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Research Paper

Candida species biofilm and *Candida albicans ALS3* polymorphisms in clinical isolates

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Abstract

Over the last decades, there have been important changes in the epidemiology of *Candida* infections. In recent years, *Candida* species have emerged as important causes of invasive infections mainly among immunocompromised patients. This study analyzed *Candida* spp. isolates and compared the frequency and biofilm production of different species among the different sources of isolation: blood, urine, vulvovaginal secretions and peritoneal dialysis fluid. Biofilm production was quantified in 327 *Candida* isolates obtained from patients attended at a Brazilian tertiary public hospital (Botucatu, Sao Paulo). *C. albicans ALS3* gene polymorphism was also evaluated by determining the number of repeated motifs in the central domain. Of the 198 total biofilm-positive isolates, 72 and 126 were considered as low and high biofilm producers, respectively. Biofilm production by *C. albicans* was significantly lower than that by non-*albicans* isolates and was most frequently observed in *C. tropicalis*. Biofilm production was more frequent among bloodstream isolates than other clinical sources,in urine, the isolates displayed a peculiar distribution by presenting two distinct peaks, one containing biofilm-negative isolates and the other containing isolates with intense biofilm production. The numbers of tandem-repeat copies per allele were not associated with biofilm production, suggesting the evolvement of other genetic determinants.

Key words: *Candida* spp., biofilm, *ALS3* gene.

Introduction

Candida species are human commensals that can cause since superficial infections to systemic disease (Kojic and Darouiche, 2004) and have emerged as important agents of opportunistic infections worldwide, primarily in immunocompromised persons (Aperis *et al.*, 2006; Tumbarello *et al.*, 2007; Chaves *et al.*, 2013; Kwamin *et al.*, 2013). Although *Candida albicans* is considered the most common fungal pathogen, an increasing number of non-*albicans Candida* species infections have been described (Redding, 2001; Krcmery and Barnes, 2002; Nucci *et al.*, 2013). *Candida* species can colonize human tissues and medical devices, such as central venous catheters, prosthetic heart valves and other devices, resulting in biofilm formation and biofilm-related infections (Andes *et al.*, 2004; Kojic and Darouiche, 2004; Douglas, 2013). *Candida* infections can also be attributed to the use of invasive procedures or endogenous source (Douglas, 2002).

Biofilms are microbial communities of surface-attached cells embedded in a self-produced extracellular polymeric matrix (Donlan and Costerton, 2002). They can cause significant problems in many areas, mainly in medical settings as persistent and recurrent device-related infections (Kumar and Anand, 1998; Flemming, 2002; Fux *et al.*, 2005). It is remarkable that biofilms are more resistant than planktonic cells, and in most cases, antimicrobial ther-

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apy is not effective (Kumamoto, 2002; Douglas, 2003; Tobudic *et al.*, 2012).

Biofilm formation in *Candida* spp. is a complex process involving multiple regulatory mechanisms (Nobile and Mitchell, 2006) and once established, *Candida* biofilms serve as a persistent reservoir of infection and, in addition, offers greater resistance to antifungal agents compared to planktonic phase yeasts (Chandra *et al.*, 2001a, 2001b; Samaranayake *et al.*, 2005; Parahitiyawa *et al.*, 2006).

Several in-vitro systems have been developed to study and quantify biofilm, including yeast development on intravascular catheter discs, acrylic discs, cylindrical cellulose filters, microtiter plates and others (Douglas, 2002; McLean *el al.*, 2004). Crystal violet staining, a basic dye that binds to negatively charged surface molecules and polysaccharides in the extracellular matrix, is commonly utilized to quantify biofilms formed by a broad range of microorganisms, including yeasts (Christensen *et al.*, 1985; Jin *et al.*, 2003; Li *et al.*, 2003; Cobrado *et al.*, 2013; Cooper, 2013), with highly correlation with other methods (Peeters *et al.*, 2008).

