Adhesion and biofilm formation in artificial saliva and susceptibility of yeasts isolated from chronic kidney patients undergoing haemodialysis

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Yeasts of the genera Candida and Saccharomyces are opportunistic pathogens and cause oral lesions, especially in immunocompromised patients. This study assessed yeasts isolated from chronic kidney patients undergoing haemodialysis for their adhesion capacity, biofilm formation and susceptibility to antifungal agents. Ten isolates of Candida spp. and one isolate of Saccharomyces cerevisiae were tested for adhesion to buccal epithelial cells (BECs), adhesion and formation of biofilm in artificial saliva and their susceptibility profile to antifungal agents. Adhesion and biofilm formation were undertaken in polystyrene plates with artificial saliva, whilst susceptibility to antifungal agents was evaluated by broth microdilution. Candida parapsilosis had the highest adhesion index in BECs (154.55 ± 22.13) and Candida rugosa was the species with the highest adhesion capacity (18 398 Abs cm⁻²) in abiotic surface with artificial saliva. Candida albicans provided the greatest biofilm formation (2035 Abs cm⁻² ± 0.09) but was revealed to be susceptible to the five antifungal agents under analysis. However, some non-albicans Candida isolates showed a lower susceptibility for the antifungal agents itraconazole, fluconazole and voriconazole. All of the species were sensitive to amphotericin B and nystatin. The current analysis showed that yeasts isolated from the mouth of chronic kidney patients undergoing haemodialysis varied significantly with regard to their capacity for adherence, biofilm formation and susceptibility to antifungal agents, underscoring the high virulence of non-albicans Candida species.

INTRODUCTION

Yeasts of the genera Candida and Saccharomyces are opportunistic yeasts and belong to the human microbiota, causing lesions in the mouth, especially under immunocompromising conditions. The genus Candida comprises an extremely heterogeneous group of fungal organisms with more than 17 different species implicated in human candidosis. However, over 90% of invasive infections are caused by Candida albicans, followed by non-albicans Candida (NAC) species, such as Candida glabrata, Candida parapsilosis, Candida tropicalis and Candida krusei; the number of infections due to NAC species has increased significantly in recent years (Guinea, 2014; Ortega et al., 2011; Tamura et al., 2007). Saccharomyces cerevisiae is also known to be an emergent micro-organism in immunocompromised patients, which may be associated with the use of probiotics. It is generally associated with yeasts of the genus Candida, with high mortality rates (Enache-Angoulvant & Hennequin, 2005; Eren et al., 2014; Silva et al., 2011a).

Patients with chronic kidney insufficiency and kidney transplant patients are the populations with the highest risk of oral lesions and evolution to fungaemia (Serefhanoglu et al., 2012). Owing to their exposure to various procedures and to constant changes in their metabolic conditions, chronic kidney insufficiency patients are susceptible to opportunistic infections. Haemodialysis is indicated as one of the main risk factors for fungaemia in these patients (Conde-Rosa et al., 2010).

Fungal proliferation and the development of infections are also potentiated by other factors: host factors (use of medicines, diabetes mellitus), local factors (dental prosthesis, saliva components) and yeast intrinsic factors.
(morphological transition, phenotypic variability, hydrophobicity, adhesion capacity, formation of biofilm and susceptibility to antifungal agents). These all contribute towards the persistence of colonization and the development of opportunistic fungal infections.

The investigation of virulence factors is important to prevent systemic complications in risk populations through support in clinical interventions and control strategies with antifungal agents for improvement of the patient’s clinical conditions. The objective of this study was to evaluate the ability for adhesion in buccal epithelial cells (BECs) and adhesion and biofilm formation in artificial saliva, as well as assessing the profile of antifungal susceptibility of yeasts isolated from the oral cavity in chronic renal failure patients undergoing haemodialysis.

