ORIGINAL ARTICLE

Study of Azole Susceptibility and Virulence Patterns of Vulvovaginal Candida Species in Menoufia, Egypt

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ABSTRACT

Background: *Vulvovaginal candidiasis (VVC) is one of the most common forms of superficial fungal infections. Azoles are the first line antifungals used in the treatment and prevention of VVC.* ***Objectives:*** *This study aimed to determine the prevalence rate of VVC with evaluation of the potential risk factors. Also, to assess azoles susceptibility patterns with association to biofilm formation and to detect candidal virulence genes (HWP1, ALS1 and INT1) and ERG11 gene expression.* ***Methodology:*** *Evaluation of vaginal infection risk factors was achieved by a designed questionnaire. Candida strains isolated from high vaginal swab samples were identified up to species level by conventional methods. Assessment of antifungal susceptibility patterns (by disk diffusion method) and phenotypic detection of biofilm formation were also performed followed by molecular detection of virulence genes (HWP1, ALS1 and INT1) by multiplex PCR and ERG11 gene expression by real time PCR.* ***Results:*** *VVC represented 33.9% of vaginal infections. C. albicans was the predominant isolated species (62.4%). The highest resistance rate (40%) was observed to itraconazole and the lowest (12.9%) was to voriconazole. Biofilm formation rate was 51.8% by cultivation on Congo red agar and 57.6% by microtiter plate method. About, 79.6%, 71.4% and 81.6% of biofilm producers carried HWP1, ALS1 and INT1 virulence genes respectively. ERG11 gene Overexpression was detected in 31% of fluconazole resistant Candida isolates.* ***Conclusion:*** *Evaluation of VVC risk factors can help in the implementation of appropriate preventative measures. The elevated azoles resistance rates among Candida spp necessitates the critical need for new alternative therapeutic approaches*

INTRODUCTION

Candida species are major components of the human microbiome. In spite of their being commensals in healthy humans, they are considered opportunistic pathogens in immunocompromised patients causing variable infections ranging from superficial fungal infections (SFI) to systemic invasive life-threatening infections[1].

Vulvovaginal candidiasis (VVC) is one of the most common forms of SFIs. [2] Nearly, 75% of women will have at least one VVC episode throughout their lifetime, 50% of them will have second episodes. Moreover, 5-10% of them will suffer from recurrent vulvovaginal candidiasis (RVVC) that is defined as four or more episodes in 12 month period.[3]

Some pregnancy-related factors (e.g, weakened immunity; elevated level of sex hormones, glycogen deposition; low vaginal pH; decreased cell-mediated immunity), antibiotic overuse, diabetes, immunodeficiency disorders, contraceptives and several other hygienic and behavioral factors can be suggested as potential risk factors of candidiasis[4].

Candida albicans is the predominant cause of VVC. Furthermore, a significant shift in candidiasis etiology regarding non-albicans *Candida* (NAC) species (*C. krusei*, *C. parapsilosis*, *C. tropicalis*, and *C. dubliniensis)* has been reported [5]

Specific virulence factors encoded by *Candida* genes attribute the pathogenicity of *Candida* spp. These virulence factors include, transition from the yeast to hyphal form, adhesions, biofilm formation and secretion of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and hemolysins [6].

In *Candida* spp, hyphal wall protein1 (*HWP1*), agglutinin-like sequence 1 (*ALS1*) and integrin like protein (*INT1*) genes mediate adherence, hyphal and biofilm formation [7].

Azoles are the most common antifungal drugs and the first line therapy used in treating and preventing candidal infections including both imidazoles (e.g.

ketoconazole, clotrimazole and miconazole) and triazoles (e.g. fluconazole, voriconazole, itraconazole, ..). Azoles bind to the enzyme 14-α-demethylase which is encoded by *Erg11* gene, an important enzyme in ergosterol biosynthesis, thereby lowering the cell ergosterol levels. Fluconazole is the most common azole used for prophylaxis and treatment of VVC. However, its extensive empirical use resulted in evolution of resistant strains, therapeutic failures and recurrent infections. [1]

Overexpression of *ERG11* is linked with azole resistance in many fungi [8]. Several point mutations in *ERG11* have been identified in resistant clinical isolates. Many of these point mutations lower azole binding in the active site of the enzyme. [9]

This study aimed to determine the prevalence rate of VVC with evaluation of the potential risk factors. Also, to assess azoles susceptibility patterns with their association to biofilm formation and to detect candidal virulence genes (*HWP1*, *ALS1* and *INT1*) and *ERG11* gene expression among *Candida* spp.

