Dynamics of in vitro acquisition of resistance by Candida parapsilosis to different azoles

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Abstract

Candida parapsilosis is a common isolate from clinical fungal infectious episodes. Resistance of C. parapsilosis to azoles has been increasingly reported. To analyse the development of resistance in C. parapsilosis, four azole-susceptible clinical strains and one American Type Culture Collection type strain were cultured in the presence of fluconazole, voriconazole and posaconazole at different concentrations. The isolates developed variable degrees of azole resistance according to the antifungal used. Fluconazole was the fastest inducer while posaconazole was the slowest. Fluconazole and voriconazole induced resistance to themselves and each other, but not to posaconazole. Posaconazole induced resistance to all azoles. Developed resistance was stable; it could be confirmed after 30 days of subculture in drug-free medium. Azole-resistant isolates revealed a homogeneous population structure; the role of azole transporter efflux pumps was minor after evaluation by microdilution and cytometric assays with efflux pump blockers (verapamil, ibuprofen and carbonyl cyanide 3-chloro-phenylhydrazone). We conclude that the rapid development of azole resistance occurs by a mechanism that might involve mutation of genes responsible for ergosterol biosynthesis pathway, stressed by exposure to antifungals.

Introduction

During the last decade Candida species have emerged as major opportunistic pathogens, mainly due to the increase in the number of immunocompromised patients (Pfaller & Diekema, 2007). Among Candida species causing nosocomial infections, the opportunistic yeast Candida parapsilosis is at present the second or third most common blood culture fungal isolate in Europe, Canada and Latin America, outranking Candida albicans in some European hospitals (Pfaller & Diekema, 2007).

Although sometimes considered a member of the indigenous microbial population in healthy individuals, C. parapsilosis can be also recovered from distinct environmental sources and from the hands of health care workers, thus suggesting its role as a potential route for nosocomial transmission (Levin et al., 1998; Kojic & Darouiche, 2004).

Unfortunately, fungal pathogens acquire resistance to azoles, particularly after prolonged exposure, as is the case with its prophylactic overuse. The widespread use of antifungal molecules, especially fluconazole, has selected Candida species with easily inducible resistance, such as Candida glabrata (Wingard et al., 1993) and Candida tropicalis (Barchiesi et al., 2000) or species that show intrinsic resistance, such as Candida krusei (Wingard et al., 1991). Candida albicans resistance to fluconazole was promoted following incubation with subinhibitory concentrations of the drug (Marr et al., 2001). In the case of an antifungal drug being more prone to induce in vitro resistance in comparison with others, this might represent a hypothetical drawback for its use in vivo, at least for prophylactic treatment.

Three distinct mechanisms of azole resistance have been described so far in C. albicans: (1) failure to accumulate the drug intracellularly, which may be caused by the lack of drug
penetration due to changes in membrane lipids or sterols (Hitchcock et al., 1986) or (2) by active efflux of drugs resulting from overexpression of genes CDR1, CDR2 and MDR1 (Albertson et al., 1996; Sanglard et al., 1996; White, 1997; Marr et al., 1998); and (3) increased production of the azole target enzyme and point mutations in genes that codify for this enzyme, the products of which have reduced affinity to azoles (Sanglard et al., 1998). Lanosterol-14α-demethylase is a common target for all azole antifungals. This enzyme belongs biochemically to the group of cytochrome P450 enzymes and is involved in the synthesis of ergosterol, which is a major and essential lipid constituent of the cell membrane of fungi (Akins, 2005). All azoles, including posaconazole (Munayyer et al., 2003; Hof, 2006), inhibit the production of ergosterol, causing a depletion of this compound. Mutations in other enzymes involved in the synthesis of ergosterol may also contribute to azole resistance.

Although several resistance mechanisms may operate in fungal pathogens, efflux-mediated drug tolerance is the major factor responsible for clinical resistance (Kontoyianis & Lewis, 2002; Sanglard & Odds, 2002; White et al., 2002). Previous studies demonstrated that resistance related to overexpression of efflux pumps can be reverted by modulators, particularly verapamil or sex hormones (Ford & Hait, 1990) and more recently by ibuprofen (Pina-Vaz et al., 2005). Inhibition of the efflux pumps that are H+−dependent in yeasts by carbonyl cyanide 3-chlorophenylhydrazone (CCCP) has also been demonstrated (Prudêncio et al., 2000; Guinea et al., 2006).

