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ORIGINAL ARTICLE

Antifungal Susceptibility of Planktonic Cells and Biofilms of *Candida tropicalis* Isolated from Hospital Acquired Infections in Pediatric Intensive Care Units.

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ABSTRACT

Background: *Candida tropicalis is one of the main non albicans Candida which is implicated in many serious infections. Biofilm formation is an important virulence factor in Candida species. This study was designed to determine the ability of C. tropicalis isolated from infected patients in Pediatric Intensive Care Units (PICUs) to form biofilm, also to test the antifungal susceptibility of Candida tropicalis planktonic cells and biofilms.* ***Methodology:*** *Candida tropicalis isolates were collected from patients suffering from hospital acquired infections in PICUs of Mansoura University Children hospital. The isolates were identified by Analytic Profile Index (API) 20 C. The ability of the isolates to form biofilm was measured by crystal violet assay. The susceptibility of planktonic cells and the biofilms to amphotricin B and fluconazole was determined according to Clinical and Laboratory Standards Institute (CLSI) M27 A2 guidelines and by crystal violet assay respectively.* ***Results****: Thirty seven isolates of C. tropicalis were detected during period of study. About sixty percent of the isolates (23 isolates) were biofilm producers. All planktonic cells were susceptible to amphotricin B and 27 isolates (73%) were susceptible to fluconazole. All biofilm cells were resistant to amphotricin B and fluconazole.* ***Conclusion:*** *The biofilm cells expressed higher resistance to the tested antifungal agents more than planktonic cells. Formation of biofilm may represent an important cause of the poor response of infections caused by C. tropicalis to amphotricin B and fluconazole therapy.*

INTRODUCTION

Candida tropicalis is one of the frequent non albicans species which cause infections in immunocompromised and critically ill patients [1-3]. *C. tropicalis* is considered one of the main causes of invasive candidal infections in pediatric intensive care units (PICUs) [4].

Biofilm production is one of the main virulence factors in *Candida tropicalis* [5]. Biofilm formation helps the adherence of *C. tropicalis* and other *Candida* species to medical devices like urinary and intravascular catheters [6]. The use of these devices is especially important and inevitable in patients of intensive Care Units (ICUs)[7]. These biofilms are candidal microcolonies embedded in polymeric matrix which act as a protective barrier from the effect of antifungal agents [2,8]. In addition, they represent an important site for *Candida* colonization which acts as a source for serious infections like candidemia [9]. So, biofilms producers candidal cells are highly virulent than planktonic cells. Biofilm production may cause therapeutic failure increasing morbidity and mortality of *C. tropicalis* infections [10-12].

Amphotricin B (AMB) and fluconazole (FLC) are main antifungal agents used in treatment of candidal infections[13]. These agents are active against planktonic *C. tropicalis* cells [14-15].

Little data is available about the susceptibility of *C. tropicalis* planktonic and biofilm cells to commonly used antifungal agents in pediatric critical care patients.

This study aimed at the assessment of the ability of *C. tropicalis* causing hospital acquired infections in patients of PICUs to form biofilm. Also, identify the activity of AMB and FLC antifungal agents against planktonic and biofilm *C. tropicalis* cells.

METHODOLOGY

This study was carried out including pediatric patients (age <18 years) admitted to PICUs of Mansoura University Children Hospital during period extending from March 2014 to March 2016 and presented with signs and symptoms of hospital acquired infections according to CDC criteria [16]. Urine and blood samples were collected. Samples were processed in department of medical microbiology and immunology, faculty of medicine, Mansoura University. *Candida* isolates were identified by conventional microbiological methods. Non *albicans Candida* were differentiated from *Candida albicans* by absence of germ tube formation [17].

C. tropicalis was identified using API 20 C according to the the manufacturer's instructions.

Antifungal susceptibility testing of the planktonic cells: was done by broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) recommendation (M27-A2) for yeast [18].

Biofilm assay:

The ability of the isolates to form biofilm was tested on polystyrene microtiter plates. Ninty micron of Sabouraud's dextrose broth supplemented with 8% glucose and10 µl of standardized cell concentration of 1 × 10^6 cells/ml were added to the wells of flat-bottom 96-well microtiter plates. The plates were incubated for 48 hours at 37°C. After the biofilm was formed, the medium was removed. The wells were washed three times by phosphate buffer saline (PBS; pH 7.2) for removal of non adherent cells [19].

Assay of bofilm was done by crystal violet staining: 385 µl of 0.4% aqueous crystal violet were added to each well. Cells were covered with crystal violet for 45 min at room temperature. The cells were washed with (PBS; pH 7.2) three times. De-staining was done with 95% ethanol for 30 min. The absorbance of the dye in solution at 595 nm was determined which refer to the strength of the biofilm formed. Wells contain broth alone were used as negative control [19].

Biofilm susceptibility assay:

The susceptibility assay of the biofilm cells was performed to AMB and FLC.

