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Original Article

Investigating the Frequency of *Candida glabrata* in Diabetic Women of Tehran with Recurrent and Non-recurrent Vulvovaginal Candidiasis Using PCR-RFLP Assay

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Introduction: Vulvovaginal Candidiasis (VVC) is one of the most common genital tract infections among women, especially in diabetic patients. The increasing prevalence of recurrent infections caused by drug-resistant non-*albicans* species necessitates further studies on diabetic patients and the identification of causative agents by reliable molecular techniques. The obtained results can assist in adopting proper treatment procedures and prevention of recurrent vulvovaginitis (RVVC). **Methods:** In a cross-sectional study, 150 vaginal discharge samples were collected from diabetic women suspected of candidiasis referring to health centers in Tehran province. Following the culture of samples on SDA, CHROMagar *Candida* and PCR-RFLP were used for presumptive and definitive identification of *Candida* species, respectively. **Results:** Out of 115 positive patients, 105 showed infection with one species, and 10 had a mixed infection with two species. The frequency of *Candida glabrata* isolated from non-mixed and mixed infections in RVVC group was higher than *Candida albicans* (27.8% vs. 9.6%), which contradicted the results of the VVC group (6.1% vs. 24.3%). In the RVVC group, therefore, the patients were more infected with non-*albicans* species than *C. albicans* (47.8% vs. 9.6%), while in the VVC group the non-*albicans* were of lower frequency (18.3% vs. 24.3%). **Conclusion:** Our findings showed a statistically significant correlation ($P<0.001$) between the frequency of *C. glabrata* and the prevalence of RVVC. On the other hand, that blood sugar, duration of diabetes, and antibiotics usage had significant correlations ($P<0.001$) with the recurrence of severe symptoms. *J Med Microbiol Infec Dis, 2019, 7 (1-2): 44-51.*

Keywords: *Candida glabrata*, Diabetes Mellitus, Polymerase Chain Reaction, Vulvovaginal candidiasis, Iran.

INTRODUCTION

Vaginitis is one of the most prevalent reproductive tract infections (RTIs) among sexually active women. Annually, about 340 million cases of curable sexually transmitted infections (STIs) occur worldwide, mostly in developing countries [1, 2]. After bacterial infections, vulvovaginal candidiasis is believed to be responsible for approximately one-third of vaginitis cases [3, 4]. However, some studies indicate a higher prevalence of *Candida* infection compared to bacterial vaginosis and trichomoniasis [5, 6, 7]. About %75 of healthy women within the age range of 25-50 years have experienced non-recurrent vulvovaginal candidiasis (VVC) at least once in their lifetime, but 5% to 20% are prone to recurrent vulvovaginal candidiasis (RVVC) at least four times during a year [7-9].

Candida species are part of normal flora in the skin, mucous membranes, and gastrointestinal tract; however, in the presence of predisposing factors, they can appear as an opportunistic agent causing severe infections. The most critical predisposing factors include immunosuppressive diseases, uncontrolled diabetes, high carbohydrate diet, pregnancy, lack of personal hygiene, estrogen contraceptives, high sexual activity or high-risk behaviors, prescription of improper drug, self-treatment and prolonged use of broad-spectrum antibiotics and corticosteroids, incomplete or extended use of antimycotics, and resistance of *Candida* species to azoles [10-13]. Diabetes mellitus (DM) is one of the major risk factors for severe infections caused by different *Candida* species. Women with VVC and DM, particularly those with poorly controlled hyperglycemia, are prone to develop RVVC caused by non-*albicans Candida* (NAC) species [14-16]. Non-recurrent vulvovaginal candidiasis is an uncomplicated and mild disease which is mainly prevalent among healthy women infected with *Candida albicans*, while recurrent VVC is a complicated and more severe disease often caused by drug-resistant NAC species in immunocompromised women [14-16]. *Candida albicans* is known as the primary cause of vulvovaginal candidiasis, however; today, NAC species, especially *C. glabrata*, which is intrinsically resistant to a vast range of azoles and antifungal compounds are on the

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increase. Some studies have estimated that %15 to %47 of RVVC cases were caused by *C. glabrata*, which is a major cause of treatment-resistant severe infections worldwide [11, 12, 17]**.**

