In vitro antifungal susceptibility of candidemia agents and detection of their biofilm production by two different methods

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Abstract

Candida bloodstream infections are a significant cause of morbidity and mortality in hospitalized patients. The most important contribution of biofilm is the higher antifungal resistance than planktonic cells. We aimed to investigate the biofilm formation rate and antifungal susceptibility characteristics of our bloodstream isolates, and evaluate two different biofilm detection methods. A total of 200 bloodstream *Candida* isolates were included. The biofilms were formed on 96-well microtiter plates and measured by spectrophotometric percent transmittance and 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium- 5-carboxanilide colorimetric assay. In addition antifungal susceptibilities of these isolates were evaluated against caspofungin, anidulafungin and amphotericin B by reference method. Biofilm production rate was considerably high among our bloodstream isolates. The most important biofilm producer species was *C. tropicalis*; *C. glabrata* had the lowest biofilm production rate. The consistency rate between biofilm detection methods was 66%. Remarkable antifungal resistance was not observed among our isolates in general. In conclusion, biofilm production in *Candida* species is an important virulence factor, and its rate is considerably high in bloodstream isolates. At present, a standardized method has not been established to detect the biofilm formation.

Introduction

Bloodstream infections (BSI) caused by various *Candida* spp. are a significant cause of morbidity and mortality especially in patients of intensive care units and patients with hematological malignancy. Although the introduction of antifungal drugs has improved the outcome of *Candida* BSIs, antifungal resistance has emerged recently as a major challenge in the management of *Candida* infections because of the increasing prophylactic use of fluconazole and the relative rise in the proportion of non-*albicans Candida* species.[1] Biofilm production on artificial or biological surfaces is an important virulence factor of *Candida* spp., because it is frequently associated with deep seated infections enhancing resistance to antimicrobial agents and protecting from host defenses, making these infections refractory to conventional therapy.[2] Therefore, the detection of in vitro antifungal susceptibility and biofilm production of the agents is critical for the appropriate management of *Candida* bloodstream infection. In this study, primarily we aimed to investigate the biofilm formation rate and antifungal susceptibility characteristics for our bloodstream isolates. Secondarily, we aimed to evaluate two different biofilm detection methods.

Materials and Methods

Isolates

A total of 200 *Candida* species isolated from blood cultures were used; *C. albicans* (n=75), *C. parapsilosis* (n=50), *C. glabrata* (n=30), *C. tropicalis* (n=20), *C. krusei* (n=10), *C. kefyr* (n=4), *C. lusitaniae* (n=4), *C. dubliniensis* (n=3), *C. guilliermondii* (n=2) and *C. pelliculosa* (n=2). All isolates were selected randomly from the agents of bloodstream infection in our clinical microbiology laboratories from January 2008 to January 2014. Species identification was made by conventional procedures such as germ tube production, microscopic morphology on corn meal Tween 80 agar, as well as commercial methods such as CHROMagar *Candida* (BD Diagnostic, Sparks, MD), API 20C AUX (bioMérieux, Marcy L'Etoile, France). *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control for antifungal susceptibility studies. All isolates were stored at −70ºC until use.

Biofilm production

The biofilms of *Candida* spp. were formed on presterilized, polystyrene, flat bottomed, 96-well microtiter plates (Nunclon; Nalge Nunc International, Roskilde, Denmark) as described previously.[3-5] All isolates were grown for 24 h at 35°C on SDA, and saline washed suspensions of each isolate were prepared. The turbidity of each suspension was adjusted to the equivalent of 3×10^7 CFU/mL with Sabouraud dextrose broth (SDB) supplemented with glucose (8% final concentration). Each well of microtiter plates was inoculated with aliquots of 20 µL of yeast cell suspension and 180 µL of SDB, three consecutive wells were used for each isolate. At least three wells included 200 µL sterile SDB only as control wells in each plate. All plates were then incubated at 35°C without agitation. After 24 h, all wells were discharged and washed once with distilled water (BIO-TEK EL×50) to remove the planktonic cells; eventually 200 µL of distilled water was added to each well. We used two different methods for detection of biofilm production; a spectrophotometric method and a colorimetric assay by using XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium- 5-carboxanilide].[3-5]

