Jpn. J. Med. Mycol.
Vol. 46, 197−201, 2005
ISSN 0916−4804

Original Article

In vitro Investigation of Antifungal Activities of Phenotypic Variation *Candida albicans* Strains against Fluconazole, Itraconazole and Voriconazole

Zafer Cetinkaya[1], Nuri Kiraz[2]

[1]Afyon Kocatepe University Faculty of Medicine Department of Microbiology, Turkey
[2]Osmangazi University Faculty of Medicine Department of Microbiology, Eskisehir, Turkey

〔Received: 3, March 2005. Accepted: 13, May 2005〕

Abstract

The aim of this study was to investigate the susceptibility of the different phenotypes of *Candida albicans* strains isolated from clinical specimens to three antifungal agents, fluconazole, itraconazole and voriconazole. Totally 215 specimens were collected from oropharyngeal, gastrointestinal and urogenital tracts of non-neutropenic patients who had received no previous prophylactic treatment. Each of the 215 *C. albicans* strains recovered was found to express one of the six phenotypes: smooth 73%, fuzzy 10.7%, irregular 2.3%, star 2.8%, ring 6% or stipple 5.1%.

The mean MICs for the six phenotypes of *C. albicans* strains ranged between 0.25 μg/m*l* and 64 μg/m*l* for fluconazole, 0.03 μg/m*l* and 1 μg/m*l* for itraconazole and 0.03 μg/m*l* and 0.5 μg/m*l* for voriconazole. The mean minimum inhibitory concentration (MIC) of fluconazole was consistently higher for *C. albicans* strains expressing the stipple phenotype. The antifungal susceptibility of the phenotypic switching requires attention, especially in patients who are clinically unresponsive to fluconazole chemotherapy or in cases of serious *C. albicans* infections of immunocompromised hosts.

Key words: *Candida albicans*, phenotype, antifungal susceptibility

Introduction

Candida albicans is the most frequent opportunistic fungal infection of man. Although antifungal resistance in *C. albicans* is less frequent than in other species, an increasing number of resistant strains are emerging[1, 2]. The major virulence factors of *C. albicans* are proteinase secretion, hyphal formation, adhesion, and phenotypic switching[3]. The high-frequency switching of colony morphology in *C. albicans* was reported by Slutsky *et al.*[2]. Phenotypic switching in *Candida* has been shown to be effective in defence of the immune system, increase in adherence, increase in enzyme secretion, and decrease in susceptibility to antifungals[1]. Most strains of *C. albicans* and related species are capable of switching spontaneously, reversibly, and at high frequencies (10^{-4} to 10^{-1}) between a number of general phenotypes distinguishable by colony morphology[4, 5].

There is a higher degree of phenotypic variability among *C. albicans* with karyotype variations, resistance to antifungal agents and variation in adhesion to epithelia. The effects of switching on proteinase secretion were dramatic. However, despite the abundance of data on antifungal susceptibility patterns of *C. albicans* and on some factors affecting colonization of hosts by *C. albicans*, there is only limited information on the association between phenotypic variation and the antifungal susceptibility of wild-type variants[2, 4, 6]. Fluconazole, itraconazole and voriconazole are new generation azole antifungal agents which show excellent *in vitro* activity against a wide variety of yeast and molds[7].

In this study we investigated the susceptibility of the different phenotypes of *C. albicans* strains isolated from clinical specimens to three antifungal agents, fluconazole, itraconazole and voriconazole.

Corresponding author: Dr. Zafer Cetinkaya, MD, PhD,
Maresal Cakmak Mahallesi,
Adnan Menderes Bulvari No: 8/11
TR-03000 Afyon, Turkey

Materials and methods

Strains: *C. albicans* strains were isolated from all of 215 non-neutropenic patients who had had no previous antifungal prophylactic chemotherapy and in each case a single species was isolated. The presence of *C. albicans* in the oropharynx, gastrointestinal tract and urogenital tract was determined through oropharyngeal swabs, stool and urine samples, and urethral and/or vaginal swabs.