METHODOLOGY

Study design and patients:

This cross sectional study was performed in Departments of, Medical Microbiology, Clinical Pathology, Public health, Obstetrics and Gynecology, Faculty of Medicine, Menoufia University during the period from March 2021 to May 2022. High vaginal swab (HVS) samples were taken from 251 women with clinical criteria of vulvovaginal infection (vulvar pruritus, vaginal erythema, edema, burning vaginal soreness, dysuria, dyspareunia, fissures and vaginal discharge) attended to Gynecological Clinics at Menoufia University Hospitals, Egypt. Written informed consent was obtained from each patient. The protocol of this study was approved by the Ethical Committee, Faculty of Medicine, Menoufia University (8-2022CPATH). Sample size was determined by open Epi program with power of study 80% and confidence level 95%.

Risk Factors Evaluation:

For evaluation of vaginal candidiasis risk factors, every female patient involved in this study had fulfilled a designed questionnaire (by epidemiological methods) including general information (age, residence, educational level and occupation); daily living habits (drinking sweet drinks, eating sweet foods, physical training and daily emotional state); hygienic habits (underwear material, frequency of alternation, wiping direction after toilet, frequency of intravaginal douching and non-menstrual care), medical history (diabetes, immunocompromised disease, prolonged antibiotic therapy and previous vaginitis,) and reproductive history (marriage status, pregnancy , contraceptive method or intrauterine device usage and frequency of sexual life)

Specimens processing:

Collected HVS samples were directly transferred to Microbiological laboratory. Direct microscopic examination of 10% potassium hydroxide wet mounted preparation and Gram stained smears was done. Samples were cultivated on Sabouraud dextrose agar (SDA) medium supplemented with chloramphenicol (0.5 g/l) (Oxoid, USA) and incubated at 37 °C and 25°C for 48–72 h .

Identification of Candida isolates:

The isolated *Candida* strains were preliminarily identified according to conventional methods by colonial appearance (smooth, white to cream colored colonies); Gram's staining (round to oval Gram-positive budding cells with or without pseudo-hyphae) and germ tube test[10]. Identification of isolated *Candida* up to species level was performed by detecting the produced color on *Candida* differential Chrom agar media (Hi Media). For example: green, blue, white, pinkish purple, white to cream and cream to white with slight purple center were interpreted as: *C. albicans*, *C. tropicalis*, *C. glabrata,* and *C. krusei*, *C parapislosis and C. kefyr*, respectively).[3]

Antifungal susceptibility testing:

Assessment of *candida* isolates susceptibility patterns to the following antifungal agents: fluconazole, itraconazole, clotrimazole, miconazole, ketoconazole, voriconazole, and nystatin was performed using the disc diffusion method. Then results were interpreted according to Clinical and Laboratory Standards Institute guidelines [11,5]

Phenotypic detection of biofilm formation:

Congo red agar method

Every *Candida* isolate produces black colonies onto Congo red agar (CRA) after incubation for 48 h at 35 ° C, was interpreted as biofilm forming isolate.[12]

Microtitre plate method:

