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ORIGINAL ARTICLE

Candida glabrata's recurrent infections: biofilm formation during Amphotericin B treatment

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Significance and Impact of the Study: This study shows new insights regarding recurrent candidiasis. The authors demonstrated that Amphotericin B did not totally prevent the development of biofilms during *Candida glabrata*'s infection treatment and that the change in the biofilm matrices may have a high responsibility for the fail in the treatment of systemic candidiasis.

Keywords
amphotericin B, biofilm cells, biofilms, *Candida glabrata*, matrix composition.

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Abstract

Candida species are responsible for recurrent human infections, mostly in immunocompromised patients, due to their high vulnerability. *Candida glabrata* has a major role in systemic candidiasis and Amphotericin B (AmB), a polyene only used in hospitals, is frequently used to treat this disease. Lately, however, clinical evidences of *Candida* recurrent infections during these treatments are being described, probably due to biofilm (re)formation during this therapy. Thus, this work aims at inferring if *C. glabrata* biofilms are still being formed during AmB treatment. For that, *C. glabrata* biofilms were formed in the presence of AmB and analysed by dry weight. Matrix composition was analysed quantifying carbohydrates and, specifically, β-1,3 glucans. Results demonstrated that, although in a lesser extent, *C. glabrata* is able to develop biofilms in the presence of AmB, with a thick extracellular matrix, with an increase on carbohydrates, especially β-1,3 glucans. Therefore, it is confirmed that complex biofilms of *C. glabrata* can be formed during an AmB treatment.

Introduction

Infections caused by *Candida* species are a problem of increasing clinical significance, which can result in septicaemia or systemic infections, with high morbility and mortality (Lass-Flörl 2009). *Candida* can be found in the gastrointestinal, genital and urinary tract of healthy individuals as innocuous commensals. However, in immunocompromised and/or hospitalized hosts they are able to cause superficial infections, which may lead to invasive and very critical complications. In addition, *Candida* possess high capability to adapt to different niches and to invade several epithelia, resulting in septicaemia (Ellis 2002; Lass-Flörl 2009).Yet, Amphotericin B, a fungicidal polyene, has shown efficacy activity against many *Candida* species (Ellis 2002; Krogh-Madsen *et al.* 2006; Vandeputte *et al.* 2008; Baginski and Czub 2009; Laniado-Laborin and Cabrales-Vargas 2009).

Among the several *Candida* species, *Candida glabrata* is the one of the most prevalent pathogenic fungal species in humans, after *Candida albicans* (West *et al.* 2013; Angoulvant *et al.* 2016). Besides being very resistant to antifungal agents (Ellis 2002; Krogh-Madsen *et al.* 2006; Lass-Flörl 2009), and although lacking capability to produce hyphae, *C. glabrata* possess a number of virulence factors, making it very aggressive. Among these are included the adhesion to host cells or to medical devices (e.g. catheters, algaliation devices) composed of silicone, latex or polyurethane for example, the secretion of hydrolytic enzymes such as phospholipases and haemolysins, and the biofilm formation capacity (Sánchez-Vargas *et al.* 2013; Rodrigues *et al.* 2014). These biofilms can be formed on the host mucosa and/or on surfaces of medical indwelling devices and are composed by yeast cells embedded in a complex polymeric structure, which makes them much more resistant to treatments than original

planktonic cells (Sardi *et al.* 2013; Rodrigues *et al.* 2014; d'Enfert and Janbon 2016). Moreover, it was already shown that even after standard treatment of infections caused by biofilms associated with medical devices, some patients still undergo recurrent candidiasis (Mishra *et al.* 2007).

Therefore, the main goal of this work was to understand why *C. glabrata* infections are still recurrent even during the patient's treatment with AmB, by evaluation of its capacity to form biofilms.

Results and discussion

The ability of *Candida glabrata* to form biofilms in the presence of AmB was determined by dry weight (Table 1). *Candida glabrata* 534784 showed higher capacity to produce biofilms, compared with the other two strains (Table 1), which can also be noticed in the SEM images (Fig. 1I, II and III). Importantly, it can be remarked that biofilms are, in fact, still being developed by the three strains in the presence of AmB to a lesser extent and they even present a considerable amount of biomass, except for *C. glabrata* 562123, in the presence of the highest concentration of AmB (Table 1). It should also be highlighted that this corresponds to usual therapeutic doses.