Firstly, biofilm was measured directly by spectrophotometric readings at 405 nm with a microtiter plate reader (BIO-TEK, EL×800, USA). The percent transmittance (%T) was calculated by subtracting the %T value for each test sample from the %T value for the reagent blank to obtain a measure of the amount of light blocked passing through the wells (%Tbloc). Biofilm production by each isolate was scored as negative (%Tbloc, <5), 1+ (%Tbloc, 5 to 20), 2+ (%Tbloc, 20 to 35), 3+ (%Tbloc, 35 to 50), or 4+ (%Tbloc, > 50).[4,5] Each isolate was tested in duplicate.

In the second method, XTT (Sigma-Aldrich, St. Louis, MO, USA) was prepared

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in a saturated solution at 0.5 g/L in Ringer's lactate and sterilized through a 0.22 µm pore size filter. Menadione (Sigma-Aldrich, St. Louis, MO, USA) was prepared as 10 mM in acetone and added to XTT solution to be a 1 mM final concentration. A 100-µl aliquot of the XTT-menadione solution was then added to each prewashed biofilm and control wells (for the measurement of background XTT-reduction levels). The plates were then incubated at 37°C in the dark chamber for 2 h. A colorimetric change in the XTT reduction assay, a direct correlation with the metabolic activity of the biofilm, was then measured in a microtiter plate reader at 490 nm.[3] The absorbance values of the controls were then subtracted from the values of the test wells to eliminate spurious results due to background interference.[6] To compare the two different detection methods, net absorbance values of XTT reduction assay were classified as negative (<0.2), 1+ (0.2 to 0.49), 2+ (0.5 to 0.99), 3+ (1.0 to 1.49), or 4+ (1.5≤).

Antifungal susceptibility testing

Antifungal susceptibility testing was made by using reference broth microdilution (BMD) method, Clinical and Laboratory Standards Institute.[7] First, caspofungin (CAS; Merck, Rahway, NJ, USA), anidulafungin (AND; Pfizer, New York, NY, USA) and amphotericin B (AmB; Sigma, St. Louis, MO, USA) were obtained as standard powders from their manufacturers. CAS was dissolved in distilled water; AND and AmB were dissolved in dimethyl sulfoxide. Stock solutions were prepared in RPMI 1640 medium (Sigma-Aldrich, Steinheim, Germany) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma-Aldrich, Steinheim, Germany), and serial twofold dilutions ranging 0.03-16 µg/mL of each drug were performed in 96 well microtiter plates.

All isolates were subcultured twice on Sabouraud dextrose agar (SDA) plates to ensure viability and purity. After 24- to 48-h incubation, a standard 0.5 McFarland fungal suspension was prepared with 0.85% sterile saline by spectrophotometric methods. It was diluted with RPMI 1640 broth medium to obtain a starting inoculum which results in 1-5×10^3 cells per mL. Microtiter plates were inoculated and incubated at 35ºC. The minimum inhibitory concentrations (MICs) were read at 24 h for echinocandins, both 24 and 48 h for AmB. Endpoints for echinocandins were defined as the lowest concentration of drug that resulted in a prominent reduction (approximately 50% inhibition) of visual growth compared with the growth control wells. MICs of AmB were defined as the lowest concentration of drug which resulted in total inhibition of visual growth. For the analysis of antifungal susceptibility test results, interpretive species-specific MIC breakpoints were used for CAS and AND according to recently revised criteria by CLSI.[8] All tests were carried out in duplicate.

Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 20. All tests were two sided, and a P value of <0.05 was considered statistically significant.

