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Biofilm Formation by and Antifungal Susceptibility of *Candida* Isolates from Urine▽

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Biofilm formation (BF) in the setting of candiduria has not been well studied. We determined BF and MIC to antifungals in *Candida* spp. isolates grown from urine samples of patients and performed a retrospective chart review to examine the correlation with risk factors. A total of 67 *Candida* spp. isolates were grown from urine samples from 55 patients. The species distribution was *C. albicans* (54%), *C. glabrata* (36%), and *C. tropicalis* (10%). BF varied greatly among individual *Candida* isolates but was stable in sequential isolates during chronic infection. BF also depended on the growth medium and especially in *C. albicans* was significantly enhanced in artificial urine (AU) compared to RPMI medium. In nine of the *C. albicans* strains BF was 4- to 10-fold higher in AU, whereas in three of the *C. albicans* strains and two of the *C. glabrata* strains higher BF was measured in RPMI medium than in AU. Determination of the MICs showed that planktonic cells of all strains were susceptible to amphotericin B (AMB) and caspofungin (CASPO) and that three of the *C. glabrata* strains and two of the *C. albicans* strains were resistant to fluconazole (FLU). In contrast, all biofilm-associated adherent cells were resistant to CASPO and FLU. The biofilms of 14 strains (28%) were sensitive to AMB (MIC_{50} of <1 μg/ml). Correlation between degree of BF and MIC of AMB was not seen in RPMI grown biofilms but was present when grown in AU. A retrospective chart review demonstrated no correlation of known risk factors of candiduria with BF in AU or RPMI. We conclude that BF is a stable characteristic of *Candida* strains that varies greatly among clinical strains and is dependent on the growth medium. Resistance to AMB is associated with higher BF in AU, which may represent the more physiologic medium to test BF. Future studies should address whether in vitro BF can predict treatment failure in vivo.

Candiduria is rare in otherwise healthy people (16) but relatively frequent in hospitalized patients. The clinical significance of candiduria is unclear (33, 34). Urinary tract infection (UTI) is the most common type of nosocomial infection (37), and 10 to 15% of UTIs are caused by *Candida* species (2). Candiduria is even more common in the setting of indwelling catheters. Although the majority of infections are caused by *Candida albicans*, *C. glabrata* is emerging as a nosocomial pathogen with a predilection for the urinary tract (17). Of concern is that candiduria is associated with higher mortality (20), especially in patients with comorbidities. Most studies report a relatively low percentage for concomitant candidemia in patients with candiduria. Hence, it has been suggested that candiduria represents a surrogate marker that is associated with, but not causative, of increased mortality (19). Alternatively, candiduria may reflect a relevant infection and appropriate aggressive treatment could improve the outcome. Treatment recommendations are largely based on expert opinion and anecdotal reports. A large prospective study (41–43) demonstrated that fluconazole (FLU) was effective for short-term eradication of candiduria. The precise reason for the inefficacy of therapy in long-term eradication has not been thoroughly investigated. Higher eradication rates were achieved with urinary catheter removal. Another study in renal transplant patients also did not demonstrate an effect of treatment on survival (35). *Candida* readily forms biofilms that attach to solid surfaces (23, 24). Biofilm formation (BF) can be affected by growth conditions and coinfection with other pathogens (11). In addition, several studies have demonstrated that BF can interfere with antifungal therapy (30, 32).

Although candiduria is associated with indwelling devices and low eradication rates with antifungal therapy, systematic studies that measure BF in urine *Candida* isolates and compare it to clinical data are lacking. To date, biofilm studies have been carried out primarily with laboratory strains, and some variability in BF among *Candida* isolates and species has been reported (22, 26). The goal of the present study was to broaden our understanding of the epidemiology and the natural history of candiduria. The BF of *Candida* isolates isolated from candiduric patients was compared in different media. In addition, the response of biofilms to antifungal agents and the effect of known risk factors on BF were assessed.

