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Molecular Investigation of Etiologic Agents Causing Vulvovaginal Candidiasis

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Abstract

Background: Vulvovaginal candidiasis (VVC) is an ordinary infection caused by *Candida* species. Meanwhile, a shift towards non-albicans *Candida* (NAC) species has been detected in VVC patients.
Objectives: This study aimed at molecular identification of *Candida* isolates, causing VVC.
Methods: Vaginal secretion samples of 320 non-pregnant vaginitis patients at Shahid Akbar-Abadi Obstetrics and Gynecology Hospital in Tehran (Iran) were collected. Samples were evaluated using mycological and molecular approaches. Vaginitis isolates were analyzed with the PCR using NL1 and NL4 primers, and the D1/D2 region of the large-subunit rRNA gene was amplified and sequenced.
Results: In total, 100 *Candida* isolates were identified from VVC and recurrent vulvovaginal candidiasis (RVVC). *Candida albicans* was the most frequent (51%), followed by *C. glabrata* (36%), *C. krusei* (*Pichia kudriavzevii*) (8%), and *C. kefyr* (*Kluyveromyces marxianus*) (5%). 51 and 49% of isolates had *C. albicans* and NAC, respectively.
Conclusions: *Candida albicans* and *C. glabrata* were the most common agents of vulvovaginal candidiasis. NAC spp. (49%) was found as an important agent associated with VVC.

Keywords: vulvovaginal Candidiasis, Molecular Investigation, *Candida* Species, D1/D2 Region

1. Background

Vulvovaginal candidiasis (VVC) is an uncomfortable infection that occures up to 75% of women at least once in their life. Moreover, for a small percentage of women, it occurs more than one episode (1, 2). Due to increased resistance of *Candida* spp. to current antifungal drugs, the challenge of recurrent vulvovaginal candidiasis (RVVC) is on the rise (2, 3). Both pregnant and non-pregnant women are at risk of vaginal colonization of *Candida* spp. (4, 5). *Candida albicans* is the most important cause of VVC, while non-*albicans Candida* (NAC) species is due to *C. glabrata* (5, 6). Also, NAC species including *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, are reported as causative agents of VVC, and these species often no longer respond to antimycotic drugs (1, 7).

Furthermore, the difficulty of definitive identification of *Candida* species using conventional methods has increased, particularly for unusual and closely related species. Amplification of genomic DNA and sequencing of the resulted amplicons is one of the most commonly used molecular diagnostic techniques. Nucleotide sequence assessment of the internal transcribed spacer section of the rRNA gene (8) and the D1/D2 region of the large-subunit rRNA gene are the most widely used techniques for definitive identification of pathogenic fungal species (9, 10).

In the *Candida* complexes, such as the *C. albicans* complex, amplification of the hyphal wall protein 1 (*HWP1*) gene is the appropriate molecular approach for distinguishing *C. albicans*, *C. dubliniensis*, and *C. africana* (11). Various *Candida* spp. have different levels of susceptibility to antimycotic drugs. Therefore, the exact identification of *Candida* vaginal isolates is important for providing effective care.

2. Objectives

This study aimed at molecular identification of *Candida* isolates, causing VVC using nucleotide sequences of

the D1/D2 region of the large-subunit rRNA gene.

3. Methods

3.1. Patients and Collecting Isolates

This cross-sectional study was performed on 320 non-pregnant vaginitis patients referred to Shahid Akbar-Abadi Obstetrics and Gynecology Teaching Hospital in Tehran (Iran) from 2016 - 2017. Besides, information on age, occupation, medical history, symptoms, and recurrent episodes (recurrent VVC was defined as $\geq$ 4 episodes within a year) were also collected (12). Vaginal secretions were collected from the posterior fornix with a sterile cotton swab and put into 3 mL of sterile saline.

3.2. Exclusion Criteria

Infection with *Mycoplasma* spp., *Trichomonas vaginalis*, *Chlamydia*, or any bacterial vaginosis or vulvar skin infection. History of using local or systemic antifungal compounds during the past two weeks.