**METHODS**

**Clinical isolates.** Ten clinical isolates of *Candida* spp. and one isolate of *S. cerevisiae* from the oral cavity of chronic kidney patients undergoing haemodialysis, from the fungal collection of the Medical Mycology Laboratory, State University of Maringá, Maringá, PR Brazil, were analysed. The isolates were: three *C. albicans* (JP01, JP02 and JP03), two *C. glabrata* (JP04, JP05), three *C. tropicalis* (JP06, JP07 and JP08), one *C. parapsilosis* (JP09), one *Candida rugosa* (JP10) and one *S. cerevisiae* (JP11). Strains from the reference American Type Culture Collection (ATCC) were also tested for *C. albicans* (ATCC 90028), *C. tropicalis* (ATCC 750), *C. glabrata* (ATCC 2001) and *C. parapsilosis* (ATCC 22019). Samples were reactivated in Sabouraud dextrose agar medium (SDA; Merck). After growth, they were tested in CHROMagar *Candida* (bioMérieux) to assess the culture’s purity.

**Adhesion capacity and biofilm formation on an abiotic surface.** Adhesion capacity and biofilm formation were analysed in a 96-well polystyrene plate (Nunclon Delta; Nunc), with artificial saliva (Sali-form; Formula and Action). Standardized yeast suspensions (200 µl comprising 100 µl 1 × 10^6 c.f.u. ml⁻¹ and 100 µl artificial saliva) were placed in contact with the polystyrene microplate for 2 h (adhesion) or 24 h (formation of biofilm) at 130 r.p.m. at 37°C. Adhesion capacity (2 h) and biofilm formation (24 h) were subsequently quantified by the crystal violet (CV) coloration method (Silva et al., 2009). The absorbance of the obtained solution was read in quadruplicate in a microtitre plate reader (WaveX5 power; Biotech) at 570 nm. The mean absorbance of CV retained by yeasts adhering or in biofilm was expressed as absorbance per unit of well area (Abs cm⁻²). Experiments were repeated as part of three to five independent assays.

**Determination of adherence in BECs.** BECs were obtained from healthy volunteers by softly swabbing the oral mucus with sterilized swabs and dispensing in 10 ml PBS (pH 7.2). All subjects were free of any oral lesions, and none had received antibiotics or antifungals. The BEC suspension was washed three times in PBS by centrifugation at 430 g for 5 min. The sediment was checked under an optical microscope to ensure any micro-organisms present had been removed. The BECs were resuspended in PBS and adjusted to a concentration of 2 × 10⁶ cells ml⁻¹ with a Neubauer chamber. A recent yeast culture in SDA (16–24 h) was recultivated in a yeast nitrogen base broth (Difco) supplemented with 50 mM galactose and incubated for 24 h at 37°C. The yeast suspension was washed three times in PBS and resuspended in the same buffer to obtain and adjust the inoculum to 5 × 10⁶ c.f.u. ml⁻¹. Adherence assays were performed according to the method of Taguti Irie et al. (2006).

The number of yeasts adhering to BECs was determined by light microscopy at × 400 magnification. The following criteria were used to standardize the counts: isolated yeasts with or without germination tubes were the fungal units and were assessed in microscopic fields without the interference of coloration. Adherent yeasts had a clear interaction with BECs shown by a surrounding bright halo or with clear signs of cell injury. A budding yeast cell with daughter cells smaller than the mother was considered a unit cell. Overlapping and folded BECs, as well as cells not seen entirely in the selected field, were excluded (Pizzo et al., 2001).

*Candida* spp. adhering to BECs were quantified according to Taguti Irie et al. (2006) with some modifications. We evaluated three parameters in 100 cells counted consecutively: (i) the percentage of cells with adhered yeast cells; (ii) the number of yeast cells adhering per cell; and (iii) the adherence index (AI) calculated by multiplication of parameters (i) and (ii) to account for the overall adherence to BECs for each isolate.

**Antifungal susceptibility profile.** The antifungal susceptibility profile was determined against nystatin, amphotericin B, itraconazole, fluconazole and voriconazole all from Sigma-Aldrich. The test was performed by a microdilution assay in broth, according the guideline of the Clinical Laboratory Standards Institute (CLSI, 2008). Concentrations ranged between 0.125 and 64 µg ml⁻¹ for fluconazole and nystatin and between 0.03 and 16 µg ml⁻¹ for voriconazole, itraconazole and amphotericin B. The suspensions were tested with antifungal solutions in 96-well microplates (Nunclon Delta; Nunc) incubated for 48 h at 35°C. *C. parapsilosis* ATCC 22019 was used as a control and the reading of microplates was carried out at 490 nm (Expert Plus Microplate Reader; ASYS). The MICs of polyene antifungals (amphotericin B and nystatin) were defined as the lowest concentrations that caused complete inhibition of growth. The MICs of the azoles (fluconazole, itraconazole and voriconazole) were defined as the lowest concentrations that inhibit growth by 50%.