MATERIALS AND METHODS

Study population and study design. The study was conducted from September 2004 to April 2005 at a teaching hospital of the Albert-Einstein College of Medicine in the Bronx, NY. During that time all urine samples positive for yeast on microscopy were sent that same day to a research laboratory for determination of the fungal burden (i.e., the CFU count) and species identification. No attempts were made to influence the physicians' responses to the report of urine culture or analysis, yielding yeast. The data on patient demographics, underlying diseases, therapy, and hospital outcome were collected by retrospective chart review, which was done according to Albert-Einstein College of Medicine inter-

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nal review board regulations. A total of 137 urinalysis samples were obtained from 117 patients during this time. The distribution of isolates in the overall cohort was 46% *C. albicans*, 40% *C. glabrata*, 10% *C. tropicalis*, and 4% other species, including *C. parapsilosis* and *C. dubliensis*. BF was measured in a subgroup of these isolates. To avoid artifact from variation in storage conditions, we determined the BF for the latter half of this cohort because these strains were all frozen under identical conditions.

***Candida* strains and molecular strain characterization.** Urine samples were reexamined for the presence of bacteria and hyphae by microscopy at ×400 magnification in the research laboratory. Aliquots of urine were plated on Sabouraud agar plates to determine the CFU per milliliter of urine. *Candida* speciation was determined by plating the isolates on BBL CHROM-agar. In some unclear cases where this technique produced ambiguous results, the *Candida* isolates were speciated by VITEK using biochemical methods. To investigate the strain relatedness of sequential isolates, molecular typing was performed by sequencing of multiple PCR-amplified loci (multilocus sequence typing) as previously described (8, 44). For *C. albicans* the loci *AAT1a*, *AAT1b*, *MP1*, *ZWF1*, Ca*VPS13*, Ca*ADP1*, Ca*SYA1*, and Ca*RPN2* were amplified with published primers. For *C. glabrata* the loci *FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1*, and *URA3* were amplified.

Measurement of BF. BF was examined by adherence to polystyrene in pH-balanced RPMI and in artificial urine (AU). Streaked colonies were grown up overnight in SD broth and washed three times in phosphate-buffered saline (PBS). Cells were prepared at a concentration of 10^6/ml in RPMI and AU. AU has a pH of 5.8 and was slightly modified that described previously (7, 15); it was composed of $CaCl_2$ (0.65 g/liter), $MgCl_2$ (0.65 g/liter), NaCl (4.6 g/liter), Na_2SO_4 (2.3 g/liter), sodium citrate (0.65 g/liter), sodium oxalate (0.02 g/liter), KH_2PO_4 (2.8 g/liter), KCl (1.6 g/liter); NH_4Cl (1.0 g/liter), urea (25.0 g/liter), creatinine (1.1 g/liter), 5% (vol/vol) YNB broth, and 8% dextrose (38). In selected experiments 3.25 and 32.5 μM nicotinic acid was added. A 100-μl volume of suspension was plated on 96-well plates (polystyrene, nontreated) and incubated for 48 h at 37°C for adherence and BF. The plates were washed three times manually with PBS to remove nonadherent cells. A semiquantitative measure of BF and viability was detected by an XTT [2,3-bis(2-methoxy-4nitro-5-sulfo-phenyl)–2H–tetrazolium-5-carboxanilide] reduction assay as described previously (31). Briefly, XTT at 0.5 g/liter was added with menadione (10 mM in acetone) to a final concentration of 1 μM in PBS. A 100-μl portion of the XTT-menadione suspension was added to each well, followed by incubation in the dark at 37°C for 2 h. Biofilm was observed by calorimetric changes in each well by determining the optical density at 490 nm. SC5314, a standard *C. albicans* strain, was used as a control strain in each experiment that allowed comparison of the BF between plates. We performed the measurement of BF in three to six wells per sample depending on the test.

Determination of the MIC_{50} in biofilm-associated and planktonic cells. The MICs for amphotericin B (AMB), caspofungin (CASPO), and FLU were determined by microdilution method as described by Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) M27-A methodology (28) with RPMI 1640 buffered to pH 7 by using MOPS. As a quality control measure, the results were considered valid only when the MICs of the quality control isolates fell within the prespecified ranges: *C. parapsilosis* ATCC 22019 (FLU, 2 to 8 mg/liter) and *C. krusei* ATCC 6258 (FLU, 16 to 64 mg/liter). The MIC_{50} was determined as the lowest concentration that produced at least 50% inhibition compared to the growth of the control well for FLU and the absence of visible growth in the case of AMB. The turbidity was then compared both visually and spectrophotometrically.