After culturing of *Candida* isolates in the Sabouraud broth (SDB) at 28°C for 18 hours and adjusting to be 10^7 cfu / mL, they were distributed as 100 µL followed by distributing of 100 µl synthetic dextrose liquid (SDL) medium containing 2.5% glucose in each well of the 96-well microtitre plate and incubated for 48 h at 35 ° C. Negative control wells were inoculated with SDL only without *Candida*. The plate was emptied and wells were washed 3 times with 200 µl phosphate buffer. The wells were fixed with 200µL 95% ethanol for 45 minutes. Subsequently, wells were emptied, air dried and stained with 110 µL crystal violet (0.4%) for 45 minutes. After wells washing with sterile ultra-pure water, 200 µl of acetic acid (30%v/v) were added to release and dissolve the stain then optical density was measured at 595 nm and biofilm formation was interpreted regarding OD values: $0 \leq OD \leq 0.120$ (-), $0.121 \leq OD \leq 0.240$ (+),

0.241 ≤ OD ≤ 0.500 (++), OD ≥ 0.500 (+++), as negative, weak, moderate and strong respectively[7,12].

Molecular study

Detection of virulence genes (HWP1, ALS1 and INT1) by multiplex PCR:

Candidal DNA was extracted using QIAamp DNA Mini Kit (Qiagen-Germany). The used primers sequences and amplicon sizes were: ***HWP1*F:** 5'ATGACTCCAGCTGGTTC3'**R:** 5'TAGATCAAGAATGCAGC3'**(572bp),***ALS1***F:**5'GACTAGGAACAACAAATACCAGA3'**R:**5'CCAGAAGAAACAGCAGGTGA3'**(318bp) and *INT1*F:** 5'AAGCTCTGATACCTACACTAGCGA3' **R:**5'GTTAGGTCTAAAGTCGAAGTCATC3' **(239bp).**[7] The PCR reactions were as follows: initial denaturation at 95°C for 5min, followed by 32 cycles of 1min at 95°C, 1 min at 55°C, 1min at 72°C, and a final extension of 10min at 72°C. The products were visualized by electrophoresis on 1% agarose gels for 45 min at 70v.[13]

Evaluation of *ERG11* gene expression by real time PCR (RT-PCR):

Detection of *ERG-11* gene expression among *candida* spp was done by RT-PCR. The RNA extraction steps were carried out using the RNeasy Plus Mini Kit (Qiagen, Germany) as recommended by the manufacture instructions. RNA purification was performed using RNase-Free DNase Set Kit (Qiagen) followed by RNA qualitative assessment using a Nanodrop device (Thermo Scientific, USA). The OD A260/A280 nm ratio of 1.9–2 for all samples was obtained, indicating 90%-100% nucleic acid purity. All RNA was transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to kit instructions. cDNA was used as a real-time PCR template, utilizing SYBR Green II master blend (QuantiTect SYBR Green PCR Kit) in Applied Biosystems 7500, software version 2.0.1. (Applied Biosystems, USA). The reaction solutions included 10 µl of SYBR Green II Master Mix, 5 µl of cDNA and 1µl of each forward and reverse primer. The final reaction volume reached 20 µl by adding nuclease free water. The nucleotide sequence of *ERG11* gene primers was **F:** 5'-GTT GAA ACT GTC ATTGAT GG 3' **R:** 5'-TCAGAA CAC TGA ATC GAA AG 3'. *ACT1* gene with the following nucleotide sequence was used as a housekeeping gene **F:** 5' ACTGCTTTGGCTCCATCTTCT3' **R**: 5' TGTGGTGAACAATGGATGGAC3'. The thermal conditions were as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation for 30 sec at 93ºC, annealing and extension at 60°C for 1 min.[14,15]

Statistical analysis:

Data coding, validation and analysis were conducted by the Statistical Package for the Social Sciences (SPSS), version 22 (SPSS Inc, Chicago, IL, USA). Frequencies and proportions were used to present the data.

RESULTS

This study included 251 sexually active female patients (mean age=32±5.7years) with vulvovaginal infections attended to Gynecological clinics, Menoufia University Hospitals, Egypt from March 2021 to May 2022. Microbiological examination of obtained HVS samples revealed that 85 of them (33.9%) were infected with *candida* spp (VVC).

Risk factors of vulvovaginal infections were demonstrated in table 1. There were statistical significant differences between women with VVC and other women with *Candida* negative vaginal infections regarding: age, history of diabetes, prolonged antibiotic therapy, previous vaginal infection, pregnancy and methods of contraception.