The accurate diagnosis of the causative agent of infection can be helpful in timely appropriate treatment of vulvovaginal candidiasis. Due to the similar clinical symptoms (*e.g.*, pruritus, inflammation and irritation of vulva and vagina, and smelly discharges) in vulvovaginitis caused by *Trichomonas vaginalis*, bacteria and *Candida* spp., clinical examinations, and routine microbial procedures are not very reliable for identification of the real causative agent [1, 18-21]**.** Therefore, the use of precise and specific molecular techniques such as PCR-RFLP, Real-Time qRT-PCR, RAPD-PCR, multiplex-PCR can be useful for solving diagnostic limitations. Many researchers have introduced PCR-RFLP as a reliable and cost-effective method for rapid recognition of *Candida* species in research centers and diagnostic laboratories [18-22]**.**

Regarding the increasing prevalence of *C. glabrata*, and limited studies on diabetic women, we prompted to investigate the frequency of this species in two groups of diabetic patients with VVC and RVVC using the PCR-RFLP technique. Besides, the association between recurrence of infection symptoms with some important predisposing factors such as blood sugar, duration of diabetes, and antibiotics usage was evaluated.

MATERIAL AND METHODS

Sample collection and culture. In a cross-sectional study, we investigated 150 diabetic patients referring to several gynecologic clinics in the province of Tehran from April 2015 to September 2017. The inclusion criteria for the study included married non-pregnant women aged 25 to 45 year old with at least high school education, diabetes mellitus, and not being on corticosteroids and steroid contraceptives. The patients showed mild to severe vulvovaginal symptoms, *e.g.* inflammation, irritation, erythema, fissures, pruritus, dyspareunia, and smelly white-yellow discharges. They were divided into two groups: women with recurrent infection (at least 3 to 4 recurrent infection during one year) and those with a non-recurrent infection. Informed consent was obtained from all participants or their guardians, and the ethical committee of the Islamic Azad University of Science and Research Branch (Tehran) approved the study (code: 131339).

Vaginal discharges were obtained using two sterile swabs by gynecologists and immediately stored in Falcon tubes containing sterilized PBS. The clinical specimens were transferred to the university laboratory. The vaginal discharge from one of the swabs was mixed with a drop of PBS, spread on a clean glass slide and covered with a coverslip, and examined for yeast cells under a microscopic with 100x magnification. Another swab was used for culture on Sabouroad's dextrose agar (SDA, Merck KgaA Darmstadt Germany) supplemented with chloramphenicol followed by incubation at 37ºC for 24-48 h. All newly-grown colonies were cultured on CHROMagar *Candida* (CHROMagar, France) at 37ºC for 48-72 h. The initial identification of *Candida* species was performed based on color and surface characteristics of colonies (glossy or dry) [1, 13, 18-20]. *Candida glabrata* (ATCC90030) and *Candida albicans* (ATCC10231) were included as controls in the study.

DNA extraction and PCR. Extraction of genomic DNA was performed by a DNP™ kit (Sinaclon, Iran) according to the manufacturer's instruction. The extracted DNAs were stored at -20ºC until used. The ribosomal DNA (ITS1, ITS2, and 5.8 S rRNA gene) of *Candida* species were amplified by universal fungal primers (ITS-1:5´-TCCGTAGGTGAACCTGCGG-3´and ITS-4:5´-TCCTCC-GCTTATTGATAT GC-3´) [20-22].

PCR mixture was prepared according to the instruction for the *Taq* 2X Master Mix RED (Ampliqon, Denmark). The reaction included 12.5 µl 2X Master Mix RED, 2 µl DNA, 10 pmol of each primer, and molecular grade dH_2O (Thermo Scientific, Lithuania) to the 25 µl final volume. The thermal cycler (Technogen, United Kingdom) was programmed for an initial denaturation at 95ºC for 5 min followed by 30 cycles of denaturation at 95ºC for 30 s, annealing at 56ºC for 30 s, and extension at 72ºC for 60 s, and a final extension at 72ºC for 5 min.

RFLP. Digestion of the amplicons was performed with *Msp*I and *Avr*II (*Bln*I) enzymes (Thermo Scientific, Lithuania) according to manufacture's instruction. The 31 µl digestion reactions contained 1 µl digestion enzyme (*Msp*I or *Avr*II), 2 µl digestion buffer, 10 µl PCR product, and molecular grade dH_2O to the final volume followed by incubation at 37ºC for 3 h.

