Adherence and Biofilm Formation in *Candida albicans* Strains Isolated from Different Infection Sites in Hospitalized Patients

OMAR SADIK[1,2], LIA MARA DITU[1,2]*, IRINA GHEORGHE[1,2], GRATIELA GRADISTEANU PIRCALABIORU[1,2], CORALIA BLEOTU[3], OTILIA BANU[4], NICOLETA MEREZEANU[5], ANDREI CRISTIAN LUPU[6], VERONICA LAZAR[1,2], MARIANA CARMEN CHIFIRIUC[1,2]

[1]University of Bucharest, Faculty of Biology, 91-95 Splaiul Independentei, 050095, Bucharest, Romania

[2] Research Institute of the University of Bucharest, 34-36 Mihail Kogalniceanu Blvd., 050107, Bucharest, Romania

[3] Institute of Virology St.S. Nicolau, 285 Mihai Bravu Road, 030304, Bucharest, Romania

[4] Institute for Cardiovascular Diseases Prof. C.C. Iliescu, 258 Soseaua Fundenui, 022328, Bucharest, Romania

[5] University of Oil and Gas, 39 Bucuresti Blvd.,100680, Ploiesti, Romania

[6] Ion Ionescu de la Brad University of Agricultural Sciences and Veterinary Medicine of Iasi, Faculty of Veterinary Medicine, 3 Mihail Sadoveanu Alley, 700490, Iasi, Romania

Adherence of Candida albicans to the cellular and inert substratum contributes to its commensal status, but also plays an essential role in the development of fungal infections, particularly in hospitalized and immunodepressed patients. This study evaluated the adherence capacity and biofilm formation of 109 C. albicans strains isolated from upper respiratory tract secretions, wound secretions, urine culture, blood culture and stool culture taken from patients hospitalized for cardiovascular surgery. The strains were originally identified as C. albicans, based on their morphological characteristics and then confirmed by the Vitek II automatic system. All tested strains adhered to the cellular substratum, the isolates from stool culture, urine and thrush secretion exhibiting the most intensive adhesion capacity, the predominant adherence pattern being the aggregative one. Patient age and gender did not exhibit a significant influence on the adhesion process. The strains with the highest biofilm production capacity were the ones isolated from respiratory tract secretions and urine cultures. Statistically significant correlations could be established among a high number of yeast cells adhered to HeLa cells and i) the aggregative adherence pattern and ii) the moderate to high capacity to form biofilms on the inert substratum. These results could suggest the implication of common fungal structures in the colonization of inert and cellular substrata, while the elucidation of the molecular mechanisms involved in these processes could bring an important benefit to the appropriate management of fungal infections, depending on the isolation source.

Keywords: Candida albicans,adherence, biofilm

Candida infections are particularly prevalent among the hospital-acquired infections [1, 2]. The evolution of *Candida* spp. into an important nosocomial pathogen is related to specific risk factors associated with modern medical therapeutics, such as the use of broad spectrum antibiotics, cancer chemotherapy, immunosuppressive agents following organ transplantation and implanted medical devices [3]. While biofilm formation by pathogenic yeasts has been recognized as a potentially important medical issue, relatively little is known about the variation and evolution of this virulence factor within the yeast populations [4]. Biofilms are represented by aggregates of unicellular micro-organisms forming multicellular structures that adhere to surfaces. Their formation occurs in response to a variety of cues, including high cell density, nutrient deprivation and environmental stress factors [5]. *Candida* spp. strains can colonize a wide variety of host tissues by developing biofilms. For example, *Candida albicans* colonizes several sites such as the vaginal and the oral epithelia, developing a biofilm that, in immunocompromised patients, can disseminate into the bloodstream and cause fatal systemic infections [6]. *Candida* spp. strains also form biofilms on inert surfaces such as urinary or central venous catheters, dental prostheses, and other medical devices. It has been shown in different experimental models that *C. albicans* adherence to plastic surfaces and biofilm formation are favored in certain conditions, such media with low glucose concentration [7]. The yeast cells grown in biofilms are often resistant to drug therapy and can lead to reccurent infections [8]. *C. albicans* biofilm formation is initiated when planktonic cells adhere to a surface, presumably through cell wall-located adhesion molecules, and begin to aggregate into a microcolony. Proteins encoded by the *ALS* gene family and are involved in the adhesion of *C. albicans* to host tissues [4, 9]. Once a basal layer of cells is formed, an extracellular matrix of proteins and polysaccharides is produced to consolidate the new biofilm [4]. *C. albicans* biofilms produce small amounts of extracellular material under static conditions, but higher quantities are found in the case of *in vivo* biofilm associated infections [10].

We aimed a phenotypic characterization of adherence and biofilm formation capacity of *C. albicans* strains isolated from fungal infections occurred in patients hospitalized for cardiovascular surgery, during September-November 2015.