Table 1: Risk factors of vulvovaginal infections:

Risk factors	Vulvovaginal Infections			
	Candida positive (n=85)	*Candida* negative (n= 166)	*P* value	Total (n=251)
General information				
Age				
<40 years	64 (75.3%)	98 (59%)	< 0.05	162 (64.5%)
>40 years	21 (24.7%)	68 (41%)		89 (35.5%)
Residence:				
Town	34 (40%)	78 (47%)	>0.05	112 (44.6%)
Village	51 (60%)	88 (53%)		139 (55.4%)
Educational level				
Secondary and below	46 (54.1%)	103 (62%)	>0.05	149 (59.4%)
College and above	39 (45.9%)	63 (38%)		102 (40.6%)
Occupation				
Employee	53 (62.4%)	95 (57.2%)	>0.05	148 (60%)
House wife	32 (37.6%)	71 (42.8%)		103(40%)
Daily living habits				
Drinking sweet drinks				
Frequently	44 (51.8%)	72 (43.4%)	>0.05	116 (46.2%)
Occasionally or never	41 (48.2%)	94 (56.6%)		135 (53.8%)

Risk factors	Vulvovaginal Infections			
	***Candida* positive (n=85)**	***Candida* negative (n= 166)**	***P* value**	**Total (n=251)**
Eating sweet foods				
Frequently	48 (56.5%)	86 (51.8%)	>0.05	134 (53.4%)
Occasionally or never	37 (43.5%)	80 (48.2%)		117(46.6%)
Physical training:				
Frequently	40 (47.1%)	99 (59.6%)	>0.05	139 (55.4%)
Occasionally or never	45 (52.9%)	67 (40.4%)		112 (44.6%)
Daily emotional state				
Comfort	11 (12.9%)	34 (20.5%)	>0.05	45 (17.9%)
Negative emotions	74 (84.1%)	132 (79.5%)		106 (82.1%)
Medical history				
Diabetes				
Yes	28 (32.9%)	19 (11.4%)	< 0.05	47 (18.7%)
No	57 (67.1%)	147 (88.6%)		204 (81.3%)
Immunocompromised disease				
Yes	7 (8.2%)	15 (9%)	>0.05	22 (8.8%)
No	78 (91.8%)	151 (91%)		229 (91.2%)
Prolonged antibiotic therapy				
Yes	54 (63.5%)	59 (35.5%)	< 0.05	113 (45%)
No	31 (36.5%)	107 (64.5%)		138 (55%)
Previous vaginitis				
Yes	46 (54.1%)	63 (38%)	< 0.05	109 (43.4%)
No	39 (45.9%)	103 (62%)		142 (56.6%)
Hygienic habits				
Underwear material				
Pure cotton	14 (16.5%)	43 (25.9%)	>0.05	57(22.7%)
others	71 (83.5%)	123 (74.1%)		194 (77.3%)
Frequency of alternation				
One day and less	33 (38.8%)	68 (41%)	>0.05	101 (40.2%)
More than one day	52 (61.2%)	98 (59%)		150 (59.8%)
Wiping direction after the toilet:				
Forward wiping	29 (34.1%)	53 (31.9%)	>0.05	82 (32.7%)
Backward wiping	56 (65.9%)	113 (68.1%)		169 (67.3%)
Frequency of intravaginal douching				
Occasionally or never	63 (74.1%)	129 (77.7%)	>0.05	192 (76.5%)
Frequently	22 (25.9%	37 (22.3%)		59 (23.5%)
Usage of pads in non-menstrual time				
Yes	17 (20%)	47 (28.3%)	>0.05	64 (25.5%)
No	68 (80%)	119 (71.7%)		187 (74.5%)
Reproductive History				
Marriage status				
Single	9 (10.6%)	16 (9.6%)	>0.05	25 (10%)
Married	77 (89.4%)	150 (91.4%)		226 (90%)
Pregnancy:				
Pregnant	**43 (50.6%)**	**56 (33.7%)**	**<0.05**	**99 (39.4%)**
Non-pregnant	**42 (49.4%)**	**110 (66.3%)**		**152 (60.6%)**
Contraceptive method:				
Hormones	41(48.2%)	45 (27.1%)	<0.05	86 (34.3%)
Condom	7 (8.2%)	8(4.8%)		15 (5.9%)
IUD	36 (42.4%)	103 (62.1%)		139 (55.4%)
Others (ligation and so on)	1(1.2%)	10 (6%)		11 (4.4%)
Frequency of sexual life				
More than twice a week	53 (62.4%)	100 (60.2%)		153 (61%)
Less than once a week	32 (37.6%)	66 (39.8%)	>0.05	98 (39%)

