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Antifungal susceptibility pattern of *Candida* isolates against six antifungal drugs by microdilution method isolated from vulvovaginal candidiasis

Laal Kargar Melika[1], Roudbar mohammadi Shahla[2]*

1. Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
2. Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.

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ABSTRACT

Candida species are the second most common cause of vulvovaginitis worldwide. Vulvovaginal Candidiasis (VVC) is a common disease affecting more than 75% of all women at least once in their lifetime and 5 to 8% of those individuals also develop recurrent infections. Correct identification of the isolated *Candida* species is essential to direct the empirical antifungal therapy. The aim of this study were to isolate *Candida* from VVC patients, show its characterization and *in vitro* antifungal susceptibility against six antifungal drugs by the broth microdilution method. Vaginal swabs were collected from infected women. One sample was processed for direct microscopic examination and other one was used for culture on Sabouraud dextrose agar (SDA) and CHROMagar. Isolates was identified by battery of tests and antifungal susceptibility testing of *Candida* species was done by Microdilution method. A total of 145 isolates of *candida* species were obtained. *Candida albicans* was found to be the most frequently isolated species, i.e. 114 (78.6%) of the total isolates, followed by *C. glabrata* 17 (11.7%), *C. krusei* 11 (7.5 %), *C. parapsilosis* 2 (1.3 %), and *C. tropicalis* 1 (0.68%). All drugs were active against all of the isolates except for Nystatin and Econazole, two (5%) of *C. albicans* were non-susceptible to it and three (7%) of *C. albicans* isolates were non-susceptible to Tioconazole. Identification of *Candida* to species level and their antifungal susceptibility testing should be done to achieve better clinical results.

1. Introduction

Candida species are the second most common cause of vulvovaginitis worldwide (Sobel, 1986). Almost 75% of women over 25 years of age, described to have at least one episode of physician accepted vulvovaginal candidiasis (VVC) in their lifetime and 5% adapted recurrent type; which is defined by acquiring infection for at least 4 period in a one-year time (Sobel et al., 1998). Underlying factors of the VVC comprise of genetic factors, pregnancy, cancer chemotherapy, high-estrogen oral contraceptives, uncontrolled diabetes mellitus, antimicrobial therapy, Corticosteroid therapy, tight-fitting, synthetic underclothing, use of IUD, high frequency of coitus, organ transplantation, vaginal douching, and HIV infection (Denning et al., 2018). In general, *Candida albicans* (85%-95%) is the most important agent of VVC across the world (Sobel et al., 1998). Other species that cause infections, including *C. glabrata*, *C. krusei, C. tropicalis*, *C. parapsilosis*, *C. kefyr*, and *C. lusitaniae*, have also been reported (Alizadeh et al., 2017; Mirhendi SH 2008). Azoles are the treatment of

*Corresponding authors: Shahla Roudbar Mohammadi
Email address: Sh.mohammadi@modares.ac.ir

choice for VVC; however, resistance has been reported especially in non-*albicans Candida* species (Dota et al., 2011; Trick et al., 2002). Azoles have the advantage of being taken orally, which increases their potency (Salehei et al., 2012). Because of the different susceptibility of *Candida* species to antifungal agents, it is important to identify the causative *Candida* to the species level correctly (Mousavi et al., 2007). Objectives of this study were to isolate *Candida* from VVC patients, show its characterization and *in vitro* antifungal susceptibility against six antifungal drugs by the broth microdilution method.