SEM images (Fig. 1) confirmed that, although *C. glabrata* biofilm formation is reduced in the presence of AmB, an organized structure can still be observed (Fig. 1II and III). This allowed the consideration that AmB could cause structural modifications on *C. glabrata*'s biofilm, with the presence of a compact matrix. In fact, one of the major contributions to *C. glabrata* virulence is its versatility in being adapted to a variety of different habitats and the formation of biofilms (Donlan and Costerton 2002; Mishra *et al.* 2007; Sardi *et al.* 2013; Angoulvant *et al.* 2016). There is a general consensus that the biofilm matrix acts as a barrier to the diffusion of antimicrobial agents, thereby limiting the access of the antimicrobial to organisms at the lower layers of the biofilm (Donlan and Costerton 2002; Mishra *et al.* 2007; Sardi *et al.* 2013; Angoulvant *et al.* 2016). However, little is known about the effect of the antifungal agent on the matrix production and composition.

Table 1 Effect of amphotericin B on *Candida glabrata's* biofilm formation by dry weight determination

C. glabrata strain	[AmB] g l^{-1}	Dry weight of biofilm mg ± SD (*P* value)
ATCC2001	0	20 ± 0·5
	1×10^{-3}	12 ± 0·2***
	2×10^{-3}	10 ± 0·5***
562123	0	20 ± 0·4
	1×10^{-3}	10 ± 0·0***
	2×10^{-3}	0·8 ± 0·2***
534784	0	30 ± 0·2
	1×10^{-3}	13 ± 0·5***
	2×10^{-3}	16 ± 0·6***

***$P < 0{\cdot}0001$.

Thus, posteriorly, the evaluation of the matrix composition (Fig. 2) confirmed the presence of rich and mature biofilm matrices in the presence of AmB. It was possible to verify an increase in the amount of carbohydrates on the matrix on the biofilms formed in the presence of AmB (Fig. 2), especially of β-1,3 glucans (Table 2). β-1,3 glucans are polymers found in the cell wall of *Candida* and in the biofilm matrix and are related to the antifungal resistance, making it difficult for the drugs to diffuse through the biofilm matrices (Lewis *et al.* 2012; Taff *et al.* 2012; Zarnowski *et al.* 2014). In fact, the increase of these compounds in biofilm matrix is very notorious even when there is a significant reduction in the total biofilms (Table 1 and Fig. 1). Meaning that, even with a reduction on the number of biofilm cells, the increase in biofilm matrix is clear and maybe associated with the stress caused by the antifungal agent.

It can be conjectured that AmB might be triggering the production and secretion of carbohydrates, which may be related with an attempt to make a physical protection for the cells, against the antifungal aggression, as it has already been demonstrated for *C. albicans* (Taff *et al.* 2012). In fact, glucan enzymes responsible for the production of these carbohydrates seem to play a biofilm-specific role in facilitating the delivery and organization of mature biofilm matrices, being decisive for delivery of β-1,3 glucans to the biofilm matrices and for accumulation of mature matrices biomass (Taff *et al.* 2012).

Concluding, it was confirmed that *C. glabrata* can develop biofilms in the presence of therapeutic concentrations of AmB, due to the high carbohydrate and β-1,3 glucan concentrations quantified on the biofilm matrices, highlighting the capacity of *Candida* cells to rapidly overcome outside aggressions and realizing why patients undergoing AmB treatment still manifest resilient *Candida* infections. Besides, this high carbohydrate content, specially β-1,3 glucans can also justify the biofilm cells' high resistance to antifungal treatments that been typically described for *C. glabrata*.

Material and methods

Organisms

Two clinical isolates of *Candida glabrata* (*C. glabrata* 534784 from vaginal site and *C. glabrata* 562123 from urine) and one reference strain (*C. glabrata* ATCC 2001) were used in this work. The identity of all isolates was

