Efflux Pump Mediated Resistance to Fluconazole in Candida glabrata in Vulvovaginal Candidiasis Patients

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Abstract: Vulvovaginal candidiasis is a common fungal infection that affects the female genital system. Resistance to antifungals is an emergent problem worldwide. We aimed to detect the prevalence of different Candida species associated with vulvovaginal candidiasis, determine their antifungal susceptibility pattern, and evaluate the molecular mechanisms associated with Fluconazole resistance.

Methodology: This study included 300 patients. Candida species have been identified phenotypically. Antifungal susceptibility was tested using a disc diffusion method. The molecular mechanisms of Fluconazole-resistance were determined by analyzing the expression levels of Fluconazole target, efflux pump and efflux pump-regulator genes by RT-PCR.

Results: Candida spp were detected in 75/300 (25%) of cases. The most frequently isolated species was C. albicans (61.8%), whereas the predominant species of non-albicans was C. glabrata (29%). Nystatin was the most effective agent. Fluconazole-resistance was observed markedly in C. glabrata (54.5%), and efflux-pump was the predominant mechanism of resistance, which was associated with overexpressed CgPDRI, CgCDR1 and CgSNQ2 genes. Upregulation of the efflux-pump genes and their regulator were associated with cross-resistance to different azoles.

Conclusion: C. glabrata is a common cause of non-albicans vulvovaginal candidiasis. The majority of clinical resistance in C. glabrata is attributed to the upregulation of efflux-pump genes.

Keywords: Vulvovaginal candidiasis; C. glabrata; Fluconazole resistance; efflux pump.

1. INTRODUCTION:

One of the most common fungal infections affecting women of childbearing age is vulvovaginal candidiasis (VVC). Candida albicans (C. albicans) is the most frequent cause of
VVC. There is, nevertheless, a marked change in the aetiology of VVC to the non-albicans Candida (NAC) species. The majority of NAC vulvovaginitis is caused by *C. glabrata* (1). Vulvovaginal candidiasis encountered at least one time by the majority of the women of reproductive age all through their lifetime and some of them experience recurrence (2). While an acute, simple episode of vaginitis can be diagnosed and treated easily, women infected with resistant species may visit many health care workers with several tried therapeutic agents. These patients frequently act as a therapeutic confront for health care providers. Besides, pain, discomfort, anxiety, and disturbance in sexual function, vaginal candidiasis may increase a women’s risk of getting other sexually transmitted diseases (3).

Azole drugs are frequently prescribed for Candida infections. The azole antifungals inhibit Candida by targeting lanosterol 14-α-demethylase, a crucial step in biosynthesis of ergosterol. Fluconazole is the most common azole antifungal agent, while it is used as first-line for the treatment candidiasis, its efficacy has been decreased by the development of resistance, especially in *C. glabrata* (4).

Resistance to antifungal drugs is an emergent problem worldwide, resulting in increased difficulty of the choice of effective antifungal therapy (5).

Knowledge of the basis of resistance to azole antifungal is important to conserve this class of antifungal and get rid of this clinical problem (4). In the azole-resistant *C. glabrata* isolates, regular constitutive upregulation of multidrug transporters of the ATP-binding cassette transporter was detected. These transporters are encoded by several genes including *CgCDR1*, *CgCDR2*, and *CgSNQ2* and regulated by the zinc finger transcription factor *CgPdr1* (6).

Increasing reports of azole resistance in *C. glabrata* isolates from Egypt have been published over the past decade (7, 8, 9), however, literature addressing their molecular mechanisms is scarce.

2. MATERIALS AND METHOD

2.1. Study Design
Between August 2018 and June 2019, a hospital-based cross-sectional study of 300 patients presented with a clinical picture of VVC presented at the outpatient clinics of Obstetrics and Gynecology at Al-Azhar Assiut University Hospital was performed. Patients who had uterine bleeding or used vaginal douching within the last few hours were excluded from the study. The ethical approval of the research was received from the ethics committee of the Faculty of Medicine, Assiut University, and carried out in compliance with the terms of the Helsinki Declaration (approval number 17-200-164). Informed consent from all the included patients was obtained before the collection of specimens. Patients' clinical data and associated risk factors were assessed.

2.2. Sample processing
Vaginal swabs were collected from the posterior fornix of the vagina by sterile swabs through Cusco's speculum. All swabs were inoculated on SDA (Himedia, Mumbai, India) and incubated for 24-48 hours at 37°C. By colony morphology and Gram stain, Candida isolates on SDA have been defined.