Besides phenotypical assays to study biofilm formation in *Candida* species, some genotypical techniques have been used to characterize this phenomenon. The *Als* (agglutinin-like sequence) proteins have long been considered excellent candidates for biofilm adhesions (Hoyer *et al.*, 1998; Zhao *et al.*, 2003, 2004; Green *et al.*, 2004). Eight *ALS* genes (*ALS1* to *ALS7* and *ALS9*) encode large, cell surface glycoproteins, some of which promote adhesion to host surfaces (Gaur and Klotz, 1997; Hoyer, 2001; Hoyer and Hecht, 2001; Fu *et al.*, 2002; Zhao *et al.*, 2003, 2007). Although *ALS* genes share a similar three-domain structure, sequence differences among the *Als* proteins can be large, suggesting that the proteins may present different functions (Hoyer, 2001). Much of the allelic variation in *ALS* genes occurs within the tandem repeat domain (the central domain of gene) and is manifested as differing numbers of the 108-pb tandem repeats in *ALS* alleles. It has been suggested that *ALS3* is one of the most important genes associated with *C. albicans* biofilm production (Hoyer, 2001; Zhao *et al.*, 2006; Hoyer, 2008).

The aims of the present study were to quantify and to compare biofilm production in a collection of different *Candida* species, isolated from different clinical sources, as well as to detect the polymorphisms in the *ALS3* tandem repeat domain and their possible correlation with the biofilm production profiles.

Materiais and Methods

Microorganisms

A total of 327 *Candida* species isolates recovered from clinical specimens as part of routine diagnostic procedures, stored in vial tubes containing Brain Heart Infusion plus 10% glycerol, frozen at -80 °C, were re-cultured and tested for biofilm production. The isolates were obtained from patients from the Clinical Hospital, Botucatu Medical School, Sao Paulo State University (CH/UNESP), between 1998 and 2005. *Candida* species and their sources are summarized in Table 1. The identification of *Candida* species was conducted by chlamydospore formation, sugar assimilation and fermentation patterns as well as chromogenic agar (CHROMagar *Candida*, Difco).

Biofilm formation assay

Tests for biofilm quantification were performed according to previous protocols (Jin *et al.*, 2003; Li *et al.*, 2003), with slight modifications. Isolates were straked onto Yeast-Extract Peptone Dextrose agar (YEPD) plates and incubated at 37 °C for 48 h. Next, a large loop of actively growing cells was transferred to sterile Yeast Nitrogen

Table 1 - Distribution of *Candida* species obtained from different sources in a Brazilian tertiary public hospital (CH/UNESP), 1998-2005.

Species identification	Blood % (n)	Urine % (n)	Vaginal[a] % (n)	Peritoneal[b] % (n)	Total % (n)
C. albicans	22.5 (23)	34.1 (29)	80.9 (93)	32.0 (8)	46.8 (153)
All non-*C. albicans* species	77.5 (79)	65.9 (56)	19.1 (22)	68.0 (17)	53.2 (174)
C. glabrata	4.9 (5)	23.5 (20)	3.6 (4)	-	8.9 (29)
C. guilliermondii	5.9 (6)	1.2 (1)	-	8.0 (2)	2.8 (9)
C. lusitaniae	1.0 (1)	-	-	-	0.3 (1)
C. parapsilosis	43.1 (44)	8.2 (7)	4.5 (5)	40.0 (10)	20.2 (66)
C. pelliculosa	1.0 (1)	-	-	-	0.3 (1)
C. tropicalis	2.9 (3)	32.9 (28)	0.9 (1)	20.0 (5)	11.3 (37)
C. krusei	-	-	10.4 (12)	-	3.7 (12)
Candida spp.	18.6 (19)	-	-	-	5.8 (19)
Total	102	85	115	25	327

[a]Vaginal secretion; [b]Peritoneal dialysis fluid.