For the measurement of in vitro antifungal susceptibility on biofilm, the biofilm was prepared for each cell population as described above. After a washing step, the biofilm was exposed to a range of antifungal concentration for FLU (1 to 1,024 μg/ml), CASPO (1 to 1,024 μg/ml), and AMB (0.125 to 32 μg/ml) for 48 h. After 48 h, the cells were washed with PBS three times, and an XTT reduction assay was performed as described above. The calorimetric reading at 490 nm was used to compare the reduction of growth relative to the control as a result of coincubation with antifungal drugs.

Statistical methods. Chi-square, *t* tests, and linear regression were performed with SSPS version 11.0.3 (SSPS, Inc., Chicago, IL).

RESULTS

Characteristics of patients with candiduria. BF was determined in 67 *Candida* isolates recovered from 55 patients, including 2 *Candida* isolates from blood and 10 sequential isolates from urine. The species distribution was as follows: *C. albicans* (54%), *C. glabrata* (36%), and *C. tropicalis* (10%). A retrospective chart review determined that the patients exhibited the expected standard risk factors associated with candiduria, including female sex, old age, diabetes, prior antibiotic use, and indwelling urinary tract catheters (Table 1).

TABLE 1. Patient characteristics[a]

Variable	No. of patients (%)
Female patients	40 (77.4)
Race	
White	13 (36.1)
Black	19 (43.6)
Hispanic	16 (21.3)
Nursing home resident	20 (36.3)
Dialysis	6 (10.3)
Hospitalized within the last 6 mo	26 (47.2)
Diabetes	30 (54.5)
Foley catheter	34 (61.8)
Antibiotic use in the prior mo	30 (54.4)
Hospitalized in ICU	14 (26.4)
Central line	17 (32.6)
UA, >20 WBC	20 (36.6)

[a] The median age in years for the patients was 76.5. WBC, white blood cell count.

BF in *Candida* isolates from urine varies greatly and depends on the medium. The metabolic activity in preformed biofilms was measured by the XTT reduction assay after 48 h of growth in standard morpholinepropanesulfonic acid (MOPS)-buffered RPMI and in AU, a medium that better mimics the in vivo growth environment of urine. BF varied considerably among clinical isolates (Fig. 1A) under both growth conditions. Under standard growth conditions in MOPS-buffered RPMI, BF was significantly enhanced in *C. glabrata* isolates compared to *C. albicans* and *C. tropicalis* isolates (Fig. 1B). This difference, however, was not found when BF was measured after growth in AU, mainly because *C. albicans* formed more biofilm. Of note is, however, that there was no correlation between XTT values obtained in RPMI and AU in the individual *C. albicans* isolate ($R^2 = 0.026$, $P = 0.4$) (Fig. 1C). In contrast, BF in AU and RPMI of *C. glabrata* were correlated ($R^2 = 0.738$, $P < 0.001$). Nine *C. albicans* isolates produced more biofilm in RPMI than in AU, whereas three *C. albicans* and two *C. glabrata* isolates produced more biofilm in AU than in RPMI. More detailed growth studies performed with eight clinical strains established that BF in AU or RPMI reflected growth and correlated with the colony count of scraped biofilms ($R^2 = 0.9$). Also, the addition of glucose to RPMI (8%) resulted in increased BF predominantly in *C. albicans* strains but not in *C. glabrata* or *C. tropicalis* (data not shown). Previous studies reported that nicotinic acid limitation regulates the silencing of adherence genes in *C. glabrata* (9), which we hypothesized could also affect BF. We examined BF in the presence or absence of nicotinic acid in 12 *C. glabrata* strains and found no difference (0.42 ± 0.052 versus 0.44 ± 0.09 and 0.46 ± 0.08 [$P > 0.5$] for AU alone, AU with 3.25 μM nicotinic acid, and 32.5 μM nicotinic acid, respectively). In addition, BF was comparable on standard polystyrene and latex 96-well plates (data not shown) both if grown in AU and if grown in RPMI.