2.3. Phenotypic identification of different Candida species
The isolated yeasts have been identified phenotypically by Germ tube formation (10), growth at 45°C for differentiation between *C. albicans* isolates from the *C. dublinensis* isolates (12), Cornmeal agar (Himedia, Mumbai, India) (13). Hicrome Candida Differential agar (Himedia,
Mumbai, India), and KB006 HiCandida Identification Kit (Himedia, Mumbai, India) which were performed according to manufacturers’ instructions.

2.4. Antifungal susceptibility
Six antifungal agents, Fluconazole (25 μg), Itraconazole (10 μg), Voriconazole (1 μg), Nystatin (100 U), Miconazole (50 μg) and Amphotericin-B (100 U), were used to perform an antifungal susceptibility test on Candida isolates by disc diffusion method (Himedia, India). In brief, an inoculum with a turbidity of 0.5 McFarland level for each isolate in sterile 0.85% saline was streaked on Mueller-Hinton agar complemented with 0.5 μg/ml methylene blue dye and 2% glucose (11), plates were incubated at 35°C for 24h. Inhibition zones were interpreted according to CLSI interpretive breakpoints (12).

2.5. Fluconazole resistance genes expression quantification by Real time PCR

2.5.1. RNA extraction
Total RNA has been derived from C. glabrata isolates using Direct-zol™ RNA miniprep reagent (Cat.No. R2051) (Zymo research, California, USA) according to the manufacturer’s instructions. Briefly, the isolates have been grown for 24 hours at 37°C in yeast peptone dextrose (YPD) broth (Himedia, India). Then 1ml of each specimen was centrifuged for two minutes at 2000 xg, the pellet was homogenized in 600 ul of TRIzol™ reagent with vortex, the homogenate was centrifuged at 12000 xg for 2 min, and then the supernatant moved to the RNase-free Eppendorf tube, adding an equivalent volume of ethanol (95-100%) to the lysed sample in TRIzol™ reagent and was well mixed, then the mixture was moved into a Zymo-Spin Column after three washes and DNase I treatment. The RNA was eluted in an RNase-free Eppendorf tube, the RNA concentration and purity were measured using a NanoDrop spectrophotometer (NanoDrop 2000C, ThermoFisher, USA).

2.5.2. Reverse transcription
Reverse transcription has been performed on 500 ng of total RNA using COSMO cDNA synthesis Kit (Willowfort, UK) according to manufacturers’ instructions. The reaction occurred in a thermal cycler (T3 Thermocycler, Germany) with a single amplification cycle and incubation time of 5 minutes at 25°C, 15 minutes at 45°C and 5 minutes at 85°C. Under the same reverse transcription reaction conditions, all the samples tested were transcribed.

2.5.3. Real time-qPCR analysis
The expression levels of Fluconazole resistance genes (CgERG11, CgCDR1, CgSNQ2, and CgPDR1), as well as the housekeeping gene (β actin used as a normalizing gene), were carried out by using quantitative real-time PCR. Primers used were tested by primer BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and were mentioned in the table (1). The expression levels of genes were performed using the Step One Plus™ Real-Time PCR Systems (Applied Biosystems, USA) in 20 μl PCR reaction mixture containing, 10 ul HERA Plus SYBR Green Master mix (Willowfort, UK), 1 µl of each primer solution (10 µM), 5 µl cDNA sample and water was added to complete the volume to 20 μl. The conditions for thermal cycling were 95°C for 4 minutes, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C, followed by melting curve analysis. The relative expression was determined using the 2-ΔΔCt Method (13). Fluconazole susceptible C. glabrata clinical isolates were used as a calibrator isolate for gene expression analysis.

2.6. Statistical analyses:
GraphPad Prism 8.4 (GraphPad, La Jolla, CA, USA) has been used for statistical analyses. Data were expressed as mean ± standard deviation or standard error. Using the χ2 or Fisher's
exact test, categorical variables were compared, and the Mann-Whitney U test was used for continuous variables. At $P < 0.05$, the difference was statistically significant.