Results

The results of biofilm production tests by both spectrophotometric and colorimetric XTT methods were presented in Table 1. Of all isolates, 41% and 55% produced apparent (3+/4+) biofilm by spectrophotometric and XTT colorimetric methods, respectively. The most important biofilm production was observed among the isolates of *C. tropicalis* and *C. parapsilosis* by both detection methods. Although the biofilm formation in *C. krusei* isolates was very prominent, their number was low to generalize to this species in this study. In addition, *C. glabrata* had the lowest biofilm production with both methods. However, when we compared the two different detection methods, compliance was observed in 132 (66%) isolates (Table 2); negative or low (1+, 2+) biofilm production in 71 isolates and high (3+, 4+) biofilm production in 61 isolates. When the biofilm formation was evaluated among these 132 isolates, significant species specific differences were observed (P=0.035); the highest biofilm production was seen for *C. tropicalis* isolates, followed by *C. parapsilosis* and *C. glabrata* was the lowest biofilm producer (Figure 1).

Antifungal susceptibility test results were summarized in Table 3. Although MICs of CAS and AND were high against the isolates of *C. parapsilosis* as expected, the lowest MIC values were observed with CAS and AND against other species. The highest AmB MICs were observed against *C. krusei* isolates.

Discussion and Conclusions

It is known that the incidence of nosocomial fungal infections has increased over the last few decades. Candidiasis, specifically candidemia, has been shown to be the most frequent mycotic infection of hospitalized patients and associated with a significant attributable mortality and excess length of hospital stay.[9] Therefore, it is important to know the antifungal susceptibility characteristics for successfully management of these infections. Although *C. albicans* is the most frequently isolated species, recent reports indicate a trend toward an increasing prevalence of infections caused by species of non-*albicans Candida* such as *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*.[9,10] In our study, we evaluated the biofilm production rate and the susceptibility characteristics for three antifungals of our bloodstream isolates. For this reason, we selected the species reflecting our bloodstream *Candida* isolate collection and

Table 1. The results of methods used for the detection of biofilm production.

Species	Biofilm production									
	With spectrophotometric method (%)					With XTT colorimetric method (%)				
	Neg	+	++	+++	++++	Neg	+	++	+++	++++
C. albicans	15 (20)	16 (21)	14 (19)	9 (12)	21 (28)	1 (1)	16 (22)	22 (29)	22 (29)	14 (19)
C. parapsilosis	1 (2)	6 (12)	20 (40)	12 (24)	11 (22)	0	3 (6)	15 (30)	25 (50)	7 (14)
C. glabrata	11 (37)	5 (17)	8 (27)	2 (6)	4 (13)	5 (17)	4 (13)	9 (30)	11 (37)	1 (3)
C. tropicalis	4 (20)	2 (10)	4 (20)	4 (20)	6 (30)	0	3 (15)	3 (15)	6 (30)	8 (40)
C. krusei	0	1	1	8	0	0	1	2	4	3
Candida spp.	6	2	2	1	4	0	1	6	6	2
Total	37 (18.5)	32 (16)	49 (24.5)	36 (18)	46 (23)	6 (3)	28 (14)	57 (28.5)	74 (37)	35 (17.5)

three antifungal drugs in the treatment of invasive *Candida* infections.

Currently, there is no standardized method for the detection of biofilm formation; therefore, we used two different well established methods, spectrophotometric and colorimetric XTT. We observed significant species specific differences in terms of biofilm production; while the strongest biofilm producer species was *C. tropicalis*, the weakest one was *C. glabrata* by both methods. Tumbarello *et al.*[5] reported that biofilm production was most frequently observed for isolates of *C. tropicalis* (71.4%), followed by *C. glabrata* (23.1%), *C. albicans* (22.6%), *C. parapsilosis* (21.8%); the highest relative intensity of biofilm formation was seen for *C. tropicalis* isolates, followed by *C. parapsilosis* among biofilm-positive isolates. Both the highest frequency and the highest relative intensity of biofilm formation were seen for *C. tropicalis* isolates, followed by *C. parapsilosis* in our study (Table 2). Pannanusorn *et al.*[11] evaluated the biofilm formation of 393 bloodstream isolates by XTT colorimetric method with some modifications; they reported a high biofilm production rate (58.8%) for all isolates and 88.7% for non-*albicans Candida* species. In addition, while all of *C. tropicalis* and *C. krusei* isolates, 95% of *C. glabrata* isolates, 66.7% of *C. parapsilosis* isolates, 40.3% of *C. albi-*

