

**Original Article****The Phenotypic variation of *Candida albicans* and susceptibility to fluconazole and voriconazole****Nemati shirzi, L.***Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Tehran, Iran*

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**ABSTRACT**

*Candida albicans* is the most frequent opportunistic fungal agent in human being. One of its virulence factors is phenotypic switching. In this study, we investigated the susceptibility of different phenotypes of *C. albicans*, obtained from clinical specimens, to fluconazole (FLZ) and voriconazole (VRZ) with microdilution reference method. In this study, 281 *C. albicans* of six different phenotypes including 66.19% smooth, 11.38% stipple, 8.89% fuzzy, 6.40% star, 4.27% irregular and 2.84% ring form were collected. Among these specimens, 75.80% and 88.61% of the phenotypes were susceptible (s), 13.52% and 5.96% were susceptible, but dose dependent and finally 10.67% and 1.77% were resistant (r) to FLZ and VRZ, respectively. Most of (s) samples were smooth form and most (r) forms were stipple. The mean minimum inhibitory concentration of FLZ was higher than VRZ. In general, two antifungal medicines were effective on different phenotypes of *C. albicans*. Samples of (s) group had a significant difference with (r) group ( $p < 0.05$ ). The raising prevalence of candidiasis and more probability of susceptibility pattern in *C. albicans* phenotypes are the reasons to use the susceptibility tests on the antifungal drugs in clinical laboratory.

**Keywords:** *Candida albicans*, phenotypic switching, fluconazole, voriconazole, microdilution

**Etude de sensibilité des différents phénotypes de *Candida albicans* isolés à partir de patients souffrant de Candidose vis-à-vis des médicaments antifongiques Fluconazole et Voriconazole**

**Résumé:** *Candida albicans* est la cause la plus fréquente d'infections fongiques opportunistes et l'une de ses caractéristiques est son changement phénotype. Dans cette étude, la sensibilité aux médicaments antifongiques Fluconazole et Voriconazole a été déterminée chez 281 isolats de *Candida albicans* montrant 6 différents phénotypes infectieux de candidose. A cet effet, la méthode de dilution dans un liquide a été utilisée comme norme de référence. Les phénotypes avaient la forme plane (66.19%), tachetée (11.38%), duveteuse (8.89%), étoilée (6.40%), rugueuse (4.27%) et en anneau (2.84%). D'après les résultats, 75.80% et 88.61% des phénotypes étudiés étaient respectivement sensibles aux Fluconazole et Voriconazole, 13.6% et 5.96% montraient une sensibilité intermédiaire aux deux médicaments alors que 10.67% et 1.77% étaient résistants. La plus haute sensibilité aux deux médicaments était relative à la forme plane et la sensibilité la plus faible concernait la forme tachetée. Cette étude a démontré que le Voriconazole est capable d'inhiber la croissance de la plupart des phénotypes de *C. albicans* à des concentrations inférieures au Fluconazole. Dans l'ensemble, les deux médicaments montraient une bonne activité contre les différents phénotypes et la différence observée chez le groupe sensible au médicament était statistiquement significative comparée aux deux autres groupes ( $p < 0.05$ ). L'augmentation des dispositions à cette maladie, des sensibilités variables aux médicaments et la sensibilité déterminable dans les différents phénotypes de *C. albicans* ont révélé la nécessité d'utiliser en routine des méthodes de détermination et d'évaluation de la sensibilité aux médicaments dans les laboratoires cliniques afin d'améliorer le traitement de cette infection.