Determination of the different colony phenotypes in *C. albicans*: Samples were plated directly onto Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, Hampshire, United Kingdom) and phloxine B agar plates. The identification of growing yeast colony was made according to germ tube formation, microscopic appearance on Cornmeal Tween 80 Agar (Oxoid, Basingstoke, Hampshire) and API ID32 C (bioMerieux, Marcy I' Etoile, France) carbohydrates fermentation results. Phloxine B agar was used to determine the different colony phenotypes in *C. albicans*. The phloxine B agar plates were prepared according to the Anderson and Soll modification[8] of the Lee synthetic medium[9]. After inoculation, the cultures was incubated at 25°C. The phloxine B agar plates were inspected for colony morphology at 48 h and after 9 days. The number of different colonial phenotypes per culture were counted and recorded as described previously. Each of the 215 *C. albicans* strains recovered was found to express one of the six phenotypes: smooth, fuzzy, irregular, star, ring or stipple[2, 5, 10]. Smooth: A colony that has smooth surfaced convex height with smooth sides. Fuzzy: A colony type that has thorn like projections on the sides. Irregular: A colony type that has folds projecting from the center of the colony to the sides. Star: A colony type that has 6 to 12 folds that project from the center to the sides without intersecting in the center. Ring: A colony type that has a concave center with smooth sides. Stipple: A colony type that has irregular concavities and convexities on the surface.

Each of the 215 strains of *C. albicans* studied, was referred to as a wild-type phenotype. *C. albicans* strains having more than one wild phenotype were not included in the study. Wild-type variants native to each patient were sub-cultured twice onto phloxine B plates to ensure the stability of each phenotype. The different phenotypes were recorded when the subsequent cultures of the presumptive wild-type variant phenotype colonies were still distinct from those predominant in each culture. Phenotype variant colonies were obtained directly from each of the phloxine B plates. The susceptibility to antifungal agents was tested using five colonies each of the wild-type smooth, irregular, fuzzy, star, ring, and stipple *C. albicans* phenotypes.

Antifungal susceptibility testing: The susceptibility tests were performed by micro broth dilution method as described in the NCCLS M27-A2 document[11]. *C. albicans* isolates were obtained from the 48 h phloxine B cultures, and suspensions of 0.5 McFarland standard in 0.85% saline were adjusted at 530 nm wavelength. This procedure yielded a yeast stock suspension of 1×10^6 to 5×10^6 cells per m*l*. The yeast suspensions were diluted in RPMI 1640 medium (Sigma, Steinheim, Germany), with L-glutamine without bicarbonate, buffered with 0.165 μM morpholinopropane sulfonic acid (Merck, Darmstadt, Germany) at pH 7 to provide inoculum ranging between 5.0×10^2 to 2.5×10^3 colony forming units (CFU) of yeast per m*l*. The phloxine B medium contains zinc, which suppresses the formation of pseudohyphae at 25°C[12]. Several inoculum samples were prepared from the dome of each colony to be tested and were examined microscopically to ensure that the suspension contained budding and unbudded cells. The final concentrations of the antifungal agents were 0.125-64 μg ml^{-1} for fluconazole (Pfizer, Istanbul, Turkey), 0.03-16 μg ml^{-1} for itraconazole (Neuland Laboratories Limited, India) and 0.03-16 μg ml^{-1} for voriconazole, (Pfizer, Istanbul). The trays were incubated at 35°C, and MIC endpoints were read after 48 h of incubation. Following incubation, the MICs of the three agents were read as the lowest concentration inhibiting 80% of growth.

Quality control: Quality control was ensured by testing the NCCLS recommended strain *Candida parapsilosis* ATCC 22019. QC determinations made on each day of testing were within the control limits for fluconazole itraconazole, and voriconazole described by NCCLS M27 - A2[11].

Statistical methods: The statistical analysis of the collected data was made using the SPSS Program (Statistical Software Package of Social Sciences, version 10). Statistical analysis was performed using the chi-square test and the one way ANOVA test. The chi-square test was used to compare selected categorical variables. These test showed yeast growth percent and density on the media compared with working area.

