Trichosporon inkin biofilms produce extracellular proteases and exhibit resistance to antifungals

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The aim of this study was to determine experimental conditions for in vitro biofilm formation of clinical isolates of Trichosporon inkin, an important opportunistic pathogen in immunocompromised patients. Biofilms were formed in microtitre plates in three different media (RPMI, Sabouraud and CLED), with inocula of 10^4, 10^5 or 10^6 cells ml⁻¹, at pH 5.5 and 7.0, and at 35 and 28 °C, under static and shaking conditions for 72 h. Growth kinetics of biofilms were evaluated at 6, 24, 48 and 72 h. Biofilm milieu analysis were assessed by counting viable cells and quantification of nucleic acids released into biofilm supernatants. Biofilms were also analysed for proteolytic activity and antifungal resistance against amphotericin B, caspofungin, fluconazole, itraconazole and voriconazole. Finally, ultrastructural characterization of biofilms formed in microtitre plates and catheter disks was performed by scanning electron microscopy.

Greater biofilm formation was observed with a starter inoculum of 10^6 cells ml⁻¹, at pH 7.0 at 35 °C and 80 r.p.m., in both RPMI and Sabouraud media. Growth kinetics showed an increase in both viable cells and biomass with increasing incubation time, with maximum production at 48 h. Biofilms were able to disperse viable cells and nucleic acids into the supernatant throughout the developmental cycle. T. inkin biofilms produced more protease than planktonic cells and showed high tolerance to amphotericin B, caspofungin and azole derivatives. Mature biofilms were formed by different morphotypes, such as blastoconidia, arthroconidia and hyphae, in a strain-specific manner. The present article details the multicellular lifestyle of T. inkin and provides perspectives for further research.

INTRODUCTION

In recent decades, the frequency of fungal infections has increased among immunocompromised patients (Pfaller & Diekema, 2007). Although Candida spp. are the most important pathogens in such patients, Trichosporon spp. have been considered a common cause of fungaemia in patients with malignant haematological disease (Girmenia et al., 2005). Despite the fact that trichosporonosis is usually considered a benign superficial infection, invasive fungal infections caused by Trichosporon spp. are important diseases, with attributable death rates of up to 80 % in high-risk groups (Colombo et al., 2011).

Trichosporon spp. are easily recovered from soil and fresh water (Chagas-Neto et al., 2008; Vazquez, 2010), and are considered a member of the human microbiota of the

Abbreviations: AMB, amphotericin B; CAS, caspofungin; FLU, fluconazole; IFI, invasive fungal infection; ITR, itraconazole; MBIC, minimum biofilm inhibitory concentration; SEM, scanning electron microscopy; VOR, voriconazole.
skin, upper respiratory and gastrointestinal tracts (Colombo et al., 2011; Miceli et al., 2011). Although Trichosporon spp. are usually considered agents of benign superficial diseases, such as white piedra and mucous-associated infections, several reports have confirmed their pathogenic role in deep-seated infections (Colombo et al., 2011; Kustimur et al., 2002; Miceli et al., 2011).

Epidemiological data have shown that Trichosporon inkin is the second most common agent of trichosporonosis, and invasive fungal infections caused by Trichosporon spp. are commonly associated with the use of catheters (Krcmery et al., 1999; Colombo et al., 2011). The ability to form biofilms in such devices plays an important role in the development of infectious process, since sessile cells show increased antifungal resistance and are protected from host immunological defences (Di Bonaventura et al., 2006; Douglas, 2002). Despite the importance of this subject, there are few reports on Trichosporon spp. biofilms (Di Bonaventura et al., 2006; Dostálková et al., 2015; Iturrieta-González et al., 2014; Junqueira et al., 2012, Lakshmi & Das, 2013; Liao et al., 2014, 2015; Sun et al., 2012).

Therefore, the aim of the present study was to determine the experimental conditions for in vitro biofilm formation in T. inkin. Biofilms were evaluated regarding growth kinetics, proteolytic activity, antifungal resistance and ultrastructural morphology. In addition, we investigated the dynamics within the surrounding biofilm milieu, through analysis of dispersed cells and released nucleic acids.

