amplifications are carried out in a TC-3000 Thermal Cycler (Techin, USA). Reactions are as follows: initial denaturation at 94°C for 5 minute, followed by 35 cycles of denaturation at 94°C for 30 second, annealing at 55°C for 1 min and extension at 72°C for 30 second, with a final extension at 72°C for 4 min. Amplification products are separated by electrophoresis on 2% agarose gel containing 5 µg/mL ethidium bromide using a 100-bp ladder (KapaTM Express Ladder) as molecular weight marker. The tube contains all reaction components except DNA template as negative control [12].

Statistical Analysis: The data were collected, summarized, analyzed and presented using statistical package for social sciences (SPSS) version 25 and Microsoft Office Excel 2010.

RESULTS AND DISCSSION

The results of isolation and identification revealed that the most prevalent *Candida* species were *C. albicans* ;*C. glabrata* ;*C. tropicalis* ;*C. krusei* ;.The *Candida* isolates showed that their colonies appeared on SDA as white to cream, glossy, smooth, soft and circular colony. These isolates appeared as yeast cells appeared as budding cells oval to spherical or globose to ovoid with pseudo hyphae. To more biochemical and physiological confirmation, the identification of the isolates was done in Table 2 because it gives results that are more accurate in the diagnosis of the *Candida* spp.

Table 2. Biochemical And Physiological Tests Used For Candida Spp.Diagnosis

Yeast species	glucose		maltose		sucrose		lactose		galactose		trehalose		Other tests					
	fermentai	Assimilati	fermentai	Assimilati	fermentai	Assimilati	fermentai	Assimilati	fermentai	Assimilati	fermentai	Assimilati	Germtube	Cyclohe-Ximide	Surface growth	Chlamydospores	Growth In 45°C	Toba-Cco test
C.albicans	+	+	+	+	-	+	-	-	+	+	+	+	+	+	_	+	+	-
C.krusei	+	+	-	-	-	-	-	-	-	-	-	-	_	_	+	_	_	-
C.tropicalis	+	+	+	+	+	+	-	-	+	+	+	+	_	+	+	_	_	-
C.glabrata	+	+	-	-	-	-	-	-	-	-	+	+	_	_	_	_	_	-

(+): positive result ;(−): negative result

Antifungal Susceptibility Test

The results revealed that the amphotericin B is the most effective agent where the growth inhibition percent were 97 % of *Candida* isolates, followed by ketoconazole 90%, fluconazole 25% , then nystatin 5% .The amphotericin B considered the most effective antifungal agents because it is one of the polyene antifungal agents that the mechanism of action based on specific bind to ergosterol present with the fungal cell wall membrane. This process disrupts cell wall permeability by forming pores with the subsequent efflux of potassium and intracellular molecules causing fungal death [13], but Unfortunately, infusion-related toxicities, the frequent association of renal dysfunction, and the intravenous formulation of amphotericin B have limited the utility of this agent. On the other hand, the relative safety of azoles agents encourages the wide use of these agents that may play a role in the appearance of resistance strain toward these agents [5]. The results compared with standard value of diameter of inhibition zone for Candida reference strain (ATCC 10231) in Table3.

Table 3.The No. and percent of resistant and susceptible isolates toward some antifungal agents according to the diameter of inhibition zone

Antifungal agents	Values of growth inhibition zone (mm)	Resistance		Susceptible	
		No.	%	No.	%
Amphotericin B	12-28	2	5	18	95
Nystatin	0-15	19	75	1	25
Ketoconazole	6-22	2	10	18	90
Fluconazole	0-20	0	0	20	100

Minimal Inhibition Concentrations (MICs)

The results revealed that 97.3% of the tested isolates were susceptible to amphotericin B, with MICs ≤ 1µg/ml with the exception of a single isolate which was resistant with MICs value at 2µg/ml. The range of MICs to amphotericin B was (0.03-2 µg/ml). Regarding the results of susceptibility of *Candida* isolates towards antifungals, all the tested isolates were susceptible to amphotericin B , with MIC_s ≤ 1µg/ml with the exception of a single isolate of *C. krusei* which was less susceptible than any other species with MIC_s of 2 µg/ml and MIC_{50} of 2. The MIC_s to amphotericin B was from 0.125 to 2 µg/ml. In general, *Candida* sp. showed higher MIC_s than *C. albicans* isolates. The MIC_s to fluconazole was from 0.25 to 64 µg/ml. All isolates were highly sensitive to fluconazole with the exception of *C .krusei* which showed a high MIC (32) and MIC_{50} and MIC_{90} of 64 and 32 µg/ml respectively. The MIC_s to itraconazole was from 0.015 to 1.0 µg/ml. Most *C. albicans* isolates were sensitive to itraconazole. *Candida* sp. showed relatively lower susceptibility.

