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Premenopausal women without Vulvovaginal candidiasis harbor virulent and antifungal resistant strains of Vulvovaginal candida species

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Abstract

VVC is the second most common urogenital tract infection among women in reproductive age. It is caused by *C. albicans*, however lately there is changing trend in *Candida* species, especially NAC colonizing the vagina. *Candida* can exist as commensal of urogenital tract, and its pathogenic form causes infection. This study was carried out to determine the antifungal susceptibility of vulvovaginal *Candida* isolated from 201 asymptomatic women. Virulent and non-virulent *Candida* species previously identified were used for the study. Antifungal susceptibility of vaginal *Candida* isolates against polyenes (amphotericin B and nystatin) and azoles (fluconazole, voriconazole, ketoconazole, itraconazole and clotrimazole) was determined by disc diffusion assay. Of 46 *Candida* isolates, 43.5% (20) were found to be resistant and 56.5% (26) were sensitive. Isolates showed resistance against ketoconazole (18,39.1%), clotrimazole (5,10.9%), fluconazole (3,6.5%), voricanazole (3,6.5%), itraconazole (2,4.3%) and nystatin (1, 2.2%). Cross resistance against azoles were detected in four isolates of *C. glabrata* and one *C. albicans* strain. None of the virulent isolates of *C. albicans* and *C. glabrata* appeared sensitive towards any of the seven antifungals tested. Antifungal susceptibility testing of *Candida* species is crucial to establish susceptibility patterns of recovered isolates in order to guide empirical therapy. Presence of antifungal resistant virulent strains of *Candida* especially NAC in apparently healthy women underlines the need for prophylactic methods to prevent future episodes of VVC.

Keywords: Vulvovaginal candidiasis, virulent, antifungal resistance, Candida, women

Introduction

Vulvovaginal candidiasis (VVC) is the second most common mucosal infection, affecting women following bacterial vaginosis. It accounts for nearly one-third of vaginitis cases (Workowski et al, 2015). Although asymptomatic vaginal *Candida* colonization occurs in ~10-20% of healthy women, almost 75% women experience symptomatic VVC at least once during their life (Sherrard et al, 2011, Hacer et al, 2012; Adeyba et al, 2003). Symptoms are often known to affect the quality of living in women by creating uneasiness and

depression (Chew and Than, 2016). This infection if emerges during pregnancy, can lead to systemic infections in neonates (Rasti et al, 2014). *Candida* may exist as commensal or pathogen in the genital tract of women. Findings from recent studies have evidence that dysbiosis in these commensal *Candida* species might results in symptomatic infections (Cauchie et al, 2017). The pathogenicity of *Candida* species is attributed to certain virulence factors, such as the ability to switch from yeast to fungal form, evasion of host defenses defences, phenotypic switching, adhesion and biofilm formation and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and hemolysins (Chai et al, 2009; Silva et al, 2012).

The most common causative agent is *C. albicans*, but in recent studies the presence of Non-*albicans Candida* (NAC), especially *C. glabrata* appears to be increasing (Sobel, 2006; Vijaya et al, 2014). The conventional antifungal treatment generally targets *C. albicans*, ignoring the possibility of NAC. Such empirical treatment for *Candida* infections could lead to resistance due to improper and misuse of antimycotics. Besides clotrimazole and fluconazole, other azoles and polyenes (amphotericin B–based preparations) have been used for treatment of VVC. Often these antifungal treatments fail leading to recurrence. In fact recurrent VVC is observed in up to 50% women and 5% of them may have recurrence of at least thrice a year (Murina et al, 2011; Sobel, 2016). Treatment failures have been observed due to intrinsic resistance in *Candida* species such as *C. krusei* and *C. glabrata*. Resistance may be acquired in previously susceptible strains of *C. albicans* due to repeated use of antifungals in immunocompromised individuals (Groll and Kolve, 2004; Deorukkhar et al, 2014). Up to 7.5% of genital *Candida* isolates have been reported to be resistant to one or more of the commonly used azoles (Sojakova et al, 2004). Azole resistant *C. albicans* infections are extremely rare, however; the use of azoles for candidemia may contribute to the more frequent recurrent incidence of infections caused by the NAC species (Oxman et al, 2010; Lortholary et al, 2011; Fothergill et al, 2014). Most of the studies have focussed on symptomatic VVC; but very few studies have reported characterising vulvovaginal candida in apparently healthy women. In fact studying *Candida* colonization in normal women might provide some insights on possible commensal to pathogenic transition of *Candida*. Our previous study indicated the high prevalence of vulvovaginal NAC and presence of virulent *Candida* isolates in asymptomatic women. Any alteration in the vaginal microbiome of these women might encourage the growth of commensal *Candida* leading to symptomatic VVC. The current study was undertaken with the aim to study the presence of resistant *Candida* isolates and correlate this attribute to their virulence.