2. Materials and Methods

2.1. Patients and Treatment Strategies

Women who referred to the Shahid Akbar-Abadi Obstetrics and Gynecology Hospital in Tehran, Iran, between January 30, 2018 and January 30, 2019 were included in the current prospective study. Swab samples were obtained from patients with symptoms including but not limited to vulvar pruritus, burning vaginal soreness, dyspareunia and dysuria, edema, fissures, and vulvar and vaginal erythema. VVC was confirmed by microscopic detection of yeast structures and yeast/*Candida*-positive cultures (Denning et al., 2018; Gonçalves et al., 2016). Patients were treated at the discretion of the treating gynecologists. Initial antifungal treatment included two 150-mg doses of oral fluconazole (the 1st and 4th days) or 1% topical clotrimazole (for 10–12 days). If after 3 months remission persisted, patients were prescribed two 150-mg doses of fluconazole biweekly for 6 months. This study was approved by the human subject hospital review board of Shahid Akbar-Abadi Obstetrics and Gynecology Hospital (IR.MODARES.REC.1397.225). Informed consents were obtained from all patients included in the current study, and researchers were blinded to patient identifiers.

2.2. Specimen processing

Vaginal samples were taken from symptomatic patients using sterile cotton swabs and transferred immediately to Falcon tubes containing PBS. Sampling was performed in accordance with institutional safety protocols. First, the specimens were observed directly under a microscope to reveal yeast structures and then cultured on Sabouraud dextrose agar (SDA) and CHROMagar (CandiSelect, Bio-Rad, Hercules, CA, United States) at 35°C for 48 hrs.

2.3. DNA extraction and 21-plex PCR technique

DNA extraction using CTAB and phenol-chloroform methods has been described in previous studies (Arastehfar et al., 2019). Briefly, a full loop of fresh yeasts colonies was suspended in 700 µL of CTAB buffer followed by bead beating (Tissue Lyzer II, QIAGEN, Hannover, Germany) for 3 min, 3000 beats/minute. After incubation for 60 min at 55°C, 700 µL of phenol-chloroform was added. Upon vortexing and centrifugation for 20 min at 14000 rpm, 4 °C, 400 µL of supernatant was added to isopropanol. Finally, upon washing with 70% ethanol and drying the DNA samples on air, the pellets were suspended in Tris-EDTA (10 mM Tris Base, 1 mM EDTA, pH 8.0) buffer. DNA purity and quantity was assessed using Nano Drop and Qubit Broad range kit (Invitrogen) and the quality was evaluated by electrophoretic separation of 5 µl of DNA samples on 1% agarose gel.

Identification of yeasts with 21-plex PCR was performed in three multiplex PCRs as used previously (Arastehfar et al., 2019). Briefly, the first PCR reaction identifies the most prevalent *Candida species* [*C. albicans, C. glabrata*, *Pichia kudriavzevii* (*C. krusei*), *C. parapsilosis, C. tropicalis*, *C. dubliniensis* and C. *auris*], the second PCR identifies rare *Candida* species [*Diutina rugosa* (*C. rugosa*), *Clavispora lusitaniae* (*C. lusitaniae*), *Pichia norvegensis* (*C. norvegensis*), *Debaryomyces hansenii* (*C. famata*), *Yarrowia lipolytica* (*C. lipolytica*), *Meyerozyma guilliermondii* (*C. guilliermondii*) and *Kluyveromyces marxianus* (*C. kefyr*)] and the third multiplex PCR identifies the most clinically important basidiomycetous yeast species, namely *Trichosporon spp*., *Cryptococcus spp*. and *Rhodotorula mucilaginosa* and one ascomycota yeast, *Geotrichum spp*. All the primers included in the 21-plex PCR, were comprehensively evaluated using blinded test sets implemented in other studies. PCR products and a 50bp ladder were run on a 2% agarose gel (100 V, 60min), stained with Gel Red (BioTium Corporation, USA) and visualized under UV light. Yeast species

identification was achieved by discrimination of the fragment size of the PCR products (Figure 1) (Arastehfar et al., 2019).

