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Candida tropicalis biofilms: Effect on urinary epithelial cells

Melyssa Negri [a,b], Sónia Silva [a], Diogo Breda [a], Mariana Henriques [a,*], Joana Azeredo [a], Rosário Oliveira [a]

[a] Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[b] Faculdade INGÁ, Curso de Medicina, 87070-000 Maringá, Paraná, Brazil

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ABSTRACT

Candida tropicalis infection is strongly associated with the presence of biofilms in urinary catheters. Thus, the aim of this work was to study the behaviour of *C. tropicalis* in biofilms of different ages (24–120 h) formed in artificial urine (AU) and their effect in human urinary bladder cells (TCC-SUP). Reference strain ATCC 750 and two isolates from patients with candiduria (U69 and U75) were used in this study. The adhesion to human cells was evaluated after 2 h of contact with *Candida* biofilms, using the Crystal violet staining method, and the human cells response was evaluated in terms of activity inhibition and cell damage. *Candida tropicalis* aspartyl proteinase (*SAPT*) gene expression was determined by real-time PCR. *Candida tropicalis* biofilm cells were able to adhere to TCC-SUP cells. The highest extent of yeast attachment was obtained for the 72 h old biofilm cells. Yeasts affected TCC-SUP cells, with 120 h-biofilm cells causing the highest levels of cell injury. Generally, *SAPT3* was highly expressed and *SAPT4* was only detected in the reference strain. Overall, it is important to highlight that *C. tropicalis* cells detached from biofilms are able to colonize human cells and cause some injury and reduction of metabolic activity.

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1. Introduction

Candida tropicalis ranks between the second or third non-*Candida albicans Candida* species (NCAC) most frequently isolated from patients with *Candida* infections [1,2]. According to epidemiological data, *C. tropicalis* has been related with urinary tract infections and haematological malignancy [1,2]. Furthermore, the most important causes of *C. tropicalis* candiduria are associated with antibiotic therapy and indwelling catheterization [3,4].

Several virulence factors seem to be responsible for *C. tropicalis* infections, which present higher potential for dissemination and mortality in patients admitted in intensive care units (ICUs) than *C. albicans* or any other NCAC species [4]. Included in these virulence factors are: their ability to adhere and to form biofilms onto different indwelling medical devices; their capacity to adhere, invade and damage host human tissues due to enzymes production such as proteinases [4–7].

It is known that *Candida* biofilms have strategies to be more resistant to antifungal agents and more virulent than the corresponding planktonic cells [8]. Moreover, recent studies showed the capacity of *Candida* cells or biofilm parts to detach from a biofilm and to colonize distal sites, being able to disperse into the host environment and adhere to endothelial or epithelial cells and initiating an infection [9]. However, there is little knowledge about the effect of *C. tropicalis* biofilms on epithelial cells. Thus, the aim of this work was to study the behaviour of *C. tropicalis* biofilms, formed in the presence of artificial urine, and their effect in bladder cells.

2. Material and methods

2.1. *Candida tropicalis* and growth conditions

Three strains of *C. tropicalis* were used in this study, one reference strain from the American Type Culture Collection (ATCC 750) and two clinical isolates (U69 and U75) obtained from patients with candiduria admitted to the intensive care unit and belonging to the archive collection of the University Hospital in Maringá, Paraná, Brazil. For each experiment, strains were subcultured on Sabouraud dextrose agar (SDA; Merck, Darmstadt, Germany) for 48 h at 37 °C. Yeast cells were then inoculated in Sabouraud dextrose broth (SDB; Merck) and incubated for 18 h at 37 °C under agitation in an orbital shaker (120 rev/min). After incubation, yeast cells were harvested by centrifugation at 8000 × g for 5 min at 4 °C and washed twice with Phosphate Buffer Solution (PBS; pH 7.5; 0.01 mol mL^{-1}). The remaining pellets were suspended in artificial urine (AU) and the cellular density adjusted to 1×10^7 yeasts mL^{-1}, using a Neubauer chamber. Artificial urine (pH 5.8) was prepared according to Silva et al. [6].

* Corresponding author. Tel.: +351 253604408; fax: +351 253678986.
E-mail address: mcrh@deb.uminho.pt (M. Henriques).

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2.2. *Candida tropicalis* planktonic cells

After adjust the cellular density in AU, planktonic cells were incubated for 24 h at 37 °C under agitation in an orbital shaker (120 rev/min). Then, planktonic cells were harvested by centrifugation at 8000 × g for 5 min at 4 °C and the pellets were suspended in D-MEM without Penicilyn/Streptomycin (P/S) and the cellular density adjusted to 1×10^7 yeasts mL^{-1}, using a Neubauer chamber.