This result is in agreement with many studies, which reported that resistance of *C .albicans* to amphotericin B is considered uncommon [12]. However, it differ from other parts of the world where an increasing number of isolates are reported to be amphotericin B resistant [14]. Resistance to amphotericin B may be due to the accumulation of sterol intermediates in the resistant strain, which would account for the decreased affinity of amphotericin B for membrane sterols and a decreased requirement for lanosterol demethylase activity in membrane sterol production [15]. While 73.6% of the isolates was highly resistant to nystatin with MICs >16 µg/ml, while (26.3%) of the isolates were susceptible to nystatin with MICs ≤ 16 µg/ml. In contrast, Carrillo-Munoz [16] found that the MICs value for nystatin to 55 *C. albicans* clinical isolates as 2µg/ml. In addition, Blgnaut *et al.* [17] observed that the nystatin MICs for 589 oral yeast isolates from South African human immunodeficiency virus patients and healthy individuals ranged from 2 to 16 µg/ml. In our result, nystatin exhibits a very low activity. The range of its action was (8-128µg/ml) and that may be due to the wider use of nystatin in the recent past in Iraqi; this may have contributed to the increased lack of susceptibility to that antifungal.

The range of MICs to fluconazole was from (8-64 µg/ml), 12 (31.5%) of isolates were susceptible to fluconazole at the (MICs ≤64 µg/ml), while 1(2.6%) were resistant to fluconazole at the (MICs >64 µg/ml). This result was also conducted by Uzun *et al.*[18]. who observed that there was only one case of fluconazole resistance (MICs≥64µg/ml) in *C. albicans* isolated from patient with vaginal candidiasis. Also, Dorrell and Edwards [19] found that there was fluconazole resistance among *C. albicans* isolate from vulvovaginal patients in United Kingdom. In addition, El- Din *et al.* [20] revealed that no fluconazole resistance has been identified among 75 *C. albicans* isolates. The interpretation of fluconazole susceptibility test is often complicated by occurrence of trialing growth.

Approximately the 50% of the tested isolates were susceptible to ketoconazole with MICs ≤ 2 µg\ml, while 50% of the isolates was resistance to ketoconazole with MICs≤ 2µg/ml. The rang of MICs to ketoconazole was (0.5-4 µg/ml). The study of Clayton [21] revealed that ketoconazole was less effective than amphotericin B and clotrimazole, where 74% of the tested yeast isolates were sensitive to this antifungal. The cause of azoles resistance may be due to several mechanisms including the reduction in the import of the agent into the cell, modification or degradation of the agent once it is inside the cell, changes in the interaction of the agent with the target enzyme (binding, activity), changes in other enzymes in the same enzymatic pathway and an increased efflux of the agent from the cell [22]. The result compared with standard value of MICs for reference strain (*Candida albicans* ATCC 10231) in Table 4.

Table 4 . Values of MIC (µg/ml) antifungal susceptibility test toward Candida spp

Yeast isolates	Fluconazole		Itraconazole		Amphotericin B	
	Range (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)	MIC_{90} (µg/ml)
Candida albicans	0.03-16	2.0	0.008-0.5	0.125	0.03-1	0.25
Candida tropicalis	0.25-8	2.0	0.03-0.5	0.5	0.03-1	0.5
Candida glabrata	0.125-256	128	0.008-16	16	0.008-1	0.5
Candida krusei	32-128	64	0.25-1	0.5	0.125-1	1.0

Polymerase Chain Reaction(PCR)

The amplification of target genes that encoded to resistant the azole compounds showed the detection of emergence of sterol esterase and proteinase SAP1 gene genes which related with Candida resistant to antifungal drugs in Figure 1 and 2.

