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Identify and sensitivity to antifungal drugs of *Candida* species causing vaginitis isolated from vulvovaginal infected patients in Sana'a city

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ABSTRACT

Recently, antifungal susceptibility testing has become more important because of the increasing incidence of both fungal infections and antifungal drug resistance. Candida vaginitis is a common fungal infection among adult women and it has been estimated that 75% of all adult women experience at least one period of vulvovaginal candidiasis in their lifetime. Several predisposing factors, such as diabetes mellitus, using contraceptive, pregnancy, and broad-spectrum antibiotics are reported as main risk factors for the infection. While, the main etiologic agent of vulvovaginal candidiasis is Candida albicans, more antifungal resistance has been reported among non-albicans species. The aim of the present study was toidentify Candida species anddeterminesusceptibility patterns of vaginal isolates of Candida to antifungal drugs including, clotrimazole, miconazole, terbinafine,nystatin, itraconazole, fluconazole, ketoconazole, and econazole. In the present study, 150 vulvovaginal isolates of Candida species were investigated for performing identify and susceptibility tests. The isolates were kept in sterile distilled water at 4°C in the medical Microbiology laboratory of Al-thourah hospital. All isolates were re-identified using standard methods, germ tube test, CHROMagar Candida test and microscopic characteristics on corn meal by preparation high media. Identification was based on colonies producing a green coloration on CHROMagar and presence chlamydosporse on corn meal agar test which were presumptively identified as C. albicans. Disk diffusion method was used to evaluate susceptibility patterns. Paper disks containing clotrimazole, miconazole, itraconazole, fluconazole, ketoconazole, econazole, nystatin and terbinafine were applied for susceptibility tests. A total of 141 women were included in the study and yeasts were isolated in 93 (65.95%), of them C. albicans was the most common species among the isolates followed by C. glabrata 18 (12.76%), C. tropicalis 24 (17.02%) and C. krusei 6 (4.25%) that were isolated from vaginal infected patients.In the present study, we founded that 5 isolates of C. krusei were sensitive to ketoconazole, clotrimazole and miconazole. In addition both isolates were resistant to fluconazole, nystatin, econazole and terfinafine. Only 9 isolate of C. tropicalis was sensitive to miconazole and terbinafine and two isolates to clotrimazole. Highest sensitivity of C. albicans to antifungal drugs was seen against miconazole (89 of 93 isolates) followed by, clotrimazole (81), terbinafine (38) and ketoconazole (13) whereas 73 isolates were resistant to fluconazole and econazole antifungals. 17 out of 18 isolates of C. glabrata were resistant to fluconazole, whereas all isolates were sensitive to miconazole. Antifungal sensitivity testing suggests that vaginal isolates of Candida were most sensitive to miconazole, clotrimazole, and terbinafine, and least sensitive to econazole and fluconazole.