2.3. *Candida tropicalis* biofilms formation

Biofilms, with different ages of maturation (24, 48, 72, 96 and 120 h), were formed on silicone coupons (1×1 cm^2) according to Silva et al. [6]. After the defined times of incubation, the medium was aspirated and non-adherent *C. tropicalis* cells were removed by washing the silicone coupons with PBS.

2.4. Human urinary bladder epithelial cell line

The cell line TCC-SUP, derived from human urinary bladder epithelial cells (DSMZ – German Collection of Microorganisms and Cell Cultures) was used as model for analysing the colonization by *C. tropicalis* cells when in contact with their biofilms. The cells were cultured and the 24-well plate containing human cells monolayers, prior to adhesion assay, were prepared according to Negri et al. [10].

2.5. *Candida tropicalis* biofilms and planktonic cells in contact with TCC-SUP cells

The silicone coupons containing biofilms were removed from the wells, carefully inverted and immediately put in contact with the pre-formed monolayer of TCC-SUP cells and 1 mL of D-MEM without P/S was added to each well. For planktonic cells, 1 mL of medium (D-MEM without P/S) with planktonic cells was put in contact with the pre-formed monolayer of TCC-SUP cells. After 2 h of contact at 37 °C under 5% CO_2, the coupons containing the biofilms and the suspension with planktonic cells were removed, then, each well washed once with PBS. The remaining yeasts attached to the monolayer were quantified using the CV staining method, according to Negri et al. [11]. The mean absorbance of yeasts was expressed as the absorbance per area of each well and standardized by the number of adhered yeasts per area of each well using *C. tropicalis* standard curve [11]. All the procedures were repeated in triplicate in at least three separate assays.

2.6. Effect of *C. tropicalis* on TCC-SUP cells

2.6.1. Determination of epithelial cells damage assay

After the removal of the coupons, the release of lactate dehydrogenase (LDH) by epithelial cells into the culture medium was used as a measure of cell damage, using the CytoTox-ONE™ kit (Promega, Madison, USA) following the manufacturer's instructions. The LDH activity was analysed according to Negri et al. [10]. All experiments were performed in triplicate and in three separate occasions.

2.6.2. Determination of epithelial cells activity

After the removal of the coupons and the washing step with PBS, the remaining adhered yeasts were killed according to Negri et al. [10], and the CellTiter 96® assay ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H–tetrazolium], MTS; Promega, USA) was used to determine the TCC-SUP cells activity. All the procedures were repeated in triplicate in at least three separate assays.

2.7. Analysis of *SAP* gene expression

2.7.1. RNA extraction

After 2 h of TCC-SUP cells infectivity with biofilms or planktonic cells and the washing step with PBS, *C. tropicalis* cells attached to TCC-SUP cells were scrapped from the 24-well plate into 500 μL of lysis buffer. The yeast cells were disrupted according to Negri et al. [10], the PureLink™ RNA Mini kit (Invitrogen) was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination the samples were treated with RNase-Free DNase I (Invitrogen).

2.7.2. Primers, synthesis of cDNA and real-time PCR

The primers used for real-time PCR (RT-PCR) are described in Silva et al. [5] and their sequence is listed on Table 1. The relative quantification of *SAPT1–4* gene expression was performed by the ΔC_T method. Each reaction was performed in triplicate and mean values of relative expression were analysed for each *SAP* gene.

2.8. Statistical analysis

The results obtained were analysed using the SPSS 18 (Statistical Package for the Social Sciences) program. One-way ANOVA with the Bonferroni test was used in these tests. All tests were performed with a confidence level of 95%. All the experiments were performed in triplicate and in three independent assays.

3. Results

3.1. *Candida tropicalis* biofilms in contact with TCC-SUP cells

All *C. tropicalis* biofilm yeast cells were able to adhere to TCC-SUP cells independently of the biofilm age (Fig. 1). Nevertheless, there were slightly differences in *C. tropicalis* adhesion ability to TCC-SUP cells, namely, the highest number of attached yeast cells was obtained for the 72 h-biofilm from isolate U75 and the lowest occurred for the 120 h-biofilm from isolate U69 ($P < 0.05$). The latter strain (U69) showed a higher variation in the profile of adhesion extent compared to the other two strains.