2.4. Antifungal Susceptibility Testing

Antifungal susceptibility testing (AFST) was conducted according to the fourth edition of Clinical Laboratory Standards Institute (CLSI-M27) protocol, [Clinical and Laboratory Standards Institute (CLSI), 2017]. Minimal inhibitory concentration (MIC) values were interpreted based on the second edition of CLSI-M60 [Clinical and Laboratory Standards Institute (CLSI), 2020]. Antifungal drugs were dissolved in RPMI1640 (Sigma-Aldrich). Plates were incubated at 35◦C and MICs were determined after 24 hrs. AFST included Nystatin, Clotrimazole, Econazole, Tioconazole, Miconazole and Amphotricin B (both from Sigma-Aldrich, St. Louis, MO, United States). Antifungal drugs were dissolved in RPMI1640 (Sigma-Aldrich). Plates were incubated at 35◦C and MICs were determined after 24 hrs. *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) were used for quality control purposes. MICs of different species were: *C. albicans* (Nys: ≥2, Clo: ≥16, ECO: ≥8, TIO: ≥2, MICo: ≥16, AMB: ≥2), *C. glabrata* (Nys: ≥2, Clo: ≥16, ECO: ≥4, TIO: ≥ 0.0016, MICo: ≥16, AMB: ≥2), *C. krusei* (Nys: ≥2, Clo: ≥16, ECO: ≥8, TIO: ≥2, MICo: ≥16, AMB: ≥2), *C. parapsilosis* (Nys: ≥2, Clo: ≥0.031, ECO: ≥0.0016, TIO: ≥0.0016, MICo: ≥0.0016, AMB: ≥1), *C. tropicalis* (Nys: ≥1, Clo: ≥0.5, ECO: ≥0.031, TIO: ≥0.0016, MICo: ≥1, AMB: ≥1).

2.5. Statistical analysis

Data were statistically described in terms of frequencies and percentages. Comparison between the study groups was done using Chi square (χ^2) test. Exact test was used instead when the expected frequency was less than 5. All statistical calculations were done using SPSS 15 (SPSS; SPSS Inc., Chicago, IL, USA) for Microsoft Windows.

3. Results

3.1. Patients' Characteristics

In the study, 300 case patients were monitored; 81 had diagnosed VVC and over fifty percent of them were recognized with RVVC (in 43 of the 81 patients; 53%). The average age for RVVC patients was 35 years (20–68 years) and for patients with VVC only (in 38 of the 81 patients; 47%), the median age was 39 years (19–63 years).

3.2. Candida Species Distribution

In total, 145 yeast isolates were recuperated from 43 patients with RVVC (in 107 of the 145 isolates; 73.7%) and 38 patients with VVC (in 38 of the 145 isolates; 26.2%); between them, *C. albicans* was the most widespread species (in 114 of the 145 isolates; 78.62%), followed by *C. glabrata* (in 17 of the 145 isolates; 11.72%), *C. krusei* (in 11 of the 145 isolates; 7.59%), *C. parapsilosis* (in 2 of the 145 isolates; 1.38%), and *C. tropicalis* (in 1 of the 145 isolates; 0.69%). The same trend was detected between the 81 patients: *C. albicans* was the most widespread species among both VVC (in 29 isolates of the 38 patients; 76.32%) and RVVC (in 35 isolates of the 43 patients; 81.40%) groups (Table 1). However, most of *C. glabrata* (in 14 of the 17 isolates; 82.35%) and *C. krusei* (in 8 of the 11 isolates; 72.73%) were get better from patients with between RVVC whereas *C. parapsilosis* and *C. tropicalis* were only detected between those with VVC.

3.3. Antifungal susceptibility testing

The results of drug susceptibility testing according to the fourth edition of Clinical Laboratory Standards Institute (CLSI-M27) protocol [Clinical and Laboratory Standards Institute (CLSI), 2017] and MIC of clotrimazole, Nystatin, Econazole, Tioconazole, Miconazole and Amphotericin B was obtained in clinical specimens of *Candida* species. All drugs were active against all of the isolates except for Nystatin and Econazole- two (5%) of the *C. albicans* were non-susceptible to it and three (7%) of the *C. albicans* isolates were non-susceptible to Tioconazole (Table 2).