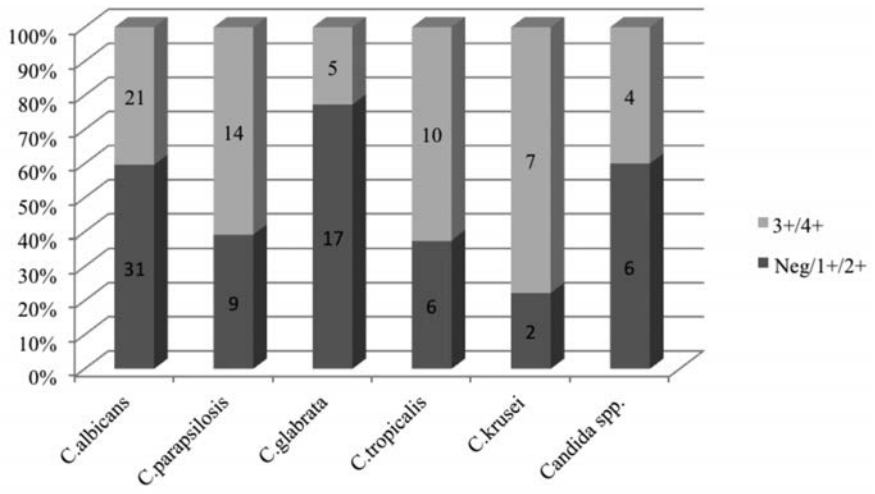

Figure 1. The distribution of biofilm production rate among *Candida* species. This Figure was constituted by using the compatible results of two different biofilm detection methods (italicized in Table 2).

Table 2. The comparison of the biofilm detection methods.

Spectrophotometric	XTT colorimetric				
	Negative	+	++	+++	++++
Negative	*3*	*8*	*24*	0	0
+	*1*	*5*	*9*	14	0
++	*2*	*9*	*10*	19	9
+++	0	8	11	*13*	*8*
++++	0	0	7	*22*	*18*

The compatible results between the two detection methods are italicized.

Table 3 Antifungal susceptibility test results by broth microdilution methods.

Candida species	Antifungal agents	Incubation time	MIC (µg/mL)			No. (%) of isolates in susceptibility category		
			Range	%50	%90	S	I	R
C. albicans (n=75)	Caspofungin	24	0.03-1	0.06	0.25	74 (98.7)	0 (0.0)	1 (1.3)
	Anidulafungin	24	≤0.03-1	≤0.03	≤0.03	73 (97.4)	1 (1.3)	1 (1.3)
	Amphotericin B	24	0.03-0.5	0.125	0.125			
	Amphotericin B	48	0.125-0.5	0.25	0.5			
C. parapsilosis (n=50)	Caspofungin	24	≤0.03-1	0.5	1	50 (100)	0 (0.0)	0 (0.0)
	Anidulafungin	24	≤0.03-2	1	1	50 (100)	0 (0.0)	0 (0.0)
	Amphotericin B	24	0.06-0.25	0.12	0.25			
	Amphotericin B	48	0.125-1	0.25	0.5			
C. glabrata (n=30)	Caspofungin	24	0.06-1	0.125	0.25	25 (83.3)	2 (6.7)	3 (10.0)
	Anidulafungin	24	≤0.03-1	0.06	0.125	27 (90.0)	0 (0.0)	3 (10.0)
	Amphotericin B	24	0.03-0.25	0.125	0.25			
	Amphotericin B	48	0.125-0.5	0.25	0.5			
C. tropicalis (n=20)	Caspofungin	24	≤0.03-0.5	0.06	0.25	19 (95.0)	1 (5.9)	0 (0.0)
	Anidulafungin	24	≤0.03-0.125	0.03	0.125	20 (100)	0 (0.0)	0 (0.0)
	Amphotericin B	24	0.03-0.25	0.25	0.25			
	Amphotericin B	48	0.06-0.5	0.25	0.5			
C. krusei (n=10)	Caspofungin	24	0.125-1	0.5	1	3	5	2
	Anidulafungin	24	0.06-0.5	0.06	0.06	9	1	0
	Amphotericin B	24	0.125-0.5	0.25	0.5			
	Amphotericin B	48	0.25-1	0.5	1			
Candida spp.* (n=15)	Caspofungin	24	0.03-1	0.125	0.5			
	Anidulafungin	24	≤0.03-2	0.125	0.5			
	Amphotericin B	24	0.03-1	0.125	0.5			
	Amphotericin B	48	0.06-2	0. 25	1			