**METHODS**

**Micro-organisms.** A total of seven clinical strains of T. inkin were used in this work: CEMM 05-6-086 (CBS Centraalbureau voor Schimmelcultures 5585; skin), CEMM 05-6-057 (hair with white piedra), CEMM 05-6-074 (urine), CEMM 05-6-075 (perigenital area), CEMM 01-1-144 (skin), CEMM 01-1-143 (skin) and CEMM 01-1-145 (urine). Upon arrival at our laboratory, the strains were plated onto potato agar and incubated for 48 h at 35 °C; and identification was confirmed by micromorphology on malt agar (De Hoog et al., 2000) and/or sequencing of the intergenic spacer region, using primers 26SF and 5SR (Messias Silvestre et al., 2010; Rodrigues-Tudela et al., 2005). The strains were deposited with the culture collection of the Specialized Medical Mycology Center of Ceará Federal University, Brazil.

**Biofilm formation assays.** Strains (n=7) were previously cultured on potato dextrose agar (PDA; Difco) for 48 h at 35 °C. Cell suspensions were adjusted to three concentrations (10⁴, 10⁵ and 10⁶ cells ml⁻¹) directly into the following media: RPMI 1640 (Sigma), Sabouraud broth (Himedia) and cystine–lactose–electrolyte–deficient (CLED) broth (casein peptone, 4 g l⁻¹; gelatin peptone, 4 g l⁻¹; beef extract, 3 g l⁻¹; lactose, 10 g l⁻¹; cystine, 0.128 g l⁻¹) at both pH 5.5 and 7.0. Aliquots of 200 μl of each suspension were transferred to flat 96-well polystyrene plates and incubated at 28 °C or 35 °C, either at 80 r.p.m. or under static conditions. Adhesion was tested at 1, 2, 3 and 6 h. After each time interval, plates were washed with PBS containing 0.05 % Tween 20 to remove non-adherent cells and then wells were filled with the respective culture medium. Plates were incubated until 72 h in each condition described above. Afterwards, plates were washed twice with PBS/Tween. Biofilms were dehydrated with methanol (Dinamica), dried at room temperature and stained with crystal violet. Plates were washed with distilled water, decolorized with acetic acid (Grupo Quimica) and absorbance was measured at 590 nm (Peeters et al., 2008). Experiments were performed in quadruplicate. Experimental conditions which formed higher-density biofilms at 590 nm were defined as standard for further tests.

**Biofilm characterization**

**Biofilm growth kinetics.** To evaluate growth kinetics, biofilms were developed over different time intervals (6, 24, 48 and 72 h). Fungal cultures were previously grown in potato agar glucose for 48 h at 35 °C as described above. Biofilms were allowed to form in RPMI medium pH 7.0 at 35 °C in a rotary shaker at 80 r.p.m. After each interval, supernatants were collected and plates were washed twice with sterile PBS/Tween to remove non-adherent cells. Viability of formed biofilms was evaluated by an XTT assay, according to Martinez and Casadevell (2006), with modifications [50 μl PBS, 75 μl XTT (1 mg ml⁻¹; Sigma) and 6 μl menadione (1 mM in acetone; Sigma)]. Plates were incubated at 35 °C for 5 h and the absorbance read at 492 nm. Biofilm biomass was assessed by the crystal violet staining method. Experiments were performed in triplicate.

**Biofilm milieu analysis.** The cells attached to biofilms and dispersed in the supernatant during biofilm growth were evaluated by c.f.u. assay. Biofilms were formed with inocula of 10⁵ cells ml⁻¹ in RPMI medium at 35 °C at 80 r.p.m. After 24, 48 and 72 h of incubation, biofilm supernatants were collected, diluted in PBS and an aliquot of 100 μl was spread onto PDA plates. Biofilms were scraped with a sterile micropipette tip, vigorously vortexed, diluted in PBS and spread onto PDA plates. To avoid counting non-adherent cells in the biofilms, wells were washed twice with PBS/Tween and refilled with fresh RPMI after every 24 h of incubation. PDA plates were incubated for 48–72 h at 35 °C, colonies were counted and numbers of c.f.u. ml⁻¹ were determined. Experiments were performed in triplicate.