Table 1. Primers for RT-qPCR analysis of target gene expression(13).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer and probe sequence (5′→3′)</th>
<th>Gene number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CgACT1</td>
<td>F: TTGGACTCTGTGTGACGGGTGTGTTA R: AAATAAGGGTGTCGGCAAGAAA</td>
<td>CAGL0K12694g</td>
</tr>
<tr>
<td>CgERG11</td>
<td>F: TGTCTTGTAGGGGTGGTGAACCA R: CTTGCTTCTACGCAACAAAGCA</td>
<td>CAGL0E04334g</td>
</tr>
<tr>
<td>CgCDR1</td>
<td>F: AGATGTGTGTGTTGTTGCTGTCTCAAGAC R: CCGGATACATGGCACAACCA</td>
<td>CAGL0M01760g</td>
</tr>
<tr>
<td>CgSNQ2</td>
<td>F: GGCGGAAGATCGACGAAG R: GGGCGCAGCGGCATGATA</td>
<td>CAGL0I04862g</td>
</tr>
<tr>
<td>CgPDR1</td>
<td>F: AACGATTATACTCAATTGCAACAG R: CCTCACAATAAGGAAAGTCTGCC</td>
<td>CAGL0A00451g</td>
</tr>
</tbody>
</table>

3. RESULTS

3.1. Prevalence of vulvovaginal candidiasis patients

Three hundred vulvovaginal swabs were obtained from 300 patients presented with a clinical picture indicative of VVC and cultured on SDA. Candida isolates were detected in 75 patients. Seventy-six Candida isolates were recovered from 75 patients as one patient had a mixed infection by two different isolates.

3.2. Risk factors associated with vulvovaginal candidiasis:

In this study the association between VVC and many risk factors (age, hormonal use, antibiotics, steroid, IUD, DM, history of vaginitis and frequent intravaginal douches) were assessed; these risk factors are listed in table (2). The age of the studied patients ranged between 18 to 55 years with mean 34.5 ± 8.9 years. Out of the 75 vulvovaginal candidiasis patients 69 (92%) aged ≤ 40 years, the remaining 6 patients (8%) aged above 40 years with mean age 32.6 ± 7.1. In this study, the statistical analysis showed that women ≤ 40 years of age had a threefold higher risk of developing VVC than older women (OR=3.1, $P=0.009$), also this study showed that patients with frequent intravaginal douching were at a higher risk for acquiring VVC. ($P = 0.00$). Moreover, ladies who had a prior history of vaginitis were more probable to have VVC than those who did not (RR: 1.9, OR: 2.3, $P= 0.002$). While no statistically significant difference was found among patients with VVC and vulvovaginitis non-candidiasis patients regarding the consumption of current hormonal contraception, the recent usage of antibiotics, taking steroid therapy, presenting with IUD in situ ($P > 0.05$).

3.3. Identification of Candida species:

Based on the results of the different phenotypic tests used for the identification of the different Candida species, the predominant isolated species was *C. albicans* (47 isolates, 61.8%) followed by *C. glabrata* (22 isolates, 29%), *C. krusei* (5 isolates, 6.6%), and lastly *C. parapsilosis* (2 isolates, 2.6%). 74 vulvovaginal swabs (98.3%) showed the growth of only one species of Candida while one sample (1.3%) showed the growth of more than one species of Candida (*C. albicans* and *C. glabrata*).

3.4. Antifungal susceptibility:

The sensitivity of 76 Candida isolates to antifungals were tested for 2 polyenes (Nystatin and Amphotericin B) and 4 azoles (Fluconazole, Voriconazole, Itraconazole, and Miconazole) by
the disc diffusion method, Nystatin exhibited an excellent efficacy against all Candida spp except one C. glabrata isolate was resistant (1.3%). A good activity of Amphotericin B was detected against C. parapsilosis, C. albicans and C. glabrata (100%, 89.8 and 81.8%) respectively, but less effect was observed against C. krusei (60%). In this study susceptibility to different azoles drugs was various among different Candida spp. Fluconazole and Voriconazole showed outstanding efficacy against 47 C. albicans isolate with a sensitivity of 100%, less susceptibility of C. albicans was detected to Itraconazole (80.9%) and notably little effect of Miconazole against C. albicans (23.4%) was recorded. Regarding C. glabrata Miconazole was the most effective azole drug with (95.5%) sensitivity. Voriconazole showed (68.1%) sensitivity, it has been observed that Fluconazole and Itraconazole had the least effect on C. glabrata with (45.5 % and 36.4%) sensitivity. Regarding the 12 Fluconazole-resistant C. glabrata isolates a detailed analysis of cross-resistance among the four tested azoles revealed that one isolate was resistant to all azoles, three isolates were resistant to three azoles (Fluconazole, Itraconazole, and Voriconazole), and 8 isolates were resistant to itraconazole and Fluconazole. C. krusei was the most resistant species to azole drugs among different Candida species in this study; all isolates were resistant to Fluconazole and Itraconazole. Miconazole and Voriconazole, resistance rates were 60% and 40%, respectively. C. parapsilosis two isolates showed complete susceptibility to all tested azoles as shown in table 3.