3.3. Yeast Identification

Microscopic assessment was used to identify yeast forms or pseudomycelium. All specimens were cultured on CHROMagar *Candida* (CHROMagar *Candida*, France) for colony count and distinguishing concomitant of *Candida* spp. infection. The identification was confirmed by germ tube formation, chlamydospore production in cornmeal agar (CMA) (Merck Germany) with 1% Tween 80, and cultured on Sabouraud dextrose agar medium with chloramphenicol at 42°C.

3.4. Molecular Methods

For DNA extraction a single colony on CHROMagar *Candida* was selected and subcultured on yeast extract peptone dextrose (Merck Germany) (YEPD) agar at 37°C for 24 - 48 h. Genomic DNA of yeast isolates was extracted using the Qiagen DNA tissue kit (Germany) and deposited at -20°C for until use. The universal primers NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) were applied for amplification of the D1/D2 region of large subunit ribosomal RNA (10) by the subsequent program: 98°C (5 min), 35 cycles of 98°C (30 s), annealing temperature 60°C (30 s), and 72°C (30 s) and 72°C for a 5-minute final extension. The amplicons were sequenced by Macrogen (Korea). Sequences were evaluated by the reference nucleotide sequences retrieved from the GenBank database with the BLAST sequence search tool (http://www.ncbi.nlm.nih.gov/BLAST), and results were deposited to GenBank. The PCR primers CR-f (5′-GCTACCACTTCAGAATCATCATC-3′) and CR-r (5′-GCACCTTCAGTCGTAGAGACG-3′) amplified the *HWP1* gene for identification *C. albicans*, *C. africana*, and *C. dubliniensis* according to Romeo and Criseo PCR condition introducing method (11). *Candida albicans* (ATCC 10231) was used as the reference strain.

3.5. Phylogenetic Analysis

The sequencing results of the D1/D2 region was examined and compared with the reference nucleotide sequences of *Candida* spp. using the neighbor-joining method by MEGA 7.

4. Results

In total, 320 non-pregnant vaginitis patients were included in the present study. That 100 patients aged 18 - 56 years showed vaginal candidiasis. Most of them were found in isolates of those aged 30 to 39 years old. 86% and 14% of cases were VVC and RVVC (Table 1). Most of the participants were housewives (27%), followed by office workers (25%), factory workers (22%), and self-employed (26%). None of the participants had underlying diseases like diabetes, urinary tract infections, immunocompromised diseases, or any other chronic diseases. Primary identification of the *Candida* spp. was conducted by germ tube test, CHROMagar *Candida*, chlamydospore production, and cultured at 42 °C. *C. albicans* produced green-colored colonies on CHROMagar *Candida*, chlamydospores on CMA medium, and appeared germ tube in the test, as well.

Candida africana generated small green colonies on CHROMagar *Candida* and did not generate chlamydospores on CMA medium. *Candida albicans* strains grew at 42°C, while the *C. africana* isolates did not. *Candida glabrata* and *C. kefyr* (*Kluyveromyces marxianus*) produced dark pink colonies with pale edges and light violet colonies respectively. The clinical isolates with green-colored colonies on CHROMagar *Candida* and reference strain were analyzed with CR-f and CR-r specific primers used to amplify the *HWP1* gene, and the amplification of the ~900-bp fragment confirmed *C. albicans* isolates (Figure 1).

The PCR of the D1/D2 region of the large-subunit rRNA gene with NL1 and NL4 primers amplified ~500 bp fragment (Figure 2). The D1/D2 nucleotide sequences of *Candida* spp. clinical strains were analyzed with reference nucleotide sequences retrieved from the GenBank database using BLAST. The species of all clinical isolates were identified. The D1/D2 sequences of *Candida* spp. clinical isolates were deposited in GenBank under accession numbers KY548531-2, KY548534-KY548548, KY548550,

Table 1. Distribution of *Candida* Isolates in Vaginal Discharge Samples According to Age Groups and Presence of VVC1 and RVVC2

Patient Characteristics	*Candida* Species				
	C. albicans	*C. glabrata*	*C. krusei*	*C. kefyr*	Total[a]
Age, y					
> 20	3	1	0	0	4 (4)
20 - 29	17	13	3	3	36 (36)
30 - 39	22	14	4	0	40 (40)
40 - 49	8	6	1	2	17 (17)
50 - 59	1	2	0	0	3 (3)
Total	51	36	8	5	100
Vaginal candidiasis					
VVC	44	32	6	4	86 (86)
RVVC	7	4	2	1	14 (14)
Total	51	36	8	5	100

Abbreviations: 1VVC, vulvovaginal candidiasis; 2RVVC, recurrent vulvovaginal candidiasis.
[a]Values are expressed as No. (%).