**Biofilm supernatants were centrifuged for 10 min at 10 000 r.p.m. and then nucleic acid concentration was estimated using a Nanodrop 2000 device (Thermo Scientific). Total nucleic acid concentration was also estimated in planktonic cultures. Inocula of 10⁵ cells ml⁻¹ of all tested strains were cultivated in RPMI for up to 72 h at 35 °C (80 r.p.m.). After 24, 48 and 72 h of incubation, aliquots were collected, centrifuged and read in a Nanodrop device. Analyses were performed in triplicate.

**Proteolytic activity.** Total protease production in T. inkin biofilms and planktonic cultures was detected using azoalbumin as substrate (Charney & Tomarella, 1947). Biofilms were allowed to form for 6, 24, 48 and 72 h; after each period, biofilms were scraped with a sterile micropipette tip and then centrifuged at 10 000 r.p.m. for 10 min. The supernatant was mixed with 0.3 % azoalbumin and the mixture was incubated in at 37 °C for 3 h. The enzymic reaction was stopped by adding 5 % trichloroacetic acid followed by addition of 0.5 M NaOH. Reading was performed at 440 nm in an Epoch spectrophotometer (Biotek). For planktonic cells, a starting inoculum of 10⁵ cells ml⁻¹ was incubated in RPMI for 72 h at 35 °C (80 r.p.m.). After 6, 24, 48 and 72 h of incubation, aliquots were collected, centrifuged and analysed for proteases as described above. Negative controls were performed without fungal cells. The experiments were conducted in triplicate.

**Antifungal biofilm resistance.** Biofilms were formed as described above and after 48 h of incubation, plates were washed twice with PBS/Tween. Aliquots of 200 μl RPMI containing amphotericin B (AMB; Sigma), caspofungin (CAS; Merck Sharp & Dohme), fluco-
nazole (FLU; Pfizer), itraconazole (ITR; Janssen Pharmaceutica) or voriconazole (VOR; Pfizer), at concentrations that ranged from to 8 to 128 \( \mu \text{g ml}^{-1} \) were added to mature biofilms. Plates were incubated at 35 °C for 24 h for CAS and 48 h for the remaining antifungals. Metabolic activity was assessed by the XTT assay. Experiments were conducted in duplicate and controls were grown in RPMI without antifungals. Minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration able to inhibit 100 % of biofilm growth for AMB (Di Bonaventura et al., 2006) and 50 % for the remaining antifungals (Liao et al., 2014), when compared to drug-free controls. Susceptibility testing of planktonic cells was performed in accordance with CLSI (2008). Candida parapsilosis ATCC 22019 was included as a quality control. All the isolates were tested in duplicate.

**Scanning electron microscopy (SEM).** Ultrastructural characterization of biofilms was carried out by SEM according to Di Bonaventura et al. (2006) with modifications. Biofilms were formed on glass slides covered with poly-\( \text{i} \)-lysine and on catheter fragments. After incubation for 6, 24, 48 and 72 h, biofilms were fixed with 2.5 % glutaraldehyde in 0.15 M sodium cacodylate buffer and incubated overnight at 4 °C. To evaluate the presence of matrix material, biofilms were also fixed with glutaraldehyde-cacodylate buffer with alcian blue (0.1 %). Afterward, biofilms were washed twice with 0.15 M cacodylate buffer for 5 min and dehydrated in an ascending ethanol series [50, 70, 85 and 100 % (twice); 10 min each] before 30 min of drying with hexamethyldisilazane (Polysciences Europe) and finally were air-dried overnight. Slides were coated with 10 nm gold (Emitech Q150T), and observed with an FEI Inspect S50 scanning electron microscope, in the high-vacuum mode at 15 kV. Images were processed with Photoscape software v3.6.5 (MooiITech).

**Statistical analysis.** Results were evaluated by ANOVA and Tukey’s multiple comparisons post-test. A \( P \) value <0.05 was considered significant. The statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software).