Base (YNB) broth containing 0.9% D-glucose, and incubated at 37 °C for 24 h. Yeast cells were twice centrifuged (5000 x *g* for 5 min) and washed with 0.5 mL PBS (0.14 M NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). After, cells were re-suspended in 1 mL YNB broth and adjusted to concentration of 10^7 cells/mL (0.5 McFarland). Next, 100 μL of the each isolate suspension was inoculated into individual wells of polystyrene 96-well plates (TPP), in four repetitions. YNB broth containing no inoculum was used as negative control. Plates were incubated at 37°C for 90 min (adhesion period). Supernatant including planktonic cells and liquid medium was then discarded and wells were gently washed twice with PBS to eliminate any non-adherent cells. For biofilm growth, 100 μL of fresh YNB broth was then added to each well. The plates were incubated at 37 °C for 48 h. After incubation, planktonic cells were discarded through three rounds of washing with 200 μL sterile PBS buffer, and the plates dried at room temperature for 45 min. For staining with Crystal Violet (CV), 150 μL of 0.4% CV, diluted in water, was added to each well, and, after 45 min at room temperature, all the supernatant was discarded before adding 150 μL of 95% ethanol and maintained for 45 min, to dissolve and/or elute the dye from the biofilm cells. Next, 100 μL of each well was transferred to a new 96-well microplate and the absorbance determined using a microplate reader at 540 nm filter (MultisKan EX, Labsystems). The absorbance values were converted into transmittance percentages (%T). The %T values for each test was subtracted from the %T for the reagent blank to obtain a measure of light blocked when passing through the wells ($\%T_{bloc}$), and the biofilm production scored as either negative ($\%T_{bloc}$, < 10), positive 1+ ($\%T_{bloc}$, 10 to 20), positive 2+ ($\%T_{bloc}$, 20 to 35), positive 3+ ($\%T_{bloc}$, 35 to 50) or positive 4+ ($\%T_{bloc}$, ≥ 50), and the positives further categorized as low-biofilm (1+) or high-biofilm producers (2+, 3+, or 4+) (Tumbarello *et al.*, 2007).

ALS3 characterization

The gene *ALS3* was studied in all isolates from bloodstream cultures (23 *C. albicans* and 16 *C.* non *albicans* isolates). DNA was extracted according to McCullough *et al.* (2000) with slight modifications. Colonies of yeasts with growth of 24 h/37 °C onto Sabouraud dextrose agar plates were suspended in 1 mL of 1 M sorbitol and 125 mM of EDTA; suspension was centrifuged (10 min, 13000 x *g*, 25 °C), the supernatant was discarded, and the pellet was resuspended in 0.5 mL of lysing solution (1 M Tris-HCl, pH 8.0, with 250 mM of EDTA and 5% SDS) plus 10 μL of proteinase K (Invitrogen) and incubated for 1 h at 65 °C. Next, 500 μL 5 M potassium acetate was added, incubated on ice for 2 h and then centrifuged (10 min, 13000 x *g*, 25 °C). Supernatant was transferred to an Eppendorf tube containing 1 mL of absolute ethanol and mixed by inversion and centrifuged (10 min, 13000 x *g*, 4 °C). The supernatant was discarded; the pellet was washed with 500 μL of cooled 70% ethanol and centrifuged (10 min, 13000 x *g*, 4 °C). Finally, supernatant was discarded and the pellet was resuspended in 0.5 mL of sterilized MilliQ water. The size of the tandem repeat domain in each *ALS* allele was determined by PCR using two independent primer pairs (Oh *et al.*, 2005). When the first pair of primers provides no clear amplification, the second pair was used. PCR products were separated on 0.7% agarose (TBE) gels stained with ethidium bromide. The gels were analyzed in the equipment AlphaImager[R] EC, and sizes of the amplicons were determined by the software AlphaEase[R] FC. To estimate the numbers of motifs present in the tandem repeats in the *ALS3* gene, the primers positions were aligned with the deposited genomic sequences of strain SC5314 DNA (GenBank Accession No. AY223552.1), large and small alleles that present twelve and nine motifs, using Mega software. The numbers of motifs for each isolate evaluated were calculated considering 108 bp the mean size for each motif. The amplicon of one homozygous isolate was purified (GFX PCR DNA and Gel Band, GE, Healthcare), sequenced using the DYEnamic ET Dye Terminator Kit (with Thermo Sequenase II DNA Polimerase) in a MegaBACE 1000 DNA Analysis System, and the chromatogram visualized by the Chromas program. The consensus sequence was sent to blastn for comparison with the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

Chi-square analysis was used to compare biofilm positivity among different *Candida* species or among isolates recovered from different sources. The Kolmogorov-Smirnov test was used to compare the numbers of tandem repeat copies between biofilm-positive and biofilm-negative isolates. Differences between groups were considered to be significant for $p < 0.05$.