Keywords: *Candida*; Candidiasis, Vulvovaginal; Antifungal Agents; Sensitivity

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INTRODUCTION

Because of alterations in immune status and invasivehospital procedures [1], infections caused by opportunistic pathogens, such as yeasts,are becoming important causes of morbidity and mortality inimmunocompromised patients. In the past 2 decades,nosocomial yeast infections have increased significantlyworldwide.Vulvovaginal candidiasis (VVC) or *Candida* vaginitis is a common fungal infection among adult women during reproductive ages. It has been estimated that 75% of all adult women experience at least one period of vulvovaginal candidiasis in their lifetime [2].Fortunately, the infection is rarely life threatening, whereas it is usually associated with such morbidities like discomfort, pain, sexual dysfunctions, vulvar dryness, cracks, itching, burning, soreness and finally health care costs [3, 2].Known predisposing host factors, which include uncontrolled diabetes mellitus, using contraceptive, compromised immune system, neutropenia, pregnancy, hormone replacement therapy and broad-spectrum antibiotics are risk factors for VVC [4, 5].Several antifungaldrugs have been applied to render the situation, and as aresult of broad prophylactic usages and long-term treatmentswith those drugs, the prevalence of drug sensitivity and resistance hasbecome an important issue in various yeast infections, whichhave profound effects on human health [6].*Candida* species have various degrees of susceptibility tofrequently used antifungal drugs. For instance, *Candida*lusitaniae is relatively resistant to amphotericin B (6). *Candida krusei* is intrinsically resistant tofluconazole, and *Candida glabrata* is less susceptible or hashigher MICs to it than other *Candida* species [7]. Thisphenomenon illustrates the importance of identification andsurveillance of *Candida* species in the clinical settings [8].However, there are a few reports about susceptibility of vaginal isolates to antifungal agents in vitro circumstances. In addition some studies have shown that there are different results from treatment of vulvovaginal candidiasis [9].There are also several reports indicating that resistance to antifungals, and infection recurrent is a serious problem among Iranian patients [10, 6].In a study performed in others authors believed that there is no significant difference between fluconazole and clotrimazole resistance in recurrent candidiasis [10].Prolonged therapy and increased use of antifungals for recurrent candidiasis are the most common risk factors for azoles resistance among *Candida* isolates from vulvovaginitis candidiasis patients. Azoles have the advantage of being taken orally, which increase their potency [10, 11].The inappropriate use of antifungal drugs and introduction of over-the-counter antimycotics in countries worldwide predispose development of antifungal resistance [12, 11].In a study conducted by Richter et al., fluconazole resistance was observed among 15.2% and 41.7% of vaginal isolates of *C. glabrata* and *C. krusei*, respectively [13]. Whereas resistance to itraconazole was observed in *non-albicans species, C. glabrata, C. parapsilosis, C. krusei,* and *S. cerevisiae* isolates [14, 1]. In another study, vaginal isolates of *Candida* were more dose-dependent susceptible to nystatin and ketoconazole [15]. *Candida* species are the normal microbiota within the oral cavity, gastrointestinal tracts, respiratory tracts, vaginal area and the mouth (4). The majority of cases of vulvovaginal candidiasis are caused by *C. albicans*, other etiologic agents are *C. glabrata, C. tropicalis*and *C. krusei*[16]. However, other study reported that *C. glabrata* as the main etiology of vulvovaginal candidiasis [17].

Objectives

This study was carried out to identify and determine susceptibility patterns of vaginal isolates of *Candida* species to antifungals including, clotrimazole, miconazole, itraconazole, fluconazole, ketoconazole, econazole, terbinafine, and nystatin**.**

MATERIALS AND METHODS

1- Isolates and Identification

In the present study, 150vulvovaginal isolates of *Candida* species were investigated for performing identify and susceptibility tests. The isolates were kept in sterile distilled water at 4°C in the medical Microbiology laboratory of Al-thourahhospital. All isolates were re-identified using standard methods, germ tube test,CHROMagar*Candida*test and microscopic characteristics on corn meal by preparation high media. Identification was based on colonies producing a green coloration on CHROMagarand presence chlamydosporse on corn meal agar test which were presumptively identified as *C. albicans*[17].Germ tube test, production of chlamydoconidia and growing at 37°C were also a confirmation for the isolates [18].*C. glabrata* produced pink colonies on CHROMagar*Candida* which microscopic characteristics on corn meal agar confirmed it with no presence chlamydosporse[17].Dark blue coloration on CHROMagar*Candida* and microscopic features confirmed *C. tropicalis.* Pale pink and spread colonies on CHROMagar*Candida* and microscopic features on Cornmeal agar were identical for *C. krusei*.

2. Antifungal Disks

Qualitative methods that require measurement of zone diameters also provide reproducible estimates of the susceptibility of *Candida spp*. to an antifungal agent. This procedure uses paper disks impregnated with 100µg/disk of fluconazole to test the susceptibility of yeasts to fluconazole.Paper disks also containing clotrimazole at 50µg/disk, miconazole at 10µg/disk, itraconazole 50µg/disk, ketoconazole 10µg/disk, econazole 10µg/disk, and ystatin 100U/disk were obtained from Liofilchem Bacteriology Products (Italy). Terbinafine disks were also prepared at 50µg/disk.