Sequential *Candida* isolates from patients. Sequential urinary *Candida* spp. isolates were available from nine patients

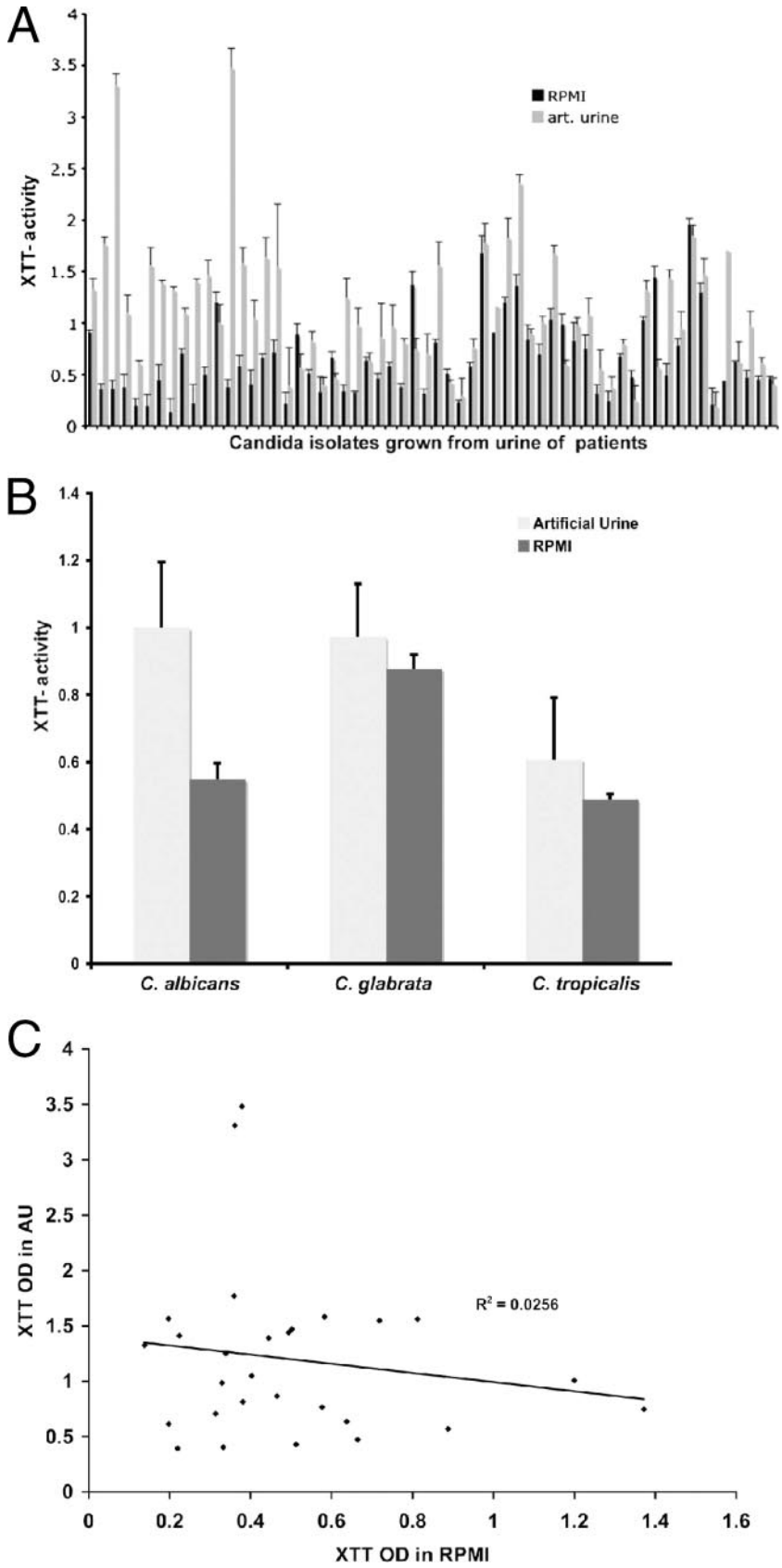

FIG. 1. (A) BF is highly variable among clinical isolates and is dependent on the growth medium. "High" and "low" biofilm formers were defined as clinical isolates that exhibited XTT activity greater than or less than the geometrical mean (optical density of 0.55). (B) Species-dependent differences in BF were only observed if biofilms were grown in RPMI. Here *C. glabrata* isolates produced significantly ($P = 0.003$) more biofilm than did *C. albicans* and *C. tropicalis* ($P = 0.01$) isolates. (C) No correlation was found between BF in AU and RPMI.

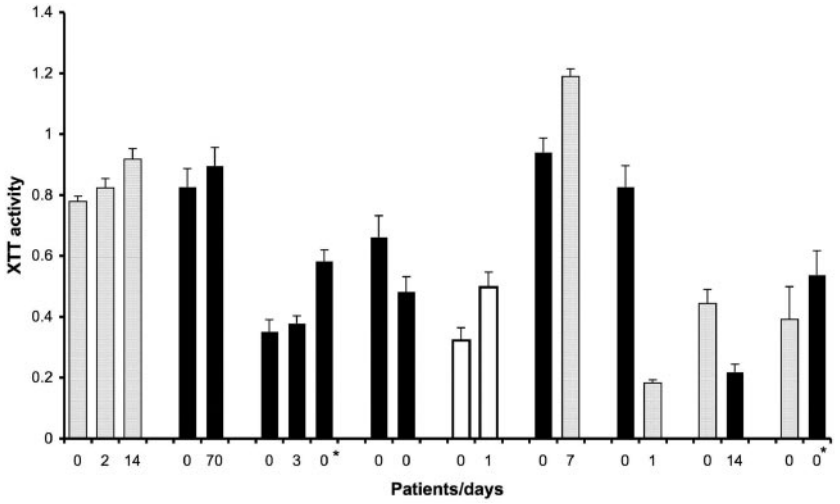

FIG. 2. BF for sequential isolates is stable unless there were more than one species in the sample. Gray bars indicate *C. albicans* isolates, black bars indicate *C. glabrata*, and white bars indicate *C. krusei.* ⋆, *Candida* isolated from blood.

(Fig. 2). In two cases blood isolates were also available. Molecular typing determined that the sequential isolate in five patients represented the same strain, whereas in four patients both *C. albicans* and *C. glabrata* were recovered from urine. The time between the first and last urine samples ranged from 0 to 70 days. The fungal burden in urine (log CFU) between sequential urine samples did not differ significantly (<10% of total log change) in patients with identical strains but varies in infections with more than one strain. In one patient two different *Candida* species were grown from blood and