Electrophoresis. The products obtained from three steps, *i.e.*, DNA extraction, PCR, and digestion with restriction enzymes were resolved by electrophoresis on 1.8% agarose gels (Sinaclon, Iran) stained with 0.5 µl/ml of ethidium bromide (Sigma-Aldrich, Germany). The bands were visualized by UV light using a Gel documentation instrument (Lourmat-VX2 Viber, Germany), photographed, and interpreted accordingly authentic and standard data.

Statistical analysis. The statistical results were analyzed using SPSS version 19 software; *P*-values<0.05 were considered statistically significant. Data was presented in terms of numbers and percentages. Comparison between the results of two patient groups was performed using chi-square (X^2) test, correlation, and independent t-test.

RESULTS

SDA culture and microscopy. Culture of vaginal samples on SDA medium containing chloramphenicol (Fig. 1A) and microscopic examination showed that out of 150 samples collected from diabetic women suspected of vulvovaginitis, 115 (76.7%) were positive for fungal infection, while 35 (23.3%) showed no infection. Based on the severity and recurrence of clinical manifestations (at least three recurrent infections in a year), 66 patients (57.4%) were identified as RVVC group and 49 (42.6%) as VVC.

CHROMagar Candida culture. For identification of *Candida* species, the colonies grown on CHROMagar

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Candida medium were evaluated based on color and surface characteristics of colonies such as being glossy or dry (Fig. 1B, C, and D).

We had difficulty in distinguishing the pale to dark pink/purple colonies of *C. glabrata* from other species producing similar colors such as *Candida krusei*, *Candida guillermondii*, and *Candida kefyr*, particularly in mixed *Candida* infection. This problem was also observed to differentiate the light to dark green colonies of *C. albicans* from *Candida dubliniensis*.

PCR-RFLP. PCR amplification of the ribosomal DNA (ITS1, ITS2, and 5.8 S rRNA gene) region yielded bands of 510 bp to 871 bp size (Table l).

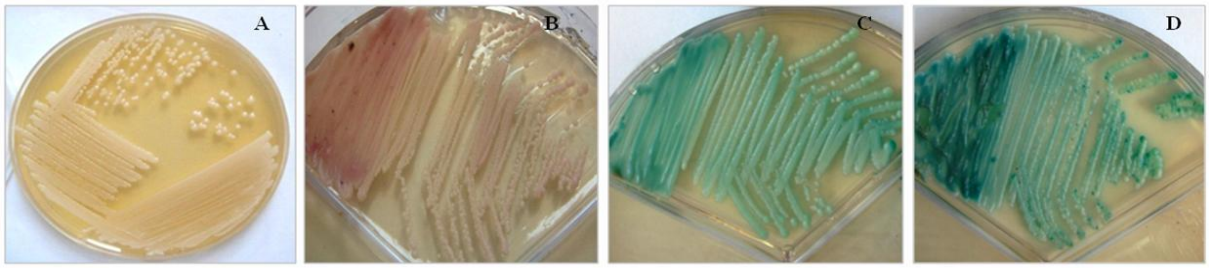


Fig. 1. Isolation and identification of *Candida* species by culture method. (A) Colonies of *Candida* spp. on Sabouroad's dextrose agar containing chloramphenicol, (B, C, and D). Colonies of *Candida* spp. on CHROMagar *Candida* medium. (B) *C. glabrata*, (C) *C. albicans*, and (D) *C. dubliniensis*

Table 1. Characteristics of the colonies of *Candida* species on CHROMagar *Candida* medium, the size of PCR-amplified ITS sequence, and fragments obtained following digestion with restriction enzymes

***Candida* species**	**Colony Characteristics on CHROMagar *Candida* medium**	**Size of ITS1-ITS4 (bp)**	**Size(s) of restriction enzyme products (bp)**	
			*Msp*I	*Avr*II
C. albicans	Light or dark green	535	238 - 297	535
C. dubliniensis	Dark green	535	238 - 297	200 -335
C. glabrata	Pale or dark pink/purple, glossy colonies	871	314 - 557	NP*
C. tropicalis	Dark bluish purple with light purple edges	524	184 - 340	NP
C. krusei	Rose pink, dry surface with whitish edges	510	249 - 261	NP
C. kefyr	Light or gray pink	721	721	NP
C. guilliermondii	Violet or mauve lavender	608	371-155-82	NP