Surprisingly, very few data are yet available regarding the mechanisms of azole resistance in *C. parapsilosis*. The initial aim of our study was to assess and characterize the in vitro induction of resistance by distinct azole antifungals at different concentrations in four blood culture isolates and one American Type Culture Collection (ATCC) type strain of *C. parapsilosis*. Additional aims of this study were to elucidate whether such induced resistance is stable and to assess the involvement of efflux pumps.

**Materials and methods**

**Strains**

Four blood culture isolates of *C. parapsilosis* (BC014, BC011, BC237 and BC190) isolated from patients admitted to Hospital S. João in Porto, Portugal, were used in this study. They were characterized by Vitek YBC identification cards (BioMérieux, Paris, France). Until testing, the strains were stored in Brain–Heart broth (Difco) with 5% glycerol at −70 °C. For each experiment, each strain was subcultured twice on Sabouraud agar (Difco) at 35 °C for 48 h. *Candida parapsilosis* type strain ATCC 22019 was included. To study the role of efflux pumps on antifungal-induced resistance, two *C. albicans* strains with well-characterized mechanisms of resistance were also included as controls: *C. albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* genes (ATP-dependent efflux pumps), and *C. albicans* strain 2-76, showing lower levels of resistance gene expression (a kind gift from Dr Ted White).

**Chemicals and culture media**

Standard powders of fluconazole (Pfizer, Groton, CT), voriconazole (Pfizer, New York, NY), posaconazole (Scher-Plough, Kenilworth, NJ), amphotericin B (AMB; Bristol Myers Squibb, New York, NY) and caspofungin (CAS; Merck, Rahway, NJ) were obtained from the respective manufacturers. A stock solution of fluconazole and CAS were prepared in distilled water; voriconazole, posaconazole and AMB were prepared with dimethyl sulfoxide. Antifungal drugs were diluted afterwards with Roswell Park Memorial Institute 1640 medium (RPMI 1640; Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid buffer (MOPS; Sigma) and stored at −70 °C until use. Verapamil, ibuprofen and CCCP were purchased from Sigma. The culture medium used for induction assays of resistance and evaluation of its stability was RPMI 1640 with 0.165 M MOPS, pH 7.0. Rhodamine 6G (Rh-6G) used in flow cytometric assays was purchased from Sigma.

**Strategy for induction of antifungal resistance and for assessing its stability**

A single, randomly selected colony from each *C. parapsilosis* strain was incubated in 10 mL of RPMI 1640 overnight in a rotating drum at 150 r.p.m., 35 °C. An aliquot of this culture, containing 10⁶ blastoconidia, was transferred to different vials, each containing 10 mL of culture medium with or without an antifungal drug (fluconazole, voriconazole or posaconazole), and incubated overnight as described above. The following day, aliquots from each culture containing 10⁶ blastoconidia were again transferred into fresh medium containing the same antifungal and reincubated as described. Each day, for the 30 days of the assay, a 1-mL aliquot from each subculture was mixed with 0.5 mL of 50% glycerol and frozen at −70 °C for later testing. In this type of experiment two different approaches were taken: (1) incubation for 30 days with constant concentrations of fluconazole (16 μg mL⁻¹), voriconazole (2 μg mL⁻¹) or posaconazole (1 μg mL⁻¹); these concentrations correspond to therapeutic serum levels obtained during antifungal treatment (Azanza et al., 2007) and (2) incubation with fluconazole, voriconazole and posaconazole at twice the concentration of the minimal inhibitory concentration (MIC) value, for 2 weeks, and afterwards at four times the concentration until the 30th day.
To assess resistence stability, the resistant isolates obtained were subcultured daily in the absence of the drug for 30 days. A single colony from each isolate was incubated in 10 mL drug-free RPMI 1640 at 35 °C, 150 r.p.m. The following day, aliquots were transferred into fresh medium. At each subculture, a 1-mL aliquot of the suspension was mixed with 0.5 mL of 50% glycerol, and frozen at −70 °C for later testing.

