**In vitro** biofilm production of *Candida* bloodstream isolates: any association with clinical characteristics?

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*Candida* spp. are a leading cause of bloodstream infection (BSI) and are associated with high mortality rates. Biofilm production is a virulence factor of *Candida* spp., and has been linked with poor clinical outcome. The aim of our study was to assess biofilm production of *Candida* bloodstream isolates at our institute, and to determine whether *in vitro* biofilm production is associated with any clinical characteristics of infection. During the four-year study period, 93 cases of *Candida* BSI were analysed. The most frequently isolated species was *C. albicans* (66.7 %), followed by *C. glabrata* (9.7 %), *C. parapsilosis* (9.7 %), *C. tropicalis* (9.7 %) and *C. krusei* (4.3 %). Biofilm production was more prevalent among non-*albicans* *Candida* spp. (77.4 %) than *C. albicans* (30.6 %) (*P* = 0.02). Abdominal surgery was identified as a risk factor of BSI caused by biofilm producing non-*albicans* *Candida* isolates. No risk factors predisposing to bloodstream infection caused by a biofilm producing *C. albicans* isolate were identified. Biofilm production was not verified as a risk factor of mortality.

**INTRODUCTION**

*Candida* spp. are a leading cause of bloodstream infection (BSI) and are associated with a high mortality rate ranging from 30.4 to 53.6 %, especially among immunocompromised patients and those with severe underlying disease (Kett *et al.*, 2011; Puig-Asensio *et al.*, 2014; Wisplinghoff *et al.*, 2014; Hii *et al.*, 2015).

Biofilm production is a well-described virulence factor of *Candida* spp. Biofilms are groups of micro-organisms encased in an extracellular matrix, adhering to an organic or inorganic surface. Biofilms attach to the surface of different medical devices, such as intravascular catheters, implants and endotracheal tubes, providing a possible source of infection (Chandra *et al.*, 2001).

Growth in biofilm form also increases the pathogenicity of micro-organisms: in a murine model of infection, *C. albicans* cells detached from the biofilm proved to be more cytotoxic than their planktonic form (Uppuluri *et al.*, 2010).

A further trait of *Candida* biofilms is *in vitro* resistance to a number of antifungal agents, such as triazoles and non-lipid amphoterin B. Amphoterin B lipid formulations and echinocandins remain effective against biofilms (Chandra *et al.*, 2001; Ramage *et al.*, 2001; Kuhn *et al.*, 2002; Mukherjee *et al.*, 2005; Estivill *et al.*, 2011). Resistance of biofilms to fluconazole and the superior efficacy of anidulafungin have also been shown *in vivo* in a rat model of biofilm infection (Kuchariková *et al.*, 2010).

The clinical significance of *in vitro* biofilm production by *Candida* spp., however, is not clear. Shin *et al.* found that non-*albicans* *Candida* bloodstream isolates in non-neutropenic patients were more likely to produce a biofilm in the presence of central venous line-related candidaemia and the use of total parenteral nutrition (TPN), but did not find any association with the outcome of *Candida* BSI (Shin *et al.*, 2002). In a study examining whether biofilm formation is a predictor of catheter-related candidaemia, and whether biofilm production is associated with higher mortality, Guembe *et al.* assessed biofilm formation in a total of 54 isolates of *C. albicans*, *C. parapsilosis* and *C. glabrata* deriving from blood samples of patients with catheter-related candidaemia (*n* = 29) and patients without catheter-related candidaemia (*n* = 25). Most of the isolates (83.3 %) proved to be biofilm producers, but no association was determined between biofilm production and...
catheter-related candidaemia or patient mortality (Guenbe et al., 2014). On the contrary, Tumbarello et al. found that the mortality rate of patients with BSI caused by a biofilm-producing isolate was significantly higher than that of patients with BSI caused by a non-biofilm-producing isolate (Tumbarello et al., 2007, 2012).

