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ANTIFUNGAL RESISTANCE IN CANDIDA SPECIES ISOLATED FROM PATIENTS PRESENTING WITH VULVOVAGINAL CANDIDIASIS DURING DIFFERENT TRIMESTERS OF PREGNANCY

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RZ did data collection, laboratory work and manuscript writing:
IU did Concept & project design, editing and final review of manuscript.
AA did data analysis and interpretation

ABSTRACT
The aim of this study was to assess frequency of *Candida* species causing vulvovaginal candidiasis among symptomatic pregnant females and their resistance pattern against azoles antifungal drugs. Specimens were taken from 176 antenatal patients having signs and symptoms of vulvovaginal candidiasis. Culture, microscopic examination and API 20C AUX system were used for isolation and identification of *Candida* species. Susceptibility testing to Azoles were performed by disc diffusion and broth microdilution method. Out of 176 samples, 73(43%) were positive for *Candida* infection. *Candida albican* were 31(42.4%) followed by *Candida krusei* 20(27.3%), *Candida parapsilosis* 18(24.6%) and *Candida glabrata* (5.4%). *Candida* infection was more frequent in 2nd trimester (54.7%) followed by 3rd trimester (27.3%) and 1st trimester 13(17.8%). A good agreement was revealed between the disc diffusion and broth microdilution methods in detecting resistance in *Candida* species. Nonalbicans *Candida* showed higher resistance against Fluconazole (P-value <0.001), Clotrimazole (P-value 0.03) and Miconazole (P-value 0.01) as compared to *C. albicans.* It is necessary to perform antifungal susceptibility tests for *Candida* species for providing resistance profile information to choose appropriate drug for treatment. Disc diffusion method can be used in diagnostic practice as it is convenient to perform.

Keywords: Broth microdilution, Fluconazole, Itraconazole, Clotrimazole, Voriconazole, Miconazole

1. Introduction

Vulvovaginal candidiasis (VVC) is caused by yeasts of the genus *Candida*. Vaginal candidiasis is the most frequent vaginal infection. Women are at higher risk for this type of infection. Approximately 75% of women suffer from (VVC) during their lifespan.[1] Risk factors for VVC are pregnancy, genetic

factors, high estrogen oral contraceptives, corticosteroid therapy, the use of over-the-counter antibiotics, diabetes. Intrauterine contraceptive device (IUCD), high glucose content diet. About 5-10% healthy women have recurrent vaginal candidiasis.[2]

Numerous studies have shown that vaginal candidiasis rises in women with pregnancy, especially during 2nd and 3rd trimesters. This increase may be due to change in the pH of the vagina, increase in the proportion of sugar in the vaginal secretions and increase level of estrogen. During pregnancy increased estrogen deposit further glycogen in the vagina. Glycogen provides carbon essential for Candida growth and germination. This leads to faster yeast growth. During pregnancy progesterone levels is also increased. The enhanced progesterone levels reduce the neutrophils power to fight Candida infection .[3] The maternal immune system induces elevated inflammatory response by producing proinflammatory cytokines to prevent immune rejection of the fetus. During pregnancy the change of immune system from high inflammatory states during first trimester of pregnancy to low inflammatory levels in later pregnancy leads to increased vulnerability to infections of the pregnant women.[4]

In Lebanon study 39% prevalence of VVC was revealed.[5] Study in Ghana showed prevalence of 30.7% conducted on 176 pregnant females.[6] In developing countries, there is limited data regarding the frequency of vaginal candidiasis. Some studies had observed the incidence of vaginal candidiasis among pregnant women in Pakistan, which was found to be between 26.9% and 56.2 %.[7]

Candida albicans is the most common among *Candida* species. Non-albicans Candida (NAC) species are also contributing pathogens and can also colonize on mucocutaneous surfaces. The significant NAC are Candida *krusei*, *Candida tropicalis, Candida parapsilosis* and *C. glabrata*. Although some NAC species like, *Candida lusitaniae, Candida kefyr* and *Candida lipolytica* can also cause VVC. Comparatively higher antifungal resistance rate of NAC species may be responsible for recurrent infections. This rise in NAC vulvovaginitis has been related to antifungal therapy overuse, that has led to the eradication of the susceptible *C. albicans.*[8]

Vaginal candidiasis may be prolonged, and symptoms are more severe during pregnancy. Long-term treatment is required due to these problems. Thus, in this situation, such studies can improve the clinical condition of women by early diagnosis and appropriate treatment.