3. Test Method

A total of 150*Candida* species were sub-cultured on Sabouraud's dextrose agar plates, SDA (Merck, Germany) and incubated at 37°C for 24h. A suspension of overnight cultures of *C. albicans*were (93), *C. tropicalis* (24), *C. glabrata*(18) and *C. krusei* (6) were prepared in sterile PBS. Turbidity was adjusted to 0.5 McFarland standard densities resulting in an inoculum containing 1-5×106 CFU/ml. 25µl of suspension inoculated on SDA plates and rolled on the surface of the agar medium. Plates were dried for 15min at room temperature in laminar hood and then antifungal disks were placed on the inoculated agar with a forceps. The plates were incubated at 37°C for 24h, and then zone diameters were measured manually**(Figure 3).**

RESULTS AND DISCUSSION

In the present study, susceptibility testing was performed on vaginal isolates of *Candida* collected during period 2012 to 2013 from patients suspected to vulvovaginal candidiasis in Sana'a City, the capital ofYemen Republic. Criteria for susceptibility to used antifungal drugs have been summarized in **(Table 1)**[20, 19].Totally, 141 out of 150*Candida* spp. were isolated from thepatients.Thus, a total of 141 women were included in the study and yeasts were isolated in 93 (65.95%), of them *C. albicans* was the most common species among the isolates followed by *C. tropicalis* 24 (17.02%), *C. glabrata*18 (12.76%), and *C. krusei*6 (4.25%) that were isolated from vaginal infected patients**(Table 2).**The main agent of vulvovaginal candidiasis as a remarkable point in our studyis*C. albicans*; however, it seems non-*albicans* species *C. krusei*, *C. tropicalis*and *C. glabrata***(Figure 1)**of *Candida* appear to be increasingwhich can pose a serious threat due to resistance to the routine antifungal agents.These observations establish the great importance of non -*albicans Candida* as a pathogen in clinical samplesand *C. tropicalis* is the second commonest agent in vaginal infections in most regions [20].

The distributions of the species are different in various regions and studies, like 50% *C. albicans*, 24.7% *C. glabrata*, and 1% *C. parapsilosis* in other studies [17, 18].Vulvovaginal candidiasis is one of the most common opportunistic fungal infections among adult women during their lifetime. Several researches have shown that the infection increases during two to three last decades. Early appropriate therapy may alter the course of fungal infections especially, in immunodeficient or immunosuppressed patients. Therefore, early determination of an organism's drug susceptibility may contribute to successful treatment [11].Nevertheless, it is important that increase in non-*albicans* species correlated with the intensive and long-term use of antifungalswhich leads to higher level of resistance of *Candida* strains to the antifungal drugs [21, 22].

In the present study, several topical and systemic antifungal drugs were evaluated against 93 isolates of *C. albicans*, 18 *C. glabrata*, 24 *C. tropicalis*and6 *C. krusei*and result was as following5 isolates of *C. krusei* were sensitive to ketoconazole, clotrimazole and miconazole. In addition all isolates were resistant to fluconazole, nystatin, econazole and terbinafine. Dose dependent was only observed about itraconazole. While 8out of 24 isolate only of *C. tropicalis* was sensitive to miconazole and terbinafine and 12 isolates were sensitive to clotrimazole**(Table 3).**

All eighteen isolates of *C. glabrata* were resistant to fluconazole and all isolates were highly sensitive to miconazole. Highest sensitivity of *C. albicans* to antifungal drugs was seen against miconazole (89 of 93 isolates) followed by, clotrimazole (81 of 93), terbinafine (47 of 93) and ketoconazole (31 of 93).While 83 of 93 isolates were resistant to fluconazole and econazole antifungals **(Table 3)**(**Figure2).**Unfortunately, it is shown that resistance to antifungal azoles has been increased in recent years (21). In addition, sources of *Candida* were also affecting on sensitivity to antifungal drugs [22, 21].Previous studyshowed that 90.2% and 91.4% of isolates of *Candida* species were sensitive to fluconazole and ketoconazole, respectively [22].Which also the effect of the pH of the medium on antifungal activity, because is knownthe susceptibility of ketoconazole activity to environmental pH [23].