Antifungal susceptibility testing

The MIC values of each antifungal drug were determined according to the Clinical Laboratory Standards Institute (CLSI, 2008) M27-A3 protocol. MIC was registered after 24 and 48 h. Interpretative criteria for fluconazole and voriconazole were those of the CLSI for fluconazole: susceptible (S)-MIC ≤ 8 μg mL⁻¹, susceptible-dose dependent (S-DD)-MIC 16–32 μg mL⁻¹, and resistance (R)-MIC ≥ 64 μg mL⁻¹; for voriconazole, S-MIC ≤ 1 μg mL⁻¹ and R-MIC ≥ 4 μg mL⁻¹. The interpretive criteria for CAS were those recently assigned by the CLSI (June 2007): S ≤ 2 μg mL⁻¹. Although susceptibility breakpoints have not yet been established for posaconazole and AMB, strains inhibited by ≤ 1 μg mL⁻¹ of each were considered to be susceptible (Pfaller et al., 2001, 2003). Every 5 days of incubation, with or without antifungal, MIC values were redetermined for the five antifungals tested. Candida parapsilosis type strain ATCC 22019 was used in each testing assay, as recommended.

Population analysis

This assay was performed as described previously by Marr et al. (2001). A single colony of the initially susceptible and of the final resistant isolates were cultured overnight in YEPD broth (1% yeast extract, 2% peptone, 2% glucose) at 35 °C; a suspension containing 10⁷ blastoconidia mL⁻¹ was prepared in RPMI 1640 and plated in Sabouraud agar with and without fluconazole (1, 4, 16 and 64 μg mL⁻¹), voriconazole (0.125, 0.5, 2 and 8 μg mL⁻¹) and posaconazole (0.25, 1, 4 and 16 μg mL⁻¹). Growth was quantified after 48 h of incubation at 35 °C. The number of colonies growing in the presence of the drug at each concentration, relative to the number growing in its absence, was calculated and plotted.

Study of efflux pumps

The role of efflux pumps in the resistance mechanism developed was evaluated using two different approaches: (1) Antifungal MIC values were determined according to the CLSI M27-A3 protocol (CLSI, 2008) in the presence of different drugs described as efflux blockers: verapamil 100 μM, ibuprofen 100 mg L⁻¹ (Pina-Vaz et al., 2005) and CCCP 0.5 μg mL⁻¹ (Guinea et al., 2006). (2) Flow cytometric analysis using Rh-6G, a specific substrate of efflux pumps was performed as described previously (Sanglard et al., 1999, Posteraro et al., 2003), to compare the induced resistant strains with susceptible strains (incubated in drug-free medium) and the induced resistant strains with and without incubation for 90 min with verapamil 100 μM, as described by Pina-Vaz et al. (2005). Briefly, yeast cells were grown to logarithmic phase in 5 mL of YEPD medium at 35 °C under constant agitation. Labelling of cells was conducted in 1 mL of YEPD, containing 10⁷ blastoconidia and 10 μM Rh-6G for 30 min at 35 °C, after which the reaction was stopped by placing the tubes on ice. The mixture was then diluted 40-fold with cold sterile 0.1 M phosphate-buffered saline (Sigma) at pH 7.0 and analysed using flow cytometry in a FACSCalibur (BD Biosciences, Sydney, Australia) equipped with a 15-nm argon laser. From each yeast suspension, 30 000–50 000 blastoconidia were analysed; cell fluorescence was determined at FL1 (515 nm). All experiments were performed in duplicate.

Results

Development of resistance

Table 1 shows the MIC values determined for fluconazole, voriconazole and posaconazole before and after induction with different azoles for all tested strains. After exposure to therapeutic serum concentrations of the different azoles, distinct patterns of resistance were observed. Following 15 days of antifungal exposure, only the fluconazole susceptibility phenotype changed. This finding was observed with most tested strains exposed to fluconazole and with a few strains exposed to voriconazole; following posaconazole exposure, only minor MIC variations were registered after this short period of time. Fluconazole and voriconazole increased MIC values to themselves and to each other, the susceptibility phenotype changing to R after 30 days, but not to posaconazole, which maintained the S phenotype. Posaconazole induced resistance to all azoles only at the very end of incubation time. Therefore, the activity of fluconazole was the most impaired and it was the fastest inducer of resistance.