Results

Biofilm production

A total of 198 of 327 (60.6%) *Candida* species isolates were biofilm-positive. Of these, 72 (36.4%) and 126 (63.6%) isolates were low and high biofilm producers, respectively. Biofilm production by *C. albicans* isolates was significantly lower than that by non-*albicans* isolates (43.1% *vs.* 75.8%, respectively; $p < 0.001$), and among the biofilm-positive isolates, the non-*albicans* isolates were classified mainly as high-biofilm producers, with *C. albicans* isolates defined as low-biofilm producers ($p < 0.001$) (Table 1).

Considering the species separately, biofilm production was most frequently observed in *C. tropicalis* isolates (94.6%, $p < 0.001$), followed by *C. parapsilosis* (80.3%),

C. glabrata (44.8%), and *C. albicans* (43.1%). Among biofilm-positive isolates, the highest biofilm production intensity (3+ and 4+) was observed in *C. tropicalis* isolates (81.1%, $p < 0.001$) (Figure 1).

In relation to the sources, biofilm production was positive in 79.4% of the isolates obtained from the bloodstream, 63.5% from urine, 37.4% from vaginal secretion and 80% from peritoneal dialysis fluid (Table 2). Biofilm production among bloodstream isolates was more frequent compared to any other source ($p < 0.001$), except peritoneal dialysis fluid isolates ($p > 0.05$), which were also prolific biofilm producers. Biofilm production was most frequent in non-*albicans* isolates ($p < 0.001$) in all sources, also with the exception of peritoneal dialysis fluid isolates ($p > 0.05$). In urine, the isolates displayed a peculiar distribution by presenting two distinct peaks, one containing biofilm-negative isolates (36.5%) and the other containing isolates (37.6%) with intense biofilm production ($\%T_{bloc}$, > 35) (Figure 1). Collinearity was not observed between species and sources of *Candida* isolates ($p > 0.05$).

ALS3 characterization

Polymerase chain reaction was performed on a total of 23 *C. albicans* isolates from bloodstream infections. In 19 of 23 *C. albicans* isolates, the expected fragments of the *ALS3* gene were amplified by using the first or the second pair of primers (Figure 2). In the 16 non-*albicans* evaluated, the PCR products were not amplified with both pairs of primers.

The consensus sequence of a fragment containing approximately 700 bp in the Blast analysis presented 93% identity with the *C. albicans* large allele *ALS3* gene (GenBank Accession No. AY223552.1; *E* value 0.0).

The numbers of copies of the central tandem repeat domain were divergent among the isolates, which three quarters presented homozygosity. Alleles in the examined isolates encoded between 7 and 14 copies of tandemly repeated 108-bp sequence (Table 3). The mean numbers ± standard deviation of tandem repeat copies per allele in biofilm-negative and biofilm-positive isolates were 11.6 ± 1.4 and 10.7 ± 1.7, respectively, which was not significant ($p > 0.05$).

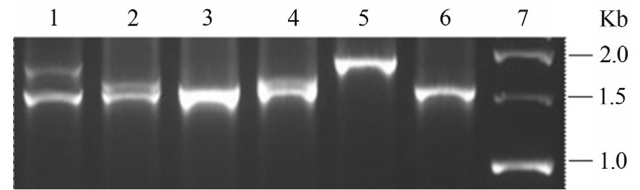

Figure 2 - Ethidium bromide-stained agarose gel highlighting amplification of PCR products from the tandem repeat region of *ALS3* gene of 6 *C. albicans* isolates with *ALS3*GenoF and *ALS3*genoR primers.1- alleles with 10 and 13 motifs; 2- alleles with 10 and 11 motifs; 3- alleles with 10 motifs each; 4- alleles with 11 motifs each; 5- alleles with 14 motifs each; 6- alleles with 11 motifs each; 7- Ladder 1 kb (Promega).