2. Materials and Methods

2.1 Study design and Settings

This cross-sectional study was conducted at the department of Institute of Pathology and Diagnostic medicine, Khyber Medical University Peshawar, Pakistan. Non-probability convenient sampling was the sampling technique. Specimens were taken from antenatal patients having signs and symptoms of vulvovaginal candidiasis attending obstetrics & gynae out-patient department of Hayatabad Medical Complex from November 2018 to May 2019. Before commencement of study approval from ethical committee of Khyber Medical University was obtained. Demographic data were collected from patients using a structured questionnaire used for collection of patient's demographic data. Two high vaginal swabs (HVS) were obtained from each patient.[9, 10]

2.2 Isolation and Identification of Candida species

The HVS were inoculated on Sabaurouds dextrose agar. For species identification *Candida* colonies were cultured on *Candida* Chromagar. Gram staining and wet film microscopy were performed. API 20C AUX system (Bio Merieux) was used for biochemical identification. The isolates were stored in micro bank at -80 °C. [10]

2.3 Disc Diffusion Method

Susceptibility testing of *Candida species* against azoles was performed following the Clinical and Laboratory Standards Institute guidelines. American Type Culture Collection (ATCC) 90028 was used for quality control. Fluconazole (25µg), Miconazole (50µg), Clotrimazole (10µg), Itraconazole (10µg) and Voriconazole (1µg) were the antifungals used for testing by disc diffusion method (DD).

The isolates were labeled as resistant R, susceptible S, and susceptible dose dependent (SDD) according to M44A document of Clinical and Laboratory Standards Institute (CLSI)[11].

2.4 Broth Microdilution Method & MIC

Broth micro-dilution (BMD) was conducted according to M27A CLSI guidelines. Colonies of *Candida* on 24 hours culture on SDA was used to prepare the inoculum. The colonies were transferred to 5ml of 0.85% saline. The suspension was adjusted to 0.5 McFarland standards and was diluted 1:50. Followed by 1:20 dilution in Roswell Park Memorial Institute Medium RPMI 1640 (M1972 HI media part A, D glucose part B). Stock solutions of Voriconazole, Itraconazole, Miconazole and Clotrimazole (Sigma, USA) were made in dimethyl sulfoxide (DMSO) at 1600 µg/ml concentration. Fluconazole stock solution was made in distilled water in 1280MG/L concentration. Drug dilutions of Fluconazole was prepared in 64-0.125 µg/ml range and Clotrimazole, Itraconazole, Miconazole and Voriconazole was in16- 0.03mg/L range. A 100µl of the drug dilutions were transferred to the first ten columns of the 96-well plate. Column 11 was growth control and column 12 was sterility control. After 24-48 hours Minimum inhibitory concentration (MIC) reading was recorded. MIC break-points for Fluconazole, Voriconazole and Itraconazole were determined according to M27A document. For Clotrimazole and Miconazole, interpretive breakpoints are not present in CLSI document. For Miconazole and Clotrimazole breakpoints of ≥16mg/L and ≥1mg/L were used.[11,12]

2.5 Statistical Analysis

Data was analyzed by SPSS version 20. Qualitative data was presented as frequencies and percentages. Chi-square test was applied for statistical significance ($p \leq 0.05$). Kappa value was calculated for detecting agreement between BMD and DD methods.