In a different strategy to compare the ability of the same azole drugs to induce resistance, incubation with MIC values two- and fourfold that of the antifungals were assayed. A representative example (type strain ATCC 22019) of the development of resistance in such instances is detailed in Fig. 1. The lower concentration of fluconazole was sufficient to induce resistance to itself and to voriconazole but not to posaconazole; the exposure of the yeast cells to the lower concentration of voriconazole only changed the fluconazole phenotype (S strain turned to S-DD), as only minor variations on MIC values were observed with
the other tested azoles. Posaconazole had the least effect on the MIC values of all azoles in such experimental conditions. Incubation with these three azoles, independently on the tested concentration, did not induce resistance to AMB and CAS.

Regarding the strains successively incubated without antifungals, the MIC values of the five antifungals tested remained constant throughout the study period.

### Stability of azole resistance in vitro

Regarding the stability of the developed resistance by resistant organisms subcultured in drug-free medium for 30 days, the resistance pattern remained stable; no decreases greater than one dilution were observed in MIC values of all azoles tested.

### Analysis of cellular populations

To determine whether the inducedazole resistance in *C. parapsilosis* isolates was associated with selection of a resistant subpopulation, analysis of resistance within the cellular population was performed with susceptible and induced resistance strains. Population analysis clearly demonstrated the existence of a homogeneous population both for the resistant-induced strains and for the susceptibility strain. A representative example of these studies is shown for BC014 strain in Fig. 2.

### Activity of efflux pumps

#### Effect of efflux blockers on MIC value of azoles

After incubation with ibuprofen, verapamil and CCCP, a decrease of MIC values of no more than one dilution was registered, and the resistance pattern remained stable. Regarding the control strains, *C. albicans* 95-68 in the presence of verapamil and ibuprofen, MIC values of fluconazole, voriconazole and posaconazole decreased drastically; as expected for strain *C. albicans* 2-76, the MIC values of the three antifungals did not change.

### Rh-6G staining

The steady state regarding Rh-6G fluorescence of the susceptible phenotype (strains incubated in drug-free medium) and of resistant phenotypes obtained after 30 days of antifungal exposure (fluconazole 16 μg mL⁻¹, voriconazole 2 μg mL⁻¹ and posaconazole 1 μg mL⁻¹) were compared. Similar intensity of fluorescence was obtained with induced resistant strains and susceptible strains (Fig. 3a). This intensity did not increase in the presence of the efflux blocker verapamil (data not shown). *Candida albicans* strain 95-68, with overexpression of *CDR1* and *CDR2* resistance genes, behaved as expected, showing an increase in Rh-6G staining when incubated with verapamil (Fig. 3b), whereas *C. albicans* strain 2-76 did not (data not shown), which validates the assays.

### Discussion

Mechanisms of azole resistance due to antifungal exposure have been extensively investigated in clinical strains of *C. albicans* (Sanglard et al., 1995, 1996, 1998; White, 1997; Franz et al., 1998). Other studies have also elucidated the mechanisms of azole resistance in *C. glabrata*, *C. krusei* and *C. tropicalis* (Marichal et al., 1997; Barchiesi et al., 1998; Orozco et al., 1998).

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### Table 1. MIC values (μg mL⁻¹) and phenotypes (Phen) of Candida parapsilosis type strain (ATCC 22019) and BC014, BC011, BC237 and BC190 clinical strains before and after induction with fluconazole (16 μg mL⁻¹), voriconazole (2 μg mL⁻¹) and posaconazole (1 μg mL⁻¹)