urine. The degree of BF among sequential isolates remained stable even in isolates that were recovered 70 days apart. In contrast, in patients infected with more than one strain the capacity for BF among the recovered strains was more variable. In one patient the *C. glabrata* blood isolate produced more biofilm than did the urine isolate. We conclude that BF is a stable characteristic of a *Candida* strain that is affected by the chronicity of infection.

MIC in biofilm-associated and planktonic *Candida*. The MICs of CASPO, AMB, and FLU were measured in 50 urine *Candida* isolates that were grown as planktonic or biofilm-associated cell phenotypes (Table 2). As expected, the majority of the planktonic phenotypes of *Candida* isolates were sensitive to standard antifungal drugs. Two *C. albicans* and three *C. glabrata* strains were found to be resistant to FLU. In contrast, biofilm-associated yeast cells were not inhibited by FLU, and only 30% of them were inhibited by AMB. Most of them also exhibited inhibition by CASPO but the MICs were greater than the drug concentrations that are expected in serum. Because BF was dependent on medium, we grew the isolates in both AU and RPMI and then compared the MICs to biofilm-associated cells under standard conditions (Fig. 3). These experiments determined that the degree of BF in AU correlated ($R^2 = 0.29$, $P = 00.1$) with inhibition by AMB, whereas there was no correlation in RPMI ($R^2 = 0.0024$, $P = 0.29$) in the *Candida* isolates. We conclude from these data that BF in AU may be a better growth medium to predict the response to antifungal treatment.