3.Results

3.1 Isolation of Candida

A total of 176 patients were included in the study. *Candida* species were detected in 73(43%) cases. Among seventy-three isolated *Candida* species 31(42.4%) were *C. albicans* while 42(57.5%) were NAC species. *C.albican* was the frequent specie followed by *C. krusei* 20(27.3%), *C. parapsilosis* 18(24.6%) and *C. glabrata* 4 (5.4%).

3.2. *Candida* infection in different trimesters of pregnancy

Candida infection was more frequent in 2nd trimester (54.7%) followed by 3rd trimester (27.3%) and 1st trimester 13(17.8%) as shown in figure 1. Comparison of 2nd trimester distribution of *Candida* infection to 1st and 3rd trimesters showed significant statistical difference (*p*value<0.001 & *p*value <0.01) respectively.

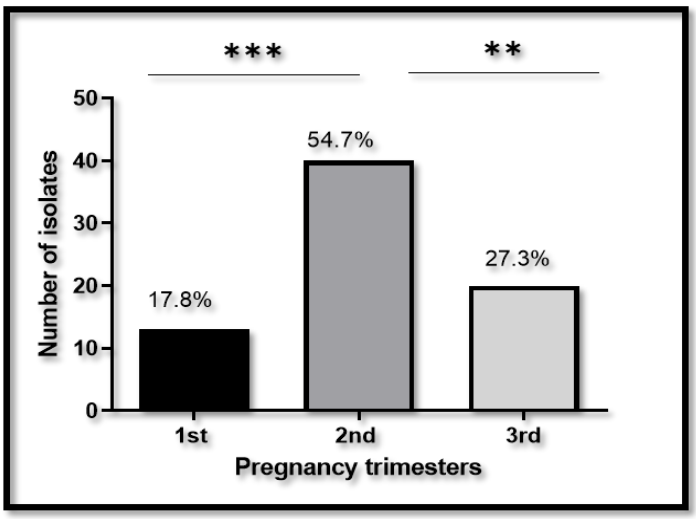

Figure 1: Frequency of *Candida* infection in trimesters of pregnancy Candida

3.3 Species among different trimesters of pregnancy

Candida albicans was the most frequent specie detected among all the three trimesters as compared to other species. This difference was found statistically significant (P value <.001). *C. krusei* was the second common specie in 1st (23%) and 3rd (35%) trimesters while in 2nd trimester *C. parapsilosis* (27.5%) was the second common specie as shown in table1.

Table1: Distribution of Candida species among different pregnancy trimesters *n (%)*

Trimester	*C.albicans*	*C.krusei*	*C.parapsilosis*	*C.glabrata*	P value
1st	7(53.8)	3(23.1)	2(15.4)	1(7.7)	<.001
2nd	17(42.8)	10(25.0)	11(27.5%)	2(5.0)	<.001
3rd	7(35)	7(35)	5(25.0)	1(5.0) -	<.001

3.3. *Candida albicans* and *non albican Candida* (NAC) distribution

Figure 2 shows *Candida albicans* and *non albican Candida* (NAC) distribution among pregnancy trimesters. The rate of NAC was high in 2nd (57.5%) and 3rd (65%) trimester. In 1st trimester *C.albicans* (53.8%) were found frequent.

