Detection of Causative Agents, Antifungal Susceptibility Profile and Cross-Resistance of *Candida albicans* isolated from oral and Vaginal Candidiasis.

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الخلاصة

صممت هذه الدراسة للكشف عن المسببات المرضية و الحساسية الدوائية للمضادات الفطرية والمقاومة العرضية لخميرة المبيضات البيضاء لأربعة من المضادات الفطرية (الامفوترسين ب والنستاتين و الفلوكونازول و الكيتوكونازول) تم دراسة 208 حالة مرضية (108 نساء و 100 رضيع) شخصت كحالة مرضية من قبل الأطباء الاختصاص يعانون من الإصابة بداء المبيضات البيضية الفموي والمهبلي للمراجعين إلى مستشفى الصويرة العام .

تضمنت الدراسة مجموعتين الأولى نساء يعانين من إفرازات مهبلية غير طبيعية وحكة والثاني رضع يعانون من طفح فموي بالإضافة لأصابتهم بأمراض أخرى مثل (التهاب القناة التنفسية العليا و تسمم الدم و الحمى السوداء و التهاب المعدة ولأمعاء و ذات الرئة) أوضحت الدراسة أن نسبة الإصابة لدى النساء كانت (35.1%) بينما كانت نسبة الإصابة لدى الرضع (70%) و نسبة عزل *C. albicans* في كلا المجموعتين كانت (76.8%) و *C. krusei* (1.85%) و *C. Tropicalis* (5.5%) و *C. glabrata* (11.1%) و *C. dublinances* (0.9%) و *Trichosporon* (1.85%) و *Cryptococcus neoformans* (1.85%) . تم قياس الفعالية الدوائية لبعض المضادات الفطرية على عزلات *C. albicans* باستخدام طريقة التركيز المثبط الادنى.

اوضحت نتائج ال MICs ان مدى فعالية المضادات كانت الامفوترسين ب (0.03-2µg/ml) و الكيتوكونازول (0.5-4µg/ml) و الفلوكونازول (8-64µg/ml) و النستاتين (8-128µg/ml). بالإضافة إلى ذلك تم التحقق من وجود المقاومة العرضية بين المضادات الفطرية من خلال تطفيرها وأظهرت الدراسة أن هناك مقاومة عرضية بين مضادي النستاتين و الامفوترسين ب بحيث زادت قيم MICs للامفوترسين من (0.5-1µg/ml) الى (4-16µg/ml) عند تطفير العزلات لمضاد النستاتين و كذلك ظهرت مقاومة عرضية بين مضادي الفلوكونازول والكيتوكونازول حيث زادت قيمت MICs للكيتوكونازول من (0.5-2µg/ml) الى (1-8µg/ml) عند تطفير العزلات لمضاد الفلوكونازول ولم تظهر مقاومة عرضية بين المضادات البولين والازول وان قيم ال MICs للسلالات المطفرة عادت للقيم الأصلية ما عدا السلالات المطفرة للنستاتين حيث قلت القيم مـــــــن (600-1200µg/ml) الى (200-600µg/ml) ولكنها لم تعد للقيم الأصلية (64-128µg/ml).

Abstract

This study was designed to investigate antifungal susceptibility and cross resistance of *C. albicans* isolates toward four antifungal drugs; Amphotericin B, nystatin, fluconazole and ketoconazole.

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The study groups included 208 patients(108 women and 100 children) who attented the Al-Suwayra Hospital/ Kut Province during the period from December 2008 to April 2009. Patients groups were represented by women who were complaining of vaginal discharge and itching and children who were complaining of oral thrush and infected with different type of disease include upper and lower respiratory tract infection, gastroenteritis, kala-azar and septicemia. The study showed that the infection percentage of vaginal candidiasis was 35.1% while oral thrush was 70%. The yeast isolates in two groups were *C. albicans* (76.8%), *C. krusei* (1.85%), *C. tropicalis* (5.5%), *C. glabrata* (11.1%), *C. dublinances* (0.9%), *Trichosporon* (1.85%) and *Cryptococcus neoformans* (1.85%). The activity of certain antifungal agents were tested against *C. albicans* isolates by using minimal inhibitory concentration (MICs). The results of range of MICs for amphotericin B was (8-128µg/ml), ketoconazole (0.5-4µg/ml), fluconazole (8-64µg/ml), Nystatin (0.03-2µg/ml). The MICs of amphotericin B to the isolate that mutant to Nystatin tended to rise in parallel from (0.5-1µg/ml) to (4-16µg/ml). There was no correlation between MICs of these strains with fluconazole and ketoconazole. In addition, The MICs of ketoconazole to the isolates that mutant to fluconazole tended to rise in parallel from (0.5-2µg/ml) to (1-8µg/ml). There was no correlation between MICs of these strains with Nystatin and amphotericin B MICs. All the MICs values of the mutant strain return to the original values after remove the drug effect except the Nystatin . The MICs value for the isolate mutant to Nystatin dropped from the range (600-1200µg/ml) to (200-800µg/ml) but not returned to the original range (64-128µg/ml).