*NP, Not performed

PCR identified the species *C. guilliermondii*, *C. kefyr*, and *C. glabrata* by amplification of definitive bands of 608 bp, 721 bp, and 871 bp sizes, respectively (Fig. 2 A, B, and C). However, it yielded amplicons of almost similar size for *C. krusei* (510 bp), *C. tropicalis* (524 bp), and *C. albicans*/*C. dubliniensis* (535 bp). RFLP analysis by *Msp*I, which cut the DNA at CCGC sequence, created two bands for *C. albicans*/*C. dubliniensis*, *C. glabrata*, *C. tropicalis*, and *C. krusei*, and three bands for *C. guillermondii*. However, digestion with *Msp*I revealed a band of 721 bp that was equal to the size of PCR-amplified ITS for *C. kefyr*. In addition to amplification of bands of similar size in *C. albicans* and *C. dubliniensis* by PCR, the *Msp*I enzyme also produced similar bands in these two species. However, *Avr*II enzyme which cut CCTAGG sequence produced two definitive bands of 200 bp and 335 bp for *C. dubliniensis* (Table 1, Fig. 3 A, B, C, and D).

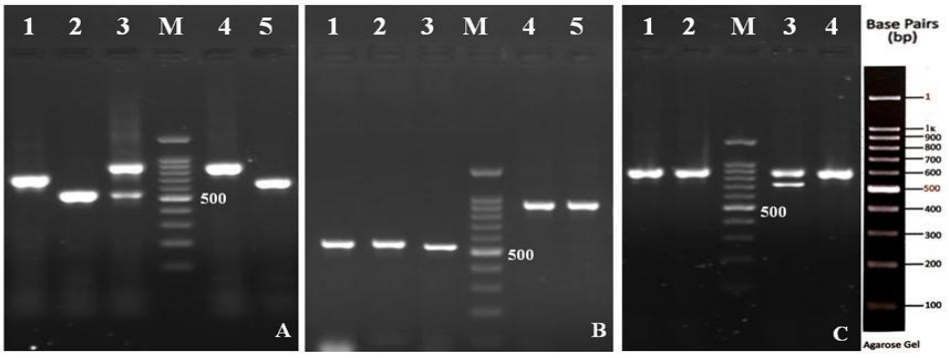


Fig. 2. Gel electrophoresis of PCR-amplified ITS sequences of *Candida* species. A) Lane 1, *C. kefyr*; lane 2, *C. krusei*; lane 3, *C. glabrata* and *C. krusei*; lane 4, *C. glabrata* and lane 5, *C. guilliermondii*. B) Lane 1, *C. albicans* (ATCC 10231); lane 2, *C. albicans or C. dubliniensis*; lane 3, *C. tropicalis*; lane 4, *C. glabrata* and lane 5, *C. glabrata* (ATCC 90030). C) Lanes 1 and 2, *C. glabrata*; lane 3, *C. glabrata* and *C. kefyr* and lane 4, *C. glabrata* (ATCC 90030). M, 100 bp ladder molecular size marker

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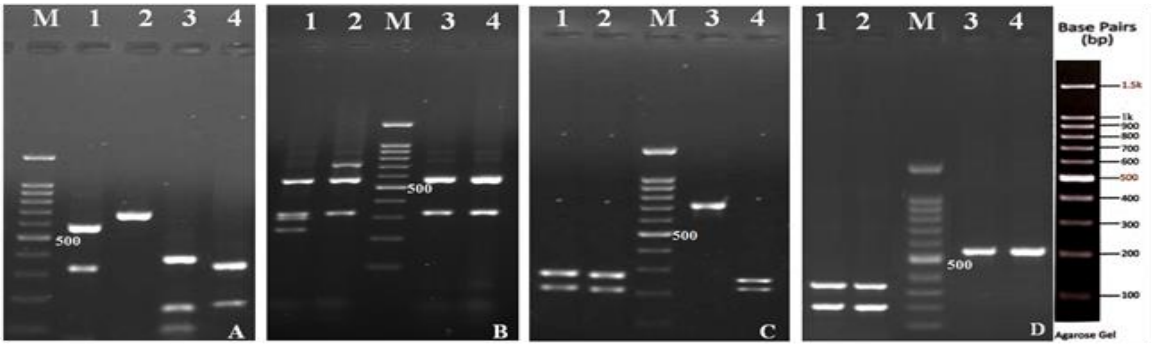