Table 2. Risk factors of vulvovaginal candidiasis:

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Total No. of patients (300)</th>
<th>No. of VVC</th>
<th>% of infection</th>
<th>Relative risk</th>
<th>OR* (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 40</td>
<td>272</td>
<td>69</td>
<td>28</td>
<td>2.5</td>
<td>(1.27-7.61)</td>
<td>0.009*</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>28</td>
<td>6</td>
<td>11.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormonal use</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>45</td>
<td>15</td>
<td>33.3</td>
<td>1.42</td>
<td>(0.82-3.22)</td>
<td>0.161</td>
</tr>
<tr>
<td>No</td>
<td>255</td>
<td>60</td>
<td>26.6</td>
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<td>Antibiotics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>25</td>
<td>5</td>
<td>20</td>
<td>0.79</td>
<td>(0.265-2.024)</td>
<td>0.55</td>
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<tr>
<td>No</td>
<td>275</td>
<td>70</td>
<td>25.5</td>
<td></td>
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<tr>
<td>Steroid</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>9</td>
<td>4</td>
<td>44.4</td>
<td>1.82</td>
<td>(0.648-9.48)</td>
<td>0.17</td>
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<tr>
<td>No</td>
<td>291</td>
<td>71</td>
<td>24.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IUD use</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>2</td>
<td>10</td>
<td>0.38</td>
<td>(0.071-1.39)</td>
<td>0.18</td>
</tr>
<tr>
<td>No</td>
<td>280</td>
<td>73</td>
<td>26.1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>1</td>
<td>14.3</td>
<td>0.566</td>
<td>(0.058-4.1)</td>
<td>0.44</td>
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<tr>
<td>No</td>
<td>293</td>
<td>74</td>
<td>25.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of vaginitis</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>146</td>
<td>48</td>
<td>32.9</td>
<td>1.9</td>
<td>(1.34-3.95)</td>
<td>0.002*</td>
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<tr>
<td>No</td>
<td>154</td>
<td>27</td>
<td>9.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency of intravaginal douching</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occasionally or never</td>
<td>156</td>
<td>54</td>
<td>34.6</td>
<td>0.42</td>
<td>(0.183-0.569)</td>
<td>0.00*</td>
</tr>
<tr>
<td>Frequently</td>
<td>144</td>
<td>21</td>
<td>14.5</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*OR: odds ratio     *p-value <0.05: statistically significant
Table 3. Analysis of antifungal sensitivity pattern in different Candida spp. by disc diffusion method:

<table>
<thead>
<tr>
<th>Species (No.)</th>
<th>Antifungal</th>
<th>Susceptible</th>
<th>Susceptible dose dependent</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N0.</td>
<td>%</td>
<td>N0.</td>
</tr>
<tr>
<td>C. albicans (47)</td>
<td>Fluconazole</td>
<td>47</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>47</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>47</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Amphotericin</td>
<td>42</td>
<td>89.8</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>38</td>
<td>80.9</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>11</td>
<td>23.4</td>
<td>17</td>
</tr>
<tr>
<td>C. glabrata (22)</td>
<td>Fluconazole</td>
<td>10</td>
<td>45.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>15</td>
<td>68.1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>21</td>
<td>95.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Amphotericin</td>
<td>17</td>
<td>77.3</td>
<td>2</td>
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<td></td>
<td>Itraconazole</td>
<td>8</td>
<td>36.4</td>
<td>2</td>
</tr>
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<td></td>
<td>Miconazole</td>
<td>21</td>
<td>95.5</td>
<td>0</td>
</tr>
<tr>
<td>C. kurzi (5)</td>
<td>Fluconazole</td>
<td>3</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>3</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Amphotericin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>2</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>C. parapsilosis (2)</td>
<td>Fluconazole</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Voriconazole</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Amphotericin</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Itraconazole</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

3.5 Expression of Fluconazole resistance genes by quantitative real-time PCR:

The expression levels of Fluconazole resistance genes (ERG11, CDR1, SNQ2, and PDR1) in 22 C. glabrata isolates were quantified and normalized relative to the housekeeping gene, B-actin.

C. glabrata isolates were the most frequent Fluconazole-resistant candida species in this study; hence they were selected for the evaluation of the molecular mechanisms of resistance to Fluconazole.

Quantitative RT–PCR experiments revealed that the mean relative gene expression levels of ERG11 was 1.43 ± 0.53 (mean ± standard error). No statistically significant difference was found in the levels of ERG11 expression in resistant isolates compared to susceptible isolates (P-value 0.159) (Fig 1a).