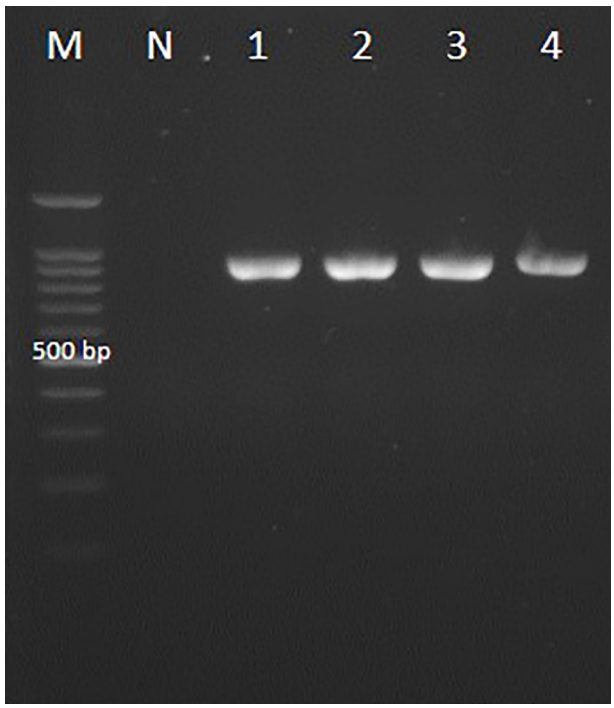

Figure 1. The clinical isolates were analyzed with CR-f and CR-r specific primers used to amplify the *HWP1* gene; lanes 1-3, *C. albicans* (~ 900 bp); lane 4, positive control *C. albicans* (ATCC10231) ~ 900 bp. N, negative control; M, marker 100 bp.

MG025904-MG025928, MG025930-MG025933, MG966211-MG966257, and MG967349-MG967353. *C. albicans* to be the most strain (51%) in total of VVC patients, followed by *C. glabrata* (36%), *C. krusei* (*Pichia kudriavzevii*) (8%), and *C. kefyr* (*K. marxianus*) (5%). Among 14 RVVC cases, the most common pathogen was *C. albicans* (50%), followed by *C. glabrata* (28.6%), *C. krusei* (*P. kudriavzevii*) (14.3%), and *C. kefyr* (*K. marxianus*) (7.1%) (Table 1). The nucleotide sequences of the D1/D2 region were aligned for phylogenetic evaluation. *Candida albicans* isolates showed similarity with KU729162 (*C. albicans* strain ATCC 28121), KY825125.1 (*C. albicans* isolate DA46), and MG859668.1 (*C. albicans* isolate HCM-NM58) reference strains. *Candida glabrata* strains matched completely with MG859667.1 (*C. glabrata* isolate HCM-NM56) and MG228366.1 (*C. glabrata* isolate DMic 154894) reference strains in gene bank databases. *Candida krusei* (*P. kudriavzevii*) indicated 100% similarity to MG859665.1 (HN-NM19 isolate), *C. kefyr* (*K. marxianus*) displayed 100% identity with MH595097.1 (UCDFST: 49-27 isolate) and CP023460 reference strain (Figure 3).