The ability of Candida isolates to form biofilms varies by species and strain (Pannanusorn et al., 2013). The aim of our study was to assess biofilm production by Candida bloodstream isolates at our institute, and to examine whether in vitro biofilm production can be implicated as a risk factor of poor clinical outcome. We also examined whether any risk factors could be associated with BSI caused by a biofilm-producing isolate.

METHODS

The study was carried out at a 2250-bed tertiary care hospital with approximately 120,000 admissions per year. The hospital provides highly specialized medical care including haematology, solid organ transplant services and advanced medical and surgical facilities. Candida bloodstream isolates deriving from independent episodes of candidaemia were collected during a four-year period, from January 2010 to December 2013. An episode of candidaemia was defined as the isolation of Candida spp. from at least one blood culture in a patient. If Candida spp. were isolated on more than one occasion in a single patient, a subsequent positive culture was considered a new episode of candidaemia if more than 30 days had elapsed since the isolation of the previous Candida sp. Patients yielding multiple Candida spp. (n=7) were not included in the study. Isolates were identified by phenotypic methods and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) analysis.

Qualitative and quantitative analysis of biofilm production was based on methods described in previous reports (Chandra et al., 2001; O'Toole, 2011). Isolates were cultured in 96-well polystyrene plates. A 20 µl aliquot of Candida cell suspension containing 10^7 c.f.u. ml^{-1} was inoculated into wells containing 180 µl Sabouraud glucose broth (Sigma-Aldrich) with 8 % glucose. After 24 h incubation at 37 °C, planktonic cells were discarded and the biofilm was washed twice with saline. Biofilm production was quantified by XTT [2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide, Sigma-Aldrich] reduction assay and crystal violet staining. In the first method, 100 µl of XTT salt solution (1 mg ml^{-1} in PBS) containing 1 µl menadione solution was added to each well followed by incubation in the dark for two hours. The amount of XTT formazan produced was measured at 490 nm with a microplate reader (Bio-Rad PR 3100 TSC microplate reader). Results ≥0.1 were considered biofilm producers. In the second method, the biofilms were stained with 100 µl of 0.2 % crystal violet (CV) dye for 15 min, the excess dye was rinsed off and 100 µl of 96 % ethanol was added to each well to solubilize bound CV. Absorbance was measured at 570 nm. Isolates with results ≥0.09 were considered biofilm producers. All isolates were measured in triplicate. Candida parapsilosis ATCC 22019 was used as control strain in each experiment.

For antifungal susceptibility testing of the biofilms, 48 h biofilms were washed and reincubated for 48 h in serial dilutions of fluconazole and anidulafungin. For determination of sessile minimum inhibitory concentrations (sMIC) of the antifungal agents, the sMIC50 (defined as the antifungal concentration that results in 50 % growth inhibition compared to the control well with no antifungal agent) was determined by XTT assay (Ramage et al., 2001).

Patient data were collected retrospectively using a standardized case report form which included demographic data, underlying diseases, comorbidities (diabetes mellitus, organ failure, malignancy, immunosuppression), presence of intravascular devices, mechanical ventilation, administration of parenteral nutrition and isolate characteristics (Candida species, biofilm production, antifungal susceptibility profile). An ethics approval was not considered necessary since the reviewed patient records were anonymous and no additional samples were obtained besides those collected during routine laboratory practice.

ANOVA was used to compare differences in biofilm production by the groups of isolates. Fisher’s exact test was used to investigate whether any of the risk factors of Candida BSI predisposed to infection with a biofilm-producing isolate, and to assess whether biofilm production of the isolates had any connection with 30-day mortality. The t-test with Welch correction was used to evaluate the association of age with infection by a biofilm-producing isolate. P-values <0.05 were considered significant.