Discussion

Biofilm production in 327 clinical isolates clearly confirms that different *Candida* species have different abilities to produce biofilm in vitro. Biofilm production was also associated with source of *Candida* isolation. Biofilm production has been considered an important virulence factor among *Candida* species (Pitts *et al.*, 2003). A broad range of methods has been employed to evaluate this phenomenon in yeasts, mainly in *Candida* species (Peeters *et al.*, 2008). Herein, we quantified the biofilm production in 327 clinical isolates of *Candida* species by the crystal violet assay, a widely used method to quantify biofilm production in several microorganisms, including yeasts (Christensen *et al.*, 1985; Stepanovic *et al.*, 2000; Li *et al.*, 2003; Peeters *et al.*, 2008, Cobrado *et al.*, 2013; Cooper, 2013). Although this method is not able to differentiate between living and dead cells (Peeters *et al.*, 2008, Pitts *et al.*, *2003*), the results obtained by the crystal violet assay have correlated highly with other assays that differentiate between living and dead cells, such as the 2,3-bis (2-methoxy-4-nitro-5-sulfo-

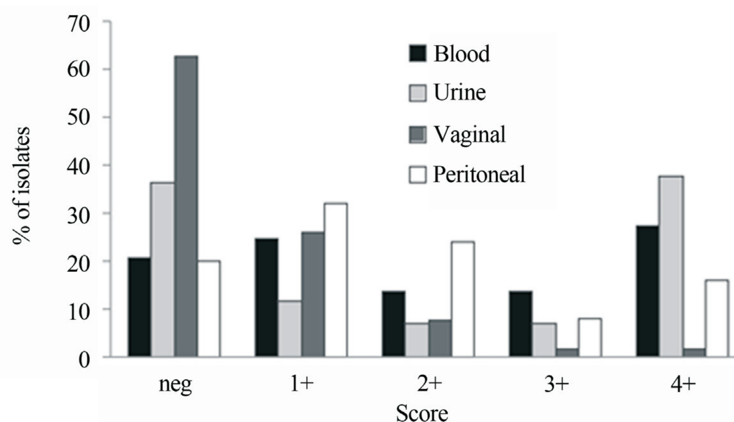

Figure 1 - Frequency distribution of *Candida* species in relation to the clinical sources and biofilm production scores, categorized according to Tumbarello *et al.*, 2007.

Table 2 - Comparison of biofilm production by *Candida* species isolates from blood, urine, vaginal secretion and peritoneal dialysis fluid obtained in a Brazilian tertiary public hospital (HC/UNESP), 1998-2005.

Candida species	No. of biofilm positive/no.total (%)				
	Total	Blood	Urine	Vulvov	Perit
C. albicans	66/153 (43.1)	13/23 (56.5)	12/29 (41.4)	34/93 (36.6)	7/8 (87.5)
All non-*albicans* species	132/174 (75.9)[a]	68/79 (86.1)[a]	42/53 (79.2)[a]	9/22 (40.9) [a]	13/17 (76.5)
C. parapsilosis	53/66 (80.3)	40/44 (90.9)	5/7 (71.4)	1/5 (20.0)	7/10 (70.0)
C. tropicalis	35/37 (94.6)	3/3 (100.0)	28/28 (100.0)	0/1 (0.0)	4/5 (80.0)
C. glabrata	13/29 (44.8)	3/5 (60.0)	10/19 (52.6)	1/4 (25.0)	-
Other *Candida* species	31/42 (73.8)	24/27 (88.9)	-	7/12 (58.3)	2/2 (100.0)
Total	198/327 (60.6)	81/102 (79.4)	54/85 (63.5) [b]	43/115 (37.4)[c d]	20/25 (80.0)

[a]$p < 0.001$, *C. albicans* vs. *non-albicans*.
[b]$p < 0.02$, blood *vs.* urine.
[c]$p < 0.001$, blood *vs.* vaginal secretion.
[d]$p < 0.001$, peritoneal dialysis fluid *vs.* vaginal secretion.