Materials and methods

Isolates maintenance

Candida isolates (n = 46) that had been isolated previously from vaginal swabs of healthy asymptomatic women of reproductive age group of 19 to 45 years old from Mumbai, India, were studied. Species identification of *Candida* isolates was previously carried out using phenotypic and genotypic methods and the virulence of the different *Candida* species determined by ability to switch from yeast to fungal form, biofilm formation and production of phospholipase. Tested *Candida* species included *C. albicans* (10, 21.743%), *C. glabrata* (30, 65.22%), *C. krusei* (4, 8.69 %), and *C. tropicalis* (2, 4.34%). The isolates were stored at -80^{o}C in Sabourauds broth containing 20% glycerol.

Antifungal susceptibility assay

Antifungal susceptibility testing was done using the disk diffusion method as per the Clinical and Laboratory Standards Institute (CLSI - M44-A2) guidelines (C.L.S.I. document M44-A2, 2009). Briefly, the *Candida* species were cultured on potato dextrose agar (Hi Media) twice for 48h at 35°C and confirmed for purity. The antifungal disks used were amphotericin B, fluconazole, voriconazole, ketoconazole, nystatin, clotrimazole and itraconazole (HiMedia). Inhibition zones were interpreted after 24 hrs of incubation using validated CLSI interpretive break points for fluconazole, itraconazole and voriconazole and amphotericin, while for other drugs, the interpretive break points were adopted from published studies (Table 1).

Antifungals	Potency	Susceptible (S) (mm)	Susceptible-Dose Dependent (S-DD) (mm)	Resistant (R) (mm)	Reference
Amphotericin B	100 mcg	≥15	14-10	<10	Jabeen et al ,2016
Clotrimazole	10 mcg	>20	19–12	≤11	Pakshir et al, 2009; ElFeky et al,2016
Fluconazole	25 mcg	≥19	18-15	≤14	Monroy-Pérez et al,2016; ElFeky et al,2016
Itracanazole	10 mcg	≥ 16	10-15	< 10	Zarei Mahmoudabadi et al, 2013
Ketoconazole	10 mcg	≥30	23-29	≤22	Zarei Mahmoudabadi et al, 2013; Monroy-Pérez et al,2016
Nystatin	100 U.I	≥25	17-24	≤16	Zarei Mahmoudabadi et al, 2013; Monroy-Pérez et al,2016
Voriconazole	1 mcg	≥17	16-14	≤13	Zarei Mahmoudabadi et al, 2013; ElFeky et al, 2016

Table-1 : Antifungals used in the study and the End points Criteria for Interpretation of the Results

		Amphotericin B n (%)			Fluconazole n (%)			Voriconazole n (%)			Ketoconazole n (%)			Nystatin n (%)			Clotrimazole n (%)			Itraconazole n (%)		
Candida species	No. of isolates	S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R	S	SDD	R
C. albicans	10	9 (90)	1 (10)	0 (0)	9 (90)	0 (0)	1 (10)	9 (90)	0 (0)	1 (10)	2 (20)	3 (30)	5 (50)	0 (0)	10 (100)	0 (0)	4 (40)	6 (60)	0 (0)	9 (90)	1 (10)	0 (0)
C. glabrata	30	23 (76.7)	7 (23.3)	0 (0)	28 (93.3)	0 (0)	2 (6.7)	28 (93.3)	0 (0)	2 (6.7)	5 (16.7)	12 (40)	13 (43.3)	4 (13.3)	26 (86.7)	0 (0)	12 (40)	13 (43.3)	5 (16.7)	22 (73.3)	6 (20)	2 (6.7)
C. krusei	4	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)	2 (50)	2 (50)	0 (0)	4 (100)	0 (0)	0 (0)
C. tropicalis	2	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	1 (50)	0 (0)	1 (50)	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
Total	46	38 (82.6)	8 (17.4)	0 (0)	43 (93.5)	0 (0)	3 (6.5)	43 (93.5)	0 (0)	3 (6.5)	9 (19.6)	19 (41.3)	18 (39.1)	5 (10.9)	40 (86.9)	1 (2.2)	20 (43.5)	21 (45.7)	5 (10.9)	37 (80.4)	7 (15.2)	2 (4.3)