The mean relative gene expression level of CDR1 was 3.45 ± 0.69, a statistically significant difference in the CDR1 expression level was detected between resistant isolates and susceptible isolates (P < 0.0001) (Fig 1 b). The mean relative gene expression levels of SNQ2 gene was 3.71±1.32, with a statistically significant difference in the SNQ2 expression levels between resistant and susceptible isolates (P = 0.038) (Fig 1c).
As regards, PDR1 gene the mean relative gene expression levels was 1.56 ± 0.17, the PDR1 expression levels showed a statistically significant difference among resistant and susceptible isolates (P = 0.0002) (Fig 1d).

It was observed that the most frequent overexpressed gene in Fluconazole resistant C. glabrata isolates was PDR1 (91.7%), followed by CDR1 (83.3%) and SNQ2 (75%), while EGR11 was overexpressed only in (33.3%).

Simultaneous upregulation of more than one transporter genes of efflux pump mechanism (CgCDR1 and CgSNQ2) and their regulator gene CgPDR1 was noticed among the studied Fluconazole-resistant C. glabrata isolates. It was found that CgCDR1, CgSNQ2, and CgPDR1 were simultaneously expressed in six isolates, while CgCDR1 and CgPDR1 were concurrently overexpressed in three isolates, two isolates upregulated CgPDR1 and CgSNQ2, and only one isolate upregulated CgCDR1 and CgSNQ2 Concurrently.

**Figure 1.** The mean fold change in the expression levels of the different tested genes.
a) CgERG 11  b) CgCDR1  c) CgSNQ2  d) CgPDR1.

4. DISCUSSION

Vaginal candidiasis is a widespread fungal infection of the female genital system induced mainly by C. albicans that may influence large numbers of reproductive-age women (14).
Though vulvovaginal candidiasis is extensively distributed all over the world, some socioeconomic factors can impact its incidence. So, service planning managers should use updated epidemiological data from each area (15). In the current research, the incidence of vulvovaginal candidiasis was 25% of all enrolled vulvovaginitis patients. A nearly similar prevalence rate (26%) was detected in Iran (16) and in Egypt 27.8% (8).

Also, this result is well compared to many other researches showing incidence rates of VVC among 20 % and 30 % (17, 18, 19, 20, 21). Other research, however, recorded a much higher VVC occurrence which accounted for prevalence rates of VVC between 41% and 84.5% (22, 23, 24, 25). In contrast, a much lower frequency of VVC was recorded in Brazil (5.44%) (26). The difference in the numbers of patients for each area, socioeconomic background of specimen patients, patient awareness of personal hygiene, and self-treatment could be attributed to this variation (15).

As the occurrence of VVC between women of reproductive age from different ethnicities and areas is different, it is essential to examine the risk factors for VVC between women of childbearing period in order to provide a reference for the treatment and prevention of VVC (2).

In this study, the association between VVC and many risk factors including age, hormonal use, antibiotics, steroid, IUD, DM, history of vaginitis, and frequent intravaginal douches were assessed.

Out of the 75 Vulvovaginal candidiasis patients 69 (92%) aged ≤ 40 years, the remaining 6 patients (8%) aged above 40 years with mean age 32.6 ± 7.1.

In this study, the statistical analysis found that women ≤ 40 years of age had a threefold higher risk of developing VVC compared to older women (OR=3.1, P=0.009). This outcome was agreed with Zeng et al, who found that women under the age of 40 had a twofold higher risk of CVV (OR= 2.431, P= 0.032) than older women (2), also other studies reported that the peak of vaginal candidiasis occurs among 20 and 40 years of age (27, 28). This might be attributed to higher sexual activity, use of various contraceptives hormonal and physiological changes in this age group. From the other hand, age progression decreases the influence of the estrogen hormone in ladies that may result in decreased rates of infection in older ladies. The majority of ladies older than 46 years have entered menopause and did not use contraceptives to prevent pregnancy (29).

Also, this study showed higher rates of VVC in patients with frequent intravaginal douching (P =0.00), this result of previous studies agreed with this finding (30, 31). The explanation may be that intravaginal douches may trigger vaginal tissue damage and disrupt the vaginal microecosystem, leading to a decrease in vaginal homeostasis, encouraging yeast growth and thus triggering VVC. In contrast to this, one study detected that the association between intravaginal practices and VVC was not statistically significant (32).