5. Discussion

In the present study, *Candida* spp. was the most important agent of the fungal vaginitis. That is in line with the findings of the previous studies that reported *C. albicans* and *C. glabrata* as the most frequent pathogenic yeasts in VVC patients (13-15). Several studies reported that the percentage of NAC isolates in vaginitis (5, 16) and also infections caused by unusual yeasts is on the rise (17). Traditional methods cause difficulties for definitive diagnosis of the multiplicity of yeast species. Molecular approaches can improve the differentiation of less common yeast isolates and strongly similar yeast species such as those in *Candida* complexes. DNA based approaches are most valuable for

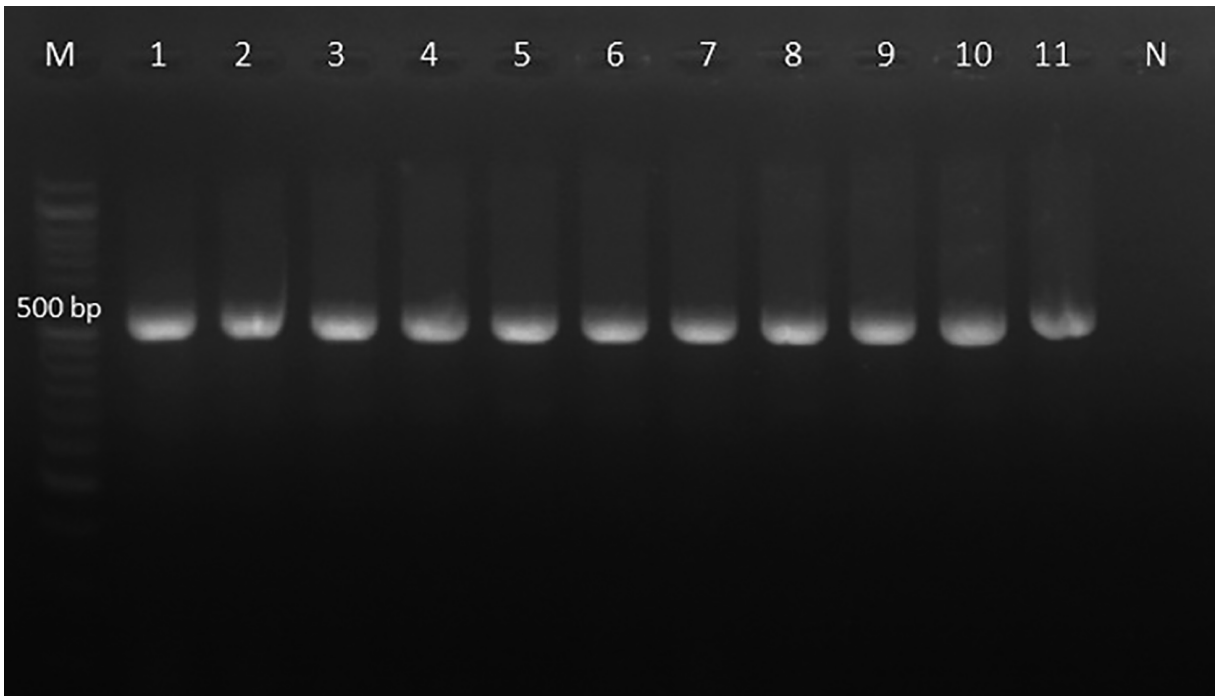

Figure 2. The D1/D2 region of clinical isolates amplified with NL1 and NL4 primers, lanes 1-4: *C. albicans*; lanes 5-7: *C. glabrata*; lanes 8, 9: *C. krusei*; lanes 10, 11: *C. kefyr*. N, negative control; M, marker 50 bp.

large epidemiological studies on unusual yeasts. Although it rarely occurs, several studies have reported the *Saccharomyces cerevisiae* as the agent of genitourinary infection. In the present study, we couldn't find this yeast. However, since it's less susceptible to the currently available antifungal drugs, *S. cerevisiae* is an important pathogenic yeast (18, 19).

In this study, routine mycological methods and DNA sequencing were used to identify yeast isolates. It's proved that sequencing the D1/D2 or ITS regions is an accurate approach for definitive identification of pathogenic yeast species, particularly the *C. glabrata* (17, 20), and the *C. parapsilosis* complexes (21). The phylogenetic tree was built using the sequences of the D1/D2 region of different *Candida* strains and indicated the creation of distinct branches for *Candida* species. The phylogenetic analyses of the D1/D2 region showed that all *C. albicans* isolates had 100% homology with KU729162 (*C. albicans* strain ATCC 28121), KY825125.1 (*C. albicans* isolate DA46), and MG859668.1 (*C. albicans* isolate HCM-NM58) reference strains. *C. glabrata* strains matched completely with MG859667.1 and MG228366.1 reference strains in gene bank databases. *C. krusei* (*P. kudriavzevii*) showed 100% homology with MG859665.1, and *C. kefyr* (*K. marxianus*) showed 100% identity with MH595097.1 and CP023460 reference strains.