Biofilm formers in the previous step were tested to determine the antifungal susceptibility. Biofilms were formed as described before. After 24h, the medium was aspirate and wells were washed three times with sterile PBS.

Serially double diluted concentrations of AMB and FLC in RPMI 1640 medium were prepared. For AMB concentrations were range from 0.03 to 32 µg\ml, and for FLC concentrations were range from 0.5 to 1024 µg\ml. Aliquot (200 µL) of each concentration was added to the wells. The plates were then incubated for 48h at 37 °C. Antifungal free wells: containing inoculums and RPMI 1640 and biofilm free wells contain RPMI 1640 are considered as controls.

Minimum biofilm eradication concentrations (MBECs): were defined as the minimum concentration of antifungal agent (FLC and AMB) required for 80 % biofilm reduction compared with antifungal-free control well.

The degree of inhibition was determined using the crystal violet assay. The percentage of biofilm eradication was calculated using the following equation [1−(A595 of the test/A595 of nontreated control)]×100, where A595 is the absorbance at 595 nm [20].

RESULTS

A total of thirty seven isolates were identified as *Candida tropicalis* during the period of study. *Candida tropicalis* were identified by API 20 C.

Distribution of *Candida tropicalis* isolates: Sex and age group distribution of enrolled patients are shown in table (1). About sixty percent (59.5%) of the isolates were from urine samples (22 isolates). Fifteen isolates were detected in bloodstream infections.

Table 1: Epidemiological features of patients

Sex	***NO (%)***
Male	16 (43.2)
Female	21 (56.8)
Age (month)	
Mean ±SD (min-max)	22.8 ± 33.7 (2 m-12 years)
Samples	**NO (%)**
Urine	22 (59.5)
Blood	15 (40.5)

Antifungal susceptibility of the planktonic cells is detailed in table 2. All isolates were sensitive to AMB. The MICs of AMP were ranged from 0.031 µg/ml to1 µg/ml. About 73% of the isolates were susceptible to FLC with MICs ranged from 4 µg/ml to 64 µg/ml. Three isolates (8.1%) were SDD and seven isolates (18.9%) were resistant to FLC.

Table 2: Activity of fluconazole and amphotericin B against *Candida tropicalis* planktonic cells isolated from blood and urine samples

Clinical sample	***Antifungal drug***	***MIC (µg/ml) Range***	***MIC 50***	***MIC 90***	***No (%) of isolates***		
					S	**SDD**	**R**
Urinary isolates	AMB	0.062-1	0.25	1	22(100%)	0	0
(22)	FLC	4-64	8	64	16(72.7)	2(9.1%)	4(18.2%)
Bloodstream isolates	AMB	0.031-1	0.125	1	15(100%)	0	0
(15)	FLC	4-64	16	64	11 (73.3%)	1(6.7%)	3(20%)
Total (37)	AMB	0.031-1	0.25	1	37 (100%)	0	0
	FLC	4-64	8	64	27 (73%)	3(8.1%)	7(18.9%)

S: susceptible SDD: susceptible dose dependant R: resistant

Biofilm formation and antifungal susceptibility of biofilm cells: Twenty three isolates (62.2%) were biofilm formers. The susceptibility pattern of biofilm formers and non bifilm formers is described in figure (1). All biofilm cells were resistant to AMB and FLC table (3). There is a statistically significant difference of the FLC susceptibility profile between biofilm producers and non biofm producers (P value < 0.05).

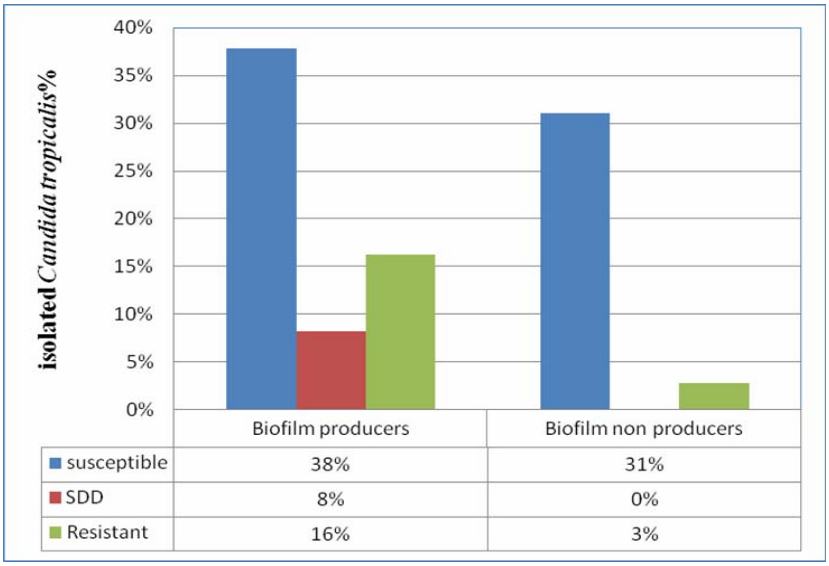

Fig. 1: Fluconazole susceptibility profile of biofilm producers and non producer the two groups were compared by Chi-square test P value= **0.03**