TABLE 2. MICs of planktonic and biofilm-associated *Candida*

Species	MIC (μg/ml) in biofilms grown in RPMI or AU				MIC_{50} (μg/ml) for planktonic *Candida*		
	AMB AU	AMB RPMI	CASPO AU	CASPO RPMI	AMB	FLU	CASPO
C. albicans	16	8	512	256	0.125	0.25	0.125
	8	8	512	512	0.25	0.25	0.125
	8	8	512	512	0.06	0.25	0.125
	0.125	0.25	16	8	0.06	32	0.125
	32	2	256	256	0.06	0.25	0.125
	8	8	512	512	0.06	0.5	0.125
	4	4	512	512	0.25	1	0.125
	8	8	512	512	0.125	1	0.125
	16	8	512	512	0.25	>64	0.125
	4	2	512	512	0.06	1	0.125
	0.25	0.125	32	32	0.25	0.25	0.125
	16	4	256	512	0.06	4	0.125
	4	2	512	512	0.125	8	0.125
	16	8	512	512	0.06	0.5	0.125
	0.5	1	16	16	0.125	0.125	0.125
	8	4	512	256	0.06	2	0.125
	4	4	32	16	0.125	64	0.125
	4	2	512	512	0.06	0.25	0.125
	8	4	32	16	0.5	8	0.125
	0.5	0.5	16	8	0.25	16	0.125
	8	1	128	512	0.125	16	0.125
	2	0.5	32	32	0.06	2	0.125
	8	4	1024	1024	0.25	4	0.5
	0.5	0.25	512	512	0.125	4	0.125
	0.25	0.25	4	4	0.25	2	0.125
C. glabrata	0.5	0.25	16	8	0.25	8	0.125
	4	16	512	512	0.5	1	0.5
	0.25	0.25	32	32	0.25	>64	0.125
	0.5	0.25	32	32	0.25	>64	0.125
	0.5	0.5	32	16	0.25	>64	0.125
	1	0.5	16	8	0.25	4	0.125
	2	4	512	512	0.125	2	0.125
	4	2	16	16	0.125	4	0.125
	2	0.25	4	2	0.125	4	0.125
	8	2	512	256	0.25	32	0.25
	0.25	0.25	16	8	0.25	2	0.125
	1	1	32	32	0.06	0.5	0.125
C. tropicalis	2	4	32	32	0.125	4	0.125
	32	32	512	512	0.125	2	0.125
	2	2	16	32	0.5	0.125	0.125