Hasanvand et al. (7) investigated *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* isolated from vaginitis patients and reported *C. albicans* as the main cause of vaginal candidiasis. Several studies conducted in Iran and other countries reported *C. albicans* as the predominant pathogen in VVC (2-4, 6, 7, 12-16, 18, 22-24). Another study has reported *C. tropicalis* as the main NAC species that cause VVC (25), which is not consistent with the current study that found *C. glabrata* as the first most frequently encountered NAC species.

Several studies mentioned *C. glabrata* as the important NAC species in *Candida* vaginitis patients (5, 12, 14). The current study revealed that *C. krusei* (*P. kudriavzevii*) and *C. kefyr* (*K. marxianus*), as NAC species, are associated with vulvovaginitis. Clinical isolates like *C. krusei* and *C. glabrata* were resistant or showed low susceptibility to azole drugs, as reported by other studies (5, 18). *Candida glabrata* is reported as a common fungal pathogen in many countries and appears to be intrinsically resistant to fluconazole.

Another study reported *C. lusitaniae* isolates as the second most common *Candida* species have also shown susceptible to all tested antifungal drugs in VVC patients (24), although this yeast was not reported in the current study. Other NAC species such as *C. tropicalis*, *C. parapsilosis*, *Meyerozyma guilliermondii*, *C. dubliniensis*, *C. famata*, *C.*