**C. kefyr*, *C. lusitaniae* , *C. guilliermondii*, *C. pelliculosa* and other *Candida* spp. I, intermediate; R, resistant; S susceptible.

cans isolates were found biofilm positive, high biofilm production capacity was observed in the rates of 87% in *C. tropicalis*, 69% in *C. glabrata*, 45.5% in *C. parapsilosis*, and 11% in *C. albicans* in their study.[11] Although the rates of biofilm production showed some variations depending on the detection method used, it was species specific and the most frequent biofilm producer species was *C. tropicalis*. The consistency between biofilm detection methods was found low in our study. In another study using the same biofilm detection methods, authors reported that the results of spectrophotometric method correlated well with XTT absorbance measurements for all isolates tested.[5] Dhale *et al*.[12] compared three different methods, spectrophotometric and colorimetric methods, and crystal violet assay; they did not find significant difference in proportion of biofilm positive cases detected by the three methods. However, Kuhn *et al*.[13] evaluated the species- and strain-related tetrazolium metabolism in *C. albicans* and *C. parapsilosis* by using XTT. They reported that the use of colorimetric XTT method for the comparison of different *Candida* isolates may have some limitations, for instance different strains metabolize XTT with different capabilities, a linear relationship may not appear between organism number and colorimetric signal, and the relationship between the XTT concentration and the resultant colorimetric signal is not necessarily proportional.[13] Although XTT reduction assay is seen as a convenient method for investigating the behavior of yeast, it may not always be appropriate.[13] We think that the use of more than one test for biofilm detection may be useful because it seems that the detection of biofilm formation is still problematic by using these tests.

A remarkable resistance was not observed among our isolates against all antifungals in our study. We observed the resistance to echinocandin in a low proportion among the isolates of *C. albicans, C. glabrata* and *C. krusei*. In a study evaluating the antifungal susceptibility of 1214 bloodstream yeast isolates by using a commercial colorimetric microdilution plate, the susceptibility rates of echinocandins were very high, resistance was reported only among the isolates of *C. albicans* (0.2% for both CAS and AND) and *C. krusei* (9.1% for CAS only).[14] Pfaller *et al*.[15,16] have reported that both species distribution and antifungal resistance patterns vary across geographic regions; resistance to caspofungin (0-1.6%) and anidulafungin (0-2.4%) was low and most prevalent among *C. glabrata* isolates. Tan *et al*.[17] evaluated the antifungal susceptibilities of 861 invasive bloodstream isolates of *Candida* species in the Asia-Pacific region, they reported that the resistance to echinocandins remained very low, reduced susceptibility or resistance was the most prominent for CAS against *C. glabrata* isolates. The MIC breakpoint values for AmB were not established yet; however, the most of our isolates had low MIC values for this antifungal. As a result, biofilm formation is an important virulence factor of *Candida* species, and the rate of biofilm production among our bloodstream isolates was found considerably high. The most important biofilm production was observed among the isolates of *C. tropicalis* and *C. parapsilosis*; *C. glabrata* had the lowest biofilm production rate. A certain standardized method has not been established to detect the biofilm formation for yeasts, and the consistency rate between biofilm detection methods was found 66% in this study. Therefore we think that further studies are necessary for the standardization of detection the biofilm formation in *Candida* spp.

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