**RESULTS**

**Biofilm formation**

Data analysis of biofilm formation of *T. inkin* in all tested conditions showed that more abundant biofilms were formed with an initial inoculum of \( 10^6 \) cells ml\(^{-1} \) when compared to biofilms produced by smaller inocula (Fig. 1a–c). Regarding the pH of the culture media, for
the CLED broth, the pH that allowed greatest biofilm formation was 5.5. However, for both RPMI and Sabouraud, the highest biofilm formation was at pH 7.0 (P<0.05) (Fig. 1d). Shaking incubation was positively associated with increased biofilm formation, since biofilms produced at 80 r.p.m., both at 35 and 28 °C, showed higher biomass production than the biofilms grown at the same temperatures, but in static conditions. Agitation overcame temperature, since biofilms produced at 28 °C under shaking showed higher biomass than biofilms produced at 35 °C in static incubation (P<0.05). There was no significant difference between biofilms produced under static conditions at the two temperatures, but when biofilms produced at 80 r.p.m. were compared, increased biofilm formation was observed at 35 °C (P<0.05) (Fig. 1e). Significantly lower amounts of biofilm were formed in CLED broth (P<0.05) than in the other culture media. The maximum biofilm formation occurred with an initial inoculum of 10^6 cells ml^{-1} in Sabouraud or RPMI medium, at 35 °C under agitation at 80 r.p.m. (P<0.05).

Biofilm growth kinetics

Following the adhesion time (6 h), biofilm formation increased over time, reaching maturity (for both cell biomass and cell viability) at 48 h (Fig. 2a, b). There was no significant difference in biomass and metabolic activity of biofilms at 48 and 72 h (P>0.05). SEM images showed microcolonies after 6 h and increase in biofilm biomass over time. After 72 h of incubation, SEM images showed higher biofilm biomass; mature biofilms consisted of a thick network of cells (Fig. 2c).

Biofilm milieu analysis

Biofilms were formed with a starting inoculum of 10^6 cells ml^{-1} in RPMI medium at 35 °C in a rotatory shaker at 80 r.p.m. Counting c.f.u. of adherent cells at 6 h revealed that approximately 10^6 c.f.u. ml^{-1} were able to attach to the surface of microplate wells (data not shown). Dispersion of viable cells to supernatant occurred during every stage of the biofilm developmental cycle (Fig. 2c) and the number of cells attached to biofilms increased over time (P<0.05).

The detection of nucleic acids in biofilm supernatant as well as in planktonic growth cultures is shown in Fig. 2(d). Nucleic acid release was observed in the supernatant in both conditions evaluated (biofilm and planktonic growth). It was not possible to observe any statistical difference between nucleic acid release at 24 and 48 h, for both conditions (P>0.05); however, it was possible to observe that higher nucleic acid release occurred at 72 h than at 24 and 48 h, for both conditions (P<0.05).

Extracellular protease production

The strains were able to produce extracellular proteases in both planktonic growth and biofilms (Fig. 3). Protease production was observed in biofilms after 6 h of incubation, the period of cell adhesion on the surface of the microplate wells. Over time, production of extracellular proteases increased only in biofilms, with higher protease production at 48 and 72 h when compared with planktonic growth (P<0.05).

Antifungal resistance of biofilms

Most of the planktonic cultures (7/8) showed resistance to AMB, and all isolates were resistant to CAS (Table 1). However, all isolates of T. inkin tested were susceptible to azoles. Regarding susceptibility of biofilms, MBIC ranged from 16 to >128 μg ml^{-1} for AMB and CAS. For FLU, MBIC ranged from 64 to >128 μg ml^{-1} and from 128 to >128 μg ml^{-1} for ITR. For VOR, MBIC were all >128 μg ml^{-1}.

SEM

Mature T. inkin biofilms consist of masses of blastoconidia, arthroconidia, pseudohyphae and long and short hyphae, featuring multi-layer cell structures closely associated (Fig. 4a–f). No difference was observed in the morphological pattern of biofilms formed in catheter devices when compared to biofilms formed on glass slides covered with poly-l-lysine. The biofilm matrix was clearly visible when sessile cells were treated with alcian blue (Fig. 4g, i).

DISCUSSION

Biofilms are structures composed of cells attached to a surface and surrounded by an extracellular matrix. The ability to form biofilms is important in the host–parasite relationship and the development of infectious processes, since cells associated with biofilms exhibit distinct phenotypic characteristics, such as increased production of enzymes and antifungal resistance (Di Bonaventura et al., 2006; Douglas, 2002; Hasan et al., 2009, Nailis et al., 2010). Although the importance of biofilms in the pathogenesis of fungal infection is clear, few reports have been published about biofilms produced by Trichosporon spp. and only one concerning T. inkin biofilm formation (Di Bonaventura et al., 2006; Dostálková et al., 2015; Iturrieta-González et al., 2014; Junqueira et al., 2012, Lakshmi & Das, 2013; Liao et al., 2014, 2015; Sun et al., 2012). Biofilm formation varied greatly between different strains of T. inkin, which accounted for the high standard deviation of the presented data.