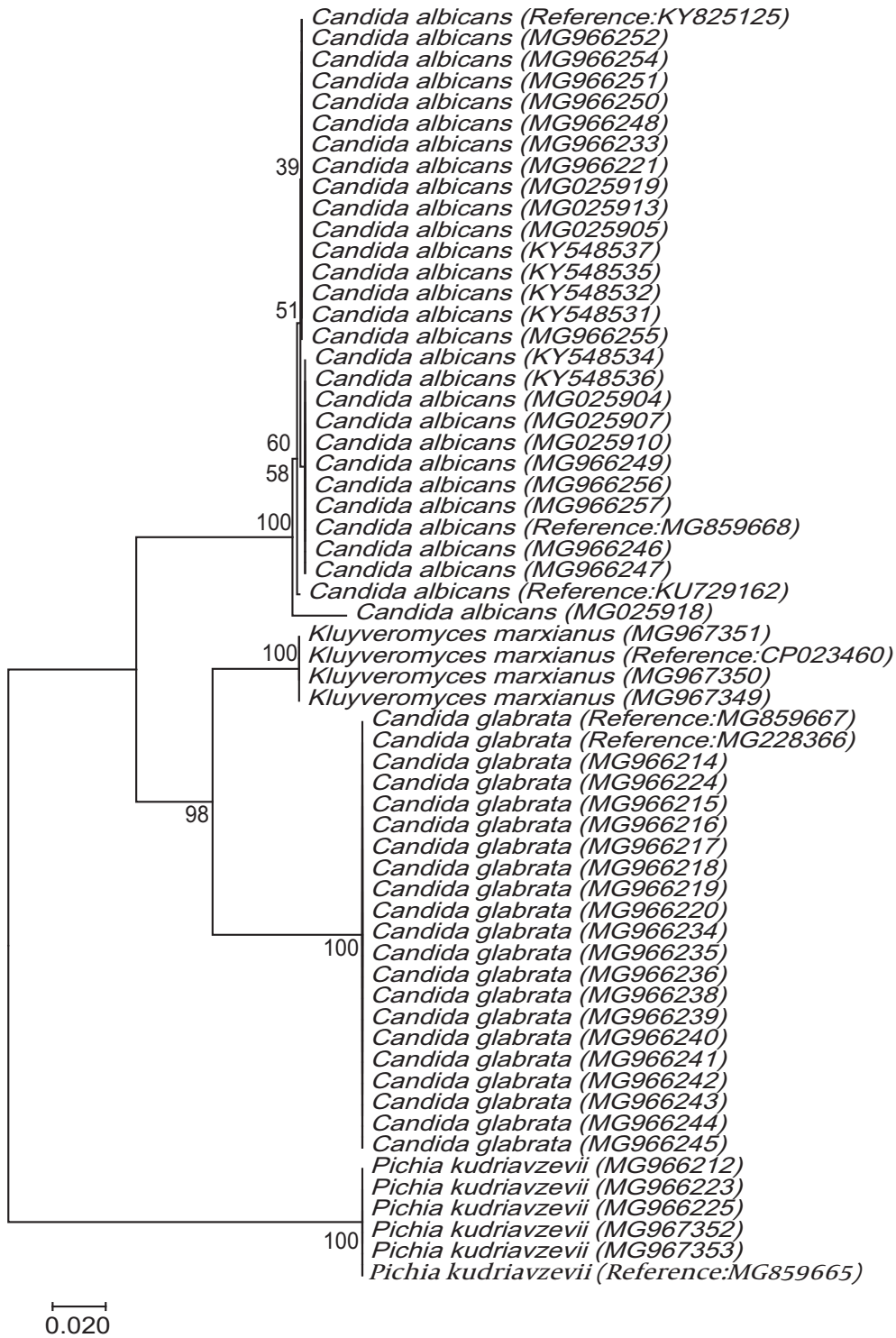

Figure 3. The phylogenetic tree of *Candida* spp. was inferred based on the nucleotide sequences of the D1/D2 region. The evolutionary relationship of *Candida* spp created by the neighbor-joining method, based on the nucleotide sequences of D1/D2 region retrieved from this study (KY548531-KY548532, KY548534-KY548537, MG025904-MG025905, MG025907, MG025910, MG025913, MG025918-19, MG966212, MG966214-MG966221, MG966223- MG966225, MG966233-MG966252, MG966254-MG966257, MG967349-MG967353) compared with reference sequences of *C. albicans* strains (KU729162, KY825125 and MG859668), *C. glabrata* strains (MG859667 and MG228366), *C. krusei* (*Pichia. kudriavzevii*; MG859665), and *C. kefyr* (*Kluyveromyces marxianus*; CP023460) from GenBank. Bootstrap values achieved from 1000 replicates are indicated on branches in percentage. Evolutionary evaluates were presented in MEGA7.

africana, and *C. orthopsilosis* have frequently been reported from VVC patients (7, 22, 24). In this study, *C. albicans* and *C. glabrata* were the most important agents in VVC and RVVC.

Candida kefyr (*K. marxianus*) and *C. krusei* (*P. kudriavzevii*) were isolated from VVC and RVVC patients, as well. In the present study VVC and RVVC patients were healthy women without a history of exposure to known risk factors such as diabetes, pregnancy, immunodeficiency disorders, or any chronic disease. According to the clinical studies, most of RVVC patients are healthy women, and multiple recurring infections are often idiopathic without known potential risk factors (26). Numerous studies reported *C. albicans* as the most prevalent species, even more than NAC species, in vaginal candidiasis patients (2, 6, 7, 13, 15, 18, 22). Overall, *C. albicans* and non-*albicans* species had almost similar rates of prevalence (51% versus 49% respectively). Since NAC species often can be find in RVVC patients and do not respond to standard antifungal therapies, their identification is of crucial importance. The results of this study indicated that *C. albicans* and *C. glabrata* are still the principal pathogenic agents in recurrent and non-recurrent Candidal vaginitis.

5.1. Conclusions

Candida albicans and *C. glabrata* are the most important *Candida* species isolated from VVC and RVVC, which confirms the dramatic incidence of NAC species in VVC patients. Accurate identification of *Candida* spp. is important for treatment administration because of some strains revealed different levels of resistance to antimycotic medicines.

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Footnotes

Authors' Contribution: Study concept and design: SF and ER. Collected samples, interpretation of data, and drafting of the manuscript: ZGM. Performed the experiments: MR and AF. Visited patients: MR.

Conflict of Interests: The authors declare no competing interest.

Ethical Approval: The research protocol was approved by the Ethics Committee of Iran University of Medical Sciences (code: IR.IUMS.REC 1394.9313385002).

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Informed Consent: Informed consent form was signed by participants.

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