Experimental part

A number of 109 *C. albicans* strains were isolated from different clinical specimens as follows: 23 urine cultures , 23 tracheal secretions , 20 wound secretions ,19 sputum, 10 bronchial secretions, 5 blood cultures, 3 stool culture , 2 thrush secretions, and 2 ATCC strains .

The strains were initially identified after seeding on the Sabouraud Dextrose agar mediums, based on their morphological characteristics. Afterwards, the strains were biochemically confirmed by the VITEK II automatic

* email: lia_mara_d@yahoo.com

analyzer. The attachment to the inert substratum and the subsequent biofilm production was assessed by using a micro-titer method. The *C. albicans* strains were grown overnight in Sabouraud broth solution at 37^{0}C and centrifuged at 150 rpm. The cells were harvested, washed with phosphate buffered saline (PBS) and standardized to 1 × 10^7 yeast cells/ml. Afterwards 100 µL from the yeast cell suspension were placed in the wells of a 96-well micro plate and incubated for 96 h at 37^{0}C. After incubation the presence of the biofilm in each well was quantified by two distinct methodologies: a semi-quantitative method based on the microscopic examination with an inverted microscope and violet crystal (CV) staining, followed by reading the optic density at a 490 nm with an ELISA reader. The experiments were performed in triplicate.

For the adherence to the cellular substratum represented by HeLa cells, the Cravioto adapted method was used [11]. Adhesion to cellular substratum was investigated using 24 h HeLa cells monolayers of 80% confluence grown in 6 multi-well plastic plates, in DMEM medium supplemented with antibiotics. The growth medium was removed by washing the wells three times with PBS. A 1 mL aliquot of the final fungal suspension was aseptically added to each well containing the cellular monolayer. The plates were incubated at 37°C for 2 h, allowing the yeast cells to adhere to the cellular surface. Then, the cells were washed three times with PBS and fixed with methanol for 5 min. Afterwards the wells were stained with the Giemsa solution (1:10) (Merck, Darmstadt, Germany) for 20 min, washed with tap water and dried at room temperature, examined microscopically (magnification, ×2500) with I.O. and photographed with a Contax camera adapted for Zeiss microscope, in order to establish the adherence index values. The adherence index was calculated as the ratio between the number of the eukaryotic cells with adhered yeast cells and 100 eukaryotic cells counted on the microscopic field. The adherence patterns were defined as: localized adherence (LA) when tight clusters of microorganisms were noticed on the HeLa cell surface, aggregative adherence (AA) when a microbial stacked brick pattern characterize the attachment, and diffuse adherence (DA) when the bacteria adhered diffusely, covering the whole surface of the cell [12].

Results and discussions

C. albicans is an important pathogen being the fourth most common organism isolated from bloodstream infections [13] and continues to be responsible of a high mortality rate, particularly among hospitalized, immunodepressed patients. The *Candida* spp. colonization occurs in up to 80 % of critically ill patients after 1 week in intensive care [14-17]. To the best of our knowledge, this is the first report on the correlation between different parameters characterizing the adherence capacity to the cellular substratum and the ability of a significant number of *C. albicans* strains to develop biofilms on the inert substratum nosocomial strains isolated from clinical infections in patients hospitalized for cardiovascular surgery.

The analyzed strains were isolated from 109 patients aged from 20 to 85 years with an average age of 61.86. There were no significant differences in isolation rates depending on gender (male:female ratio:1.08, p>0.05). The analyzed strains were equally distributed among sexes, while the isolation rate was among the established age groups was not statistically significant, although a slightly increased rate was observed for the 60-69 years age group (fig. 1).

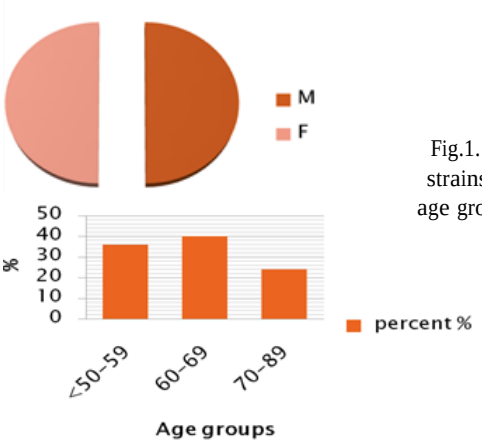
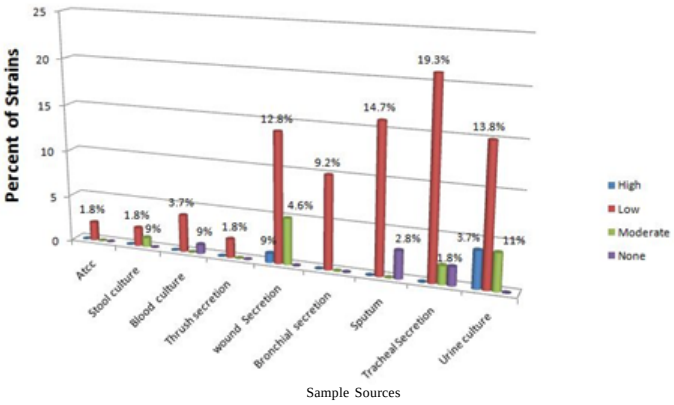


Fig.1. Distribution of strains depending on age groups and gender.