**Mots clés:** *Candida albicans*, les changements dans le phénotype, Fluconazole, Voriconazole, dilution dans liquide

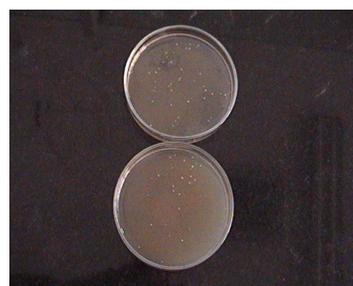
## INTRODUCTION

*Candida albicans* is normal habitat of the mucosal membranes including the gastrointestinal tract, vagina, and skin's normal flora. This opportunistic fungi is pathogen and a prevalent cause of mucosal and systemic infections. Besides, the prevalence of significant opportunistic diseases is raising due to the increment of the individuals with immunodeficiency predisposing conditions such as diabetes mellitus, prolonged antibiotic treatment, cancer treatment and other immunocompromising procedures (Antony et al., 2007; Tong et al., 2014). One of the virulence factors is phenotypic switching of the colony morphology. These cells switch at frequencies as high as  $10^{-4}$  to  $10^{-1}$  among seven distinct colony phenotypes including smooth, star, ring, stipple, irregular wrinkle, hat, and fuzzy (Cetinkaya and Kiraz, 2005; Huang, 2012). Although antifungal resistance in *C. albicans* is less frequent than the other species, but the amount of drug susceptibility is reported with the different phenotypic forms of *C. albicans*. This is a particular morphological type with a distinct advantage over the other types, in causing candidiasis. This takes part by this pathogen by: variation in the shape and size of the cells, the ability to form hyphae, the surface properties e.g. adhesion and permeability, membrane composition, range of secretory products, sensitivity to neutrophils and oxidants, antigenicity, and resistance to antifungal agents (Xie et al., 2013). These features increase the need to use the susceptibility tests to detect the predictable susceptibility patterns in *C. albicans* phenotypes to determine the best antifungal medicines (Kiraz et al., 2000; Lan et al., 2002). However, despite the abundance of data on antifungal susceptibility patterns of *C. albicans* and the factors affecting its colonization in the hosts, there is only limited evidence on the association between the phenotypic variation and the antifungal susceptibility of its wild-type variants (Sachin et al., 2014). The aim of the present study was to investigate the relationship between different phenotypes of *C. albicans* strains isolated

from clinical specimens and the susceptibility of these strains to FLZ and VRZ.

## MATERIALS AND METHODS

**Isolates.** The study strains included *C. albicans* were isolated from 281 patients who had different types of candidiasis through oropharyngeal swabs, nail and urine samples, and urethral and/or vaginal swabs. The patient were recruited from the Shariyati and Razi hospitals of Tehran, the medical mycology department of pasture institute, dental clinics in Tehran and Karaj and one mycology laboratory in Karaj. The subjects had no history of previous antifungal prophylactic chemotherapy and their invasive fungal forms were identified by direct smear. The samples were plated directly onto Sabouraud dextrose agar (SDA) and corn meal tween 80 agar (Merck, Germany) at 37 °C. The *C. albicans* isolates were approved based on the germ tube and chlamydospore formation, the microscopic appearance and carbohydrate fermentation results by using API ID32 C kit (Biomérieux, France) (Table 1).



**Figure 1.** Colony count (MIC90%) after drug confrontation. (Top plate: VRZ, Sole plate: FLZ)

The phloxine B agar plates were prepared according to the Anderson and Soll modification of the Lee synthetic medium (Cetinkaya and Kiraz, 2005). The *C. albicans* isolates were inoculated in this medium and the cultures were incubated at 25 °C. The phloxine B agar was used to determine the different colony phenotypes in *C. albicans*. The isolates were inspected in this medium at 48h and after nine days. The different colony phenotypes of the *C. albicans*, including the smooth, irregular, fuzzy, star ring and stipple forms,

were detected in the studied strains of *C. albicans*. (Table 2).

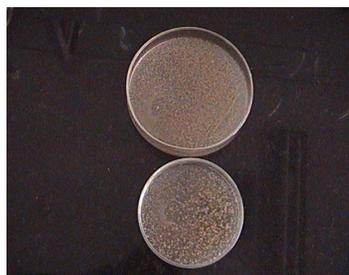


**Figure 2.** Colony count of susceptible standard strain after drug confrontation. (Top plate: VRZ, Sole plate: FLZ)

**CLSI susceptibility testing procedure.** By using the procedures specified by the M27A3 method (CLSI 2010). The stock solutions of VRZ and FLZ were prepared and diluted in dimethyl sulfoxide and water, respectively. Then, they were diluted for testing at final concentrations from 0.03 to 16mg/ml (VRZ) and 0.125 to 64mg/ml (FLZ) with RPMI 1640 medium (Sigma Chemical Co.). Finally, they were buffered to pH 7.0 with 0.165M morpholinopropanesulfonic acid (Sigma, Tehran, Iran), as outlined in document M27-A3. The drug dilutions were dispensed into 96-well round-bottom microdilution plates that were sealed and stored frozen at minus 70 °C until needed. The strains of *C. albicans* inoculums were adjusted to a concentration of  $0.5 \times 10^3$ - $2.5 \times 10^3$  CFU/ml and an aliquot of 100 $\mu$ l was added to each well of the microdilution plate. The plates were incubated at 35 °C for 48h.

**Endpoint determination.** After 48h, the contents of the wells to be inoculated to SDA and the endpoints were determined by colony counting after 48h incubation at 35 °C. The MICs was defined as the lowest concentration of drug that produced a 90% decrease of growth compared with drug-free control (Figure1). Following incubation, the MFC (Minimum fungicidal concentration) also were detected. (Table 3)

**Quality control isolates:** *C. albicans* ATCC 10231 was used as a susceptible standard isolate and *C. albicans* ATCC 64550 as a resistant standard isolate to both drugs. (Figure 2, 3)