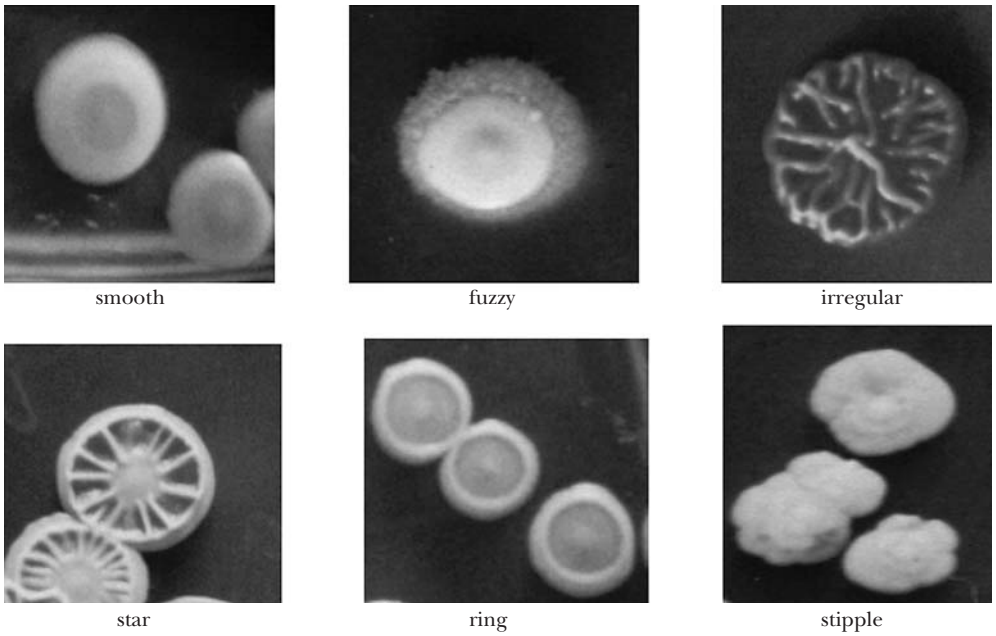

Fig. 1. Colony phenotypes of *Candida albicans*

Table 1. Mean MIC values (±SE) of fluconazole, itraconazole and voriconazole for *Candida albicans* wild-type phenotype by the micro dilution method

	Fluconazole					Itraconazole				Voriconazole			
Phenotype	n	Range μg/ml	Mean	MIC 50	MIC 90	Range μg/ml	Mean	MIC 50	MIC 90	Range μg/ml	Mean	MIC 50	MIC 90
Smooth	157 (73%)	0.25-32	3.96± 3.50	4	8	0.03-0.5	0.15± 0.08	0.125	0.25	0.03-0.25	0.07± 0.04	0.06	0.125
Fuzzy	23 (10.7%)	2-16	8.43± 4.51	8	16	0.03-0.25	0.13± 0.06	0.125	0.25	0.03-0.25	0.07± 0.05	0.06	0.125
Irregular	5 (2.3%)	4-8	7.20± 1.79	8	8	0.03-0.25	0.12± 0.08	0.125	0.25	0.03-0.06	0.04± 0.01	0.06	0.06
Star	6 (2.8%)	2-16	8.33± 6.25	4	16	0.03-0.25	0.10± 0.08	0.06	0.125	0.03-0.06	0.05± 0.01	0.06	0.06
Ring	13 (6%)	2-16	8.61± 4.78	8	16	0.03-0.25	0.12± 0.08	0.125	0.25	0.03-0.125	0.05± 0.02	0.06	0.06
Stipple	11 (5.1%)	8-64	28.36± 20.35	32	32	0.125-1	0.40± 0.24	0.25	0.5	0.06-0.5	0.19± 0.12	0.125	0.25
			$p<0.001$ F: 38.4				$p<0.001$ F: 14.54				$p<0.001$ F: 11.65		

Results

The specimens from which *C. albicans* strains were isolated were obtained from the oropharynx (n=57), genitourinary (n=94) and gastrointestinal tracts (n=64) of patients. Each of the 215 strains recovered was found to express one of the six phenotypes: smooth 73%, fuzzy 10.7%, irregular 2.3%, star 2.8%, ring 6% or stipple 5.1% (Fig. 1).