Moreover, in the current work, women who had a previous history of vaginitis were more likely to suffer VVC than those who did not (RR: 1.9, OR: 2.3, P= 0.002). This finding was concordant with some previous epidemiological studies which believed that there was an association between symptomatic episodes of VVC and a history of lower genital tract infection (30, 33).
There was no statistically significant difference between VVC patients and other vulvovaginitis patients regarding the consumption of current hormonal contraception, the recent usage of antibiotics, taking steroid therapy, or presenting with IUD in situ (P :> 0.05).

Comparable results were detected by Ozcan et al. as they found no statistical association between these risk factors and VVC (34). Na et al also found no relation between oral contraceptive pills and VVC (31). In contrast to these results, Jacob et al. detected a significant association between VVC and the use of gynecological/systemic antibiotics and oral/vaginal contraceptives also Na et al stated that there was a correlation among the use of IUD and VVC (31, 35).

As a result of increased prevalence of non-albicans species and their different antifungal susceptibility profiles, precise detection of Candida at the level of the species is essential (36).

Among VVC samples, 74 (98.3%) showed the growth of only one species of Candida while one sample (1.3%) showed the growth of two different species of Candida (C. albicans and C. glabrata), this result was in accordance with Amouri et al who mentioned that typically in VVC, One species is defined, however in some ladies two or more species have been isolated (1-10 %) (30), Mahmoudi et al, who have indicated that most vulvovaginal mixed infections are triggered by interactions among C. albicans and C. glabrata, also agree with this result (37).

In the present study, the predominant isolated species was C. albicans (47 isolates, 61.8%) accompanied by C. glabrata (22 isolates, 29%), C. krusei (5 isolates 6.6%), and lastly C. parapsilosis (2 isolates 2.6%).

The most prevalent isolated species became C. albicans (61.8 %), while the total prevalence of non-albican species became (38.2 %). Similar result was obtained from Egypt by ElFeky et al. as they reported 60.3% of VVC caused by C. albicans while the overall frequency of non-albicans species was 39.7% (38).

A higher rate of C. albicans in VVC (86.6%) was reported from a former study in Egypt by El-Sayed and Hamouda, (39), also from Kuwait by Alfouzan et al who reported (73.9%) rate (40).

In contrast to these results regarding the predominance of C. albicans, Deorukhkar et al and Jimoh et al detected a higher rate of NAC in VVC (60% and 51.5%), respectively (41, 42).

Recently, there has been a significant change in the etiology of candidiasis among NAC species. In some studies, NAC species now represent 10 % to 45 % of VVC cases. The most frequent reason of NAC-VVC is C. glabrata. (43).

C. glabrata is often isolated as a one of the natural flora of healthy individuals. For a long time, it was not considered a significant etiological agent of infections in humans. However, during the last few decades, the occurrence of mucosal and systemic infections induced by C. glabrata has risen markedly. This is a consequence of the extensive and widespread usage of broad-spectrum antibiotic therapy and immunosuppressive agents along with (44).
C. glabrata became the second most frequent isolate in the present research (29%) in VVC; similar findings were detected in studies in Saudi Arabia (31%), in Turkey (34.5%), and in Australia (20%) (45, 46, 47).

In contrast to the preceding reports, in the Bitew and Abebaw study, C. krusei became the dominant non-albicans of Candida species, representing 17.2% of the total isolates (48). In the present study C. krusei represented 6.6% of the isolated Candida, many studies have reported C. krusei rates ranging from 3% to 15.7% (38), (24, 45, 46, 49).

In agreement with the prevalence of C. parapsilosis in this study (2.6%), Bitew and Abebaw reported a similar rate (2.3%) of isolation of C. parapsilosis in VVC (48).

Due to the widespread usage of over-the-counter antifungal agents and the recovery of clinical isolates which show inherent or acquired resistance to antifungal medicines in vitro susceptibility testing of antifungal agents has become highly significant (50).

The 76 Candida isolates were tested for their sensitivity to 2 antifungal belonged to the polyenes group (Nystatin and Amphotericin B) and 4 antifungal belonged to the azoles group (Fluconazole, Voriconazole, Itraconazole, and Miconazole ) by the disc diffusion method; Nystatin exhibited an excellent efficacy against all Candida spp otherwise, only one C. glabrata isolate was resistant, this result agreed with other results reported by Fan et al and Choukri et al (51), (52) who reported the sensitivity rates of different Candida species to Nystatin were 100%. This can be explained by Wang et al mentioned that the excellent antifungal efficacy of Nystatin was correlated with comparatively low frequency of use in the clinical environment, in addition to the peculiar mechanism of altering cell membrane permeability (53).