<table>
<thead>
<tr>
<th>Induction with</th>
<th>Strains</th>
<th>Day 0</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 0</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 0</th>
<th>Day 15</th>
<th>Day 30</th>
<th>Day 0</th>
<th>Day 15</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>ATCC</td>
<td>0.5/S</td>
<td>16/S-DD</td>
<td>64/R</td>
<td>0.015/S</td>
<td>0.25/S</td>
<td>2/S-DD</td>
<td>0.065/S</td>
<td>0.06/S</td>
<td>0.125/S</td>
<td>BC014</td>
<td>1.0/S</td>
<td>16/S-DD</td>
</tr>
<tr>
<td></td>
<td>BC014</td>
<td>1.0/S</td>
<td>8/S</td>
<td>128/R</td>
<td>0.03/S</td>
<td>0.25/S</td>
<td>2/S-DD</td>
<td>0.03/S</td>
<td>0.25/S</td>
<td>0.5/S</td>
<td>BC011</td>
<td>1.0/S</td>
<td>32/S-DD</td>
</tr>
<tr>
<td></td>
<td>BC237</td>
<td>1.0/S</td>
<td>32/S-DD</td>
<td>128/R</td>
<td>0.03/S</td>
<td>0.25/S</td>
<td>4/R</td>
<td>0.065/S</td>
<td>0.125/S</td>
<td>0.5/S</td>
<td>BC190</td>
<td>0.5/S</td>
<td>16/S-DD</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>ATCC</td>
<td>0.5/S</td>
<td>16/S-DD</td>
<td>64/R</td>
<td>0.015/S</td>
<td>0.065/S</td>
<td>2/R</td>
<td>0.065/S</td>
<td>0.065/S</td>
<td>0.125/S</td>
<td>BC014</td>
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<td>32/S-DD</td>
</tr>
<tr>
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<td>8/S</td>
<td>128/R</td>
<td>0.03/S</td>
<td>0.065/S</td>
<td>2/R</td>
<td>0.03/S</td>
<td>0.125/S</td>
<td>0.5/S</td>
<td>BC011</td>
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</tr>
<tr>
<td></td>
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<td>1.0/S</td>
<td>4/S</td>
<td>128/R</td>
<td>0.03/S</td>
<td>0.125/S</td>
<td>2/S-DD</td>
<td>0.065/S</td>
<td>0.125/S</td>
<td>0.5/S</td>
<td>BC190</td>
<td>0.5/S</td>
<td>2/S</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>ATCC</td>
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<td>1/S</td>
<td>64/R</td>
<td>0.015/S</td>
<td>0.03/S</td>
<td>8/R</td>
<td>0.065/S</td>
<td>1/S</td>
<td>16/R</td>
<td>BC014</td>
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<tr>
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<td>0.03/S</td>
<td>16/R</td>
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<td>32/R</td>
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<tr>
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<td>0.03/S</td>
<td>0.03/S</td>
<td>2/S-DD</td>
<td>0.065/S</td>
<td>0.065/S</td>
<td>8/R</td>
<td>BC190</td>
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<td>16/R</td>
<td>0.065/S</td>
<td>0.125/S</td>
<td>32/R</td>
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</table>
et al., 1998). However, very few data are yet available regarding azole resistance in *C. parapsilosis*. This *Candida* species is an opportunistic yeast responsible for nosocomial infections, especially in immunocompromised or debilitated patients.

Resistance to fluconazole among *Candida* spp. has been reported to emerge whenever this drug is used extensively (Richardson, 2005). The results of our study stress several relevant characteristics of the *in vitro* acquisition of resistance by *C. parapsilosis* after prolonged exposure to antifungals. When incubated with a therapeutic fluconazole concentration (aiming to reproduce *in vivo* antifungal treatment), azole resistance quickly developed; similar results were obtained with a lower concentration of the same antifungal, showing that of the three azoles tested, fluconazole is an easy and fast inducer of resistance. The two different approaches used for induction of resistance corroborate this conclusion. This finding underlines the need to review therapeutic protocols. Both voriconazole and posaconazole induce resistance at concentrations of 2 and 1 µg mL⁻¹, respectively, but at lower concentrations the susceptibility pattern did not change drastically. As expected, incubation with any of the three azoles did not induce resistance to AMB and CAS, as azole targets are distinct and selective.