Mean MIC values (±SE) of fluconazole, itraconazole and voriconazole for *C. albicans* wild-type phenotype by the micro dilution methods are shown in the Table. The MICs for the six phenotypes ranged between 0.25-64 μg/ml for fluconazole, 0.03-1 μg/ml for itraconazole and 0.03-0.5 μg/ml for voriconazole. The mean minimum inhibitory concentrations (MICs) of fluconazole, itraconazole and voriconazole were consistently higher for *C. albicans* strains expressing the stipple phenotypes (Table 1).

The mean MIC values of fluconazole, itraconazole, and voriconazole corresponding to each phenotype of the 215 *C. albicans* strains, as determined by the reference method, showed statistically significant differences between the stipple phenotype

and the others ($F=38.43$, $p<0.001$, F: 14.54, $p<0.001$, F: 11.65, $p<0.001$), respectively.

Discussion

C. albicans phenotypic switching occurs spontaneously in the absence of environmental signals and can give rise to several different colony morphologies. Switching has been demonstrated to occur at sites of infection and to occur between recurrent episodes of infection in selected cases[13, 14]. The majority of *C. albicans* strains have the ability to exhibit switching, which recent studies have shown is often associated with genetic variation and which may provide non-sexual *C. albicans* strains with the ability to adapt rapidly to their natural environment[10].

We studied *C. albicans* strains isolated from clinical specimens of non-neutropenic patients who had had no previous prophylactic antifungal therapy. Thus, we studied strains which had not recently encountered antifungals. Fluconazole is frequently used in the therapy for *C. albicans* infection. These strains, which are fluconazole-sensitive at the beginning of an infection, may become fluconazole-resistant with long-term use of the drug[15, 16]. Itraconazole is a triazole antifungal agent. In comparison with fluconazole, the advantage of itraconazole is its spectrum of activity[17]; however, absorption problems of itraconazole limit its clinical usefulness. Voriconazole is active against all *Candida* species, including *Candida krusei*, strains of *Candida glabrata* that are inherently fluconazole-resistant, and strains of *C. albicans* that have acquired resistance to fluconazole[7].

There are very limited studies on the relation between phenotypic switching and antifungal susceptibility of *C. albicans*. The colony morphology variation *in vitro* can generate derivatives with stable, reduced azole susceptibility over without prior exposure to azoles[18]. In another study, different phenotypes of *C. albicans* strains were isolated from neutropenic patients who had received no previous prophylactic treatment[19]. Smooth, irregular, fuzzy and stipple phenotypes of *C. albicans* strains were found and the MIC levels of the stipple phenotypic for fluconazole and itraconazole were consistently higher than MICs of the other phenotypes[19].

Vargas *et al.*[4] reported dramatic differences in susceptibility among the switch phenotypes of individual colonizing strains for fluconazole and voriconazole. Kiraz *et al.*[10] reported that fluconazole susceptibility of the stipple phenotype showed statistically significant difference from other phenotypes. Calvet *et al.*[20] found that *C. albicans* isolates which were initially fluconazole sensitive developed high-level fluconazole resistance (50% inhibitory concentration $>256\,\mu g\ ml^{-1}$) after serial passage in medium containing 8, 16 or 128 μg of fluconazole per m*l*. Reduced susceptibility was noted within four to seven passages, which was equivalent to 14-19 days of exposure to the drug. However, all isolates returned to the susceptible phenotype after 8 to 15 passages in medium lacking the drug; thus, fluconazole resistance was reversible *in vitro*.

We compared antifungal activities of phenotypic variation *C. albicans* strains against fluconazole, itraconazole and voriconazole. In our study, fluconazole, itraconazole, and voriconazole mean MIC levels of the stipple phenotype were found to be higher than that of the smooth, irregular, star, fuzzy and ring phenotypes. We found no significant difference between fluconazole, itraconazole and voriconazole mean MIC levels for the non-stipple phenotypes. These findings were in accord with the results of our study. The phenotypic characters of selected colonies to test susceptibility of azole group drugs for *C. albicans* isolates may affect their MIC levels. Random selection of phenotypes other than the stipple may cause a low MIC level and this condition may produce inappropriate *in vitro* and *in vivo* results. We suggest that investigation of the colonial phenotypes on phloxine B agar and evaluation of the antifungal susceptibility of these phenotypes separately may be useful in therapy. However, more studies should be conducted to determine the antifungal activities of phenotypic variation of *C. albicans* strains.