Whereas, our results show that 86.6% and 82.6% of tested isolates were resistant to fluconazole and econazole, correspondingly**(Table 3)(Figure3).**On the other hand,we founded that the best choice for the treatment of isolates was miconazole followed by clotrimazole, terbinafine and ketoconazole.Other study reported that clotrimazoleand fluconazole are the two antifungal drugs that are widely used in the treatment of vulvovaginal candidiasis [23].Several researches have shown that terbinafine is the first choice for the treatment of dermatophytosis[24, 25], However, few details are available about its effects on vaginal isolates of *Candida.* Despite the fact our results show that terbinafine is effective against 76.1% of vaginal isolates. Therefore, terbinafine therapy can be considered as a good therapeutic option in the management of vaginal candidiasis.

Susceptibility patterns of *Candida speciesC. albicans* was isolated 83 of 93from patients with (89.2%) of these isolates were susceptible to fluconazole while *C. tropicalis*was 23 out of 24 with (95.8%)susceptible to fluconazole. The fluconazole-nonsusceptible*Candida* isolates included *C. glabrata* 11 out of 18 with (65%), *C. krusei* was 6 with (100%), *C. tropicalis*was 1 (4%) and *C. albicans* was 1 (4%) isolates withfluconazole-nonsusceptible**(Table 4).**The sensitivity a pattern of *Candida* isolates varies among studies in different countries [26, 27].Other study showed that all tested *Candida* were susceptible to nystatin, miconazole, ketoconazole and fluconazole and *C. albicans* isolates were more susceptible to azoles than was *C. glabrata*[28].In previous study wedid not find a significant statistical difference in the fluconazole susceptibility between *C. albicans* and non-*C.albicans*candidiasis[29].

Table 1- Criteria of Susceptibility and Resistance of Antifungal Disks

	Zone Diameter in mm		
	Sensitive	**Dos Dependent**	**Resistance**
Nystatin	≥ 25	17-24	≥ 16
Fluconazole	≥ 19	15-18	≥ 14
Ketoconazole	≥ 30	23-29	≤ 22
Clotrimazole	≥ 20	12-19	≤ 11
Miconazole	≥ 20	12-19	≤ 11
Itraconazole	> 16	10-15	< 9
Econazole	≥ 30	23-29	≤ 22
Terbinafine	≥ 20	12-19	≤ 11

Figure1-*Candida* species isolates from patients were sub-cultured on Sabouraud's dextrose agar plates

Figure2-Antifungal susceptibility testing in *C. albicans* by disk diffusion methodat 24 hours

Table 2. Distributions of *Candida* species isolates from patients

Species	No. of isolates	% of isolates
Candida albicans	93	65.95%
Candida krusei	6	4.25%
Candida glabrata	18	12.76%
Candida tropicalis	24	17.02%

Table 3 - Sensitivity of Tested Isolates against Several Antifungal Drugs

Antifungal Drugs.	*C. albicans*			*C. glabrata*			*C.tropicalis*			*C. krusei*			Total		
	S[a]	DD[a]	R[a]	S[a]	DD[a]	R[a]	S[a]	DD[a]	R[a]	S[a]	DD[a]	R[a]	S[a]	DD[a]	R[a]
Itraconazole	11	41	8	0	8	0	0	4	0	0	6	0	11 (14.1%)	59(75.6%)	8(10.3%)
Fluconazole	12	5	83	0	0	17	0	0	4	0	0	6	12 (9.48%)	5 (3.93%)	110(86.6%)
Ketoconazole	31	17	23	6	9	3	0	3	1	5	0	0	42(42.9%)	29 (29.6%)	27(27.6%)
Terbinafine	47	18	7	3	6	9	8	0	3	0	0	6	58 (54.2%)	24 (22.4%)	25(23.4%)
Clotrimazole	81	11	6	9	9	0	12	0	2	5	0	0	107 (79.3%)	20 (14.8%)	8(5.9%)
Miconazole	89	4	1	18	0	0	8	2	0	5	0	0	120(94.5%)	6 (4.7%)	1 (8%)
Nystatin	17	46	0	5	16	0	0	4	0	0	0	6	22(23.4%)	66 (70.2%)	6(6.4%)
Econazole	0	10	83	0	9	9	0	2	2	0	0	6	0 (0.0%)	21 (17.4%)	100 (82.6%)