Both Nystatin and Amphotericin B are belonging to the same drug class (polyenes) and have a similar mechanism of action, but Amphotericin B has the advantage that can be taken systemically, in the present study a good activity of Amphotericin B was detected against C. parapsilosis, C. albicans and C. glabrata (100%, 89.8%, 81.8%), respectively. But less effect was observed against C. krusei (60%). Similar findings were reported by many studies who revealed that resistance to Amphotericin B is less frequent in the isolates of different Candida spp. (38, 49, 54, 55).

In this study susceptibility to different azoles drugs was various among different Candida spp. Fluconazole and Voriconazole showed outstanding efficacy against all C. albicans isolates, lower susceptibility of C. albicans isolates was detected to itraconazole (80.9%) while the notably little effect of Miconazole against C. albicans (23.4%) was observed.

No resistance to Fluconazole or very low resistance (0.6%) between vaginal C. albicans isolates has been documented in Australian studies in accordance with these results (56) and Kuwait (40). In contrast to these results, lower susceptibility rates of C. albicans to Fluconazole were recorded in Brazil studies (68%), India (84%), and from Egypt (89.5%) (57, 49, 38) respectively.

Many studies detected the resistance of C. glabrata to many azole antifungals, especially Fluconazole (58, 59, 60, 61, 62, 63).

In this study, C. glabrata is less susceptible to azole, which is the most popular agent used to treat VVC, high resistance rate was detected to Fluconazole (54.5%). In line with these
findings, studies from Egypt have documented a similar rate of Fluconazole resistance (50%) (38). On the other hand, Wang et al. detected a marked higher resistance rate of C. glabrata to Fluconazole 90.8% (53).

It was observed in this research that Miconazole was the most effective azoles drug (91.1%), followed by Voriconazole (68.1%). This are in agreement with Salehei et al, who demonstrated the highest sensitivity of C. glabrata to Miconazole and Voriconazole 100%, 60%, respectively (64). In contrast to these findings, ElFeky et al detected lesser susceptibility rates against Miconazole and Voriconazole 38.5% and 50%, respectively (38).

C. krusei, recorded to be intrinsically resistant to Fluconazole (65), was 100% resistant to Fluconazole and Itraconazole in the current research. In accordance with these findings, several studies reported 100% resistance of C. krusei to Miconazole and Voriconazole (48, 64, 66).

Therefore, these in vitro susceptibility results warrant clinicians dealing with cases of C. krusei vaginitis, that has intrinsic resistance to Fluconazole may use other alternative antifungals for treatment (48).

C. krusei isolates were 60% susceptible to Voriconazole, the sensitivity of Voriconazole to C. krusei vaginal isolates in the present research was compatible with Bitew and Abebaw results who found that Voriconazole was the most effective azole against C. krusei 60%. Rex and Stevens reported that there is no obvious reason for the disparity in susceptibility among Fluconazole and voriconazole to C krusei, because all azole medications have a similar mechanism of action, i.e. ergosterol synthesis inhibition (67).

In the present study the 2 C. parapsilosis isolates were susceptible to all tested azoles in accordance with this finding Xiao et al mentioned that because of its high sensitivity to nearly all antifungal agents, C. parapsilosis vaginitis is easy to cure (68).

Mainly because of the wide use of Fluconazole in the prophylaxis and treatment of fungal infections, Candida spp. has developed several mechanisms for resistance toazole antifungals (69). The occurrence of antifungal resistance is generally moderate in normally sensitive fungi, particularly as relative to antibiotic resistance in bacteria. Nevertheless, due to the limited number of antifungal agents available, antifungal resistance is a fundamental issue. So that, understanding the different mechanisms of antifungal resistance is essential. This can aid in the design and developing alternative treatments. In addition, understanding the mechanisms of molecular resistance can define the resistance genes that can be used for diagnosis of resistance by molecular diagnostic tools (70).

Common overexpression of ATP-binding cassette transporters has been shown in studies ofazole-resistant C. glabrata isolates, these transporters are encoded by different genes including CgCDR1, CgCDR2, and CgSNQ2 (71), the zinc finger transcription factor CgPdr1 regulates these transporters (72). However, it is well documented that the most significant factor involved in C. glabrata resistance to azole antifungal agents is the overexpression of the CDRI gene (73).

This has been also confirmed in this study as ten out of twelve Fluconazole-resistant C. glabrata (83.3%) expressed CDRI gene at higher levels than the susceptible control isolates with the differences between the two groups being statistically significant (p-value < 0.0001). In agreement with this result, many studies detected a statistically significant difference
in CDR1 expression level between Fluconazole resistant and susceptible groups (71, 74, 75, 76).