Our data strongly suggest that the dynamics of *in vitro* development of azole resistance in *C. parapsilosis* is quite different from that described for *C. tropicalis* and *C. albicans* (Calvet et al., 1997; Barchiesi et al., 2000; Marr et al., 2001). For *C. albicans*, the time required to develop fluconazole resistance is longer and it could be fully reverted to baseline when the antifungal exposure stopped. *Candida albicans* isolates that became resistant showed an increase of mRNA...
specific for a CDR ATP-binding cassette transporter efflux pump (Marr et al., 1998, 2001). The in vitro induction of resistance in C. tropicalis has been documented with associated increased expression of CDR1 and MDR1 genes (Barchiesi et al., 2000), coding for a specific fluconazole transporter.

Distinct antifungal resistance mechanisms have been described among Candida spp., active efflux being the most relevant mechanism of azole resistance; its molecular base is relatively well known for C. albicans (Sanglard & Odds, 2002). Overexpression of these efflux proteins confers resistance to most azoles but in the same degree. Whereas ketoconazole, fluconazole, itraconazole and voriconazole are transported readily, posaconazole is transported to a much lesser extent (Chau et al., 2004). Apparently, this compound is not the ideal substrate for these transporters.

If the underlying resistance mechanism induced after antifungal exposure were overexpression of efflux pumps, we would expect that in the presence of both types of efflux blockers MIC values would show a decrease and Rh-6G staining in resistant cells exposed to antifungal would be lower when compared with nonexposed cells. According to these results, the efflux seems to play a minor role on the resistance induced; however, the hypothesis of the existence of specific C. parapsilosis efflux pumps blocked by different modulators or with fluorescence substrate other than Rh-6G cannot be excluded.

In this study, the hypothesis that induced resistance in C. parapsilosis may be due to mutation in genes that codify enzymes of the ergosterol biosynthesis pathway gains considerable strength. This mutation is probably induced by the antifungal and does not result from selection of an initial resistant clone. This last hypothesis was not supported by the results from our population studies, as both the susceptible population (without antifungal exposure) as well as the resistant population, obtained following induction, were shown to be homogeneous.

The azoles bind in the vicinity of the haeme group of lanosterol-14α-demethylase. Although there are silent mutations scattered throughout the gene, at least a dozen different mutations in the active region result in a lower affinity for azoles, resulting in a greater influence on the antimicrobial activity of fluconazole and voriconazole than of posaconazole and itraconazole (Levin et al., 1998). Moreover, the latter two drugs are able to bind to an additional domain of the enzyme by means of their long side chain (Li et al., 2004; Akins, 2005), meaning that these two antifungals can still inhibit the target at a point when fluconazole and voriconazole are no longer active (Sabatelli et al., 2006). Accordingly, such mutation should result in a lower impact on the susceptibility profile to posaconazole than to fluconazole or voriconazole. Because of the two different binding sites of posaconazole, the occurrence of large-spectrum cross-resistance is highly unlikely.

Different assumptions support the occurrence of drug-induced mutation during the described assays: the rapidity of development of the resistant phenotype; the fact that the three azoles are affected, although to a different extent; the insignificant role of efflux; and the stability of resistance following prolonged absence of drug.

Spontaneous mutations are very infrequent, occurring at frequencies of $10^{-6}$–$10^{-8}$ per gene. The constancy of the MIC values in time when strains were incubated in drug-free medium strongly supports the assumption that the occurrence of spontaneous mutations is uncommon.

Diminished sensitivity of the target is probably the main resistance mechanism to azoles in C. parapsilosis. Likewise, phenotypically stable resistance to azole antifungals in C. albicans can result from mutations in genes involved in the ergosterol synthesis pathway (including the target enzyme 14α-demethylase). Regarding C. parapsilosis, the ERG sequences of the biosynthetic pathway of ergosterol need to be determined in resistant strains and compared with susceptible strains to clarify whether mutations are associated with azole resistance, as well as to assess changes in membrane...
lipid content. Studies involving a large number of isolates of C. parapsilosis, aiming to characterize the cellular and molecular factors conferring resistance are being pursued.

In summary, we described the rapid and stable development of azole resistance in C. parapsilosis after growth in the presence of azole antifungals, fluconazole being the fastest inducer while posaconazole was the slowest.

References