In general, *C. tropicalis* biofilm cells affected TCC-SUP cells after 2 h of contact (Table 2), and in a similar way for the three strains. It is important to highlight that TCC-SUP cells exhibited the highest ($P < 0.05$) percentage of damage after contact with 96 h-biofilm U75 cells and with 120 h-biofilms from strains U69 and ATCC 750. Overall, no direct correlation was observed between the profile of TCC-SUP cells damage and activity inhibition caused by the different *C. tropicalis* biofilms. For instance, the human cells response induced by 72 h-biofilms is contradictory once there was a decrease in cell activity inhibition, but an increase in cell damage ($P < 0.05$). However, it is interesting to notice that when human

Table 1
Primers used for real-time PCR analysis of *SAP* and control gene expression.

Sequence (5′→3′)	Primer	Target	PCR product size (bp)
GGAAGATCTGATGTGCCAACTACATTGA	Forward	*SAPT1*	1005
CGTGCGGCCGCTCTACAAAGCCGAGATGTCT	Reverse		
TTCTTCTAGTGGTACCTGGGTCAAAG	Forward	*SAPT2*	762
CATAGATCTCTAAACAATAGTGACATTAGA	Reverse		
ACTTGGATTTCCAGCGAAGA	Forward	*SAPT3*	165
AGCCCTTCCAATGCCTAAAT	Reverse		
GTACTCGAGCTCCTACAACTTCACCTCCT	Forward	*SAPT4*	1130
CATGGATCCCTATGTAAGTGGAAGTATGTT	Reverse		
GACCGAAGCTCCAATGAATC	Forward	*ACT1*	181
AATTGGGACAACGTGGGTAA	Reverse		

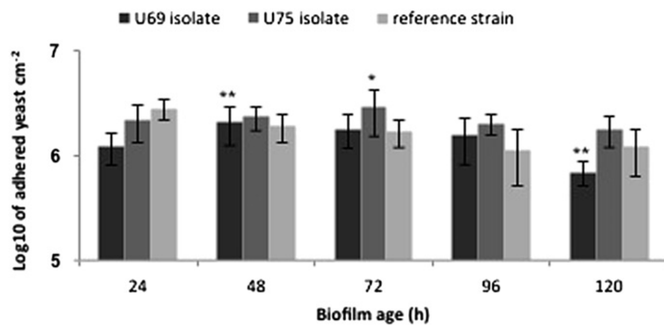

Fig. 1. Number of *Candida tropicalis* from different biofilms time point attachment to human epithelial cells. * Statistical difference among strains ($P < 0.05$); ** Statistical differences from 24 to 48 h and 96 h to 120 h of U69 isolate biofilms' time points ($P < 0.05$).

cells injury caused by biofilms (120 h-biofilm of strain U69) was more notorious, both tests were concordant, $P < 0.05$, (50.0% and 27.7% of cell damage and inhibition of cellular activity, respectively).

3.2. *Candida tropicalis SAP gene expression*

Analysing *C. tropicalis SAP* gene expression (Table 3) when in contact with TCC-SUP cells, it can be noticed that *C. tropicalis* suspended cells grown in AU were not able to express *SAPT1* gene. However, when grown in the sessile form, strains U69 and ATCC 750 from 48 h-biofilms were able to express *SAPT1*. As regards *SAPT2* gene expression, for U69 and ATCC 750 strains from 24 to 48 h-biofilms and U75 from 120 h-biofilms, *SAPT2* was detected in similar amount.

In opposition to the other *SAPT* genes, *SAPT3* was expressed and in the great majority of situations.

Interestingly, *SAPT4* was only expressed by the reference strain, and in few situations (adhered yeasts grown in suspension and from 24 h-biofilms).

4. Discussion

It is known that during the development of *Candida* biofilms, some dispersion/detachment of cells or dissolution of biofilm pieces can occur [7,9]. This is an important phenomenon, since the dispersed and detached cells could be responsible for the subsequent establishment of disseminated candidiasis at distal organs [9,12]. Thus, it is extremely relevant to study the ability of *C. tropicalis* cells detached from pre-formed biofilms to colonize human epithelial cells and the consequent degree of damage.

All *C. tropicalis* biofilms (independently of their age) were able to detach and colonize TCC-SUP cells (Fig. 1), although in a strain dependent way. Other studies have shown that *C. tropicalis* planktonic cells extent of adhesion to human cells is also strain specific [5,10,13,14].