[a] *Abbreviation: S, Sensitive; DD, Dose dependent; R, Resistant.*

Table 4- Susceptibility patterns of *Candida* species

Candida species (No. of isolates)[b]	Fluconazole-susceptible isolates			Fluconazole-nonsusceptible isolates		
	No. (%)	MIC (µg/ml)		No. (%)	MIC (µg/ml)	
		Range	50%		Range	50%
C. albicans(93)	83 (89.2)	0.12–1	0.5	1 (2)	256	256
C. glabrata(18)	6 (35)	0.12–8	8	11 (65)	16–256	16
C. tropicalis(24)	23 (95.8)	0.06–4	2	1 (6)	64	64
C. krusei(6)	0 (0)	NA[c]	NA	6(100)	32–128	64

[a] *Susceptibility breakpoints were determined by using the CLSI guidelines recommended at the time of the study [12].*
[b]*A total of 114Candida species were tested.;* [c]*NA, not applicable.*

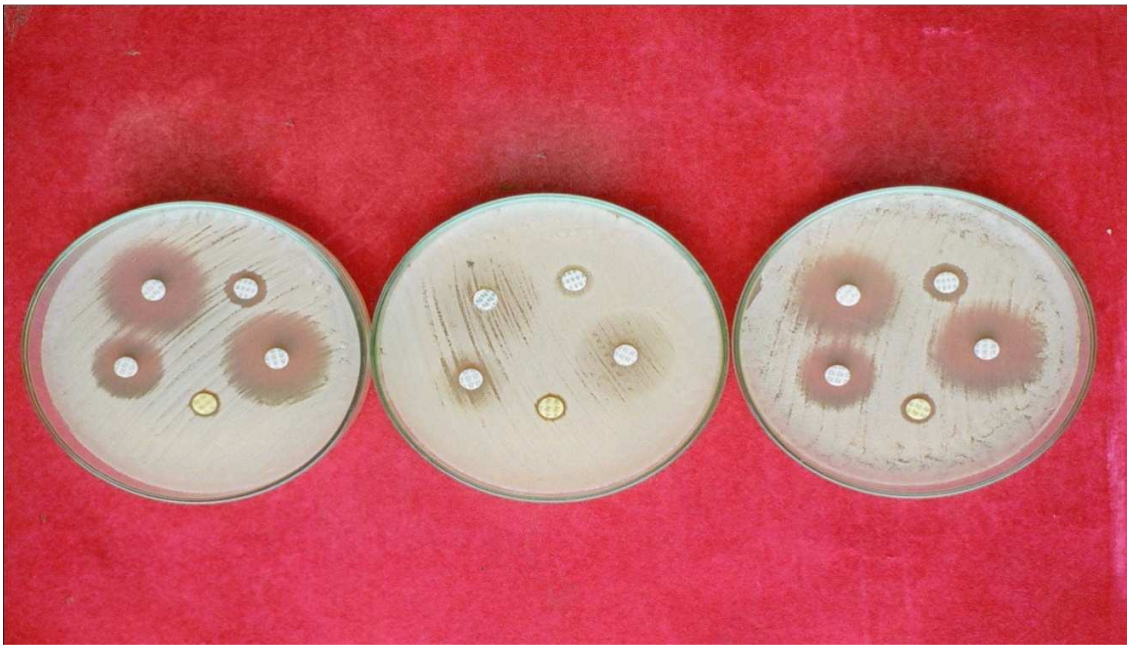

Figure2- Antifungal susceptibility testing in non-*C.Albicans*species by disk diffusion method

CONCLUSION

It is concluded that antifungal susceptibility testing should be performed before prescribing treatment. Antifungal sensitivity testing reveals that vaginal isolates of *Candida* were most sensitive to miconazole, clotrimazole, and terbinafine, and least sensitive to econazole, followed by fluconazole and ketoconazole. Mostisolates of *C. glabrata* were resistant to fluconazole, whereas all isolates were sensitive to miconazole.

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