RESULTS

During the study period, 93 cases of Candida bloodstream infection were investigated. The most frequently isolated species was C. albicans (66.7 %), followed by C. glabrata (9.7 %), C. parapsilosis (9.7 %), C. tropicalis (9.7 %) and C. krusei (4.3 %).

Forty-six per cent (n=43) of all isolates proved to be biofilm producers. There was no discrepancy between the two methods in determining whether an isolate was a biofilm producer or not. Biofilm-producing isolates were significantly more prevalent among non-albicans Candida spp. (77.4 %) than C. albicans (30.6 %; P=0.02). The results of the CV and XTT assays for positive isolates are presented in Fig. 1.

When the degree of biofilm formation was evaluated, however, the two biofilm assessment methods yielded different results with several of the isolates. Based on the CV assay, a C. tropicalis isolate produced the highest amount of biofilm (OD 570 nm: 3.86), but this strain did not have the highest metabolic activity as measured by XTT assay. According to the CV assay, the OD values for positive isolates could be categorized into two distinct groups: values between 0.09 and 0.45 (low-biofilm producers) and values ≥0.9 (high-biofilm producers). The distinction between the groups was verified by ANOVA: there was a significant difference between the values in the two groups (P<0.01). All C. albicans isolates produced low amounts of biofilm, while high-biofilm producers were detected among non-albicans isolates (C. parapsilosis n=3, C. krusei n=1, C. tropicalis n=6, C. glabrata n=2). Fig. 2 shows the number of biofilm-producing isolates among the different species.

Biofilm susceptibility testing yielded fluconazole sMIC values at least 100-fold greater than the planktonic MIC (pMIC) for every biofilm-producing isolate, while anidulafungin remained active against the biofilms. Fig. 3 shows fluconazole and anidulafungin pMIC and sMIC distribution of the isolates.
Patient demographic data and clinical characteristics are presented in Table 1. Overall 30-day mortality was 46.2 %, with the highest (100 %) being for \textit{C. tropicalis} BSI and the lowest (22 %) for \textit{C. parapsilosis}. One clinical characteristic, prior abdominal surgery, was associated with infection by a biofilm-producing non-albicans \textit{Candida} spp. isolate \((P<0.012)\). No risk factors were associated with infection by any biofilm-producing \textit{C. albicans} isolate.

There was no statistically significant difference in 30-day mortality between patients infected with a biofilm-producing \textit{C. albicans} \((P=0.18)\), \textit{C. parapsilosis} \((P=0.44)\) or \textit{C. glabrata} \((P=1.0)\) isolate compared with patients infected with a biofilm non-producing isolate of the same species. All \textit{C. tropicalis} isolates \((n=9)\) in our study were biofilm producers, and all cases resulted in death within 30 days. All four \textit{C. krusei} isolates were biofilm producers, one patient dying within 30 days.

DISCUSSION

In our study, biofilms were assessed with two different methods, CV and XTT assays. While the methods gave results consistent with each other in regard to qualitative analysis of biofilm formation, the XTT assay has some limitations regarding its usefulness in the evaluation of biofilm formation by \textit{Candida} spp. The XTT assay measures the metabolic activity of isolates and the results may vary from strain to strain; therefore it is not suitable for interstrain comparisons (Kuhn \textit{et al.}, 2003). While XTT activity correlates with the number of viable cells in the biofilm, CV binds to viable and non-viable cells along with extracellular matrix components, and is suitable for the quantification of the biofilm mass itself.

It appears that biofilm production has a role in the pathogenesis of BSI by non-\textit{albicans Candida} species, since biofilm-producing isolates are recovered more frequently from the blood than from other, non-sterile sites. The percentage of biofilm-producing \textit{C. albicans} isolated from the bloodstream, on the other hand, is no higher than isolates recovered from other sites, suggesting that other factors also have an important role in virulence of the species (Shin \textit{et al.}, 2002). Almost half of our isolates (46 %) were biofilm producers, and we detected significantly more \((P=0.02)\) biofilm-producing isolates among non-\textit{albicans Candida} spp. (77.4 %) than among \textit{C. albicans} (30.6 %) isolates. Furthermore, isolates able to produce a large amount of biofilm were found exclusively among non-\textit{albicans Candida} spp., demonstrating the importance of the biofilm-forming capability of invasive non-\textit{albicans Candida} spp. isolates.