Results were given as: S, sensitive; DDS, dose-dependent sensitive; and R, resistant. Cut-off points were: S, ≤ 8; DDS, 16–32; and R, ≥ 64 for fluconazole; S, ≤ 1; DDS, 2; and R, ≥ 4 for voriconazole; and S, ≤ 0.125; DDS, 0.25–0.5; and R, ≥ 1 for itraconazole, according to CLSI (2008). CLSI (2008) has not determined breakpoints for amphotericin B, ‘resistant’ isolates were defined as isolates with MICs > 1 µg ml⁻¹ (Furlaneto et al., 2011; Montagna et al., 2014). For nystatin, isolates were considered sensitive with an MIC of ≤ 4 µg ml⁻¹ (Carrillo-Muñoz et al., 1999; Choukri et al., 2014; Hamza et al., 2008).

**Statistical analysis.** Data distribution was given as means ± SD of three independent assays. Significant differences between multiple comparisons were identified with Graph Pad Prism version 5.0, using Student’s t-test and Bonferroni Method. Rates were considered significant for P < 0.05.

**RESULTS AND DISCUSSION**

Virulence factors of micro-organisms and the immunological conditions of the host are parameters closely linked to the development of infections by yeasts. The development of oropharynx candidiasis in immunocompromised patients, such as chronic kidney patients, may cause malnutrition and disturb the adsorption of drugs (Sardi et al., 2013). Consequently, the characterization of the main virulence factors of yeasts isolated from the oral cavity of chronic kidney patients is highly relevant to
explain how these micro-organisms adhere to the tissues and form biofilms, coupled to the relationship between these factors and the susceptibility of the yeasts to antifungal agents that are clinically employed.

The adhesion capacity and in particular the formation of biofilms and the consequent production of the matrix are highly dependent on environmental conditions such as medium, pH and oxygen (Silva et al., 2010). In the oral cavity, as most of the surfaces are covered with saliva, the interaction of micro-organisms with salivary proteins is of fundamental importance for colonization (Seabra et al., 2013). In current assay, all the yeasts adhered and formed biofilms on abiotic surfaces with artificial saliva, a solution with all the mineral salts found in natural saliva, mimicking in vitro the environment found in vivo (Fig. 1).

C. rugosa (JP10) showed significantly (P<0.05) the greatest adhesion capacity (18398 Abs cm⁻²) (Fig. 1a). C. rugosa is a less frequently isolated in the clinic, although recent studies have shown that its incidence is increasing, especially in immunocompromised patients. C. rugosa has been detected in the oral cavity of kidney haemodialysis patients and diabetes patients (Pfaller et al., 2007; Pires-Gonçalves et al., 2007; Sardi et al., 2013). The current assay emphasizes the ability of this species to demonstrate high adhesion capacity on abiotic surfaces.

Inter-species analyses showed that C. tropicalis (JP06, JP07, JP08 and ATCC 750) and C. albicans (JP01, JP02, JP03 and ATCC 90028) varied greatly with regard to adhesion, with a significant difference (P<0.05) among some isolates (data not shown). Variability may be actually associated with the development of hyphae and pseudo-hyphae for the species, as the surface’s antigen variety and mycelium format may affect the adhesion capacity of the yeast (Ribeiro et al., 2004).

According to Fig. 1(a), C. glabrata showed the lowest adhesion capacity on abiotic surfaces (2844 Abs cm⁻²). C. glabrata is unable to form filaments and always maintains its yeast shape, with different metabolic activities and lower contact surface than yeasts capable of filament formation. These factors inherent to the species may contribute towards the low adhesion capacity of this species on abiotic surfaces (Silva et al., 2011b).