Fig. 3. RFLP pattern following digestion with *Msp*I (A-C) and *Avr*II (D) enzymes. A) lane 1, *C. glabrata*; lane 2, *C. kefyr*; lane 3, *C. guilliermondii* and lane 4, *C. tropicalis*. B) Lane 1, *C. glabrata* and *C. krusei*; lane 2, *C. glabrata* and *C. kefyr*; lane 3, *C. glabrata* and lane 4, *C. glabrata* (ATCC 90030). C) Lane 1, *C. albicans* (ATCC10231); lane 2, *C. albicans* or *C. dubliniensis*; lane 3, *C. kefyr* and lane 4, *C. krusei*. D) Lanes 1 and 2, *C. dubliniensis*; lane 2, *C. albicans* and lane 3, *C. albicans* (ATCC 10231). M,100 bp ladder molecular size marker

PCR-RFLP results showed that out of 115 patients, 105 including 47 with VVC and 58 with RVVC were infected with one *Candida* species, and 10 patients including 2 with VVC and 8 with RVVC had infection two species (Tables 2 and 3).

Table 2. Absolute and relative frequency of *Candida* species in patients with vulvovaginal candidiasis

Type of disease	*C. glabrata* No (%)	*C. krusei* No (%)	*C. kefyr* No (%)	*C. tropicalis* No (%)	*C.dubliniensis* No (%)	*C.guilliermondii* No (%)	Total non-*albicans* species No (%)	*C. albicans* No (%)	Total species No (%)
RVVC [1]	24 (20.9)	**7** (6.1)	5 (4.3)	6 (5.2)	4 (3.5)	1 (0.9)	47 (40.9)	11 (9.5)	58 (50.4)
VVC [2]	5 (4.3)	4 (3.5)	4 (3.5)	3 (2.6)	3 (2.6)	0 (0.0)	19 (16.5)	28 (24.4)	47 (40.9)
Total	29 (25.2)	11 (9.6)	9 (7.8)	9 (7.8)	**7** (6.1)	1 (0.9)	66 (57.4)	39 (33.9)	105 (91.3)

[1]Recurrent vulvovaginal candidiasis [2]Non-recurrent vulvovaginal candidiasis

Table 3. Absolute and relative frequency of mixed infections in patients with vulvovaginal candidiasis

Type of disease	***C. glabrata*+*C. krusei*** **No (%)**	***C. glabrata*+*C. kefyr*** **No (%)**	Total species **No (%)**
RVVC[1]	5 (4.4)	3 (2.6)	**8 (7.0)**
VVC[2]	0 (0.0)	2 (1.7)	**2 (1.7)**
Total	3 (2.6)	7 (6.1)	**10 (8.7)**

[1]RVVC: Recurrent vulvovaginal candidiasis [2]VVC: Non-recurrent vulvovaginal candidiasis

In RVVC group, the number of patients with non-*albicans* species to *C. albicans* was 55 vs. 11 cases, while in the VVC group the number of them was 21 vs. 28 cases (Table 4). Based on the chi-square test (χ^2=20.557; *df*=1; *P*<0.001) and Spearman's correlation (ρ=0.423; *P*<0.001) our results indicated a significant relationship between NAC species and the severity of the fungal infection ($P_{\text{Chi-square}}$<0.001, ρ=0.4; P_{Spearman}<0.001). The frequency of *C. glabrata* was higher than other NAC species in both groups and even higher than *C. albicans* in the RVVC group. In fact, in the RVVC group, the number of total isolated *C. glabrata* from patients with mixed and non-mixed infection to *C. albicans* was 32 vs. 11, while in the VVC group was 7 vs. 28. There was a significant association between *C. glabrata* frequency and incidence of recurrent vulvovaginal candidiasis ($P_{\text{Chi-square}}$<0.001, ρ=0.5; P_{Spearman}<0.001; Tables 2, 3, and 4).