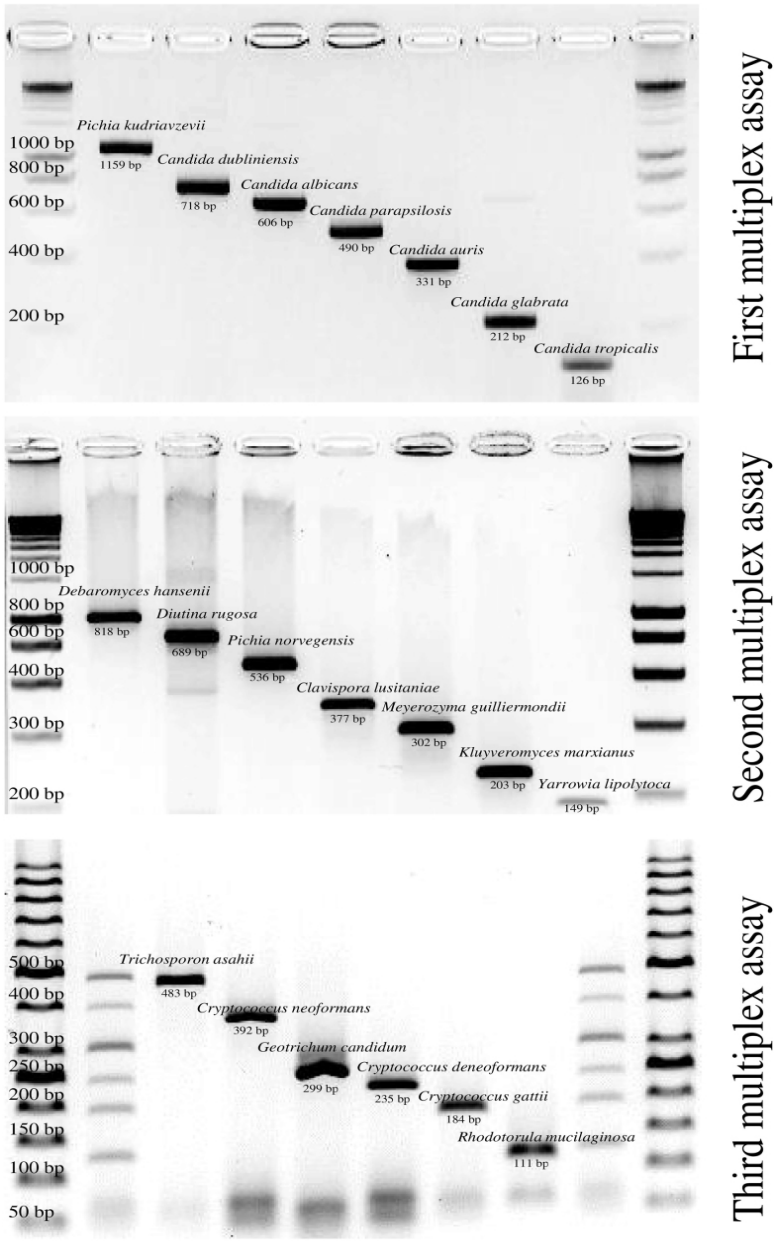

Figure 1. Multi plex PCR identifies the species based on the PCR product size. The PCR product for each species has a distinct length. Adapted from (Arastehfar et al., 2019).

Table 1. The Number of isolates and *Candida* species distribution among patients with VVC and RVVC

Species	isolates				VVC		RVVC		Total number of Patients	
	VVC	RVVC	VVC & RVVC							
C. albicans	29	85	114	78.62%	29	76.32%	35	81.40%	64	79.01%
C. glabrata	3	14	17	11.72%	3	7.89%	5	11.63%	8	9.88%
C. krusei	3	8	11	7.59%	3	7.89%	3	6.98%	6	7.41%
C. parapsilosis	2	0	2	1.38%	2	5.26%	0	0.00%	2	2.47%
C. tropicalis	1	0	1	0.69%	1	2.63%	0	0.00%	1	1.23%
Total	145			100.00%	38	100.00%	43	100.00%	81	100.00%

Table 2. Antifungal susceptibility profile of the different isolated *Candida* species