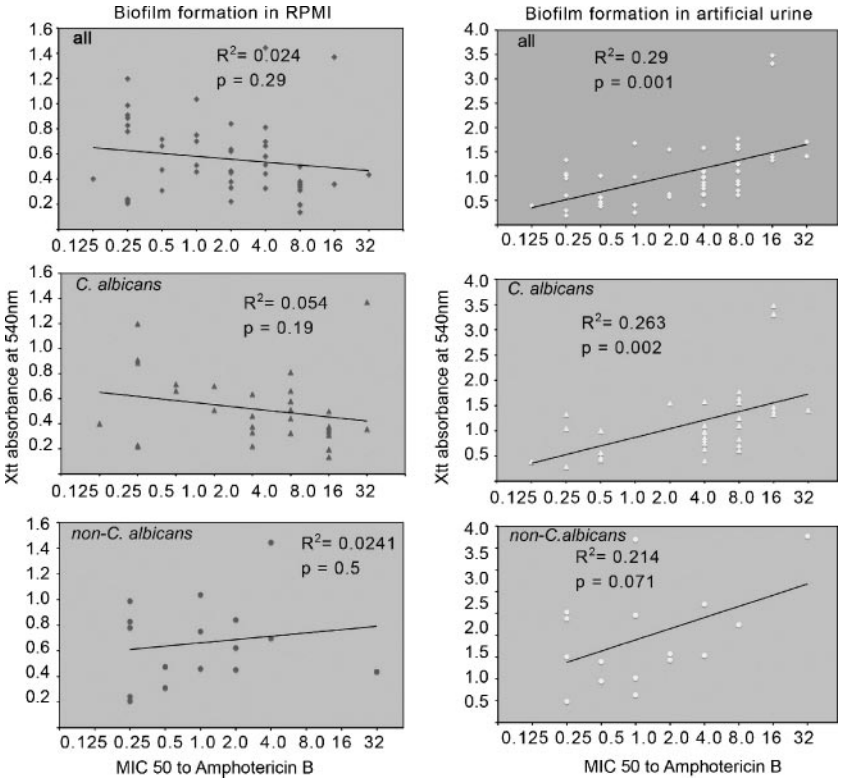

FIG. 3. The degree of inhibition by AMB correlated with the degree of BF only if the biofilms were grown in AU and not in RPMI (*R* as determined by linear regression).

Association of patient characteristics and BF. Because of the extensive variability of BF observed among strains and because environmental factors such as glucose, coinfection, and adherence can affect BF, it is reasonable to hypothesize that certain patient characteristics may predispose a patient to be infected or colonized with a high or low biofilm former. To examine this hypothesis, a retrospective chart review was performed. We determined that BF in *Candida* isolates of patients with potential risk factors, including urinary catheter, diabetes, or antibiotic treatment, did not differ from the BF of *Candida* isolates of patients that did not exhibit these risk factors (data not shown). Furthermore, the degree of BF was not associated with a higher CFU in urine, leucocyturia (white blood cell count of >20) or concomitant bacterial infection. We conclude from these data that high BF in *Candida* isolates appears to be a stable but highly variable characteristic of an individual *Candida* strain that does not appear to be associated with specific conditions or characteristics in the host. This was irrespective of the medium (AU or RPMI) the BF was measured in.

DISCUSSION

BF is associated with adherence to surfaces and presumed to promote persistence of infection (10, 27). In the present study we investigated BF in *Candida* isolates of patients with candiduria. We can draw several conclusions from our data. (i) BF varies greatly among clinical *Candida* isolates from urine and is highly dependent on growth conditions. (ii) Biofilm production by *C. albicans* is enhanced if it is grown in AU. (iii) The degree of BF and the inhibition by AMB correlate better if the *Candida* isolate is grown in AU than if grown in standard RPMI. (iv) BF is stable during chronic infection. (v) No association between individual host factors and the capacity for BF in the *Candida* spp. was observed.

Studies of BF often use *Candida* strains passaged in laboratories (3, 31) that have been adapted to culture media, and thus they may not be representative of BF in clinical strains. Microevolution of fungal pathogens occurs in vitro and in vivo and can affect major virulence factors (5, 6, 13, 14, 36). In the present study we determined that BF among clinical *Candida* strains varied significantly and ranged from high to low. These findings differed from previous studies in which 26 *Candida* strains isolated from patients with oropharyngeal candidiasis, where most isolates manifested comparable BF (18). Overall, we found that a larger percentage of *Candida* isolates produce biofilm in the conditions described here than has previously been described (38), although more recent studies have also found BF to be common especially in non-*albicans* strains (25). It is not known whether this finding could be specific to urine isolates, although some studies suggest that there is no difference between the BF of *Candida* isolated from blood versus that isolated from other sites (39). Several studies have documented that BF correlates with XTT absorbance (26, 31), although one study also reported limitations of the XTT assay in studies comparing different *Candida* species (21). We found that more CFU is recovered from biofilms that exhibit higher XTT values. However, most studies determine the BF after growth in RPMI or in YNB growth medium (30, 31), whereas