The participants' age ranged from 25 to 45 years. No significant difference was between the mean age of the two groups (RVVC=36.4±5.6 vs. VVC=35.0±5.5, $P_{t\text{-}test}$=0.195). The association between age and disease incidence was not statistically significant ($P_{\text{Chi-square}}$=0.276, ρ=0.1; P_{Spearman}=0.2; Table 4). The incidence of infection also showed no significant association with the educational status of participants ($P_{\text{Chi-square}}$=0.547, ρ=-0.03; P_{Spearman}=0.800). Nevertheless, the negative correlation coefficient indicates that an inverse correlation may exist between these variables (Table 4).

The results illustrated that the FBS levels could be significantly related to the incidence of infection in the RVVC group ($P_{\text{Chi-square}}$<0.001, ρ=0.8; P_{Spearman}<0.001). The mean FBS level was also significantly higher in the RVVC than the VVC group (322.0±91.2 vs. 166.0±29.5, $P_{t\text{-}test}$<0.001). There was also a significant positive association between diabetes duration and recurrence of severe fungal infection ($P_{\text{Chi-square}}$<0.001, ρ= 0.6; P_{Spearman}<0.001). The mean duration of diabetes was significantly higher in the RVVC group than the VVC (10.6±5.7 vs. 4.0±2.1, $P_{t\text{-}test}$ <0.001). We also found a significant positive association between antibiotic use and RVVC incidence ($P_{\text{Chi-square}}$<0.001, ρ= 0.7; P_{Spearman}<0.001; Table 4).

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Table 4. Distribution of samples according to the parameters and type of vulvovaginal candidiasis

Variables	RVVC	VVC	Statistics data	*P*-Value
Total number of patients with vulvovaginitis	66 (57.4)[a]	49 (42.6)	-	-
Number of patients with NAC species (mixed & non-mixed) vs. *C. albicans*	55 (47.8) vs. 11 (9.6)	21 (18.3) vs. 28 (24.3)	χ^2=20.557[b] ρ=0.423[c]	<0.001 <0.001
Total number of *C. glabrata* isolated from mixed & non-mixed samples vs. *C. albicans*	32 (27.8) vs. 11 (9.6)	7 (6.1) vs. 28 (24.3)	χ^2=22.856 ρ=0.541	<0.001 <0.001
Age (years)				
25-30	11 (16.7)	12 (24.5)		
31-35	14 (21.4)	15 (30.6)	χ^2=3.864	0.276
36-40	28 (42.4)	13 (26.5)	ρ=0.131	0.161
41-45	13 (19.7)	9 (18.4)		
Range of age (years) (Mean ± SD[e])	25 - 45 (36.39±5.61)	25 - 45 (34.98±5.47)	*t*=1.304[d]	0.195
Education				
Under diploma	11 (16.7)	5 (10.2)	χ^2=1.207	0.547
Diploma –Bachelor's degree	30 (61.2)	35 (53.0)	ρ= -0.029	0.754
Master's degree & higher	14 (28.6)	20 (30.3)		
FBS[f] (mg/dl)				
<200	9 (13.6)	46 (93.9)	χ^2=72.701	<0.001
200 - 400	46 (69.7)	3 (6.1)	ρ= 0.772	<0.001
>400	11 (16.7)	0 (0.0)		
Range of FBS level (Mean ± SD)	119 - 488 (321.98±91.2)	115 - 217 (165.96±29.48)	*t*=13.012	<0.001
Duration of diabetes (years)				
<5	6 (9.1)	27 (55.1)	χ^2=40.303	<0.001
5 - 10	34 (51.5)	22 (44.9)	ρ= 0.592	<0.001
>10	26 (39.4)	0 (0.0)		
Range of diabetes duration (years) (Mean ± SD)	1 - 27 (10.62±5.67)	1 - 8 (4.03±2.9)	*t*=8.663	<0.001
Treatment of vulvovaginitis				
Antibiotics (with or without antifungals)	62 (93.9)	14 (28.6)	χ^2=53.915	<0.001
Antifungals (without antibiotic)	4 (6.1)	35 (71.4)	ρ= 0.683	<0.001
Severity of vulvovaginitis symptoms				
Extensive & severe	+	-	-	-
Mild to moderate	-	+	-	-
Incidence of vulvovaginitis in patients (in a year)				
1	-	+		
>1	+	-		
Corticosteroids	No	No	-	-
Estrogen contraceptives	No	No	-	-
Pregnancy	No	No	-	-
Other diseases	No	No	-	-

[a]Number (%) [c]Spearman's correlaton [e]Standard deviation
[b]Chi-square [d]Independen t-test [f]Fasting blood sugar level

DISCUSSION

With the increase of antifungal-resistant NAC species in recent years, the RVVC has become a significant health threat to women, especially those with diabetes [2, 14]. However, the data on the prevalence of RVVC caused by NAC species among diabetic women is not much, as most studies were more focused on *C. albicans* and non-recurrent VVC [7, 9].