Table 3: Antifungal susceptibilities of planktonic and biofilm cells of *Candida tropicalis* isolates

Antifungal agent	*Planktonic cells*			*Biofilm cells*		
	MIC (µg/ml) Range	**MIC 50**	**MIC 90**	**MBEC (µg/ml) Range**	**MBEC 50**	**MBEC 90**
AMB	0.062-1	0.25	1	2-16	4	8
FLC	4-64	8	64	128- >1024	512	>1024

DISCUSSION

Hospitalized pediatric patients especially those in PICU are susceptible to invasive candidal infections. In addation to *C. albicans*, *C. tropicalis* represents one of the main non albicans species in pediatric setting [1,21]. Candidiasis in critical care units is usually associated with indwelling devices like urinary and vascular catheters. These devices act as a target for the formation of biofilm [11,22]. The present study was designated to investigate the ability of *C. tropicalis* to form biofilm and the antifungal susceptibility pattern of planktonic cells and biofilm cells of *C. tropicalis* causing infections in PICU.

In this study, *C. tropicalis* was isolated from blood and urine samples. This may be due to the access of *Candida tropicalis* to blood stream and urinary tract via venous and urinary catheter which used widely in these patients. Higher prevalence of *C. tropicalis* was from urine samples(60%). This agrees with other studies [23-24]. *C. tropicalis* was described as the most common non albican species that cause candiduria especially in catheterized critical care patients [23]. Also, the use of broad spectrum antibiotics in PICU patients suppresses the bacterial flora in the gut and lower urogenital area which promote *Candida* colonization [25].

Biofilm production is considered one of the important virulence factors of the *C. tropicalis*. It plays a relevant role in serious infections like bloodstream infections [2,6]. Biofilm is responsible for the persistence of these infections in spite of the proper antifungal therapy to which *Candida tropicalis* is susceptible by antifungal testing [2,9].

This study was designed to test the ability of *C. tropicais* isolates from PICU to form biofilm. In this study, crystal violet assay was used to measure the ability of the isolates to form biofilm. Twenty three isolates (62.2%) were biofilm producers. Our result agrees with other studies like Goe et al. and Deorukhkar et al.[26-27], they found in their study on non albicans *Candida* that only about 60% of *C. tropicalis* were

biofilm producers. However, this result disagrees with other result like Negri et al. [28], they found in their result all *C. tropicalis* were able to form biofilm. Also, this result disagrees with result of Aslan and Gülmez,[29], they found in their study none of *C. tropicalis* isolates were biofilm producers. This difference may be due to the different detection method and the medium for testing biofilm formation, Negri et al. [28] search in the ability of *C. tropicalis* to form biofilm in artificial urine.

In this study, all *C. tropicalis* isolates were susceptible to amphotricin B. this finding match with results of other studies [30-34]. These results conclude that resistance of AMB is still uncommon in *C. tropicalis.*

Azole antifungal group represents an important option for treatment of candidal infections. The extensive use of azole agents especially in high risk patients like ICU patients leads to the emergence of resistance especially to FLC[35-36].

Regarding the susceptibility of isolated *C. tropicalis* to FLC, about 73% of the isolates were susceptible to FLC. Three isolates (8.1%) were SDD and seven isolates (18.9%) were resistant to FLC. This result is concurrent with the result of Singla et al. [33] and with the result of Punithavathy et al.[36], these studies found the FLC resistance were about 20%. However, our result concerning fluconazole resistance in *C. tropicalis* candidemia is much higher than the results of Bassetti et al.[37], they found the resistance of *C. tropicalis* candidemia only (4.5%). This higher rate of resistance may be due to the frequent use of FLC in treatment of fungal infections in our hospital.

The antifungal susceptibility testing of biofilm cells was performed on microtitration plate. The quantitation of the biolfim was performed by crystal violet assay. Biofilm is measured in different concentration of AMB and FLC. The degree of eradication is measured as parentage from the biofilm formed in the drug free well at OD 595nm [21]. There was a great difference in the MICs and MBECs values of the planktonic and biofilm cells respectively. All planktonic cells were susceptible to AMB and 73% of the isolates were susceptible to FLC. However, all biofilm cells were resistant to both antifungal agents with higher MBECs values. This result agrees with many reports before [14,29]**.** Also, this result was in match with results of other studies on biofilm formed by other species *C. albicans* [38-39].

CONCLUSION

The majority *C. tropicalis* causing hospital acquired infections in PICU has the ability to form biofilm. The current used antifungal agents are not able to eradicate biofilm and cannot treat these infections caused by biofilm producers in spite of having activity against planktonic cells. Further studies on other agents that can have the capacity to eliminate candidal cells in biofilm agents are recommended.

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