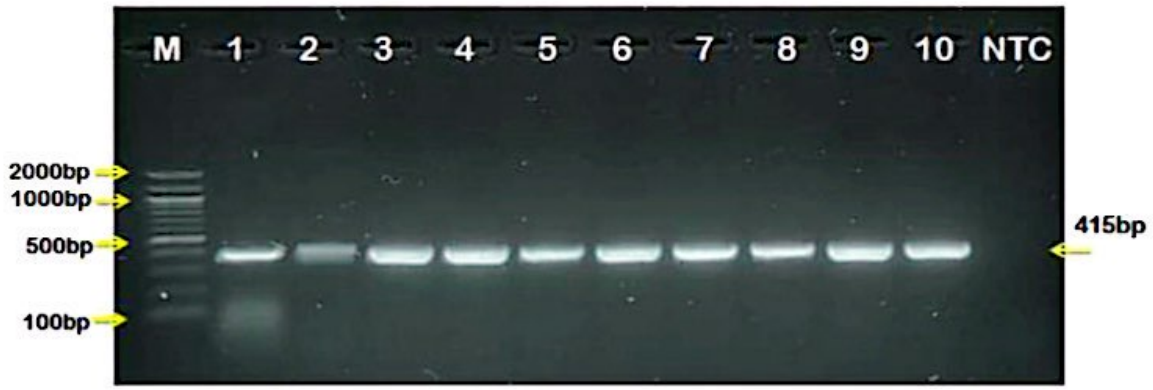

Figure 1. Agarose gel electrophoresis image that show the PCR product analysis of pathogenic Candida isolates ,Where Marker ladder (2000-100bp), lane (1-10): Positive sterol esterase gene at 415bp. Lane (NTC): Non template negative control

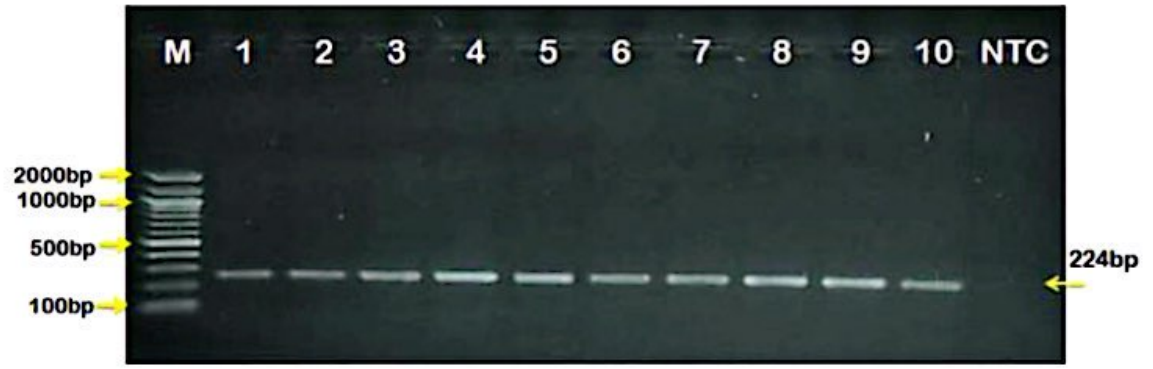

Figure 2. Agarose gel electrophoresis image that show the PCR product analysis of pathogenic

Candida albicans. Where Marker ladder (2000-100bp), lane (1-10):Positive proteinase SAP1 gene at 224bp. Lane (NTC): Non template negative control.

This emergence may be attributed to its related with another conditions that originated genetically to resist antifungal agents [22].These changes are related to some genes that encoded to proteins and enzymes regarding to their resistance to antifungals and how diminish it such as efflux pump systems which then determine the ability of microorganisms to grow again or not. In addition to ability the fungus to continuous biosynthesis of cell membrane through the over-expression or high regulation of these genes in the presence or absence of antifungals [23,24].

CONCLUSION

The present data emphasized the emergence of drug resistant phenomena against azole agents in *Candida* spp and these resistance are correlated with genetic origin of two genes encode to diminish or exclude the antifungal agents by having the efflux pump system.

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