Candida albicans was the predominant isolated *Candida* spp (53/85, 62.4%), followed by *C.glabrata* (14/85, 16.47%), *C.tropicalis* (10/85, 11.8%), *C. parapsilosis* (5/85, 5.9%) and *C.krusei* (3/85, 3.53%). (figure1).

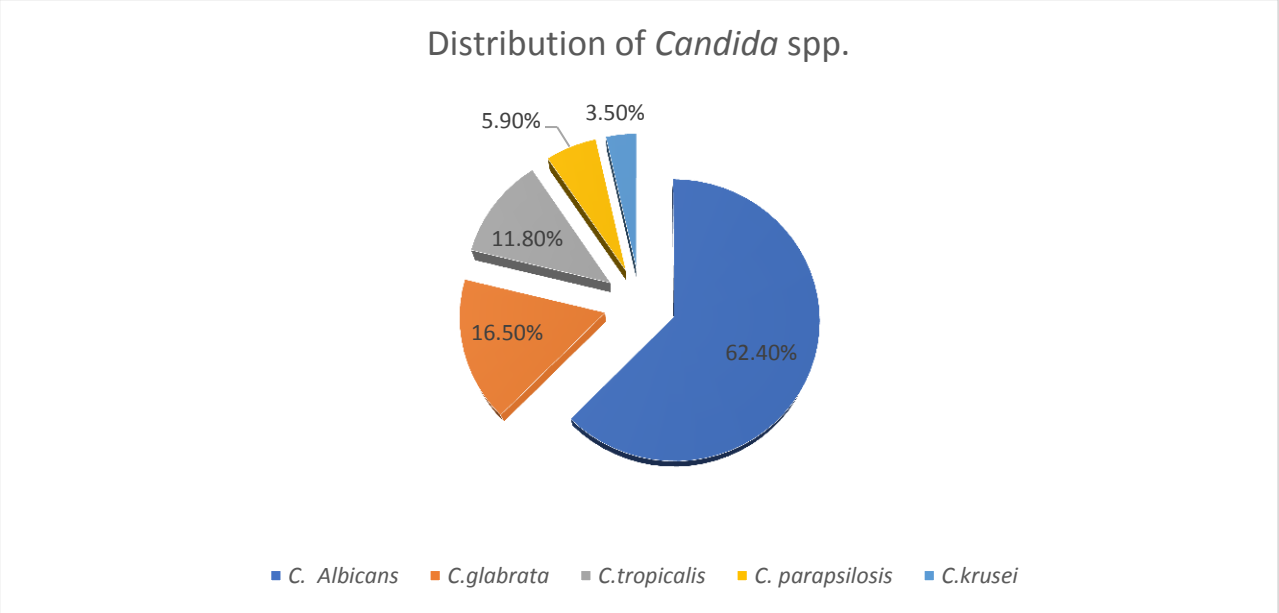

Figure 1: Distribution of *Candida* species

Antifungal resistance patterns by disk diffusion method were demonstrated in table 2. The highest antifungal resistance rate was observed to itraconazole (40%) followed by ketoconazole (38.8%), miconazole (35.3%), fluconazole (34.1%) and clotrimazole (31.8%). Taking in consideration that *C. krusei* has intrinsic resistance to fluconazole. While, the lowest antifungal resistance rate was observed to voriconazole (12.9%) followed by nystatin (18.8%).