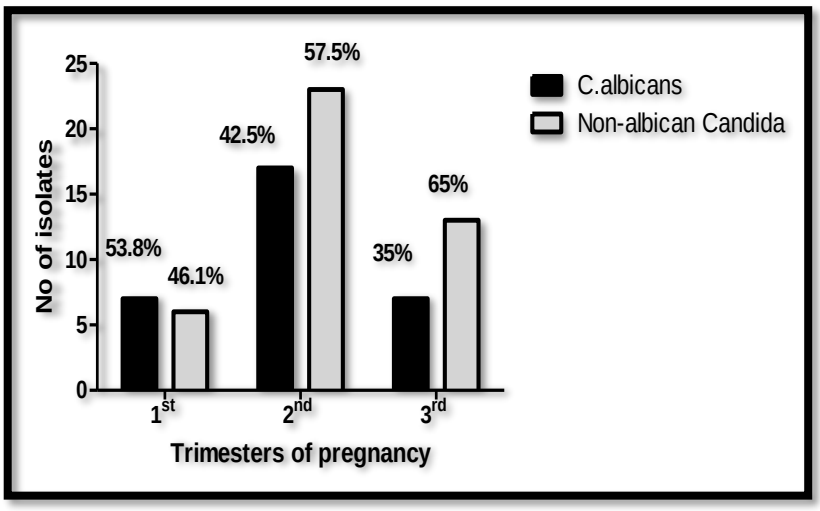

Figure2: *Candida albicans* and nonalbicans *Candida* (NAC) distribution among pregnancy trimesters.

3.5 Antifungal resistance profile

3.5.1. Broth-microdilution method

Resistance pattern of Candida species against azoles by Broth-microdilution method showed least resistance in species against Itraconazole (19.2%) followed by Voriconazole (50.7%), Miconazole (60.3%) and Clotrimazole (67.1%). Resistance in Candida species was found high against Fluconazole (74.0%) as shown in table 2

Antifungal susceptibility profile of Candida species showed that *Candida albican* revealed high resistant to Clotrimazole (54.8%) as shown in table 2. *Candida krusei* showed high resistance against Fluconazole (100%) followed by Clotrimazole (70%), Miconazole (70%) and Voriconazole (55%) while no resistance to Itraconazole was observed. *Candida parapsilosis* showed high resistance to Fluconazole (88.9%) followed by Clotrimazole (83.3%) Miconazole (77.8%), Voriconazole (61.1%) and Itraconazole (33.3%). *Candida glabrata* revealed a high resistance of 75% against Fluconazole and Clotrimazole followed by 50% resistance to Miconazole. A resistance of 25% was observed in *C. glabrata* to Voriconazole and Itraconazole (table2).

Table2: Antifungal resistance profile of Candida species by Broth Micro Dilution method n(%)

Candida Species	Fluconazole	Clotrimazole	Miconazole	Voriconazole	Itraconazole
C.albicans	15 (48.4)	17 (54.8)	14 (45.2)	14 (45.2)	7 (22.6)
C.krusei	20 (100)	14 (70.0)	14 (70.0)	11 (55.0)	0
C.parapsilosis	16 (88.9)	15 (83.3)	14 (77.8)	11 (61.1)	6 (33.3)
C.glabrata	3 (75.0)	3 (75.0)	2 (50.0)	1 (25.0)	1 (25.0)
Total	54 (74.0)	49 (67.1)	44 (60.3)	37 (50.7)	14 (19.2)

3.5.2. Minimum inhibitory concentration (MIC) of Resistance *Candida* isolates

The resistant *Candida* species had the MIC ranges as follows: for Fluconazole the resistant *Candida* species had MIC of 64 µg/ml. For Clotrimazole the resistant *C. albican. C.parapsilosis and C. glabrata* had MIC in range of 1-8µg/ml. *C. krusei had 1*-4 µg/ml of MIC range. For Miconazole the resistant *C. albican, C. krusei, C. parapsilosis and C. glabrata* had MIC of 16µg/ml. For Voriconazole the resistant *C. albican. C. krusei and C. parapsilosis* had MIC in range of 4- 16µg/ml. *C. glabrata* had 4µg/ml of MIC range. For Itraconazole the resistant isolates *C. albican* had MIC in range of 1-2µg/m, *C. glabrata* had MIC of 4µg/ml. *C. parapsilosis had* 1-4 µg/ml of MIC rang. All of *C. krusei* isolates were susceptible to Itraconazole and had MIC in range of 0.03-0.5 µg/ml (table 3)

