



## Drug Resistance and Biofilm Formation in *Candida* Species of Vaginal Origin

Tahereh Alikhani<sup>1</sup>, \*Roshanak Daie Ghazvini<sup>1</sup>, Mehdi Mirzaii<sup>2</sup>, Seyed Jamal Hashemi<sup>1</sup>,  
Mozhgan Fazli<sup>2</sup>, Zahra Rafat<sup>4</sup>, Davoud Roostaei<sup>5</sup>, Pegah Ardi<sup>1</sup>,  
Hasti Kamali Sarvestani<sup>1</sup>, Mahdi Zareei<sup>1</sup>

1. Department of Medical Parasitology and Mycology, School of Public Health, Tebran University of Medical Sciences, Tebran, Iran
2. Department of Microbiology, School of Medicine, Shabroud University of Medical Sciences, Shabroud, Iran
3. Food Microbiology Research Center, Tebran University of Medical Sciences, Tebran, Iran
4. Department of Medical Microbiology, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran
5. Department. of Pharmacology, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran

\*Corresponding Author: Email: rdaie@tums.ac.ir

(Received 15 Apr 2021; accepted 10 Jun 2021)

### Abstract

**Background:** *Candida* species are normal vaginal flora in healthy women, which can cause vulvovaginal candidiasis (VVC). The formation of biofilm is a cause of drug resistance in *Candida* species of vaginal origin. We aimed to specify *Candida* species cause VVC, detect their biofilm-forming ability, and antifungal susceptibility pattern.

**Methods:** Overall 150 vaginal samples were collected from suspected cases of referring to Bahar Hospital of Shahroud, Iran between Jan 2018 and Jan 2019. Samples were cultured on Sabouraud dextrose agar (SDA), Chrome gar *Candida* and Corn meal agar (CMA). PCR-RFLP was performed to confirm the identification. Biofilm formation of the identified species was measured by the Crystal Violet method. The susceptibility to fluconazole, clotrimazole, and miconazole was determined based on the CLSI document M27-A3.

**Results:** Of 50 women (33.3%) were suffering from VVC. *C.albicans* was the predominant species isolated in this study (n=39, 78%) followed by *C. glabrata* (n=11, 22%). In addition, in 25 (50%) of positive samples, biofilm formation was determined. The mean MIC of fluconazole and clotrimazole for *C. albicans* was 5.02 µg /mL and 3.92 µg /mL, respectively. Furthermore, the mean MIC related to these drugs for *C. glabrata* was 12.45 µg / mL and 4.1µg / mL, respectively. The mean diameter of miconazole inhibition zone for *C. albicans* and *C. glabra* isolates was 25.13 mm and 24.5mm, respectively and all of them were susceptible to this drug.

**Conclusion:** *C.albicans* was the predominant *Candida* species isolated from patients with VVC and also was the predominant biofilm producer species.

**Keywords:** Vulvovaginal candidiasis; Biofilm; *Candida albicans*; *Candida glabrata*; Azole antifungals



## Introduction

Vulvovaginal candidiasis (VVC) often referred to a mucosal infection and a common gynecologic problem caused by *Candida* species (1, 2). About 75% of all women will experience an episode of VVC in their lifetime (3). This infection affects most women of reproductive age and is one of the main reasons for referral to a doctor.

*C. albicans*, a dimorphic normal vaginal flora, is responsible for 85–90% of clinical significance in patients with positive vaginal fungal infections (4). However, it seems that there is an increase in the frequency of other *Candida* species (*C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and others) in certain populations (5). In fact, where the balance between the normal vaginal flora and host cells is disturbed, they can cause vulvovaginal infections. Socio-demographic characteristics, use of antibiotics and oral contraceptives, diabetes mellitus, dietary practices, personal hygiene, sexual activity, and specific immunological defects have been identified as potential risk factors (6).