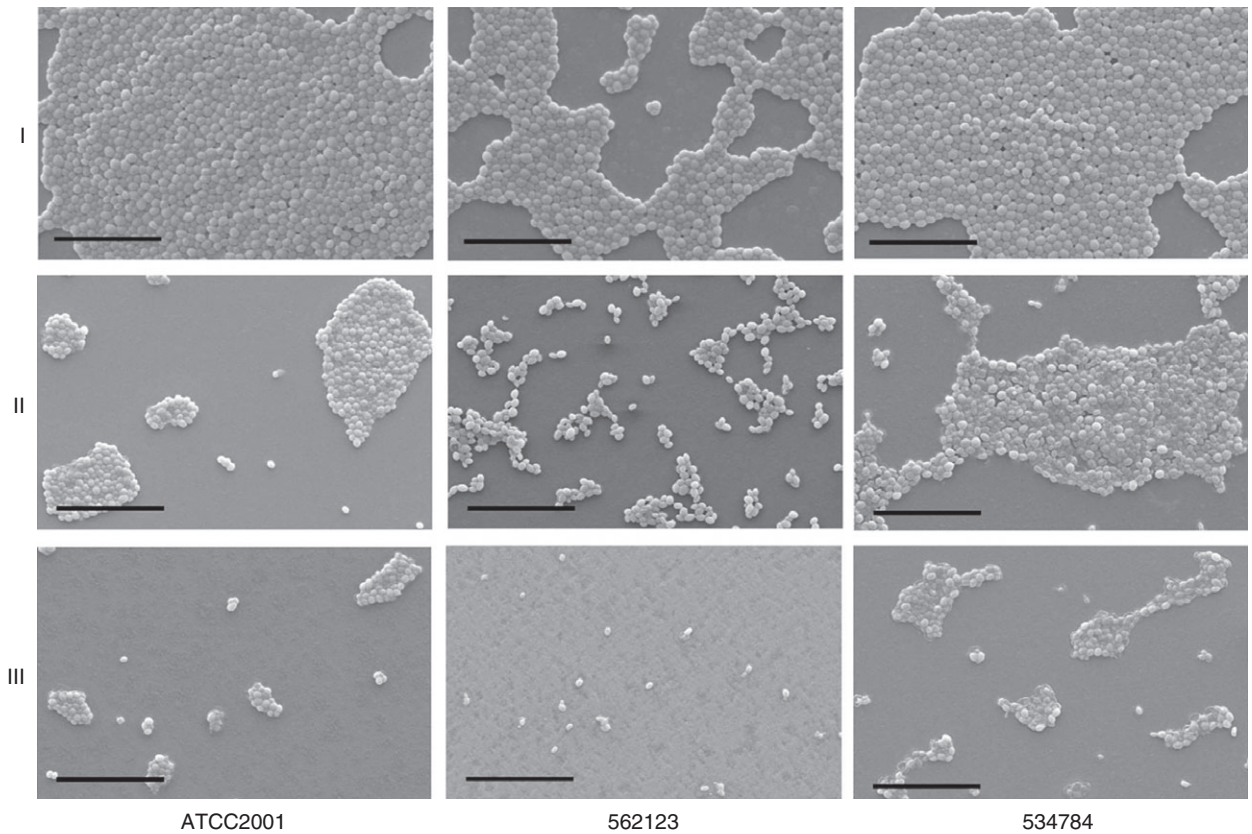

Figure 1 SEM images of *Candida glabrata's* biofilms. Magnification: 1000×. I – Biofilm grown without AmB; II – Biofilm grown with 1×10^{-3} g l^{-1} of AmB; III – Biofilm grown with 2×10^{-3} g l^{-1} of AmB. (Measure bar = 20 μm).

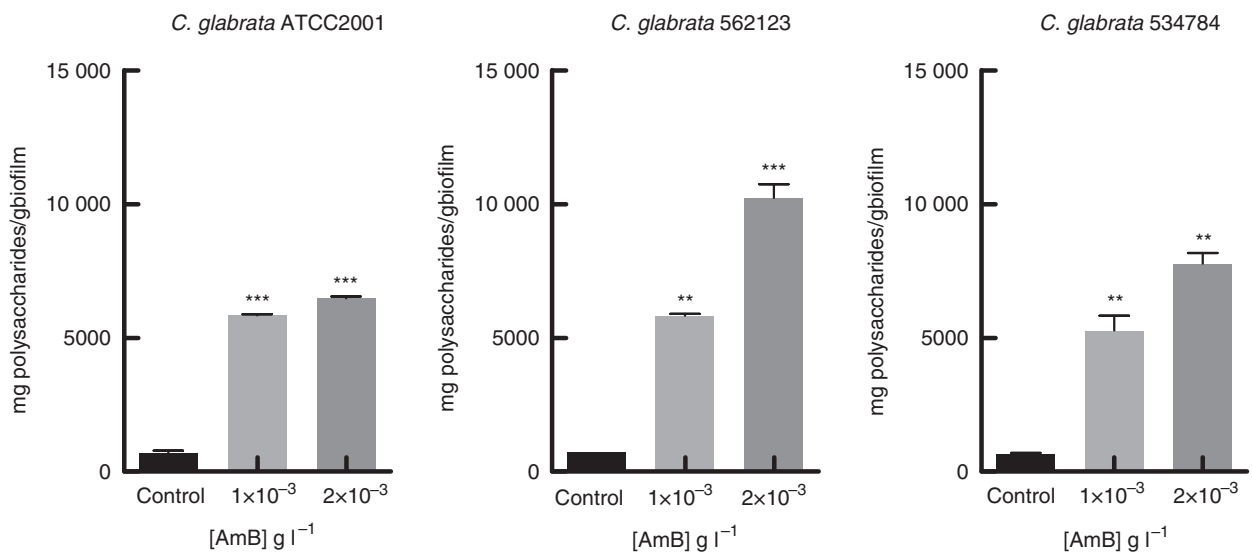

Figure 2 Carbohydrate content on *Candida glabrata's* biofilms matrices with and without Amphotericin B (**$P < 0{\cdot}001$, ***$P < 0{\cdot}0001$).