It is interesting to highlight that biofilm yeast cells adhered to TCC-SUP cells (Fig. 1) in a similar extent of their planktonic counterparts [14]. Uppuluri et al. [9] reported a different behaviour for *C. albicans* adhesion to endothelial cells, since yeast cells dispersed from biofilms adhered in larger numbers than planktonic ones. Maybe, this fact can be related with differences between the two yeast species or the differences between the human cells used, which determine very specific interactions with yeast cells [15,16].

Human cells damage is reported to be dependent on the contact time and yeast infectivity rate [5,9]. Overall, the present results point out that older biofilms (96 h or 120 h) induced higher cell damage (Table 2) than younger ones, however in a strain dependent manner. Therefore, these data highlight the virulence potential of *C. tropicalis* cells dispersed from biofilms, since they seem to be a causative agent of an increased LDH release by TCC-SUP cells.

Opportunistic yeast pathogens, such as *C. tropicalis*, are able to promote cell host immune response and progressive cell damage during infection [5,13,17]. However, in this study no direct correlation was observed between cell damage and inhibition of cellular activity, namely adhered yeast cells from 72 h-biofilm induced high damage but a low extent of inhibition of cell activity. Nevertheless, it is very interesting to notice that when the inhibition of cellular activity was significantly higher (for U69 120 h-biofilm) cell damage was also the highest. This underlines that these two factors are only in direct relation when the degree of injury is very high. The lack of a direct relation between cell activity inhibition and cell damage at low levels of injury can be explained by the fact that, during the first stage of human cells colonization by *Candida*, the cellular defence mechanisms are initiated, which may cause an increase of metabolic activity before the onset of any damage [17–19].

It has been widely reported that, during the adhesion and invasion processes of host tissues, *Candida* species are able to secrete hydrolytic enzymes that cause damage on host cells membrane integrity, leading to dysfunction or disruption of host structures [20,21]. *Candida* secreted aspartyl proteinases (SAPs) have been associated with tissue invasion and their secretion is also associated with the inflammatory host response [22]. Additionally, the expression of *SAP* genes by *C. tropicalis* (*SAPT1*–*SAPT4*) has been associated to the dissemination of infection and evasion from macrophages after yeast cells phagocytosis [5,20,23].

Real-time PCR analysis (Table 3) revealed that *SAPT* gene expression by *C. tropicalis*, grown in AU (in planktonic and biofilm form), and colonizing TCC-SUP cells showed, in general, a higher level for *SAPT3* expression followed by lower levels of *SAPT2*, *SAPT1* and *SAPT4*. These features were similar to those described by Silva et al. [5] who studied the expression profiles of *SAP* genes by seven *C. tropicalis* strains in contact with reconstituted human oral epithelium: all strains also expressed the 4 genes (*SAPT1* − *4*), moreover *SAPT2* and *SAPT4* transcripts were detected in a similar extent to *SAPT3* and the majority of strains did not express *SAPT1*. Similarly, in the present study, only two strains (U69 and ATCC 750 strains) from 48 h-biofilms were able to express *SAPT1*, but in planktonic form this gene expression was not detected.

Table 2
Percentage of cell activity inhibition and damage evaluated by MTS and LDH, respectively, after *Candida tropicalis* adhesion to human TCC-SUP cell line. *P* values obtained from the comparison among the three strains and among biofilms with different ages (24, 48, 72, 96 and 120 h) are also presented.

Reaction	Time (h)	Isolate		
		U69	U75	Reference
% Activity inhibition (MTS)	24	16.0 ± 2.4	11.0 ± 1.6	5.2 ± 1.6[a]
	48	16.6 ± 2.4	15.9 ± 3.6[b]	19.5 ± 4.4[b]
	72	8.7 ± 2.4[b]	4.5 ± 1.1[b]	5.9 ± 1.6[b]
	96	9.6 ± 0.9	8.7 ± 1.4	10.4 ± 2.8
	120	27.7 ± 3.4[a,b]	7.7 ± 1.6	6.1 ± 0.8
% Damage (LDH)	24	9.0 ± 2.6	14.8 ± 4.1	8.3 ± 1.6
	48	12.3 ± 2.6	14.1 ± 3.5	14.8 ± 1.0
	72	25.4 ± 4.6[b]	18.4 ± 3.5[b]	18.8 ± 2.9[b]
	96	7.4 ± 1.1[b]	26.6 ± 4.9[a,b]	12.4 ± 2.3
	120	50.0 ± 10.5[b]	20.6 ± 1.3[a]	40.3 ± 5.0[b]

All values are means ± Standard deviations.
[a] Effect on human epithelial cells statistically different from the other strains ($P < 0.05$).
[b] Effect of biofilm age on human epithelial cells statistically different from the others ($P < 0.05$).