Few studies have investigated the risk factors associated with BSI caused by biofilm-forming isolates. Tumbarello \textit{et al.} reported that diabetes mellitus, TPN and the presence of an intravascular catheter were associated with BSI caused...
Anidulafungin (b)

Fluconazole (a)

0
5
10
15
20
25
30
35
mg ml\(^{-1}\)

0.125 0.25 0.5 1 2 4 8 16 32 64 128 256 512 ≥1024

No. of isolates

≤0.008 0.015 0.03 0.06 0.125 0.25 0.5 1

No. of isolates

Fig. 3. Fluconazole (a) and anidulafungin (b) planktonic MIC (pMIC) and sessile MIC (sMIC) distribution of biofilm-producing Candida bloodstream isolates.

Table 1. Comparison of clinical characteristics of biofilm-producing and -non-producing Candida bloodstream isolates

<table>
<thead>
<tr>
<th>Candida albicans</th>
<th>All (n=62)</th>
<th>Biofilm positive (n=19)</th>
<th>Biofilm negative (n=43)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>62.6 (22–86)</td>
<td>61.9 (44–81)</td>
<td>62.9 (22–86)</td>
<td>0.777</td>
</tr>
<tr>
<td>Male</td>
<td>40 (64.5 %)</td>
<td>14 (73.7 %)</td>
<td>26 (60.5 %)</td>
<td>0.395</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>12 (19.3 %)</td>
<td>6 (31.6 %)</td>
<td>7 (16.3 %)</td>
<td>0.192</td>
</tr>
<tr>
<td>Abdominal surgery</td>
<td>34 (54.8 %)</td>
<td>9 (47.4 %)</td>
<td>25 (58.1 %)</td>
<td>0.581</td>
</tr>
<tr>
<td>Solid malignancy</td>
<td>17 (27.4 %)</td>
<td>4 (21 %)</td>
<td>13 (30.2 %)</td>
<td>0.548</td>
</tr>
<tr>
<td>Haematological malignancy</td>
<td>4 (6.4 %)</td>
<td>2 (10.5 %)</td>
<td>2 (4.6 %)</td>
<td>0.56</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>18 (29 %)</td>
<td>6 (31.6 %)</td>
<td>12 (28 %)</td>
<td>0.77</td>
</tr>
<tr>
<td>Presence of endovascular catheter</td>
<td>43 (69.3 %)</td>
<td>13 (68.4 %)</td>
<td>30 (69.8 %)</td>
<td>1</td>
</tr>
<tr>
<td>Total parenteral nutrition</td>
<td>10 (16.1 %)</td>
<td>2 (10.5 %)</td>
<td>8 (18.6 %)</td>
<td>0.71</td>
</tr>
<tr>
<td>30-day mortality</td>
<td>28 (45.1 %)</td>
<td>11 (57.8 %)</td>
<td>17 (39.5 %)</td>
<td>0.268</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-albicans Candida spp.</th>
<th>All (n=31)</th>
<th>Biofilm positive (n=24)</th>
<th>Biofilm negative (n=7)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>61.5 (22–80)</td>
<td>63.1 (22–79)</td>
<td>56 (23–80)</td>
<td>0.428</td>
</tr>
<tr>
<td>Male</td>
<td>20 (64.5 %)</td>
<td>17 (70.8 %)</td>
<td>3 (42.8 %)</td>
<td>0.2</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>7 (22.6 %)</td>
<td>5 (20.8 %)</td>
<td>2 (28.6 %)</td>
<td>0.64</td>
</tr>
<tr>
<td>Abdominal surgery</td>
<td>17 (54.8 %)</td>
<td>14 (58.3 %)</td>
<td>3 (42.8 %)</td>
<td>0.012</td>
</tr>
<tr>
<td>Solid malignancy</td>
<td>10 (32.2 %)</td>
<td>9 (37.5 %)</td>
<td>1 (14.3 %)</td>
<td>0.379</td>
</tr>
<tr>
<td>Haematological malignancy</td>
<td>4 (12.9 %)</td>
<td>2 (8.3 %)</td>
<td>2 (28.6 %)</td>
<td>0.212</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2 (6.5 %)</td>
<td>2 (8.3 %)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Presence of endovascular catheter</td>
<td>21 (67.7 %)</td>
<td>17 (70.8 %)</td>
<td>4 (57.1 %)</td>
<td>0.652</td>
</tr>
<tr>
<td>Total parenteral nutrition</td>
<td>12 (38.7 %)</td>
<td>9 (37.5 %)</td>
<td>3 (42.8 %)</td>
<td>1</td>
</tr>
<tr>
<td>30-day mortality</td>
<td>15 (48.4 %)</td>
<td>12 (50 %)</td>
<td>3 (42.8 %)</td>
<td>1</td>
</tr>
</tbody>
</table>