Species	Amphotericin B (%)			Clotrimazole (%)			Nystatin (%)			Econazole (%)			Tioconazole (%)			Miconazole (%)		
	S	DD	R	S	DD	R	S	DD	R	S	DD	R	S	DD	R	S	DD	R
C. albicans	78	18	0	80	20	0	80	15	5	80	15	5	77	10	7	65	25	0
C. glabrata	75	25	0	75	25	0	70	30	0	90	10	0	100	0	0	88	12	0
C. parapsilosis	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
C. krusei	15	75	0	0	100	0	80	20	0	90	10	0	90	10	0	15	75	0
C. tropicalis	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0

S = sensitive, DD = susceptible dose dependent, R = resistant.

4. Discussion

Vulvovaginal candidiasis (VVC) is one of the most common fungal infections among adult women during their lifetime. The main agent of VVC is *C. albicans;* but it seems that non-*C. albicans* species (*C. glabrata* and *C. tropicalis*) of *Candida* appear to be increasing. In most regions, *C. glabrata* is the second most common agent in vaginal infections. The sensitivity patterns of *Candida* isolates varies among studies conducted in different countries (Deorukhkar & Saini, 2013; Gandhi et al., 2015). The analysis of the yeast species causing vaginitis among Iranian patients conducted in this study revealed that *C. albicans* constituted 79% of the isolates and was the most abundant *Candida* species responsible for VVC and RVVC. This observation is consistent with previous studies conducted on VVC (Ghajari et al., 2018; Sharifynia et al., 2017), and oral candidiasis in Iran (Arastehfar et al., 2019), which documented the abundance of *C. albicans* among Iranian patients, whereas *C. glabrata* was reported the most prevalent *Candida* species among Indian patients (Mohanty et al., 2007).

In this study all *Candida spp.* were sensitive to Amphotericin B. The result was the same as in another study (Zaidi et al., 2018), in which in our study Amphotericin B had the highest sensitivity, i.e. 100 % against *C. parapsilosis*, *C. tropicalis* and *C. krusei* followed by *C. albicans* (80%) and *C. glabrata* (75%), as well as 95% sensitivity was found in study of Gandhi et al. (Gandhi et al., 2015), against *C. glabrata*. In our study, Nystatin was 100% sensitive in all *Candida spp.* except for *C. albicans* resistant (5%) which is quite comparable with the study of Emam et al. (Emam et al., 2012) that showed 100% sensitivity was against *C. albicans*. In study of Arastehfar et al (Arastehfar et al., 2021), they observed that approximately 83% of *C. glabrata* and 73% of *C. krusei* isolates, which intrinsically respond to high MICs of azoles, were obtained from patients with RVVC. This result was consistent with this study.

Altogether, this epidemiological profile indicates that *C. albi*cans is the dominant yeast species in Iranian patients supering from both VVC and RVVC; however, NAC species should also be a matter of concern. The assessment of the azole, Amphotericin B and Nystatin susceptibility profiles of *Candida* isolates in this study showed complicating the management of VVC is the prescription of antifungal agents in the absence of species identification and AFST, suggesting that VVC is an underestimated complication and a growing challenge for the healthcare in Iran.

Conclusions

This study provides information on species pattern and antifungal susceptibility of *Candida* species isolated from VVC cases from Shahid Akbar-Abadi Obstetrics and Gynecology Hospital in Tehran, Iran. In this study, though C. *albicans* was the most common *spp*. isolated, there was a slight increase in the prevalence of non-*albicans Candida spp*. Among the non-*albicans Candida*, *C. glabrata* was the most common species. Antifungal susceptibility pattern showed that *Candida* isolates were more sensitive to Amphotericin –B and Nystatin, compared with that of Clotrimazole and other Azoles. *In vitro* genotypic identification of *Candida* species and susceptibility testing of the yeast to antifungal agents will play a vital role in appropriate selection of antifungal agents for the treatment of fungal infections.

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Refereces

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