Table 3: Antifungal drugs MIC ranges of Candida species

MIC(µg/ml)	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**
***C. albicans* (n=31)**												
Fluconazole	-	-	1	1	4	1	2	3	2	1	1	15
Clotrimazole	1	1	2	7	3	4	5	6	2			
Miconazole			2	1	2	3	1	5	3	14		
Voriconazole	1	3	2	2	4	3	2	7	5			
Itraconazole	3	4	5	6	7	4	3					
***C. krusei* (n=20)**												
Fluconazole												20
Clotrimazole		1	2	1	2	5	4	5				
Miconazole					2	1		1	2	14		
Voriconazole			1	1	2	4	1	5	2	4		
Itraconazole	3	5	3	4	5							
***C. parapsilosis* (n=18)**												
Fluconazole						1		1				16
Clotrimazole		1		1	1	6	3	5	1			
Miconazole					2			2		10		
Voriconazole			1		2	1	3	7	3	1		
Itraconazole	2	4	1	5		3	2	1				
***C. Glabrata* (n=4)**												
Fluconazole						1						3
Clotrimazole				1		1	1		1			
Miconazole						1	1			1	1	
Voriconazole			1	2			1	1				
Itraconazole		2		1				1				

3.5.3. Disc diffusion method

By Disc diffusion method the antifungal resistance pattern revealed resistance of 73.9% ,68.4%,65.5%,57.5% and 19.2% in *Candida* species against Fluconazole, Clotrimazole, Miconazole, voriconazole and Itraconazole respectively. Specie wise antifungal resistance pattern is depicted in table4.

Table4: Antifungal resistance profile of Candida species by Disc diffusion method n(%)

Candida Species	Fluconazole	Clotrimazole	Miconazole	Voriconazole	Itraconazole
C.albicans	16 (51.6)	17 (54.8)	15 (48.4)	14 (45.2)	7 (22)
C.krusei	20 (100)	14 (70)	15 (75)	4 (55)	0
C.parapsilosis	16 (88.9)	16 (88.9)	16 (88.9)	15 (83.3)	6 (33.3)
C.glabrata	2 (50)	3 (75)	2 (50)	2 (50)	1 (25)
Total	54 73.9	50 (68.4)	48 (65.5)	35 (57.5)	14 (19.2)

3.5.4 Agreement strength between BMD and DD Methods

The Kappa value was obtained for analysis of agreement strength between DD and BMD methods used for identification of antifungal resistance. Broth -microdilution and Disc diffusion methods had very good agreement strength for Fluconazole, Voriconazole and Itraconazole and good agreement for Clotrimazole and Miconazole. (table 5)

Table5: Antifungal resistance profile of Candida species by Disc diffusion method n(%)

Antifungal agents	Kappa value	Agreement strength
Fluconazole	0.79	Very good
Clotrimazole	0.67	Good
Miconazole	0.54	Good
Voriconazole	0.84	Very good
Itraconazole	0.93	Very good

3.5.5 Comparison of resistance pattern among *C. albican* and NAC species

Comparison of resistance pattern between *Candida albicans* and nonalbicans *Candida* species is shown in table 6. The results revealed increased resistance in nonalbicans against Fluconazole than in *C. albicans* species. Statistically the difference was found to be significant (Pvalue0.001). Nonalbicans *Candida* also showed higher resistance against Clotrimazole and Miconazole than the *C. albicans*. The statistical difference was significant. For Clotrimazole P value 0.03 was found while for Miconazole P value 0.01 was found. Against Voriconazole also high résistance was found in NAC species, but no statistical difference was found (P value 0.548). Nonalbicans *Candida* showed higher sensitivity against Itraconazole as compared to *Candida albicans* but no difference was found statistically significant.