Imidazoles and polyene macrolides are used commonly as effective drugs for eradicating fungal infections (1). The use of these drugs is topically or systemically and they are safe and efficacious (7). The Chemical structures of azole antifungal agents contain either two or three nitrogens. Therefore, classified as imidazoles (ketoconazole, miconazole, clotrimazole, econazole, and butoconazole) or triazoles (itraconazole, fluconazole, terconazole). The azoles inhibit ergosterol synthesis in the fungal cell membrane through their action on the cytochrome P450-dependent enzyme lanosterol 14 $\alpha$ -demethylase. Differences among various azoles are related to their pharmacokinetics as well as their affinity for the target enzymes (8). Therefore, there is no equal effect for all imidazoles against all *Candida* species. For example, *C. tropicalis* and *C. glabrata* are 10 times less sensitive to the action of miconazole than *C. albicans* (9). According to the report, up to 7.5% of *Candida* species isolated from the genital area are resistant to one or more of the commonly used azoles (7).

The excessive use of antifungal drugs as well as a lack of proper drug therapy has led to drug resistance in recent years. One of the resistance factors to antifungal drugs is the formation of biofilms. Biofilms, by preventing the binding of antifungals to microorganisms, produce high resistance to antifungal drugs. Therefore, for the correct diagnosis and timely treatments, it is necessary to identify the factors causing and investigate the pattern of drug sensitivity (10).

In this study, we isolated and identified *Candida* species in the vaginal area of patients referred to Shahroud Bahar Hospital and tested in vitro activities of fluconazole, clotrimazole, and miconazole against isolated *Candida* species. Therefore, this study can provide a deeper understanding and a better perspective of biofilm production and antifungal susceptibility in *Candida* spp. isolated from patients with vaginal candidiasis and assist in its control.

## Materials and Methods

### *Ethics approval*

This study was approved by the Ethical Committee of Tehran University of Medical Science (the number of Ethics Committee protocol: IR.TUMS.SPH.REC.1397.154). Written informed consent was obtained from all subjects before sample collection.

### *Sampling*

During a cross-sectional study, from Feb 2018 to Jul 2018, 150 patients with vulvovaginitis symptoms such as itching, burning sensation, pain, inflammation, and excessive and stinging discharge were enrolled in this study. Laboratory test for determining the presence of infection and evaluation the *in vitro* susceptibility to three antifungal agents was done in Shahroud University of Medical Sciences, Iran.

In the first step, demographic data of the patients were obtained through questionnaires, and then the collection of posterior vaginal fornix or vagi-

nal wall specimens by the senior clinicians was done for laboratory testing. Patients who were previously treated with anti-fungal drugs within two months ago were excluded from the study. Samples were prepared by KOH 10%, cultured on Sabouraud's dextrose agar (Merck, Germany), and kept at 37 °C for one week. Isolates were identified based on production chlamydoconidia in cornmeal agar (Becton, France) and colony color on chromogenic CHROMagar *Candida* medium (CHROMagar, Paris, France). In addition, for confirmation of species identification isolates were subjected to the polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) technique (11).

#### **Crystal violet method for assessment biofilm formation**

Growth from Sabouraud dextrose agar (SDA) was taken in 5 mL of sterile brain heart infusion (BHI) broth and incubated overnight. It was followed by 1:100 dilutions in BHI. Then, 100 µL of diluted broth was incubated at 37 °C overnight in commercially available pre sterilized, polystyrene, round-bottomed, 96-well microtiter plate (Cat no. 2101; Thermo Fischer Scientific, Waltham, MA, USA) for biofilm production. The microtiter plates were washed with distilled water. After washing with distilled water, the microtiter plate was stained with 120 µL of 0.1% aqueous crystal violet solution (crystal violet, Hi-Cert/ACS-GRM114-10G) and incubated at room temperature for 15 min. Each well was washed four times with sterile distilled water, blot dried, immediately de-stained with 125 µL of 95% methanol, and incubated for 15 min at room temperature. After de-staining, 100 µL of the de-staining solution was transferred to a new well, and the de-staining solution was measured spectrophotometrically using the enzyme-linked immunosorbent assay (ELISA) reader at 570 nm. Each sample was run in duplicates (12). The following were the values for the biofilm formation:

$OD_c = OD_{\text{Cut off}} = (\text{Mean} + 3SD) + OD_{\text{Blank}}$

$OD \leq OD_c = \text{non-adherent}$

$OD_c < OD \leq 2 OD_c = \text{weakly adherent}$

$2OD_c < OD \leq 4 OD_c = \text{moderately adherent}$

$4OD_c < OD = \text{strongly adherent}$

#### **Assessment of antifungal drug susceptibility**

Three clinically important antifungal agents were used in this study. All drug solutions were prepared immediately before use. Antifungal susceptibility testing for fluconazole and clotrimazole was determined by the broth microdilution method using the guidelines outlined in the Clinical and Laboratory Standards Institute (CLSI) document M27-A3. Both of them were dissolved in dimethyl sulfoxide (DMSO) and filtered into the growth medium. Freshly prepared stock solutions of the drugs were diluted in the growth medium (RPMI with MOPS buffer, pH 7). Testing was performed in sterile flat-bottomed 96-well microplates, containing RPMI-1640 medium with L-glutamine, without sodium bicarbonate supplemented with glucose (18 mg/mL) and buffered with M3-(N-morpholine)-propane sulfonic acid (MOPS) at pH 7.0. A final yeast concentrations after inoculation  $1 \times 10^3$  cells/ml. 50 µL of this suspension was transferred to an ampule of microplates and 100 µL of the inoculated medium was transferred into each cupule. Microplates were incubated at 35 °C for 48 h. Readings were made both visually and spectrophotometrically at 490 nm with a microplate reader.

Disk diffusion (DD) testing of miconazole was performed as described previously in the CLSIM27-A3 protocol (13). We prepared plates with Muller Hinton Agar + 2% Glucose + 0.5 mcg/mL Methylene Blue Dye (M1825) for carrying out the susceptibility of antifungal discs. The medium in the plates was sterile and had a depth of about 4 mm. The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to the turbidity of a 0.5 McFarland standard. Miconazole disks (Becton Dickinson, Sparks, MD) were placed onto the surfaces of the inoculated plates, and the plates were incubated in the air at 35 °C to 37 °C and read at 18 to 24 h. Zone diameter endpoints were read at 80% growth inhibition by using a Biomic image analysis plate reader system (Giles Scientific) (13-15).

### Statistics analysis

Data analysis was performed by *t*-test method with SPSS (Chicago, IL, USA) Statistics version 21. The level of statistical significance was set at  $P < 0.05$ . The Chi-square test was used to compare significantly percentages.

### Results

From 150-suspected cases of vulvovaginal disease, 50 women (33.3%) were suffering from VVC. *C. albicans* was the predominant species isolated in this study ( $n=39$ , 78%) followed by *C. glabrata* ( $n=11$ , 22%).

All 50 clinical isolates were subjected to biofilm production. Out of 39 *C. albicans* isolates, 19 (48.7%) strains were positive for biofilm production.

Among the total 11 *C. glabrata* isolates, 6 (54.5%) isolates were biofilm producers. We calculated the OD Cut off = 0.13.

The highest inhibitory concentration of fluconazole for *C. albicans* was 16  $\mu\text{g}/\text{mL}$ , while the lowest inhibitory concentration was 0.5  $\mu\text{g}/\text{mL}$ . Furthermore, the highest inhibitory concentration of this drug for *C. glabrata* was 32  $\mu\text{g}/\text{mL}$ , while the lowest inhibitory concentration was 1  $\mu\text{g}/\text{mL}$ . Furthermore, the highest inhibitory concentration of clotrimazole for both isolated species was 8  $\mu\text{g}/\text{mL}$ , while the lowest inhibitory concentration was 1  $\mu\text{g}/\text{mL}$  for both of them.

The diameter range of miconazole inhibition zone against *C. albicans* strains was 20-40 mm and the diameter range of miconazole inhibition zone against *C. glabrata* was 19-40 mm. Moreover mean diameter of miconazole inhibition zone for *C. albicans* and *C. glabra* isolates was 25.13 mm and 24.5 mm, respectively and all of them were susceptible to this drug.

There was a statically significant relationship between the MIC of clotrimazol and the ability of biofilm production in *C. albicans* and *C. glabrata* isolates ( $P < 0.05$ ). In addition, there was not a statically significant relationship between the MIC of fluconazole and the ability of biofilm

production in *C. albicans* and *C. glabrata* isolates. Concerning miconazole disk the results of *C. albicans* and *C. glabrata* isolates were not statistically significant.

### Discussion

Candidiasis is an infection caused by a yeast (a type of fungus) called *Candida*. *Candida* normally lives in anatomical sites of the body such as the mouth, gut, vagina, and skin without causing any problems. However, sometimes it can multiply and cause a vaginal yeast infection (16-18). VVC due to different *Candida* species is a very common type of fungal infection. It remains a significant problem worldwide estimated to be the most common fungal infection in a number of countries (19-21).