In our study, the prevalence of RVVC (57.4%) was higher than VVC (42.6%) in diabetic women, while in Brazil the prevalence of VVC and RVVC in diabetic women was the same and VVC was of a higher prevalence in non-diabetic women [23]. In other studies on non-diabetic patients, in Tehran (Iran) and Indonesia, RVVC showed a lower incidence, whereas in Zahedan (Iran) the rate of RVVC was more than VVC [8, 18, 19]. Our findings also showed that the number of patients infected simultaneously with two species was higher in RVVC group than those in VVC group, although the total percentage of single-species infections (VVC=40.9% & RVVC=50.4%) was higher than double-species ones (VVC=1.7% & RVVC=7%) in both groups. In India and Egypt, vulvovaginal candidiasis with one species was also more prevalent [24, 25]. However, in Tehran, the frequency of multiple-species infections was the same in both VCC and RVVC groups [18]. In Sari, the rate of multiple infections was lower in VVC group [12].

In the present study, the NAC species in RVVC group had a higher frequency than the VVC group. Besides, *C. glabrata* was the most abundant NAC species, and its frequency in comparison to *C. albicans* in RVVC group was significantly higher than the VVC group (P<0.001).

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Therefore, in RVVC group, the number of diabetic patients infected with NAC species compared to *C. albicans* was significantly higher than VVC group (P<0.001), which was similar to the findings in diabetic women in Brazil and non-diabetic women in Urmia [5, 23]. However, in a similar study in Sari (Iran), the frequency of NAC species in VVC group was higher than RVVC group [12]. Other reports from Nigeria [13, 15], India [3, 25, 26], Iraq [17], and Burkina Faso of Africa [27] also revealed a higher prevalence of NAC species compare to *C. albicans* among diabetic and non-diabetic women. On the contrary, in diabetic patients of Yemen [29], Kermanshah (Iran) [30], and some non-diabetic patients with VVC in Iran and other countries, the prevalence of NAC species was less than *C. albicans* [10, 20, 25, 31, 32]. However, in non-diabetic women of America and Brazil, and diabetic women of Egypt, the ratio of NAC species and *C. albicans* was equal [7, 16, 28].

Some studies have reported lower rates of *C. glabrata* compared to *C. krusei* [26, 32], *C. tropicalis* [3,5] and *C. parapsilosis* [12], while our results and other studies in Iraq and Egypt [15, 17] demonstrated *C. glabrata* as the most prevalent species in diabetic and non-diabetic women. High prevalence of *C. glabrata* was also reported in other countries among diabetic [16, 29, 30] and non-diabetic women [25, 27, 32], which indicate an increasing trend in the prevalence of infections caused by this species. In China, during an eight-year study, the prevalence of *C. glabrata* increased from 10.6% to 21.7%, while *C. albicans* declined from 82% to 77.2% [10].

Recurrence of severe vulvovaginitis symptoms depends on many predisposing factors. In the current study, the contributing factors were studied based on the data registered in the questionnaires. All women were almost of the same socioeconomic status, were not pregnant and had not taken corticosteroids and estrogen contraceptives. In both groups, the majority of cases (n=41) were among women aged 36-40 years with a high school diploma or a bachelor's degree (n=65). Some studies have reported the highest vulvovaginitis prevalence in the age range [24, 27] and education level [33] similar to our results. In India, Nigeria, and Sari (Iran), the highest incidence was in the age range 20-35 years [1, 12, 34], and in Yemen, Nepal, and Ethiopia, the women with the educational level of illiterate, primary and high school showed the highest prevalence rate [29, 31, 32]. Our results also showed no significant relationship between the incidence of infection and the age or education of the patients (P>0.05). In Ethiopia and Malaysia, age and education were not significantly correlated with the incidence of vulvovaginitis [32, 35], while in Yemen and China, the relationship was significant [29, 33].