Table 6: Comparison of antifungal resistance profile of Candida albicans and Non-albicans Candida

Candida Species	**Fluconazole**	**Clotrimazole**	**Miconazole**	**Voriconazole**	**Itraconazole**
C. albicans	15 (48.4)	17 (54.8)	14 (45.2)	14 (45.2)	7 (22.6)
Non-albican *Candida*	39 (92.8)	32 (76.1)	30 (71.4)	23 (54.7)	7 (16.6)
P value	0.001***	0.036*	0.013*	0.548^{NS}	0.153^{NS}

***** =**Highly significant * =significant NS= Nonsignificant

3. Discussion

Vulvovaginal candidiasis is one of the reasons that females consult gynecologist. VVC is a common problem, affecting women worldwide.[2] The present study revealed a frequency of 43% of vaginal candidiasis. The results of the current study are in accordance with previous study[13]. The frequency of VVC in pregnancy is reported, 26.9% and 38% in by different researchers.[14, 15]

A high prevalence of *Candida* infection in pregnancy was seen was seen in Nigeria (62.5%) and Yemen (61.5%) as compared to the current study [2, 16]. The high occurrence observed may be due to restricted diagnostic facilities, insufficient knowledge, poor hygiene and dietary habits, increased l estrogens levels and use of antibiotics that inhibits the growth of normal body flora.

High prevalence (54.7%) of *Candida* infection was seen in 2nd trimester in our study. Similar to the current study, high frequency (60.2%) was a found in 2rd trimester of pregnancy. [17] In some studies, high *Candida* infection was found in 3rd trimester of pregnancy .[13, 18] The explanation behind this is that in pregnancy, generally during the second and third trimester there are elevated estrogen levels which causes deposition of glycogen in vaginal epithelium, which help the *Candida* species to flourish. Moreover, estrogen augments the *Candida* affinity for the cytosol receptor in epithelial cells of vagina.[7]

Although NAC were the dominant species found but *Candida albicans* (42.4%) was found to be most common specie in this study which was followed by *Candida krusei* (27.3%). A study conducted by Kan etal found *C. albican* as a predominant *Candida* spp, while *C. glabrata* was frequent among the NAC. [19] Results of the current study are in agreement with other study.[20]

Antifungal susceptibility tests revealed species of *Candida* had high resistance to Fluconazole (74.0%). Followed by Clotrimazole (67%), Miconazole (60.0%) and Voriconazole (50.7%) while least resistance was shown against Itraconazole (19.2%). In a study conducted in Pakistan in 2018 a resistance rates of 40.7%, 59.2%, 62%, and 10.2% were found in *Candida* against Itraconazole, Clotrimazole, Fluconazole, Voriconazole respectively.[17] The reason for increase in resistance may be that Fluconazole and Clotrimazole are the most common azoles and excessively used for the treatment of VVC. The overuse of Azoles antifungal has caused the eradication of the more sensitive *C. albicans*, which has resulted in increase in resistant NAC. [6] A study by Zida etal also revealed resistance rates which are in accordance with our study. [21] A low resistance to azoles in *Candida* species from other regions is reported as compared to this study.[5, 8] The reason may be that in our country fungal cultures and susceptibility testing are not performed and empirical treatment of VVC is in practice.

In the current study the comparison of the Broth microdilution and Disc diffusion method revealed a good agreement for all the tested azoles antifungal drugs. The results are similar to previous study.[, 22] More particle skills are required for BMD method as it is cumbrous. Disc diffusion method can be used on daily basis for diagnostic purpose as it is easy to perform. In the current study the NAC has revealed more resistance as compared to the *C albicans*, which is in accordance with the study conducted in south India .[23]

Conclusion

The NAC species were isolated more than *C. albicans*. Which has resulted in increase in resistance to the commonly used azoles antifungal. It is necessary to perform antifungal susceptibility tests for *Candida* species for providing resistance profile information to choose appropriate drug for treatment. There is need for continuous surveillance programs to study trends of resistance. Disc diffusion method can be used for investigating antifungal resistance pattern in *Candida* species in daily practice as it is less laborious.

Declaration of interest

There is no competing interest as declared by all authors.

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