Table 2: Antifungal resistance patterns

Antifungal agent	***C. albicans* (n=53)**	**Antifungal Resistance profile**					
			Non-albicans candida (n=32)				**All candida isolates (n=85)**
		***C. glabrata* (n=14)**	***C. tropicalis*** (n=10)	***C. parapsilosis*** (n=5)	***C. krusei*** (n=3)	**Total NAC (n=32)**	
	NO (%)	**NO (%)**	**NO (%)**	**NO (%)**	**NO (%)**	**NO (%)**	**NO (%)**
Fluconazole (10µg),	15 (28.3%)	6(42.9%)	4 (40%)	1(20%)	3(100%)*	14 (43.8%)	29 (34.1%)
Itraconazole (10µg)	23(43.4%)	7 (50%)	3(30%)	0(0%)	1(33.3%)	11 (34.4%)	34 (40%)
Clotrimazole (10µg)	17(32.1%)	5 (35.7%)	2(20%)	2 (40%)	1(33.3%)	10 (31.3%)	27(31.8%)
Miconazole (30 µg)	18 (34%)	6 (42.9%)	2 (20%)	3 (60%)	1 (33.3%)	12 (37.5%)	30 (35.3%)
Ketoconazole (30 µg)	16 (30.2%)	8 (57.1%)	5 (50%)	2 (40%)	2 (66.7%)	17 (53.1%)	33 (38.8%)
Voriconazole (1 µg)	7 (13.2%)	3(21.4%)	1 (10%)	0 (0%)	0 (0%)	4 (12.5%)	11 (12.9%)
Nystatin (100 IU)	9 (17%)	4 (28.6%)	2(20%)	0 (0%)	1(33.3%)	7 (21.9%)	16 (18.8%)

*: intrinsic resistance.

Cultivation on Congo red agar revealed that 44/85 (51.8%) *Candida* isolates were biofilm producers. While by Microtitre method, 49/85 *Candida* isolates (57.6%) were biofilm producers with no statistical significant difference between the two methods as illustrated in table 3.

Table 3: Biofilm production

Candida spp.	Cultivation on Congo red agar		Microtitre method		*P* value
	Biofilm positive	Biofilm negative	Biofilm positive	Biofilm negative	
	No. (%)	No. (%)	No. (%)	No. (%)	
C. albicans (n=53)	**27 (50.9%)**	**26 (49.1%)**	**30 (56.6%)**	**23 (43.4%)**	
Non- albicans Candida					
***C.glabrata* (n= 14)**	8 (57.1%)	6 (42.9%)	9 (64.3%)	5 (35.7%)	**>0.05**
***C.tropicalis* (n=10)**	6 (60%)	4 (40%)	6 (60%)	4 (40%)	
***C.parapsilosis* (n=5)**	2 (40%)	3 (60%)	2(40%)	3 (60%)	
***C.krusei* (n=3)**	1 (33.3%)	2 (66.7%)	2 (66.7%)	1 (33.3%)	
Total (n=85)	**44 (51.8%)**	**41 (48.2%)**	**49(57.6%)**	**36 (42.3%)**	

There were statistically significant differences between biofilm producers and non- producers regarding fluconazole, voriconazole and nystatin resistance patterns and high statistically significant differences between them regarding susceptibility to itraconazole, clotrimazole, miconazole, ketoconazole and carriage of *HWP1*, *ALS1* and *INT1* virulence genes as illustrated in table 4.

Table 4: Comparison between biofilm producers and non-producers *Candida* isolates regarding antifungal resistance patterns and prevalence of virulence genes.