Adhesion of planktonic cells to a given substrate and further cell–cell interactions are prerequisites for microbial biofilm formation (Blankenship & Mitchell, 2006). The results of the present study indicate that maximum biofilm
Formation occurred after 6 h of cell attachment to the substrate. During this phase, it is well known that deep cellular and molecular events take place. Microcolonies have been observed as early as 1 h later in RPMI medium in *Trichosporon asahii* biofilms (Di Bonaventura et al., 2006) and after 90 min in *Trichosporon mucoides* growing in YNB medium supplemented with 100 µM of glucose (Junqueira et al., 2012). Iturrieta-González et al. (2014) also tested different adhesion times in RPMI medium, and showed that 90 min of adhesion with a starter inoculum of 10⁷ cells ml⁻¹ of *T. inkin* was more reproducible than other combinations. Liao et al. (2015) tested ethanol efficacy against *T. asahii* biofilms in three different adhesion times, at 30 min, 1 and 2 h, with a starter inoculum of 10⁶ cells ml⁻¹ in RPMI medium. Sun et al. (2012) evaluated the same adhesion times, at 30 min, 1 and 2 h, in *T. asahii* biofilms, with a starter inoculum of 10⁵ cells ml⁻¹. It is known that the adhesion time depends on the...
fungal species and culture conditions, and these reports showed that all different patterns of adhesion time already tested allowed biofilm formation of *Trichosporon* spp.

In the present study, maximum biofilm formation was observed with an initial inoculum of $10^6$ cells ml$^{-1}$. This cell concentration has been preferred by many authors for fungal biofilm assays (Di Bonaventura *et al.*, 2006; Liao *et al.*, 2015; Pusateri *et al.*, 2009), although Junqueira *et al.* (2012) and Iturrieta-González *et al.* (2014) analysed *Trichosporon* spp. biofilms from an inoculum of $10^7$ cells ml$^{-1}$ and Sun *et al.* (2012) analysed *T. asahii* biofilms with a starter inoculum of $10^3$ cell ml$^{-1}$. Optimal conditions for *T. inkin* biofilms were also evaluated regarding nutritional substrate, pH, temperature and agitation. *T. inkin* biofilms were stimulated by higher temperatures and agitation, independently of the substrate.

Previous studies have shown that these conditions also enhance planktonic and biofilm growth of other fungal species (Ajesh & Sreejith, 2012; Di Bonaventura *et al.*, 2006). *T. inkin* biofilms were formed in either low-glucose media (CLED, RPMI) or a nutrient-rich medium (Sabouraud) at both pH 7.0 and 5.5. These results support the physiological versatility of *Trichosporon* spp., which are able to grow in many different substrates (Magalhães *et al.*, 2008). Following attachment stage, *T. inkin* biofilms were allowed to grow for 48 h, when the structure reached maturity. Liao *et al.* (2015) evaluated different incubation times for *T. asahii* biofilms and showed that with prolonged times of incubation, the rate of germ tube formation increased. Many authors have shown that fungal biofilms mature after 48 h following adhesion (Iturrieta-González *et al.*, 2014; Lattif *et al.*, 2010; Martinez & Casadevall, 2006), although Di Bonaventura *et al.* (2006) showed that *T. asahii* biofilms exponentially increased until 72 h. It is likely that these differences are related to the experimental conditions tested.