In the current study, high FBS levels and long-term diabetes, the two most important contributing factors, were significantly correlated (P<0.001) with the RVVC prevalence. Many reports from Iran and other countries have indicated a significant relationship between the recurrence of severe infection and FBS levels [19, 24, 29, 34, 36] as well as long-term uncontrolled diabetes [29]. Therefore, diabetes mellitus can be considered as one of the most important predisposing factors for vulvovaginitis, especially in RVVC group [1, 23, 36]. Because uncontrolled hyperglycemia potentially reduces the adhesion, chemotaxis, and phagocytosis of pathogen-destroying leukocytes [16, 26]. Increased glucose in the vulvovaginal tissue along with a decreased pH can increase the capability of adhesion, colonization, and invasion of *Candida* species to the tissues [16, 26].

In RVVC group that most women were taking antibiotics (with or without antifungal), there was a significant correlation between the severity of the infection and the antibiotics usage. Similar findings are available from Iran (Zahedan), Egypt, and Iraq [19, 24, 36], whereas other studies in Iran (Shahrekord) and Nepal reported no relationship between them [31, 37].

Interviewing the patients revealed that the diagnosis of vulvovaginitis and drugs prescription was merely based on clinical examinations and routine laboratory tests. Azitromycin along with another antibiotic such as clindamycin, metronidazole, tinidazole or triple sulfa (for bacterial vaginosis and trichomoniasis) were commonly prescribed following the initial clinical examination to most patients with severe clinical symptoms. Following recurrence of more acute symptoms, antifungals, *e.g.* clotrimazole, miconazole, or fluconazole were prescribed along with two of these antibiotics. *Candida albicans* is one of the sensitive species to clotrimazole, miconazole, and fluconazole, while most NAC species, particularly *C. glabrata*, have shown an intrinsic and acquired resistance to a vast range of azoles [25, 27, 37]. Treatment failures might be due to the inaccurate diagnosis of the causative agent, as several pathogens may cause vulvovaginitis with similar clinical symptoms. Many studies have reported simultaneous infection with different *Candida* species [12, 18, 23, 24], or coinfections of yeasts, bacteria, and *Trichomonias vaginalis* [1, 6]. In China, the rate of vulvovaginitis caused by mixed infections of *Candida* and bacteria was higher than *Candida* infection alone [33]. The use of broad-spectrum antibiotics for an extended time might reduce the vaginal indigenous microbiota, and led to a remarkable increase in the colonization of drug-resistant NAC species and recurrence of the disease [7, 24, 28, 37]. The high prevalence of RVVC caused by *C. glabrata* in our study might be linked with this issue.

In our study, CHROMagar *Candida* medium was a useful method for primary isolation and identification of *Candida* species based on phenotypic features. However, it was time-consuming (48-72 h) and lacked the reliable sensitivity to discriminate the species by the color of colonies, especially in mixed infection. For example, the differentiation of some *C. albicans* colonies from dark green colonies of *C. dubliniensis*, or pale-dark pink/purple *C. glabrata* colonies from those of species with a similar color were ambiguous, which was in agreement with other studies reporting the same challenge [11, 12, 38, 39].

PCR technique along with proper restriction enzymes, has shown to be a reliable method to differentiate *Candida* species [11, 19, 22]. Some studies demonstrated that some

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restriction enzymes such as *Hepa*III, *Dde*I, and *Bfa*I did not have a reliable discriminating power to identify *Candida* species, probably due to inaccessibility of ITS1-ITS4 sequences [40, 41]. Similar to previous studies [11, 19, 22, 41, 42], we could identify *Candida* species by PCR-RFLP using the efficient *Msp*I and *Avr*II restriction enzymes.

Vulvovaginal candidiasis is still a prevalent infection, especially among diabetic women. Regarding the significant relationship between recurrent VVC and long-time hyperglycemia and antibiotics usage, controlling FBS and avoiding excessive antibiotics can be helpful in the treatment of infection. Besides, the significant frequency of *C. glabrata* in comparison with *C. albicans* in RVVC group and its resistance to treatment with common antifungals can emphasize the importance of accurate diagnosis of the causative agent using molecular techniques.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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