Table-2: Antifungal susceptibility patterns of *Candida* species to different antifungal drugs used in the study.
S: susceptible; SDD: Susceptible Dose Dependent; R: resistant.

Cross- resistance was defined as resistance to two antifungals of the same drug class. We evaluated cross-resistance to azoles, defined as resistance to any two of the azoles tested. Multi-resistance was defined as resistance to two antifungal drug classes, namely the azoles and polyenes (Orasch et al 2014).

Data Analysis

Data was entered into excel and Epi-Info software for statistical analysis. Proportions were generated to demonstrate the susceptibility patterns of *Candida* species. The chi-squared test was used to compare groups of categorical data. Findings were considered significant at $p < 0.05$.

Results

Efficacy of antifungals used in the study

Out of the 46 *Candida* isolates, 43.5% (20) isolates were found to be resistant against at least one of the tested antifungals and 91.3% (42) showed dose dependent susceptibility. Only 56.5% (26) isolates were sensitive to all the tested antifungals. Resistance was observed against ketoconazole (18, 39.1%) followed by clotrimazole (5, 10.9%), fluconazole (3, 6.5%) and voricanazole (3, 6.5%), itraconazole (2, 4.3%) and nystatin (1, 2.2%). None of the isolates showed resistance against amphotericin B; however 17.4 % (8) isolates showed dose dependent susceptibility towards it (Table 2).

Variation in susceptibility to antifungals among the species

Among the species *C. albicans* was found to be resistant against Ketoconazole (5, 50%), followed by voriconazole (1, 10%) and fluconazole (1, 10%). Of the 10 isolates of *C. albicans* studied, five (50%) were resistant and rest five (50%) showed SDD against any of the antifungals. *C. glabrata* showed resistance to ketocanozole (13, 43.3%), followed by clotrimazole (5, 16.7%), voriconazole (2, 6.7%), itraconazole (2, 6.7%) and fluconazole (2, 6.7%). Isolates of *C. krusei* and *C. tropicalis* were sensitive to all the tested azoles and polyenes, except one strain of *C. tropicalis* showing resistance towards nystatin (Table 2). Cross resistance to more than one azole was seen in four strains of *C. glabrata* and only one isolate of *C. albicans*.

Antifungal susceptibility of virulent isolates of *Candida* species

From 46 isolates, 16 (34.8%) strains were virulent, which included 10 isolates of *C. albicans*, four of *C. glabrata* and one each of *C. krusei* and *C. tropicalis*. Of these virulent isolates, 56.3% (9) exhibited resistance to at least one of the azoles and 43.7% (7) isolates were susceptible to all the antifungals. On the other hand, out of the 30 non-virulent isolates only 36.7% (11) were resistant to at least one of the antifungals. The virulent *C. albicans* isolates were either resistant (5, 50%) or showed dose dependent susceptibility (5, 50%). No significant correlation was observed with virulence and antifungals resistance displayed by the *Candida* isolates ($p<0.204$). However, all the virulent *C. glabrata* isolates were resistant (4, 100%) as compared to non-virulent isolates (10, 38.5%) (Table 3).

Discussion

Vulvovaginal candidiasis (VVC) is one of the common reproductive tract infections in women, caused by opportunistic *Candida* species (Chew and Than, 2016). *C. glabrata* is the second most cause of VCC after *C. albicans* (Sobel, 2006).