C. albicans had significantly (P<0.05) a greater capacity for biofilm formation (2035 Abs cm⁻²) when compared with other species (Fig. 1b), contrasting with the results of Seabra et al. (2013) who conducted an experiment with artificial saliva (produced by the authors) and yeasts isolated from the buccal cavity. The authors reported that C. parapsilosis formed more biofilm than C. albicans. Differences between our assay and the results of Seabra et al. (2013) may be related to the saliva composition. This fact was reported by Silva et al. (2012), who registered the influence of the artificial saliva formulation during biofilm formation by C. albicans (Seabra et al., 2013; Silva et al., 2012). It should be emphasized that C. albicans is not merely the main yeast in buccal infections but is highly isolated in hospital infections. It is currently the most important pathogenic yeast in humans and is one of the main fungaemia agents worldwide. Several studies suggest that most illnesses caused by C. albicans are related to its capacity for forming biofilm, coupled with its ability to form filaments. This virulence factor has a relevant function in the formation of biofilm, invasion of tissues and resistance to phagocytosis (Chauhan et al., 2013; Sardi et al., 2013).

Although most studies on fungus biofilms focus on C. albicans, several authors have recently reported the involvement of other yeast genera and species, such as S. cerevisiae and NAC species in biofilm formation (Sardi et al., 2014). There is actually a great interest in assessing the specific characteristics of biofilm formation by these fungi due to the relevance of in-depth studies on biofilm mechanisms for control strategies (Sardi et al., 2014).

The current analysis also found differences with regard to the capacity for forming biofilms by NAC, albeit not
statistically significant. The difference was affected by important factors inherent to each species, such as the cells’ relative size, morphology and biochemistry (Silva et al., 2011b). Furthermore, the biofilm’s extracellular matrix varies for the several species, mainly with regard to protein content and carbohydrates (Silva et al., 2011b).

According to Silva et al. (2009), C. parapsilosis, C. tropicalis and C. glabrata are also capable of producing biofilm, but the latter had a lower capacity when compared with C. parapsilosis and C. tropicalis (Silva et al., 2009). In current analysis, C. glabrata had a higher capacity for biofilm formation (1036 Abs cm⁻²) when compared with C. parapsilosis (0.803 Abs cm⁻²), albeit with a slight significant difference between the two species (P > 0.05) (Fig. 1b).

The yeast S. cerevisiae is also capable of forming biofilm (Fig. 1b), although it had the lowest level of production (0.468 Abs cm⁻²). However, there are reports in the literature that this species adheres to plastic surfaces and is capable of forming a more carpet-shaped biofilm in semi-solid agar (Sarode et al., 2014; Weiss Nielsen et al., 2011).

Table 1 and Fig. 2 showed that there was a relevant variability in the adherence to BECs of isolates with regard to the percentage of adhered yeasts (10–88%) and the number of yeasts adhering to BECs (1.64–3.51). The product of these parameters is the AI in BECs. In fact, yeasts adhering to BECs (1.64–3.51). The product of these parameters is the AI in BECs. In fact, yeasts adhering to BECs (1.64–3.51).

C. tropicalis (JP06) revealed a low percentage of cells with yeasts adhering (Table 1), but provided a high number of adhered yeasts per cell (Fig. 2a), as well as adhered pseudohyphae (Fig. 2c). Likewise, C. parapsilosis (Fig. 2b) had the highest mean number of adhered yeasts per cell (2.81) and the highest AI among the species (Fig. 3). Other NACs also varied with regard to AI (Fig. 3). The above characteristics may be associated with the intra-species variability in the adhesion capacity on the abiotic surface. There is also a possible co-relationship between the yeasts’ adhesion capacity and the surface, due, among other factors, to the adherence of micro-organisms being dependent on the roughness of the substrate surface. Areas with greater surface roughness, such as epithelial cells, are more suitable for the physical retention of micro-organisms (Carlén et al., 2001; Silva et al., 2010).

C. rugosa was the yeast with the least adherence in BECs but with the highest adherence on abiotic surfaces. On the other hand, the isolate that showed the highest adhesion capacity in BECs was C. glabrata (JP04) (Table 1), but the species C. glabrata showed lower adherence on abiotic surfaces (Fig. 1a). Therefore, the results suggest that C. glabrata has a greater affinity for biotic surfaces, whilst C. rugosa has a greater affinity for abiotic surfaces.