	Biofilm producers (n=49)	Biofilm non-producers(n=36)	P value
	No (%)	No (%)	
Antifungal agents resistance patterns			
Fluconazole (n=29)	22 (44.9%)	7 (19.4%)	<0.05
Itraconazole (n= 34)	29 (59.2%)	5 (13.9%)	<0.001
Clotrimazole (n= 27)	24 (49%)	3 (8.3%)	<0.001
Miconazole (n= 30)	26 (53.1%)	4 (11.1%)	<0.001
Ketoconazole (n=33)	31 (63.3%)	2 (5.6%)	<0.001
Voriconazole (n=11)	11(22.4%)	0 (0%)	<0.05
Nystatin (n= 16)	14 (28.6%)	2 (5.6%)	<0.05
Virulence genes			
***HWP1* (n=44)**	39 (79.6%)	5 (13.8%)	<0.001
***ALS1* (n=43)**	35 (71.4%)	8 (22.2%)	<0.001
***INT1* (n=46)**	40 (81.6%)	6 (16.7%)	<0.001

While, only 9 fluconazole resistant *Candida* isolates ((31%) showed overexpression of *ERG11* gene (table 5), there was statistical significant difference between fluconazole sensitive and fluconazole resistant *Candida* isolates regarding *ERG11* gene expression (table 6)

Table 5: The Prevalence of *ERG-11* gene overexpression in fluconazole resistant isolates

Over expression of *ERG 11* gene in fluconazole resistant isolates	No.	%
Yes	9	31%
No	20	69%
Total	29	100%

Table 6: *ERG-11* gene expression by RT-PCR.

	ERG11 gene expression	
	Fluconazole sensitive isolates (n=56)	Fluconazole resistant isolates (n=29)
Mean ±SD	2.97± 0.16	3.63±0.147
t.test	3.34	
p- value	<0.05	

DISCUSSION

Vulvovaginal Candidiasis is a common vaginal infection among sexually active young adult females. Nearly, 75% of women will experience at least one episode of VVC throughout their lifetime.[3]. Various species of *Candida i.e, C albicans and non-albicans*

Candida are associated with VVC. Empirical administration of oral or topical azoles had resulted in evolution of azoles resistance, critical issue that can be challenging to clinicians. [13].

Among 251 female patients with symptomatic vulvovaginal infections included in our study, 85 (33.9%) had VVC . Similar prevalence rate (32.7%) was observed in India[16]. On the other hand, higher rates (43%, 57.3% and 58.5%) [3,19,20] and lower rate (5%) [21] were reported in previous studies.

Regarding risk factors, VVC was more commonly observed in this study among patients: aged less than 40 years (75.3%), lived in villages (60%), educated to secondary or below (54.1%),employee (62.4%), had frequently drunk sweet drinks (51.8%), eaten sweet food (56.5%), with limited daily physical training (52.9%) had negative emotions (84.1%), , with prolonged antimicrobial therapy (63.5%), with previous vaginitis (54.1%), did not wear pure cotton underwear (83.5%), did not change underwear every day (61.2%), did not follow forward wiping after toilet (65.9%), did not use pads in non-menstrual time (80%), married (89.4%), pregnant (50.6%), using hormonal contraceptive methods (48.2%) and those had frequency of sexual life more than twice a week (64.6%) . Comparable results were reported in previous researches[16,17,18,22] .

Young women have adverse factors of risky sexual behaviors, physiological and tissue changes resulted from reproductive hormones. Diabetes mellitus, sweet drinks and sweet foods have the ability to increase the glycogen (an excellent carbon source) concentrations in vaginal mucosa and vaginal secretions which promote *Candida* spp adherence and proliferation [17]. Pregnancy suppresses immunity; elevates level of sex hormones, causes glycogen deposition; decrease vaginal pH and cell-mediated immunity [4]. Misuse of antibiotics will destroy vaginal normal flora leading to overgrowth of *candida* spp[17]. Reproductive hormones produced from ovaries in young females, oral contraceptive pills and pregnancy maintain the acidic pH and enhancing the yeast adherence to vaginal epithelial cells. Intravaginal douching disrupts the vaginal flora and causes damages to vaginal and rectal tissues Wearing tight clothes enhances friction and maceration, thereby raising the local acidity and therefore the fungal infection. [17]