References

1) Soll DR: *Candida* commensalism and virulence: the evolution of phenotypic plasticity. Acta Tropica **81**: 101-110, 2002.
2) Slutsky R, Buffo J, Soll DR: High frequency switching of colony morphology in *Candida albicans*. Science **230**: 666-669, 1985.
3) Yang YL: Virulence factors of *Candida* species. J Microbiol Immunol Infect **36**(4): 223-228, 2003.
4) Vargas K, Messer SA, Pfaller M, Lockhart SR, Stapleton JT, Hellstein J, Soll DR: Elevated phenotypic switching and drug resistance of *Candida albicans* from human immunodeficiency virus-positive individuals prior to first thrush episode. J Clin Microbiol **38**(10): 3595-3607, 2000.
5) Soll DR: High frequency switching in *Candida albicans*. Clin Microbiol Rev **5**: 183-203, 1992
6) San-Blas G, Travassos LR, Fries BC, Goldman

DL, Casadevall A, Carmona AK, Barros TF, Puccia R, Hostetter MK, Shanks SG, Copping VM, Knox Y, Gow NA: Fungal morphogenesis and virulence. Med Mycol **38**(1): 79-86, 2000.
7) Johnson LB, Kauffman CA: Voriconazole: a new triazole antifungal agent. Clin Infect Dis **36**: 630-637, 2003.
8) Anderson JM, Soll DR: Unique phenotype of opaque cells in the white opaque transition of *Candida albicans*. J Bacterial **169**: 5579-5588, 1987.
9) Lee KL, Bucklcy HR, Cambell CC: An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. Sabouraudia **13**: 148-153, 1975.
10) Kiraz N, Ang O, Akgun Y, Erturan Z: Phenotypic variation and antifungal susceptibility patterns of *Candida albicans* strains isolated from neutropenic patients. Mycoses **43**: 119-123, 2000.
11) National Committee for Clinical Laboratory Standards: Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved standard -Second Edition. NCCLS document M27-A2. Wayne, Pennsylvania, 2002.
12) Dutton S, Penn CW: Biological attributes of colony-type variants of *Candida albicans*. J Gen Microbiol **135**: 3363-3372, 1989.
13) Jones S, White G, Hunter PR: Increased phenotypic switching in strains of *Candida albicans* associated with invasive infections. J Clin Microbiol **32**: 2869-2870, 1994.
14) Soll DR, Galask S, Isley S, Rao TV, Stone D, Hicks J, Schmid J, Mac K, Hanna C: Switching of *Candida albicans* during successive episodes of recurrent vaginitis. J Clin Microbiol **27**: 681-690, 1989.
15) Sojakova M, Liptajova D, Borovsky M, Subik J: Fluconazole and itraconazole susceptibility of vaginal yeast isolates from Slovakia. Mycopathologia **157**: 163-169, 2004.
16) Van Belkum A, Malchers W, De Pauw BE, Scherer S, Quint W, Meis JF: Genotypic characterization of sequential *Candida albicans* isolates from fluconazole-treated neutropenic patients. J Infect Dis **169**: 1062-1070, 1994.
17) Gordon E, Schutze MD: Antifungal agents for the treatment of systemic mycoses Seminars in Pediatric Infectious Diseases **12**(3): 794-798, 2001.
18) Gallagher PJ, Bennett DE, Henman MC, Russell RJ, Flint SR, Shanley DB, Coleman DC: Reduced azole susceptibility of oral isolates of *Candia albicans* from HIV-positive patients and a derivative exhibiting colony morphology variation. J Gen Microbiol **38**: 1901-1911, 1992.
19) Velegraki A, Papalambrou D, Soremi S, Legakis NJ: Variable antifungal susceptibility of wild-type *Candida albicans* phenotypes from neutropenic hosts. Em J Clin Microbiol Infect Dis **15**: 854-860, 1996.
20) Calvet HM, Yeaman MR, Filler SG: Reversible fluconazole resistance in *Candida albicans*: a potential invitro model. Antimicrob Agent Chemother **41**: 535-539, 1997.