Introduction

***Candida albicans* is a dimorphic fungus that exists as a commensal of humans. It colonizes mucosal surfaces of the oral and vaginal cavities and the digestive tract and is able to cause a variety of infection, depending on the nature of the underlying host defect[1]. Candidiasis results from infection by *C. albicans*, that can infect the skin and mucosa of the mouth, intestine and genital tract. Young infants, pregnant women, diabetics, people with prosthetic heart valves, patients on broad-spectrum antibiotics and people immunocompromised by drugs or disease are especially susceptible to Candidiasis infections[2].**

The past decade has witnessed a significant increase in the prevalence of resistance to antibacterial and antifungal agents. Resistance to these agents has important implications for morbidity, mortality and health care cost in the community[3]. The increase of predisposing factors for infection with *Candida* spp., especially in the

immunocompromised patient and appearance of mutant strains capable to resist the antifungal therapy in addition to the little attention to the clinical significance of antifungal cross – resistance.

The aim of present work was to Conducting the antifungal susceptibility for *C. albicans* isolate using discs diffusion method and determining the minimal inhibition concentration (MICs), Induction of resistance to nystatin and fluconazole and detection of cross – resistance between antifungal agents, in vitro and determining the MICs values of mutant strains than wild strains (un- mutants).

Materials and Methods

Samples collection

Oral and vaginal swabs were collected from 108 women and 100 infants in Al-Suwayra city during the period from December 2008 to April 2009 by using sterile swabs, and then transported to the laboratory for diagnosis.

Isolation and identifecation

The swabs inoculated on to sabouraud dextrose agar (SDA) plates supplemented with chloramphenicol (0.05g/L),then inoculated at 37˚C for 2-7 days. Yeast isolate were identified by using the commercial identification system api candida in addition to usually identification methods including morphological, physiological and biochemical characteristics[4,5,6,7,].

Determination of minimal inhibition concentration (MICs)

The minimal inhibition concentrations (MICs) of the test agents were established using agar dilution method, described by[8] and modified by[9].

Development of Cross –Resistance

Cross- resistance was assumed for a single isolate when an elevated MIC to a given antifungal agent was corresponding to an elevated MIC against other antifungal agents[10].

Strategy for induction of resistance

This method was prepared according to Hebeka and Solotorovsky (1962) with some modification as the follow. A set of tube containing (5 ml) of sabouraud's dextrose broth was prepared in addition to a gradually increasing amount of the antibiotic prepared in step 4. These tubes were inoculated with 0.1 ml volume of cell suspension and then incubated at 37˚C. 0.1 ml volume wes made from the highest

concentration of antibiotic permitting substantial growth into another series of agent dilutions until the highest concentration of the antibiotic was obtained. That did not show any visible growth indicating that the isolate could not be mutant to this agent anymore after that MICs was preformed to the tested isolates.

Stability of Resistance

To assess the stability of resistance organism's serially subculture in SDA medium free from antifungal for 10 times were preformed to the tested isolates. Then the MICs was preformed to these isolates to determine whether the MICs value back to the original value (loss the resistance) or remain high or drop but not back to the original value (retain the resistance)[11].

Results and discussion

Isolation and Identification

Morphological examination of the suspected isolates showed that their colonies appeared on SDA as white to cream, glossy, smooth, soft and circular colony. Such characteristics come in accordance with those belonged to yeast . Yeast cells appeared as budding cells oval to spherical or globose to ovoid like *Candida* spp. with hyphae or pseudohyphae . The isolates were subjected to some biochemical and physiological tests shown in(table 1) which includes germ tube formation that is a characteristic feature of *C. albicans* and *C. dublinances* isolates, chlamydospore , surface growth, the ability to growth in 45˚C, the ability to growth in medium containing cyclohexamid, sugar fermentation test, sugar assimilation test, tobacco test which differentiates between *C. albicans* and *C. dublinances*, where the diagnosis depended on the morphology appearance on this culture. To more confirmation, the identification of the isolates was done by the use of API *Candida* kit because it gives results that are more exact in the diagnosis of the species.