Candida albicans was the predominant *Candida* spp (٦٢.4%) associated with VVC in this study. In agreement with previous studies [2,5,13,20]**.** On the other hand, *Kombade et al,* revealed that the prevalence of NAC was 81% with *Candida glabrata* being the most common *Candida* isolates (58%) [3]**.** Although, *C. albicans* may remain to be the most common VVC associated *Candida* spp but rising trend in NAC species incidences is observed especially in recurrent vulvovaginal candidiasis (RVVC) which may be due to the excessive use of antifungal agents that can suppress the growth of *C. albicans* with limited effect on NAC. [3]

In the current study, the highest antifungal resistance rate among vaginal *Candida* isolates was observed to itraconazole (40%) followed by ketoconazole (38.8%), miconazole (35.3%), fluconazole (34.1%) and clotrimazole (31.8%). This coordinates with other researches [3,5]. Structural similarity of different members of azole family had led to appearance of cross-resistance between them. Recently, researchers found that most fluconazole-resistant strains were also resistant to itraconazole and ketoconazole. [23,5,7]. While, the lowest antifungal resistance rate was observed to voriconazole (12.9%) followed by nystatin (18.8%) rendering them the most effective antifungal drugs, correlating with the studies done by *Kombade et al, Das et al,* in India, *Mohammadi et al*, in Iran and *Mokhtar et al* in Egypt. [3,24, 13,5]

In our study, 51.8% and 57.6% of VVC-associated *Candida* spp were biofilm producers by cultivation on Congo red agar and microtiter plate respectively with no statistical significant difference. Variable biofilm production rates (54.3%, 44%) were detected in previous studies. [13, 24]. Also, in our study we detected that; biofilm forming isolates were more resistant to antifungal agents than non- forming with statistical significant difference. The same result was reported in previous studies[25,7,26]. Biofilm formation is an important virulence factor due to its significant role in the pathogenesis, and adherence of *Candida* spp to host cells. Also, it is associated with antifungal resistance as it reduces drug penetration through biofilm matrix, decreases the nutritional needs in *Candida* spp which will lead to their persistence. Also, biofilm formation increases the expression rates of resistance genes through induction by surface contact and persistence of a small number of cells. [13,27]

In the current study, 79.6%, 71.4% and 81.6% of biofilm producers and 13.8%, 22.2% and 16.7% of biofilm non producers had carried *HWP1, ALS1* and *INT1* virulence genes respectively. Also, *Sherif et al* in Egypt observed high prevalence of *HWP1, ALS1*and *INT1* virulence genes, (96.6%, 96.6% and 98.3%, respectively) among biofilm –producing *C. albicans* compared to biofilm non-producing isolates (50%,21.4% and 35.7%, respectively). [7]on the other hand, *İnci et al.* in Turkey reported very low *HWP1* (3.9%) and *ALS1* (48%) frequency rates [28]. Variations in the type of clinical samples, the quantity of isolates, and the diagnosis methods may all affect the prevalence of virulence genes.

In this study, among the 29 fluconazole-resistant candida isolates, only 9 isolates (31%) showed overexpression of the *ERG11* gene when compared with sensitive isolates, indicating that there are other involved factors. This agrees with the results of *Rosana et al* [29] , *Mohamed et al* [14], *Fathi* [30] and Maheronnaghsh

et al.[31] So, further investigations of other azoles resistance mechanisms is recommended. On the contrary, *Zhang et al*[23] and *Salari et al* [32] reported that there was no difference in the expression of *ERG11* genes among the fluconazole-susceptible and resistant strains.

CONCLUSIONS

Evaluation of VVC risk factors can help in the implementation of appropriate preventative measures that, in turn, will reduce the prevalence of such infections. Proper identification of *Candida* spp and their antifungal susceptibility testing prior to initiate medical therapy is essential enhancing antifungal selection, patient response and prevent emergence of recurrent infections with multidrug-resistant strains. Structural similarity of different members of azole family has led to appearance of cross-resistance between them. Biofilm formation is associated with elevated antifungal resistance rates rendering the treatment of biofilm forming isolates more difficult and necessitates different lines of treatment with concerning new alternative therapeutic approaches. Fluconazole resistance was associated with *ERG11* gene over-expression in only 31% of isolates so, further investigations of other azole resistance mechanisms are recommended.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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