Regarding *in vitro* biofilm experimentation, different temperatures have been tested by many authors. In the case of *Candida albicans*, for instance, both 35 °C (de Vasconcellos *et al.*, 2014; Ruiz *et al.*, 2013; Shin *et al.*, 2002; Vila *et al.*, 2013) and 37 °C (Mukherjee *et al.*, 2003; Nett *et al.*, 2011; Perumal *et al.*, 2007; Pusateri *et al.*, 2009; Robbins *et al.*, 2011; Uppuluri *et al.*, 2010) have been tested successfully for biofilm assays. Although *T. inkin* is considered an opportunistic pathogen, able to grow at 37 °C, there were no differences in total biomass between biofilms grown at 37 or 35 °C (data not shown). Since we also wanted to characterize the antifungal susceptibility profile of *T. inkin* biofilms, we decided to perform experimentation at 35 °C, as this temperature is described in CLSI protocols as suitable for antifungal testing. Therefore, we chose two strategies: to evaluate biofilm formation at room temperature (28 °C) – because *T. inkin* can also be recovered from environmental sources – and at 35 °C. As pointed out by many studies, there is strong evidence that human physiological temperature (oral, rectal, tympanic, axillary) can range from 35.2 °C to approximately 38 °C (Lu & Dai, 2009; Sund-Levander *et al.*, 2002;...

### Table 1. Susceptibility patterns of *T. inkin* biofilms and planktonic cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amphotericin B</th>
<th>Caspofungin</th>
<th>Fluconazole</th>
<th>Itraconazole</th>
<th>Voriconazole</th>
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<tr>
<td></td>
<td>MBIC* MIC</td>
<td>MBIC** MIC</td>
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<tr>
<td>CEMM 05-6-074</td>
<td>16 2</td>
<td>16 8</td>
<td>64 1</td>
<td>128 0.06</td>
<td>&gt;128 0.06</td>
</tr>
<tr>
<td>CEMM 05-6-086</td>
<td>32 2</td>
<td>32 8</td>
<td>&gt;128 0.5</td>
<td>&gt;128 0.06</td>
<td>&gt;128 0.06</td>
</tr>
<tr>
<td>CEMM 01-1-144</td>
<td>&gt;128 &gt;16</td>
<td>32 8</td>
<td>&gt;128 2</td>
<td>&gt;128 0.125</td>
<td>&gt;128 0.125</td>
</tr>
<tr>
<td>CEMM 05-6-075</td>
<td>16 2</td>
<td>64 8</td>
<td>&gt;128 2</td>
<td>&gt;128 0.06</td>
<td>&gt;128 0.125</td>
</tr>
<tr>
<td>CEMM 05-6-057</td>
<td>&gt;128 8</td>
<td>&gt;128 8</td>
<td>&gt;128 0.5</td>
<td>&gt;128 0.125</td>
<td>&gt;128 0.125</td>
</tr>
<tr>
<td>CEMM 01-1-143</td>
<td>32 2</td>
<td>64 8</td>
<td>&gt;128 2</td>
<td>128 0.06</td>
<td>&gt;128 0.06</td>
</tr>
<tr>
<td>CEMM 01-1-145</td>
<td>32 1</td>
<td>128 8</td>
<td>&gt;128 1</td>
<td>&gt;128 0.06</td>
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*100 % of biofilm inhibition
MBIC, Minimal Biofilm Inhibitory Concentration (µg ml$^{-1}$).
**50 % of biofilm inhibition
Consequently, we believe that our results yield relevant findings regarding *in vitro* *T. inkin* biofilms. In order to characterize the biological events within *T. inkin* biofilms, we also studied cell dispersion throughout the formation and maturation steps. Our results show that dispersion occurs in the early stages of biofilm formation and continues during the maturation phase. According to microscopic examination, the majority of cells released in biofilm supernatant were blastoconidia and arthroconidia. Filament structures were rarely observed in the supernatant, regardless of the developmental stage. In a seminal study performed by Uppuluri *et al.* (2010) with *C. albicans* biofilms, it was shown that cell dispersion is a phenomenon that occurs uninterruptedly throughout the biofilm developmental cycle, being affected by experimental conditions such as nutritional source. The authors also found that the dispersed cells were predominantly in the yeast form and displayed increased virulence and enhanced ability to form biofilms. Further experimentation should be performed to describe phenotypical traits of the dispersed cells of *T. inkin* biofilms.