Recent evidence suggested that the majority of infections produced by these fungal elements are associated with biofilm growth. Biofilms are biological communities with a high degree of organization, in which microorganisms form structured, coordinated, and functional communities. These biological communities are embedded in a self-created extracellular matrix. Biofilm production is also associated with a high level of antimicrobial resistance of the associated organisms. The ability of *Candida* species to form drug-resistant biofilms is an important factor in their contribution to human disease.

In this study, according to other studies, *C. albicans* was the predominant *Candida* species isolated from vaginal specimens (22-27). However, one study provided evidence of the increasing prevalence of non-*C. albicans* species contribute to VVC (28).

Moreover, *C. albicans* and *C. glabrata* are capable of producing biofilms. The majority of disease produced by *C. albicans* is associated with biofilm growth (29). *C. glabrata* isolates were also capable of producing biofilms (30).

Antifungal susceptibility testing revealed that clotrimazole had the highest antimicrobial activity against isolated *Candida* species. In addition, miconazole demonstrated potent inhibitory activity against all of the *Candida* strains tested, indi-

cating that no miconazole resistant strains were detected among any of the species.

On the contrary, 2 *C. glabrata* species (18.18%) were resistant to fluconazole. Totally, with the exception of fluconazole, all of the comparator drugs demonstrated activity against the tested isolates.

## Conclusion

For the correct diagnosis and timely treatment, it is necessary to identify the factors causing drug resistance and investigating the pattern of drug sensitivity. In the present study, *C. albicans* was the predominant *Candida* species isolated from patients with VVC, which also was the predominant biofilm producer species. Resistance of vaginal *Candida* species isolates to antifungal agents was infrequent.

## Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

## Acknowledgements

The authors would like to thank Tehran University of Medical Sciences for supporting this project. This article was extracted from an MSc thesis by Tahereh Alikhani and was supported by the funding from Tehran University of Medical Sciences, Tehran, Iran (grant number: 42806).

## Conflict of interest

The authors declare that there is no conflict of interest.

## References

1. Nyirjesy, P (2008). Vulvovaginal candidiasis and bacterial vaginosis. *Infect Dis Clin North Am*, 22(4): 637-652.
2. Sobel, J. D. (1992). Pathogenesis and treatment of recurrent vulvovaginal candidiasis. *Clin Infect Dis*, 15:148-53.
3. Mendling W, Brasch, J (2012). Guideline vulvovaginal candidosis (2010) of the german society for gynecology and obstetrics, the working group for infections and infectimmunology in gynecology and obstetrics, the german society of dermatology, the board of German dermatologists and the german speaking mycological society. *Mycoses*, 3:1-13.
4. Oriel JD, Partridge B M, Denny MJ, et al (1972). Genital yeast infections. *Br Med J*, 4(5843): 761-4.
5. Sobel JD (1985). Epidemiology and pathogenesis of recurrent vulvovaginal candidiasis. *Am J Obstet Gynecol*, 152(7 Pt 2):924-35.
6. Linda O, Eckert MD (2006). Acute vulvovaginitis. *N Engl J Med*, 355(12): 1244-52.
7. Redding S, Smith J, Farinacci G, et al (1994). Resistance of *C. albicans* to fluconazole during treatment of oropharyngeal candidiasis in a patient with AIDS: documentation by in vitro susceptibility testing and DNA subtype analysis. *Clin Infect Dis*, 18(2):240-2
8. Donlan R, Murga R, Carpenter J, et al (2001). Monochloramine disinfection of biofilm-associated *Legionella pneumophila* in a potable water model system. *Legionella*, 31 (1): 406-10.
9. Sobel JD (1987). Recurrent vulvovaginal candidiasis. A prospective study of the efficacy of maintenance ketoconazole therapy. *N Engl J Med*, 315(23):1455-8.
10. Kumamoto CA (2005). A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. *Proc Natl Acad Sci*, 102(15): 5576-81.
11. Mohammadi R, Mirhendi H, Rezaei-Matehkolaei A, et al (2013). Molecular identification and distribution profile of *Candida* species isolated from Iranian patients. *Med Mycol*, 51(6):657-63.
12. Inci M, Atalay MA, KOÇ AN, et al (2012). Investigating virulence factors of clinical *Candida* isolates in relation to atmospheric conditions