confirmed using CHROMagar Candida (CHROMagar, Paris, France) and by PCR-based sequencing using specific primers (*ITS1* and *ITS4*) against the 5·8 s subunit gene reference. Genomic DNA was extracted following previously described procedures (Williams *et al.* 1985). The PCR products were sequenced using the ABI-PRISM Big

Table 2 β-1,3 glucan quantity in *Candida glabrata*'s biofilm matrices, in the presence and absence of amphotericin B

C. glabrata strain	[AmB] g l^{-1}	mg β-1,3 glucans per g of dry weight of biofilm (*P* value)
ATCC2001	0	$5{\cdot}15 \times 10^{-5}$
	1×10^{-3}	$5{\cdot}80 \times 10^{-5}$***
	2×10^{-3}	$3{\cdot}29 \times 10^{-5}$***
562123	0	$1{\cdot}79 \times 10^{-5}$
	1×10^{-3}	$5{\cdot}43 \times 10^{-5}$***
	2×10^{-3}	$6{\cdot}78 \times 10^{-5}$***
534784	0	$3{\cdot}13 \times 10^{-5}$
	1×10^{-3}	$6{\cdot}95 \times 10^{-5}$***
	2×10^{-3}	$4{\cdot}63 \times 10^{-5}$***

***$P < 0{\cdot}0001$.

Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK).

Growth conditions

For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37°C. Cells were then inoculated in Sabouraud dextrose broth (SDB) (Merck) and incubated for 18 h at 37°C under agitation at 120 rotations per minute (rpm min^{-1}). After incubation, the cells were harvested by centrifugation at 3000 ***g*** for 10 min at 4°C and washed twice with Phosphate-Buffered Saline (PBS, pH = 7·5). Pellets were then suspended in SDB and the cellular density was adjusted to 1×10^5 cells ml^{-1} using a Neubauer counting chamber (Rodrigues *et al.* 2015).

Amphotericin B

AmB (Sigma-Aldrich®, St Louis, MO), was prepared at 1×10^{-3} g l^{-1} and 2×10^{-3} g l^{-1}, from a stock of 1 g l^{-1} diluted in dimethylsulfoxyde.

Biofilm formation and analysis

Standardized cell suspensions were placed into selected wells of 24-well polystyrene microtitre plates (Orange Scientific, Braine-l'Alleud, Belgium) plus 250 μl of each duplicated concentration of antifungal agent to test (1×10^{-3} g l^{-1} and 2×10^{-3} g l^{-1}, final concentration). As negative control, SDB without cells and antifungal agent were used. As positive control, cells suspensions were tested without an antifungal agent. After 24 h, 250 μl of SDB medium was removed and an equal volume of fresh SDB, plus the respective antifungal concentration agent was added.

Dry weight was analysed to evaluate AmB effect on biofilm production (Rodrigues *et al.* 2015).

In order to examine the structure of biofilms, after formation in the presence or absence of AmB, they were observed by scanning electron microscopy. For that, biofilms formed as described above were dehydrated with ethanol (using 70% ethanol for 10 min, 95% ethanol for 10 min and 100% ethanol for 20 min) and air-dried for 20 min. Samples were kept in a desiccator until the base of the wells was removed for analysis. Prior to observation, the base of the wells were mounted onto aluminium stubs, sputter coated with gold and observed with an S-360 scanning electron microscope (Leo, Cambridge, MA).

Total carbohydrate content of the biofilm matrix was estimated according to the procedure of DuBois *et al.* (1956), using glucose as a standard. The values were normalized per g of dry weight biofilm and the values were presented as mg of carbohydrate/g of dry weight of biofilm.

β-1,3 glucans concentrations β-1,3 glucans concentrations were determined using Glucatell® kit (Cape Cod®, East Falmouth, MA). The values were normalized by mg of β-1,3 glucans per g dry weight of biofilm.

All the experiments were performed in triplicate and in three independent assays.

Statistical analysis

Results were compared using a one-way ANOVA, Dunnett's test, using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). All tests were performed with a confidence level of 95%.

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Conflict of Interest

The authors declare no competing financial interest.

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