The result of isolation and identification revealed that the most prevalent yeast isolates from vaginal and oral samples were *C. albicans* 83/108(76.8%), *C. glabrata* 12\108 (11.1%), *C. tropicalis* 6/108(5.5%), *C. krusei*, *trichosporon*, *Cryptococcus neoformans* (1.85%) for each of them, *C. dublinances* 1/108(0.9) (table 2).

Minimal Inhibition Concentrations (MICs)

Table 3. show the number and percent of resistance and susceptible isolates of C. albicans according to MICs values . 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).

This result is in agreement with many studies, which reported that resistance of *C.albicance* to amphotericin B is considered uncommon[12]. However, we differ from other parts of the world where an increasing number of isolates are reported to be amphotericin B resistant[13]. 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[14]. Complicating these issues is the fact that in vitro susceptibility testing of amphotericin B is technically difficult. The NCCLs methodology fails to detect amphotericin B resistance isolates[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. The range of MICs to nystatin was from (8-128 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 might 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 cause of resistance to polyene antibiotics may be due to decrease in the depress of its disordered selective permeability[18], in addition to the decreased in the level of ergosterol content in the resistant strain compared to those of sensitive one and the effect of that on the affinity of the agent to the cell[19].

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) (Table 3). This result was also conducted by Sober and Vazquez[20] 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 [21] found that there was fluconazole resistance among *C. albicans* isolate from vulvovaginal patients in United Kingdom. In contrast Lynch and Beighton[22] found that there was no fluconazole resistance among 100 *C. albicans* isolates from vulvovaginal patients. In addition, El- Din *et al.*[23] 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. This phenomenon will influence the outcome of the test depending on whether the incubation period is 24 or 48hours[24]. In our study, we consider the incubation time for 24hours. Many investigators have suggested that determination of MICs after 24hours of incubation results in better match with in vitro response and avoid the incorrect high MICs readings[25].

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) (Table 3). The study of Clayton and Jennifer[26] 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[27].

Cross-Resistance

The result of development of resistance to nystatin and fluconazole is presented in table (4) and (5). Isolates (4, 5, 36, 56, and 84) developed a progressive resistance to nystatin. All nystatin resistance strains were studied for development of resistance to amphotericin B, fluconazole, ketoconazole. The tested strain developed a resistance to amphotericin B. and the range of MICs rise from (0.5-1µg/ml) to (4-16µg/ml), but did not develop resistance to fluconazole and ketoconazole. All the resistant strain was serially subculture for 10 times in SDB in absence of nystatin. The level of resistance was again checked, and the level of MICs dropped but did not back to the original value for nystatin, while the value of MICs for other antifungal back to the original value. This result was also conducted by Ather and Winner [28]who succeeded in training less than half the strains tested for their ability to resist polyene antifungal and Sorenson *et al.*[29], Hamilton[30], Muller *et al.*[31] observed that there was a cross-resistance among polyene antifungal in addition Sevtap *et al.* [32] observed that there was correlation between the MICs of nystatin and the MICs of amphotericin B, which tended to rise in parallel, while there was no correlation between fluconazole and nystatin MICs. The cause of the cross-resistance between nystatin and amphotericin B may be due to structural similarity of both of them, in addition to their effect with the alteration in the membrane structure or in the sterol to phospholipids

ratio in the membrane and that may be associated with resistance to polyene[33].

Strains (24, 28, 42, 42, and 63) developed resistance to fluconazole. All fluconazole resistance strains showed a rise in the level of MICs value for ketoconazole and the range of MICs rise from (0.5-4µg/ml) to (1-8µg/ml), while the value of MICs for nystatin and amphotericin B did not show any increase in the value of MICs. The level of resistance then checked after serial subculture for 10 times in SDB in the absence of fluconazole. The value of MICs for fluconazole and ketoconazole back to the original value. These results were conducted by Ruhnke *et al.*[34], Erja Chryssanthou *et al.*[35], Frank *et al.* (2000) who found that the cross-resistance in vitro occurred between fluconazole and other azoles. These observations may establish that resistance of *C. albicans* to one azoles derivative implies cross-resistance to other azoles antifungal agents. All azoles irrespective of their distinctive chemical structure and variable biological properties interact and inhibit lanosterol 14α-demethylase needed for transforming lanosterol into ergostarol in the cell membrane of the yeast[37]. Therefore, any changes in this process alter the cell from susceptibility to resistance situation. Current studies reveal that several change occur to the cell that acquired resistance as a result to presence of agents. These changes include increased energy-dependent efflux activity of membrane transporters and that ergostarol content decrease with the accumulation of sterol intermediates in the resistant strain as compared with the susceptible strain. Furthermore, a single amino acid difference in *ERG*3 that led to the inactivation of Erg3 could account for both sterol precursor accumulation and the changes in the expression of ergostarol biosynthesis genes in this resistant strain[38].