As a rule, there was little association between adhesion capacity and biofilm formation on abiotic surfaces with artificial saliva and adhesion on BECs. Adhesion proteins and physico-chemical factors are essential in the interaction process between micro-organisms and biotic and abiotic surfaces. In fact, they are intrinsic to yeasts (specific adhesion proteins) and are influenced by the environment (physical and chemical factors). The difference in adhesion between surfaces is related to species and to the context in which the yeasts are added (Bendel & Hostetter, 1993; Negri et al., 2010; Sohn et al., 2006).

Regarding the antifungal susceptibility profile, Table 2 showed a significant variability in MICs between the five antifungal agents tested in susceptibility tests. The results demonstrated that all clinical isolates of C. albicans were sensitive to the five antifungal agents. A similar profile has been registered in Brazil on the susceptibility of the species to the antifungal agents tested (Furlaneto et al., 2011).

However, the capacity of Candida spp. to form biofilm may increase resistance to antifungal therapy. This is especially true for C. albicans with its great capacity for biofilm formation, as shown in the current assay. Although C. albicans was sensitiveness to antifungal agents, Kuhn et al. (2002) reported that, among the three classes of antifungal agents in clinical use, only amphoteracin B and echinocandins showed a consistent in vitro activity against biofilms of C. albicans. In spite of these therapeutic options, it is very difficult to eliminate biofilm in infected medical instruments and removal of the instruments is usually required (Kuhn et al., 2002; Montejo, 2011).

For amphoteracin B, isolates of C. albicans (JP01), C. glabrata (JP05) and C. rugosa (JP10) had MICs equal

| Table 1. Adhesion to BECs of yeasts isolated from the oral cavity of chronic kidney patients undergoing haemodialysis |
|------------------|------------------|------------------|------------------|
| Species | Isolate | Cells with attached yeasts (%) | No. yeasts adhering per BEC | AI |
| C. albicans | JP01 | 10 | 2 | 20 |
| | JP02 | 29 | 1.72 | 48.88 |
| | JP03 | 47 | 1.64 | 77.08 |
| | ATCC 90028 | 35 | 3.42 | 119.7 |
| C. glabrata | JP04 | 88 | 2.88 | 253.44 |
| | JP05 | 54 | 2.03 | 109.62 |
| | ATCC 2001 | 40 | 2.02 | 80.8 |
| C. tropicalis | JP06 | 20 | 3.51 | 70.2 |
| | JP07 | 38 | 1.9 | 72.2 |
| | JP08 | 23 | 2.28 | 52.44 |
| | ATCC 750 | 59 | 2.09 | 123.31 |
| C. parapsilosis | JP09 | 50 | 3.35 | 167.5 |
| | ATCC 22019 | 60 | 2.27 | 136.2 |
| C. rugosa | JP10 | 25 | 1.74 | 43.5 |
| S. cerevisiae | JP11 | 41 | 1.93 | 79.13 |

*Counted for 100 consecutive BECs.
to 1 µg ml$^{-1}$. Although no cut-off points have been established for this antifungal agent, the CLSI recommends special care in the use of MICs of amphotericin B in therapeutic decisions in clinical practice. The fungus is probably resistant when the MIC in vitro is >1 µg ml$^{-1}$ (CLSI, 2008; Furlaneto et al., 2011). An isolate of C. albicans (JP01) also showed a great capacity for the formation of biofilm (1.97 Abs cm$^{-2}$) and may not respond to antifungal therapy with amphotericin B.

All species were susceptible to amphotericin B and nystatin. Two isolates of C. glabrata (JP04 and JP05) and the isolate of C. parapsilosis (JP09) were DDS for fluconazole, with MICs equal to 16 µg ml$^{-1}$ and resistant to itraconazole, with MICs of 2 µg ml$^{-1}$. An isolate of C. tropicalis (JP08) and S. cerevisiae (JP11) showed DDS to itraconazole, whilst an isolate of C. rugosa (JP10) was DDS to voriconazole with an MIC of 2 µg ml$^{-1}$ (Table 2).

In the case of DDS, the variations suggest that treatment may cause a therapeutic flaw as the treatment of oral candidiasis is not generally started from laboratory results. Consequently, yeasts with high MIC rates may not be inhibited if treated with a subdose (Córdoba et al., 2011; Godoy et al., 2013).