Table 3 - Distribution of allele per *ALS3* gene tandem-repeat copies in *C. albicans* isolates from the bloodstream obtained in a Brazilian tertiary public hospital (CH/UNESP), 1998-2005.

Biofilm production (n)	Percent (n) alleles in each tandem repeat copy group						Mean no of repeat copies/allele[a]
	7	10	11	12	13	14	
Biofilm negative (16)	0 (0)	31.3 (5)	25.0 (4)	0 (0)	43.8 (7)	0 (0)	11.6 ± 1.4
Biofilm positive (22)	9.1 (2)	36.4 (8)	36.4 (8)	4.5 (1)	4.5 (1)	9.1 (2)	10.7 ≥ 1.7

[a]Mean number of repeat copies per allele ± standard deviation. p value not significant.

phenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay (Jin *et al.*, 2003; Peeters *et al.*, 2008). We verified that non-*albicans* species are the highest biofilm producers. Among biofilm producers, *C. tropicalis* showed the highest intensity of biofilm production. In other studies involving similar numbers of *Candida* species isolates, *C. tropicalis* was the species with the highest percentages of biofilm positivity, while *C. albicans*, ranked third or fourth among the biofilm-positive isolates (Shin *et al.*, 2002; Tumbarello *et al.*, 2007).

We also evaluated the associations between the source and the biofilm positivity of isolates. Isolates obtained from the bloodstream and peritoneal dialysis fluid typically were prolific biofilm producers. Urine isolates presenting intense biofilm production might be associated with the use of urinary catheter, or, alternatively, be originated from systemic candidiasis and not from urinary tract infections, since the isolates from invasive infections tend to produce more biofilm than those from non-invasive infections (Kuhn *et al.*, 2002). Once that we have not evaluated patient charts, this hypothesis of urinary catheter remains to be proven.

Important advances have been achieved in outlining the genetic basis of biofilm production, however, the subject is far from being completely understood. After the *C. albicans* genome was sequenced (Jones *et al.*, 2004), the biofilm production by this yeast was better comprehended, although biofilm formation in non-*albicans* species remains poorly understood (Weber *et al.*, 2008). Several genes are involved in the biofilm formation by *Candida* spp. (López-Ribot, 2005; Ramage *et al.*, 2005; Nobile and Mitchell, 2006; Yu *et al.*, 2012; Lin *et al.*, 2013; Srikantha *et al.*, 2013). It was demonstrated that the expression of several *ALS* genes is upregulated during biofilm formation; furthermore, the Als proteins have long been considered excellent candidates for biofilm adhesins (Green *et al.*, 2004; Blakenship and Mitchell, 2006). It was analyzed the association between biofilm production and polymorphisms in the *ALS3* central domain. Previous studies showed that *ALS3* gene expression is altered in *C. albicans* sessile cells compared to planktonic cells (Chandra *et al.*, 2001a, 2001b; García-Sánchez *et al.*, 2004; Nailis *et al.*, 2006). Maximal *ALS3* expression is associated with formation of germ tubes and hyphae (Hoyer *et al.*, 1998); and an overexpression of *ALS3* was observed in initial stages of biofilm formation (Nailis *et al.*, 2009). We did not observe association between the number of *ALS3* tandem-repeat copies per allele and biofilm production. However, biofilm-negative isolates showed the majority of alleles with 13 tandem-repeat copies, while the majority of alleles in biofilm-positive isolates showed 10 or 11 copies (Table 3).

In conclusion, we showed that *Candida* non-*albicans* species were more prolific biofilm producers than *C. albicans* and that the source of isolates might influence the biofilm production, in which more invasive isolates (blood and peritoneal dialysis fluid) show greater biofilm productivity. The polymorphism of the *ALS3* central domain in *C. albicans*, detected by the different numbers of tandem-repeat copies, appears not to be directly related to biofilm production.

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