we also examined BF in AU, which presumably represents a more physiologic growth environment. Interestingly, we found that BF is highly dependent on the growth medium. In particular, *C. albicans* strains produced significantly more biofilm in AU than in RPMI. To some extent this difference may be the result of increased glucose levels in artificial urine. More importantly, however, a change in growth conditions did not simply increase BF in all *Candida* isolates but could have a variable effect on the individual *Candida* strain. As such, no correlation of BF in RPMI and AU was documented. For example, some *C. albicans* strains that were identified as high biofilm producers in one medium could be classified as a low biofilm producer when grown in another medium. This finding suggests that BF is not a stable characteristic in a *Candida* strain. However, this conclusion is unlikely given that BF was consistent in repeated assays and was stable in serial isolates from individual patients. Our data raise the question as to which assay would best predict BF in vivo. The decision regarding which assay to use should take into consideration that BF in *Candida* isolates from blood and urine may differ. In one patient from which the same *Candida* strain was recovered from blood and urine, BF in the blood isolate was increased relative to the urine isolate. Especially for non-*albicans* species other studies have also reported more BF in the blood-derived *Candida* strains compared to strains from other sites (25, 38). Factors such as pH may affect BF and may in part explain these observations.

Cells in biofilms display phenotypic traits, such as increased resistance to antimicrobial agents and protection from host defenses, that are dramatically different from their planktonic counterparts. Our assays confirm previous findings that *Candida* biofilms display resistance to FLU, whereas the planktonic cells are susceptible (1, 12, 45). This is of particular importance since FLU is the drug of choice in patients with candiduria (41, 42). At the same time this may explain why FLU treatment often does not achieve long-term eradication even if indwelling urinary catheters are removed (35, 41, 42). CASPO is a commonly used antifungal drug, although it is usually not the drug of choice for UTI because less than 1% of the drug is excreted in urine. In contrast to previous studies, we did not document in vitro activity of CASPO against *Candida* biofilms (4). Our study examined MIC in a large number of clinical isolates, and this may be in part explained by differences in CASPO activity between *Candida* strains and species (4, 40). In addition, the length of treatment of biofilms differs significantly between in vitro and in vivo models of antifungal susceptibility in the setting of biofilms because in the in vivo model rabbits are treated for 7 days with antifungal medication (40). About one-third of biofilms were successfully inhibited by AMB. The MIC of AMB was comparable in biofilms grown in AU and in RPMI. However, for some strains, and especially for *C. albicans*, the degree of AMB-induced inhibition correlated only with the metabolic activity of biofilms grown in AU and not biofilms grown in RPMI. In summary, our data demonstrate that even in *Candida* isolates classified as low biofilm producers FLU is not an effective drug. The growth of *Candida* as an adherent biofilm-associated cell phenotype is not suppressed by FLU. These data further support the view that FLU resistance in *Candida* biofilms is multifactorial (29). In contrast, resistance to AMB is correlated with the degree of BF in AU and may be effective against *Candida* biofilms. Hence, our data support revisiting strategies to treat candiduric patients with brief courses of intravenous AMB rather than azoles or CASPO (12).

In vitro BF can be affected by external factors such as coinfection with other pathogens, glucose concentration, antibiotic treatment, and pH. In addition, a more adherent cell phenotype may be selected in the presence of a surface such as a urinary catheter. Hence, we examined the hypothesis that host factors and specific conditions in the local microenvironment of the bladder may select for strains with high BF and constitute a risk factor for persistent candiduria. Our retrospective analysis did not document a correlation with host characteristics, nor did our results support evidence that an indwelling urinary catheter, diabetes, or concomitant bacterial infection select for a *Candida* population that produces more BF. In contrast, we conclude from our data that BF is an inherent and stable characteristic of a *Candida* strain. Supportive of our conclusion is molecular typing data that also could not associate multilocus genotypes of *Candida* with extensive variation in BF (26).

The extent to which strain differences in BF affect the pathogenesis and outcome of candiduria is not clear. A limitation of our study was that treatment of UTI was not controlled for because it was a retrospective analysis. However, it is conceivable that the failure to eradicate candiduria in these individuals is a result of BF. We propose that controlled studies should be undertaken to optimize antifungal treatment based on strain characteristics of the *Candida* strain because candiduria is associated with decreased survival and treatment is not successful. These studies will help to shed light on the question of whether candiduria is a condition that should be treated aggressively or if it is only a surrogate marker for poor outcome.

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