- and genotype. *Turkish Journal of Medical Sciences*, 42(Sup2):1476-1483.
13. Pfaller MA, Diekema DJ, Gibbs DL, et al (2007). Results from the ARTEMIS DISK Global Antifungal Surveillance study, 1997 to 2005: an 8.5-year analysis of susceptibilities of *Candida* species and other yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J Clin Microbiol*, 45(6):1735-45.
  14. Hazen KC, Baron EJ, Colombo AL, et al (2003). Comparison of the susceptibilities of *Candida* spp. to fluconazole and voriconazole in a 4-year global evaluation using disk diffusion. *J Clin Microbiol*, 41(12): 5623-32.
  15. Pfaller M, Diekema D, Gibbs D, et al (2009). Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: 10.5-year analysis of susceptibilities of noncandidal yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J Clin Microbiol*, 47(1):117-23.
  16. Rafat Z, Hashemi SJ, Ahamdikia K, et al (2017). Study of skin and nail *Candida* species as a normal flora based on age groups in healthy persons in Tehran-Iran. *J Mycol Med*, 27(4): 501-505.
  17. Rafat Z, Hashemi SJ, Ashrafi K, et al (2020). Fungal Isolates of the Respiratory Tract in Symptomatic Patients Hospitalized in Pulmonary Units: A Mycological and Molecular Epidemiologic Study. *J Multidiscip Healthc*, 13(1): 661-669.
  18. Rafat Z, Hashemi SJ, Ashrafi K, et al (2020). Epidemiology, laboratory diagnosis and clinical aspects of fungal pulmonary infections in 384 patients hospitalized in pulmonary units in Guilan province, Iran. *Iranian Journal of Microbiology*, 12(4): 353-63.
  19. Klimko N, Kozlova Y, Khostelidi S, et al (2015). The burden of serious fungal diseases in Russia. *Mycoses*, 5:58-62.
  20. Sherry L, Kean R, McKlound E, et al (2017). Biofilms formed by isolates from recurrent vulvovaginal candidiasis patients are heterogeneous and insensitive to fluconazole. *Antimicrob Agents Chemother*, 61(9): e01065-17.
  21. Giacomazzi J, Baethgen L, Carneiro LC, et al (2016). In association with the LIFE program. The burden of serious human fungal infections in Brazil. *Mycoses*, 59(3):145-50.
  22. Jung HS, Ehlers MM, Lombaard H, et al (2017). Etiology of bacterial vaginosis and polymicrobial biofilm formation. *Crit Rev Microbiol*, 43(6):651-667.
  23. Hardy L, Cerca N, Jespers V, et al (2017). Bacterial biofilms in the vagina. *Res Microbiol*, 168(9-10):865-874.
  24. Akbarzadeh M, Bonyadpour B, Pakshir K (2010). Causes and clinical symptoms of vaginal candidiasis in patients referring to selective clinics of Shiraz University of Medical Sciences. *J Arak Uni Med Sci*, 3(3):12-20.
  25. Nazeri M, Mesdaghinia E, Moravej SA, et al (2012). Prevalence of vulvovaginal candidiasis and frequency of *Candida* species in women. *J Mazandaran Univ Med Sci*, 21(86):254-262.
  26. Gonçalves B, Ferreira C, Alves CT, et al (2012). Prevalence of vaginal candidiasis infection in diabetic women. *African Journal of Microbiology Research*, 6(11):2773-2778.
  27. Sasani E, Rafat Z, Ashrafi K, et al (2021). Vulvovaginal candidiasis in Iran: A systematic review and meta-analysis on the epidemiology, clinical manifestations, demographic characteristics, risk factors, etiologic agents and laboratory diagnosis. *Microb Pathog*, 154:104802.
  28. Gonçalves B, Ferreira C, Alves CT, et al (2016). Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. *Crit Rev Microbiol*, 42(6), 905-27.
  29. Ramage G, López-Rib JL (2005). Techniques for antifungal susceptibility testing of *Candida albicans* biofilms. *Methods Mol Med*, 118 (1): 71-9.
  30. Silva S, Henriques M, Martins A, et al (2009). Biofilms of non-*Candida albicans* *Candida* species: quantification, structure and matrix composition. *Med Mycol*, 7(1):681-9.