**Figure 3.** Colony count of resistant standard strain after drug confrontation. (Top plate: VRZ, Sole plate: FLZ)

**Statistical analysis.** All statistical analysis was done with SPSS version 16.0

## RESULTS

In this study, 281 isolates of *C. albicans* were tested. These specimens were obtained from nail (n=35), vagina (n=138), urine (n=23), oral lesions (n=12), gastrointestinal tract (n=12) and oropharynx (n=21). The mean frequency of occurrence of six common phenotypes identified in the 281 *C. albicans* strains is shown in table 1. The smooth phenotypes accounted for the majority of the colonies, that obtained from all of samples had frequency greater than 66% and the ring phenotypes was the least frequent type. Our findings, based on the microdilution reference method, revealed that 75.80% and 92.52% all of isolates were susceptible to FLZ (MICs $\leq$ 8 $\mu$ g/ml) and VRZ (MICs $\leq$ 0.125 $\mu$ g/ml), respectively. Then, 13.52% and 5.60% were susceptible dose-dependent to FLZ (S-DD, MICs $\leq$ 16 to 32 $\mu$ g/ml) and VRZ (S-DD, MICs $\leq$ 0.25 to 0.5 $\mu$ g/ml), respectively. Finally, 10.67% and 1.77% showed resistance to FLZ (MICs $\geq$  64 $\mu$ g/ml) and VRZ (MICs $\geq$  1 $\mu$ g/ml), respectively. The detail of Mean MICs values ( $\pm$ SE) of FLZ and VRZ for *C. albicans* phenotypes are shown in the table 3. The MICs for the six phenotypes ranged between 0.25-64 $\mu$ g/ml for FLZ and 0.03-1 $\mu$ g/ml for VRZ. The stipple phenotypes showed the highest resistance to both drugs and showed statistical difference to other phenotypes and the highest sensitivity was related to the smooth phenotypes (F=13.43, p<0.01, F=0.86, p<0.001)(Table 2, 3).

## DISCUSSION

*Candida albicans* is both a commensally and an opportunistic pathogen, and a prevalent cause of mucosal and systemic infections. It causes some significant opportunistic diseases in which the increase in the number of individuals with immunodeficiency and other predisposing conditions such as diabetes mellitus, prolonged antibiotic treatment, cancer treatment and invasive procedures, have increased its prevalence (Morschhauser, 2010; Pfaller et al., 2015). Most strains of *C. albicans* are capable of switching frequently and reversibly between varied phenotypes, distinguishable by colony morphology (Vargas et al., 2000; Brockert et al., 2003). A number of different switching systems have been defined according to the limited set of phenotypes in each switching repertoire, and each strain appears to possess a single system (Yang, 2003). The switching can affect many aspects of cellular physiology and morphology, so it appears to be a second level of phenotypic variability superimposed upon the bud-hyphae transition (Lohse and Johnson, 2009). The phenotypic switching influences the virulence, mating behavior, antifungal susceptibility and biofilm formation (Antony et al., 2007). FLZ is a member of the azole antifungal agents, which acts by inhibiting cytochrome P-450 sterol 14 demethylase, an enzyme involved in ergosterol biosynthesis. This azole is frequently used against *C. albicans* infection; however, the long-term administration of the drug, might lead to growth of FLZ-resistance isolates. VRZ is a new triazole antifungal agent that has potent activity against many fungal infections and FLZ-resistance *C. albicans* (Marr et al., 2001). There is limited evidence about the relationship between phenotypic switching and antifungal susceptibility of *C. albicans*. Therefore, in this study, 281 *C. albicans* strain isolates from clinical specimens with different phenotypes were identified and susceptibility testing was performed to assess the sensitivity or resistance of *C. albicans* phenotypes to FLZ and VRZ. The results showed cross-resistance between FLZ and VRZ for some *C. albicans* isolates

with higher FLZ MICs, which were associated with higher VRZ MICs ( $p < 0.001$ , linear regression). Excepting the stipple type, we found no significant difference between FLZ and VRZ mean MIC<sub>s</sub> levels for other phenotypes (Velegraki et al. 1996) determined the phenotypes of *C. albicans* strains isolates. They found smooth, fuzzy, irregular and stipple types of *C. albicans* strains and observed that the MICs levels of the stipple phenotypes for FLZ and itaconazole were consistently higher than MICs of the other phenotypes. (Vargas et al. 2000) reported dramatic differences of susceptibility among the switched phenotypes of colonizing strains of FLZ and VRZ. While, (Kiraz et al. 2000) reported that FLZ susceptibility of the stipple type showed a statistically significant difference from the other phenotypes. A high level of spontaneous variability in such populations would provide them with the advantage of rapid adaptation and this might offer a particular morphological type, with a distinct advantage over the other types in inducing the clinical disease. Therefore, the investigation of the colonial phenotypes on phloxine B agar and separate evaluation of the antifungal susceptibility of these phenotypes might be useful in the selection of the best antifungal regimen.

## Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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