

Candida tropicalis biofilms: Effect on urinary epithelial cells

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A B S T R A C T

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 × 10⁷ yeasts mL⁻¹, using a Neubauer chamber. Artificial urine (pH 5.8) was prepared according to Silva et al. [6]
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 Penicillin/Streptomycin (P/S) and the cellular density adjusted to 1 × 10⁷ 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 cm²) 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% CO₂, 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 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 independent assays.

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 ΔΔT 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

<p>| Table 1 |
| Primers used for real-time PCR analysis of SAP and control gene expression. |</p>
<table>
<thead>
<tr>
<th>Sequence (5’ → 3’)</th>
<th>Primer Target</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGAAGATCTCATATGCGCAACTACATTTG</td>
<td>Forward</td>
<td>SAP1 1005</td>
</tr>
<tr>
<td>CCTGCGGAGCGCTCTACAGGAACTGCTT</td>
<td>Reverse</td>
<td>SAP1 762</td>
</tr>
<tr>
<td>TTCTTCTAGTGGTACTCGGGAAGAAG</td>
<td>Forward</td>
<td>SAP1 165</td>
</tr>
<tr>
<td>CATAGATCTTCTAAAACATATGCACTTGA</td>
<td>Reverse</td>
<td>ACT1 1130</td>
</tr>
<tr>
<td>ACTCTGTTTCATCCACGGAAGA</td>
<td>Forward</td>
<td>ACT1 181</td>
</tr>
<tr>
<td>GCCACGATCGTATGCAATGAA</td>
<td>Reverse</td>
<td>CT1 80</td>
</tr>
<tr>
<td>CATGATCCATGTGAAATCGGAAAT</td>
<td>Forward</td>
<td>SAP1 1100</td>
</tr>
<tr>
<td>GAGCTCTTCTACAGGAACTGCTT</td>
<td>Reverse</td>
<td>SAP1 900</td>
</tr>
<tr>
<td>GTACCTGAGCTTCTACAGGAAAT</td>
<td>Forward</td>
<td>ACT1 1200</td>
</tr>
<tr>
<td>CATGATCCATGTGAAATCGGAAAT</td>
<td>Reverse</td>
<td>ACT1 181</td>
</tr>
</tbody>
</table>
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 [79]. 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.

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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.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Time (h)</th>
<th>Isolate</th>
<th>U69</th>
<th>U75</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Activity inhibition (MTS)</td>
<td>24</td>
<td>16.0 ± 2.4</td>
<td>11.0 ± 1.6</td>
<td>5.2 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>16.8 ± 2.4</td>
<td>15.9 ± 3.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.5 ± 4.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>8.7 ± 2.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.5 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.9 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>9.6 ± 0.9</td>
<td>8.7 ± 1.4</td>
<td>10.4 ± 2.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>27.7 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.7 ± 1.6</td>
<td>6.1 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>% Damage (LDH)</td>
<td>24</td>
<td>9.0 ± 2.6</td>
<td>14.8 ± 4.1</td>
<td>8.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>12.3 ± 2.6</td>
<td>14.1 ± 3.5</td>
<td>14.8 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>25.4 ± 4.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.4 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.8 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>7.4 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.6 ± 4.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.4 ± 2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>50.0 ± 10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.6 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.3 ± 5.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

All values are means ± Standard deviations.

<sup>a</sup> Effect on human epithelial cells statistically different from the other strains \( (P < 0.05) \).

<sup>b</sup> Effect of biofilm age on human epithelial cells statistically different from the others \( (P < 0.05) \).
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-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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