C. tropicalis is the most isolated species in cases of candidaemia in Brazil among NAC species (20.9%), followed by C. parapsilosis (20.5%) and C. glabrata (4.9%) (Nucci et al., 2010). C. glabrata and C. tropicalis are intrinsically more resistant to azole antifungal agents, such as fluconazole, itraconazole, voriconazole and ketoconazole, and C. parapsilosis is generally sensitive to azoles (Cuéllar-Cruz et al., 2012; Sardi et al., 2013). However, the C. parapsilosis isolate (JP09) was DDS to fluconazole and resistant to itraconazole. Cuéllar-Cruz et al. (2012) reported that the susceptibility of C. parapsilosis and C. glabrata to fluconazole has changed discretely according to the patient’s age and place (Cuéllar-Cruz et al., 2012). A correlation may exist between reduced antifungal susceptibility and the prophylactic use of fluconazole, without any basis on laboratory results. Some authors have stated that the above factor may contribute towards the emergence of CNA species as significant pathogenic agents (Bassetti et al., 2009; Premkumar et al., 2014).

The species C. parapsilosis and S. cerevisiae with high participation in normal human microbiota have recently evolved as the cause of disseminated infections due to different clinical practices and to a survival increase in patients with chronic diseases in intensive care units. These yeasts may also be associated with cases of mixed fungal infection by S. cerevisiae and yeasts of the genus Candida in intensive care units (Jensen et al., 2007; Silva et al., 2011a).

The current analysis demonstrated that yeasts isolated from the oral cavity of chronic kidney insufficiency patients undergoing haemodialysis varied considerably in their adherence capacity and biofilm formation, without any association between the two factors, underscoring NAC.

Fig. 2. Adhesion of yeasts isolated from the oral cavity of chronic kidney patients undergoing haemodialysis, on BECs, by optical microscopy. Magnification, ×400. (a) C. tropicalis adhering to BECs with a bright halo around the yeasts (arrow). (b) Adherence of C. parapsilosis indicated by cell damage (arrow). (c) Pseudohyphae of C. tropicalis adhering to cells.

Fig. 3. Mean AI for Candida spp. in BECs. Results are shown as means ± SD.
species with greater virulence. Further investigations are required to establish whether these interactions are actually isolate specific or whether they depend on other parameters.

ACKNOWLEDGEMENTS

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REFERENCES


Table 2. MICs (µg ml⁻¹) of yeasts isolated from the oral cavity of chronic kidney patients undergoing haemodialysis

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>FLUCO</th>
<th>VORIC</th>
<th>ITRA</th>
<th>AMPHO B</th>
<th>NYST</th>
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<tr>
<td>C. albicans</td>
<td>JP01</td>
<td>0.125 (S)</td>
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<tr>
<td></td>
<td>JP03</td>
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<td>0.5 (S)</td>
<td>0.03 (S)</td>
<td>0.25</td>
<td>0.125</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>JP04</td>
<td>16 (DDS)</td>
<td>0.5 (S)</td>
<td>2 (R)</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>JP05</td>
<td>16 (DDS)</td>
<td>0.5 (S)</td>
<td>2 (R)</td>
<td>1</td>
<td>4</td>
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<td>C. tropicalis</td>
<td>JP06</td>
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<td>0.0625 (S)</td>
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<td>0.03 (S)</td>
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<td></td>
<td>JP08</td>
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<td>0.5 (S)</td>
<td>0.25 (DDS)</td>
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<td>C. parapsilosis</td>
<td>JP09</td>
<td>16 (DDS)</td>
<td>1 (S)</td>
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<td>C. rugosa</td>
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<td>2</td>
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<tr>
<td>S. cerevisiae</td>
<td>JP11</td>
<td>1 (S)</td>
<td>0.06 (S)</td>
<td>0.5 (DDS)</td>
<td>0.5</td>
<td>4</td>
</tr>
</tbody>
</table>

*Criteria of interpretation (CLSI, 2008): FLUCO, fluconazole (S, ≤8; DDS, 16–32; R, ≥64); VORIC, voriconazole (S, ≤1; DDS, 2; R, ≥4); ITRA, itraconazole (S, ≤0.125; DDS, 0.25–0.5; R, ≥1); AMPHO B, amphotericin B; NYST, nystatin.