Table 3
Detection by quantitative real-time polymerase chain reaction of secreted aspartyl proteinases (*SAPT1–4*) gene expression by planktonic and biofilm cells of *Candida tropicalis* when adhered to the human epithelial TCC-SUP cell line.

Strain	*C. tropicalis* form	Relative expression of *SAP* genes[a]			
		SAP1	*SAP2*	*SAP3*	*SAP4*
U69 isolate	Planktonic	ND	0.01 ± 0.00	2.24 ± 0.32	ND
	Biofilm 24 h	ND	ND	0.24 ± 0.16	ND
	Biofilm 48 h	0.01 ± 0.00	0.01 ± 0.00	2.38 ± 0.15	ND
	Biofilm 72 h	ND	0.02 ± 0.00	0.87 ± 0.06	ND
	Biofilm 96 h	ND	ND	0.10 ± 0.03	ND
	Biofilm 120 h	ND	ND	0.21 ± 0.05	ND
U75 isolate	Planktonic	ND	ND	0.17 ± 0.05	ND
	Biofilm 24 h	ND	ND	0.12 ± 0.09	ND
	Biofilm 48 h	ND	ND	0.26 ± 0.08	ND
	Biofilm 72 h	ND	ND	0.26 ± 0.06	ND
	Biofilm 96 h	ND	ND	0.31 ± 0.09	ND
	Biofilm 120 h	ND	0.01 ± 0.00	0.56 ± 0.31	ND
Reference strain	Planktonic	ND	ND	0.01 ± 0.00	0.02 ± 0.00
ATCC 750	Biofilm 24 h	ND	0.01 ± 0.00	0.18 ± 0.05	3.04 ± 0.00
	Biofilm 48 h	0.18 ± 0.00	0.01 ± 0.00	0.34 ± 0.06	ND
	Biofilm 72 h	ND	ND	0.40 ± 0.01	ND
	Biofilm 96 h	ND	ND	0.49 ± 0.14	ND
	Biofilm 120 h	ND	ND	0.21 ± 0.16	ND

ND, indicates that no gene expression was detected.
[a] Mean arbitrary messenger RNA transcript levels based upon triplicate measurements, presented as a percentage relative to the respective *ACT1* transcript level.

Furthermore, the high expression of *SAPT3* points out its importance in the pathogenesis of *C. tropicalis* strains under these conditions.

In order to confer maximum benefits for *Candida* pathogenicity *SAP* genes family are activated efficiently and in flexible way at specific time points during colonization and infection processes. Moreover, *SAP* genes expression is strongly correlated with environment where *Candida* is grown [21,23,24]. In the present study there is a different gene expression among the different modes of growth. For instance, although adhered *C. tropicalis* from biofilms (Reference strain from 24 to 48 h-biofilms and U75 from 120 h-biofilm) exhibited *SAPT2* expression, adhered yeasts from planktonic form did not express this gene. Curiously, *SAPT4* was only detected in ATCC 750 from specific biofilm ages. Other studies indicate that there is an optimum pH for *C. tropicalis*-secreted aspartic proteinases activity, therefore making *SAP* gene expression strain and epithelium dependent [5,20,23]. It is important to highlight that there are only few studies reporting *C. tropicalis SAPT* genes expression during the adhesion to human cells. This could suggest that Saps do not play a significant role in the reduction of epithelial cells activity and damage caused by *C. tropicalis* strains.

In summary, the present study shows that *C. tropicalis* from biofilms are able to colonize human cells and cause some injury: damage and reduction of metabolic activity. Moreover, it is important to highlight that *C. tropicalis SAP* genes are expressed by cells adhered to epithelium, either from planktonic growth or biofilms. Furthermore, to our knowledge, this is the first report of *C. tropicalis SAP* genes expression when biofilm cells colonize TCC-SUP cells, and the results imply a limited role of these enzymes in human cells damage and metabolic activity reduction in the conditions assayed. Nevertheless, *SAPT3* transcript presented the highest level of gene expression by *C. tropicalis* assayed, regardless of biofilm age. However, more studies have to be performed to clarify if these *C. tropicalis SAPs* genes are associated with *C. tropicalis* virulence potential.

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