The strains mutant to nystatin do not get back to the original value of MICs after removal of the pressure of the agent, while the strains mutant to fluconazole get back to the original value of MICs. This variation may be due to the role of agent in the induction of resistance. It is hypothesized that the agent itself does not cause resistance but, rather, selects for growth more resistance cells in the population[39] or the presence of agent induce transient gene expression that renders the cell temporarily resistant. This phenomenon is called epigenetic resistance. That (a cell can alter its phenotype) happens probably through transient gene expression to become resistant in the presence of the agents, but the resistant phenotype can revert quickly to a susceptible phenotype once the agent pressure is eliminated[40,41].

Table (1)Differential characteristics of yeast species isolated from vaginal and oral candidiasis.

Yeast species	Fermentation						Other tests					
	Assimilation						Germ tube	Cyclohe-Ximide resistance	Surface growth	Chlamyd ospores	Growth In 45˚C	Toba-cco test
	glucose	maltose	sucrose	lactose	galactose	trehalose						
C.albicans	+ +	+ +	_ +	_ _	+ +	+ +	+	+	_	+	+	_
C.krusei	+ +	_ _	_ _	_ _	_ _	_ _	_	_	+	_	_	+
C.tropicalis	+ +	+ +	+ +	_ _	+ +	+ +	_	+	+	_	_	_
C.glabrata	+ +	-- _	_ _	_ _	_ _	+ +	_	_	_	_	_	_
C.dublinances	+ +	+ +	_ +	_ _	+ +	+ +	+	+	_	+	+	+
Trichosporon spp.	_ +	_ +	_ +	_ +	_ +	_ +	_	_	+	_	_	+
Creptococcus neoformans	_ +	_ +	_ +	_ _	_ +	_ +	_	_	+	_	_	_

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

Table (2) Number and percent of yeast isolated from women and infants, infected with vaginal &oral candidiasis.

Yeast species	NO. of isolates	%
C. albicans	83	76.8
C. krusei	2	1.85
C. tropicalis	6	5.5
C. glabrata	12	11.1
C. dublinances	1	0.9
Trichosporon spp.	2	1.85
Cryptococcus neoformans	2	1.85
Total	108	100

Table (3) The No. and percent of resistant and susceptible isolates of C. albicans according to the MICs values.

Antifungal agents	No. of isolates	MICs value µg/ml	Resistance		Susceptible	
			No.	%	No.	%
Nystatin	8	128	28	73.6	10	26.3
	15	64				
	5	32				
	7	16				
	3	8				
Fluconazole	1	64	1	2.6	37	31.5
	9	32				
	16	16				
	12	8				
Ketoconazole	8	4	19	50	19	50
	11	2				
	10	1				
	9	0.5				
Amphotericin B	1	2	1	2.6	37	97.3
	5	1				
	8	0.5				
	6	0.25				
	6	0.125				
	7	0.06				
	5	0.03				

Table (4) MICs values of non-mutant, mutant strains and after subculture of *C. albicans* in antifungal free medium for nystatin.

Isolated No.	MICs values(µg/ml)											
	Non- mutant strain				Mutant strain				Strains after subculture in antifungal free medium			
	Antifungal drugs											
	N	F	K	AM	N	F	K	AM	N	F	K	AM
4	128	16	2	1	1200	16	2	16	800	16	2	4
5	64	8	1	0.5	800	8	1	4	600	8	1	2
36	128	32	4	0.5	1000	32	4	8	600	32	4	2
57	64	16	1	0.5	600	16	1	4	200	16	1	2
84	128	16	2	1	600	16	2	4	200	16	2	2

Table (5) MICs values of non-mutant, mutant strains and after subculture of *C. albicans* in antifungal free medium for fluconazole.

Isolated No.	MICs values(µg/ml).											
	Non- mutant strain				Mutant strain				After subculture in antifungal free medium			
	Antifungal drugs											
	N	F	K	AM	N	F	K	AM	N	F	K	AM
24	16	8	0.5	0.06	32	70	4	0.06	16	8	0.5	0.06
28	64	16	1	1	64	80	4	1	64	16	1	1
42	32	16	2	0.125	64	90	8	0.125	32	16	2	0.125
54	32	8	0.5	0.06	32	70	1	0.06	32	8	0.5	0.06
63	64	8	2	0.125	64	80	4	0.125	64	8	2	0.125

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