Values are presented as number (%).
by a biofilm-forming isolate (Tumbarello et al., 2012). Prior abdominal surgery has been identified as a risk factor of Candida BSI (Kuhns et al., 2015), and over half the patients (54.8 %) in that study underwent major abdominal surgery in the 30 days preceding the detection of Candida BSI. We found that prior abdominal surgery predisposed to infection with a biofilm-producing non-albicans Candida spp. isolate (P=0.012), but there was no association with any of the other risk factors studied. No connection was determined between any of the risk factors and infection due to a biofilm-producing C. albicans isolate.

Biofilms readily form on various medical materials in vitro (Chandra et al., 2001), and Candida isolates capable of producing a biofilm in vitro have a significant role in the colonization of intravascular devices and catheter-associated BSI. However, we found that the presence of an intravascular device was not associated with BSI caused by a biofilm-producing isolate, supporting the findings of Guembe et al. (2014).

The impact of biofilm production on the outcome of Candida BSI is controversial. In a study performed by Shin et al., biofilm production was not associated with increased 30-day mortality, though this study included a large number of C. parapsilosis BSI associated with low mortality rates and only two biofilm-positive C. albicans isolates (Shin et al., 2002). In another study, performed by Tumbarello et al., the mortality rate in patients with BSI caused by biofilm-producing C. albicans and C. parapsilosis isolates was significantly higher than in patients with BSIs due to biofilm-negative isolates, but the association was not observed for other Candida spp. (Tumbarello et al., 2007). One possible explanation for clinical failure in BSI caused by biofilm-producing isolates is the resistance of biofilms to fluconazole, demonstrated in a number of in vitro and in vivo studies. The fluconazole sMIC of biofilms produced by the isolates in this study were at least 100 times that of the pMIC levels. Although the mortality rate of patients infected with a biofilm-producing isolate was higher than that of patients infected with a non-biofilm-producing isolate in our study (57.8 % vs 39.5 % for C. albicans; 50 % vs 42.8 % for non-albicans species), the difference did not prove to be statistically significant for any of the species.

In conclusion, this study contains valuable data to complement our knowledge regarding the clinical significance of biofilm formation by Candida spp. Abdominal surgery was identified as a risk factor of BSI caused by biofilm-producing non-albicans Candida isolates. Though biofilm production enhances the pathogenicity of Candida spp., our findings do not verify in vitro biofilm production as a risk factor of mortality. This study has some limitations due to the small sample size in case of non-albicans Candida spp. Further data are necessary to determine whether in vitro biofilm production by Candida spp. has any clinical implications.

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REFERENCES