The results of the present study showed that, along with cell dispersion, nucleic acid release occurred uninterruptedly during the *T. inkin* biofilm developmental cycle. However, microscopic analysis of biofilm supernatant did not reveal cell debris or burst cell structures, which leads us to suppose that at least part of the detected nucleic acid was actively released by growing cells in the biofilm. Previous studies have shown that high levels of extracellular DNA

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**Fig. 4.** SEM images of *T. inkin* biofilms after 72 h of incubation. (a–f) CEMM 05-6-057 (a); CEMM 05-6-086 (b); CEMM 01-1-144 (c); CEMM 01-1-143 (d); CEMM 01-1-145 (e); CEMM 05-6-074 (f). (g–j) Biofilm of CEMM 05-6-057 in catheter device treated with alcian blue (g) and without alcian blue (h); on sterile glass slide covered with poly-L-lysine treated with alcian blue – presence of matrix material (i) and without alcian blue – no matrix observed (j). Bar, 50 μm (a–h); 10 μm (i–j).
are actively incorporated into the biofilm matrix, playing an important role in biofilm structural integrity and maintenance, as well as antimicrobial resistance (Martins et al., 2010, 2012; Mulcahy et al., 2008). The results of this study show that extracellular nucleic acid, not integrated into the matrix, could reach up to 16 ng µL⁻¹ in the supernatant of T. inkin biofilms growing in RPMI. Although the amount of DNA trapped in fungal matrices is higher (Martins et al., 2010; Rajendran et al., 2013), we hypothesize that the extracellular nucleic acid released into the supernatant may have a role in the resistance of T. inkin biofilms.

Following biofilm characterization, we detected proteolytic activity in every stage of the T. inkin biofilm developmental cycle. The activity increased over time and mature biofilms produced more proteases than 24 h biofilms. Interestingly, protease activity was also detected during the 6 h adhesion time. The role of proteases in fungal biofilms has recently been studied in C. albicans. Secretory aspartic proteases genes are expressed in biofilm systems in vitro (Mendes et al., 2007; Nailis et al., 2010) and it has been hypothesized that these could be important for adhesion and nutrient acquisition in mature biofilms (Nailis et al., 2010). In the present study, we also observed that T. inkin biofilms displayed more proteolytic activity than their planktonic counterparts. Similar results were found by Mendes et al. (2007) in C. albicans biofilms. Taken together, these data suggest the importance of proteases in the development and maturation of T. inkin biofilms. To the best of our knowledge, the present study is the first to report protease production of Trichosporon biofilms.

Previous reports have described Trichosporon spp. clinical isolates resistant to amphotericin B, azoles and caspofungin, and even a multidrug-resistant phenotype (Di Bonaventura et al., 2006; Silva et al., 2008; Wolf et al., 2001). Biofilms produced by Trichosporon spp. also presented higher tolerance to antifungals (Di Bonaventura et al., 2006; Iturrieta-González et al., 2014; Liao et al., 2014; Sun et al., 2012). Data from the present study reveal that planktonic cells showed susceptibility to azoles and resistance to amphotericin B and caspofungin. On the other hand, T. inkin biofilms were tolerant to higher concentrations of all antifungals tested. Similar results were found by Di Bonaventura et al. (2006), Iturrieta-González et al. (2014) and Liao et al. (2014), who reported that, although Trichosporon spp. planktonic cells were susceptible to voriconazole and fluconazole, their biofilm counterparts were resistant to azoles, amphotericin B and caspofungin.

SEM images showed different patterns of cell ultrastructural morphology and organization. As observed in T. asahii and Trichosporon asteroides biofilms (Di Bonaventura et al., 2006; Iturrieta-González et al., 2014; Lakshmi & Das, 2013), mature T. inkin biofilms consist of a mixture of yeast and filamentous forms. The role of these different cell morphologies and arrangements associated with the presence of matrix material in biofilm phenotype, particularly regarding antifungal susceptibility, should be investigated (Di Bonaventura et al., 2006).

In conclusion, the present study showed that T. inkin biofilms can be formed under different experimental conditions, even in nutrient-deprived media. The ability to produce extracellular proteases, as well as to release viable cells and nucleic acid throughout the biofilm formation cycle, may have clinical impact during T. inkin infections. T. inkin biofilms also presented tolerance to high antifungal concentrations. Because of the clinical importance of fungal biofilms, the present article details the multicellular lifestyle of T. inkin and provides perspectives for further research.

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genotypes, virulence factors and antifungal susceptibility testing. 