It was found that 9 of 12 Fluconazole-resistant C. glabrata isolates (75%) upregulated SNQ2 another ATP-binding cassette transporter at a significant level than susceptible control (P= 0.0381). In accordance with these results, Tumbarrello et al detected a significant difference in mRNA expression of CgSNQ2 between resistant and susceptible isolates (77), in contrast Yao et al found that the expression of CgSNQ2 did not differ in resistant isolates from susceptible one (6).

Also, azole resistance may be attributed to upregulation of genes coding for enzymes responsible for biosynthesis of ergosterol mainly α demethylase, encoded by ERG11 (44).

Although overexpression of the ERG11 gene was found for some of the Fluconazole resistant isolates (33.3%), statistical analysis revealed that the mean levels of upregulation in the group of resistant strains were not statistically different than in the susceptible control group. p value = 0.159.

Similarly, Szweda et al (2015) noticed the overexpression of the ERG11 gene in some of the Fluconazole resistant isolates, despite being the statistical analysis of the mean level of upregulation between the group of resistant strains and the susceptible ones was not significant (69).

In agreement with these results Sanguinetti et al did not detect any statistically significant differences in transcription level among the population of Fluconazole resistant and susceptible clinical isolates (73). Upregulation of the ERG11 gene was also studied previously by Marichal et al who detected an eight-fold rise in ERG11 mRNA levels in clinical C. glabrata isolate, azole-resistant, which resulted from the chromosomal duplication (73, 78).

In addition, Whaley et al revealed that ERG11 does not seem to have an effective role in the resistance of clinical azole in C. glabrata. Overexpression of ERG11 has been detected in only two isolates of their clinical isolates of C. glabrata. Upregulation was subsequently observed in one isolate due to duplication of the whole ERG11-containing chromosome, and the phenotype was skipped with subsequent passage into azole-free media (4). In contrast Samaranayake et al mentioned that the overexpression of CgERG11 has a role in C. glabrata resistance to Fluconazole (79). Another azole resistance mechanism in C. glabrata seems to be the overexpression of PDR1 gene that encodes the transcriptional activator protein, involved in controlling the level of expression of genes encoding drug efflux transporters including CDR1 and SNQ2 (80). This mechanism was first revealed by Tsai et al, who demonstrated the attribution of CgPDR1 gene upregulation in clinical azole-resistant isolates and mutants to azole resistance by CgCDR1 upregulation (81).

In accordance with these findings, 91.7% of Fluconazole-resistant isolates overexpressed PDR1 at a statistically significant level than the susceptible isolates (P= 0.0002). Similar to this result many studies ensured the role of PDR1 in Fluconazole resistance (81, 75, 82, 83).

But on the contrary, Yao et al found that the expression levels of CgPDR1 among the azole-resistant and azole-susceptible isolates did not differ significantly (71).
Simultaneous upregulation of more than one efflux pump genes (CgCDR1, CgPDR1 and CgSNQ2) was noticed among the studied Fluconazole resistant C. glabrata isolates. CgCDR1, CgSNQ2, and CgPDR1 were expressed in six isolates simultaneously, while CgCDR1 and CgPDR1 were concurrently overexpressed in three isolates, two isolates upregulated CgPDR1 and CgSNQ2, and only one isolate upregulated CgCDR1 and CgSNQ2.

Of 6 Fluconazole resistant C. glabrata isolates that upregulated CgCDR1, CgSNQ2, and CgPDR1 simultaneously, 3 isolates showed cross resistance to three azole antifungals (Fluconazole, Itraconazole and Voriconazole)

Interestingly, the one C. glabrata isolate that was resistant to four tested azole antifungals did not upregulate CgCDR1 but upregulated the other tested genes. Similarly, Sanguinetti et al found the two isolates that were resistant to the four azoles (Fluconazole, Itraconazole, Ketoconazole, and Voriconazole, did not upregulate CgCDR1 but showed over expression only of CgSNQ2 (73).

CONCLUSIONS:

C. albicans was the predominant isolated species in VVC, whereas C. glabrata was the most prevalent non-albican species, and the main risk factors for VVC were the age of women ≤ 40 years old, besides using frequent intravaginal douching or a previous history of vaginitis.

Antifungal susceptibility pattern was variable among the different vulvovaginal Candida species; Nystatin, however, was the optimal option for treating VVC caused by different Candida species. Fluconazole-resistance was observed markedly in C. glabrata, most of the clinical resistance in C. glabrata was due to the upregulation of ABC transporters.

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