Original Article

_In vitro_ Investigation of Antifungal Activities of Phenotypic Variation _Candida albicans_ Strains against Fluconazole, Itraconazole and Voriconazole

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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/ml and 64 μg/ml for fluconazole, 0.03 μg/ml and 1 μg/ml for itraconazole and 0.03 μg/ml and 0.5 μg/ml 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 emerging¹, ². The major virulence factors of _C. albicans_ are proteinase secretion, hyphal formation, adhesion, and phenotypic switching³. 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 antifungals¹¹. 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 morphology⁴, 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 variants², 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 molds⁷.

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.
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 of the Lee synthetic medium. 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 and stipple. 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. 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 $1 \times 10^6$ to $5 \times 10^6$ cells per ml. The yeast suspensions were diluted in RPMI 1640 medium (Sigma, Steinheim, Germany), with L-glutamine without bicarbonate, buffered with 0.165 $\mu$M morpholinopropane sulfonic acid (Merck, Darmstadt, Germany) at pH 7 to provide inoculum ranging between 5.0 $\times 10^2$ to 2.5 $\times 10^3$ colony forming units (CFU) of yeast per ml. The phloxine B medium contains zinc, which suppresses the formation of pseudohyphae at 25°C. 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 $\mu$g ml$^{-1}$ for fluconazole (Pfizer, Istanbul, Turkey), 0.03-16 $\mu$g ml$^{-1}$ for itraconazole (Neuland Laboratories Limited, India) and 0.03-16 $\mu$g 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.

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.
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.

![Smooth](smooth.png)
![Fuzzy](fuzzy.png)
![Irregular](irregular.png)
![Star](star.png)
![Ring](ring.png)
![Stipple](stipple.png)

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

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>n</th>
<th>Range μg/ml</th>
<th>Mean ±SE μg/ml</th>
<th>MIC 50</th>
<th>MIC 90</th>
<th>Range μg/ml</th>
<th>Mean ±SE μg/ml</th>
<th>MIC 50</th>
<th>MIC 90</th>
<th>Range μg/ml</th>
<th>Mean ±SE μg/ml</th>
<th>MIC 50</th>
<th>MIC 90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth</td>
<td>157</td>
<td>(73%)</td>
<td>3.96 ± 0.25</td>
<td>0.03-1</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25 ± 0.05</td>
<td>0.15-</td>
<td>0.125</td>
<td>0.7 ± 0.04</td>
<td>0.06</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Fuzzy</td>
<td>25</td>
<td>(10.7%)</td>
<td>4.15 ± 0.18</td>
<td>0.03-0.13</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25 ± 0.06</td>
<td>0.06</td>
<td>0.125</td>
<td>0.07 ± 0.04</td>
<td>0.06</td>
<td>0.125</td>
<td></td>
</tr>
<tr>
<td>Irregular</td>
<td>5</td>
<td>(2.3%)</td>
<td>7.20 ± 4.51</td>
<td>0.03-0.12</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25 ± 0.08</td>
<td>0.125</td>
<td>0.125</td>
<td>0.04 ± 0.06</td>
<td>0.06</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Star</td>
<td>6</td>
<td>(2.8%)</td>
<td>8.33 ± 1.79</td>
<td>0.03-0.10</td>
<td>0.06</td>
<td>0.06</td>
<td>0.25 ± 0.08</td>
<td>0.125</td>
<td>0.125</td>
<td>0.05 ± 0.06</td>
<td>0.06</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Ring</td>
<td>13</td>
<td>(6%)</td>
<td>8.61 ± 4.78</td>
<td>0.03-0.12</td>
<td>0.125</td>
<td>0.125</td>
<td>0.25 ± 0.08</td>
<td>0.125</td>
<td>0.125</td>
<td>0.05 ± 0.06</td>
<td>0.06</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Stipple</td>
<td>11</td>
<td>(5.1%)</td>
<td>28.36 ± 20.35</td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
<td>0.06-1</td>
<td>0.19</td>
<td>0.125</td>
<td>0.25</td>
<td>0.5</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

*p<0.001*  
F: 38.4  
*p<0.001*  
F: 14.54  
*p<0.001*  
F: 11.65
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. 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.

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. Itraconazole is a triazole antifungal agent. In comparison with fluconazole, the advantage of itraconazole is its spectrum of activity; 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.

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. In another study, different phenotypes of *C. albicans* strains were isolated from neutropenic patients who had received no previous prophylactic treatment. Smooth, irregular, fuzzy and stipple phenotypes of *C. albicans* strains were found and the MIC levels of the stipple phenotype for fluconazole and itraconazole were consistently higher than MICs of the other phenotypes.

Vargas et al. reported dramatic differences in susceptibility among the switch phenotypes of individual colonizing strains for fluconazole and voriconazole. Kiraz et al. reported that fluconazole susceptibility of the stipple phenotype showed statistically significant difference from other phenotypes. Calvet et al. found that *C. albicans* isolates which were initially fluconazole sensitive developed high-level fluconazole resistance (50% inhibitory concentration > 256 µg mL⁻¹) after serial passage in medium containing 8, 16 or 128 µg of fluconazole per mL. 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 ofazole 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

6) San-Blas G, Travassos LR, Fries BC, Goldman