		Numbers n (%)	Resistant n (%)	SDD n (%)	Sensitive n (%)	p value
Virulent	*C. albicans*	10 (100)	5 (50)	5 (50)	0 (0)	0.204
	C. glabrata	4 (100)	4 (100)	0 (0)	0 (0)	
	C. krusie	1 (100)	0 (0)	1 (100)	0 (0)	
	C. tropicalis	1 (100)	0 (0)	0 (0)	1 (100)	
Non virulent	*C. albicans*	0 (0)	0 (0)	0 (0)	0 (0)	
	C. glabrata	26 (100)	10 (38.5)	16 (61.5)	0 (0)	
	C. krusie	3 (100)	0 (0)	3 (100)	0 (0)	
	C. tropicalis	1 (100)	1 (100)	0 (0)	0 (0)	
	Total	46 (100)	20 (43.5)	25 (54.3)	1 (2.2)	

Table-3: Antifungal susceptibility of virulent isolates of *Candida* species

Clotrimazole or fluconazole are the widely recommended antifungal treatments for VVC, however drug resistant microbes with limited therapeutic options are of serious public health concern. The increasing incidence of both fungal infections and antifungal drug resistance has pressed upon the need for antimicrobial susceptibility testing.

Our previous study had observed presence of vulvovaginal *Candida* in 30% of premenopausal asymptomatic women where many of them colonized virulent strains (unpublished data). Species identification and in vitro antifungal susceptibility testing of *Candida* before initiating therapy has been overlooked giving rise to resistant strains. In the present study azoles were found to be less effective against different species of *Candida* as compared to polyenes. Importantly, all the species studied here were susceptible to amphotericin B and nystatin which can be used as salvage therapy when other antifungals are ineffective. Absence of sensitive strains of *C. albicans, C. glabrata* to all the seven antifungals is alarming. Nearly all isolates displaying dose dependent susceptibility is a warning for emergence of resistance. A high prevalence of increased dose-dependent resistance in these isolates indicates the need to use higher therapeutic doses of these drugs in order to attain a satisfactory clinical response or a change in the routine optimal therapy of *Candida* vaginitis (Mohanty et al, 2007).

Our study throws light on the existence of virulent and resistant strains of vulvovaginal *Candida* species in healthy premenopausal women. None of the virulent *C. albicans, C. glabrata* isolates displayed sensitivity towards any of the azoles or polyenes. These observations are in conformation with studies by Chandra and co-workers (2001). Other studies have also indicated that infections caused by virulent *C. glabrata* are difficult to eradicate (Kołaczkowska and Kołaczkowski, 2016). The concomitance occurrence of virulence and antifungal resistance could be more advantageous for pathogens to colonize the host and sustain in

the niche (Beceiro et al, 2013). Presence of virulent isolates of *Candida* exhibiting high proportion of resistance to antifungals is disturbing. This information indicates the possibility of symptomatic VVC and antifungal treatment failure.

Unfortunately, reports on antifungal resistance are soaring, narrowing its scope, demanding for safe and effective alternative. Anti-candida vaccines and systemically administered antibodies have been effective in preventing vaginal candidosis in rodents, but no data are available in human beings (Vecchiarelli et al, 2012; Cassone, 2013). Although no specific strategies are on the horizon, possible future treatments could be use of vaginal *Lactobacillus* species that are capable of adhering to vaginal epithelial cells, persisting in the vagina and are capable of expressing anti-*Candida* protective factors.

The absence of VVC symptoms in our studied population, in spite of harbouring virulent *Candida* species, can be explained by the presence of beneficial *Lactobacillus* residing in the urogenital tract, that are efficiently keeping them under control (Rizzo et al, 2013, Niu et al, 2017). However, any imbalance in *Lactobacillus* population will favour the endogenous virulent *Candida* species in developing VVC. These prophylactic or therapeutics lactobacilli have been demonstrated to have anti-candida activity indicating promising strategy to against VVC (Parolin et al, 2015; Wang et al, 2017).

Conclusion

Understanding species diversity, its virulence and antifungal susceptibility of vulvovaginal *Candida* in otherwise apparently healthy women might provide an insight to host- *Candida* interaction and its interaction with other members of the vaginal microbiome, especially Lactobacillus. Considering the side-effects of prescribed antifungal drugs, rising resistance of *Candida* to these drugs and the recurrence of infection post-treatment, natural holistic treatment of these infections could be a preferred option.

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Conflict of interest

The authors declare they have no conflict of interests

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