Most strains were isolated from upper respiratory tract secretions (sputum and tracheal), followed by urine and surgical wound secretions. The significant association of *C. albicans* infections with respiratory tract, urinary tract and surgical site infections was also mentioned by other studies [18, 19].

All tested strains adhered to the cellular substratum, but with different indexes (from 38.4 to 81.69%), patterns and intensities (from 4.7 to 7.9 yeast cells/eukaryotic cells). The capacity of the isolates to colonize the cellular substratum represented by HeLa cells depended on the isolate origin source, as also revealed by other studies [14]. The isolates from stool culture, urine and thrush secretion exhibited the most intensive adhesion capacity (fig. 2).



(*) HS: Highly Sig. at P<0.01; S: Sig. at P<0.05; NS: Non Sig. at P>0.05

Fig. 2. Graphic representation of the adhesion capacity to the HeLa cells of yeast strains isolated from different sites

Adhesion represents the first step in the pathogenesis of candidal infection. After the initial colonization and local multiplication, *C. albicans* may penetrate the mucosal barrier and produce invasive infections with morbidity and mortality, especially among immunocompromised patients [20]. Therefore, the investigation of *C. albicans* interactions with epithelial cells could offer a clue for the correct management of candidal infections. The capacity of the isolates to colonize the cellular substratum represented by HeLa cells depended on the isolate origin source, as also revealed by other studies [21]. The isolates from the stool and urine cultures exhibited the highest capacity to colonize the cellular substratum. The highest ratio between the number of yeast cells/eukaryotic cell were obtained for samples isolated from stool and urine cultures and the lowest for isolates from the upper respiratory tract secretions (fig. 3).

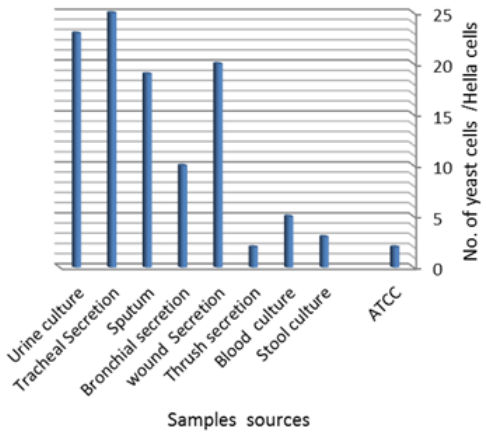


Fig. 3. (3): Graphic representation of the number of yeast cells/ HeLa cells depending on the isolation source.

(*) HS: Highly Sig. at P<0.01; S: Sig. at P<0.05; NS: Non Sig. at P>0.05

The number of yeast cells / eukaryotic cells was statistically significant correlated with the localized adherence pattern (fig. 4).

The predominant adherence pattern was the aggregative one (73.9%) ($p<0.01$) and this feature was correlated with a high adhesion index capacity of the respective strains (fig. 5).

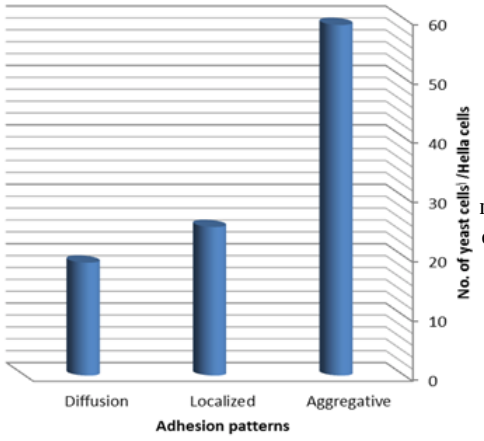


Fig 4. Graphic representation of the correlation between the number of yeast cells/HeLa cells and the adhesion patterns

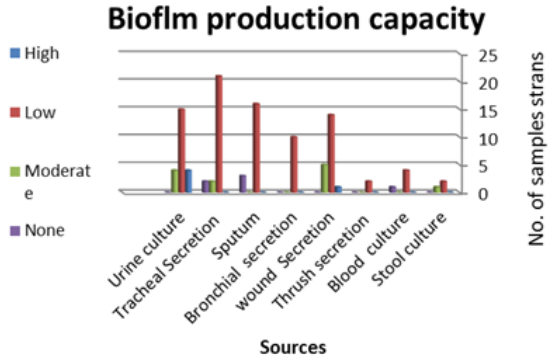


Fig. 5. Graphic representation of biofilm production capacity after staining with violet crystal and spectrophotometric reading.

Microbial biofilms are a protected mode of growth for microorganisms, including fungi, where they are safe from the antifungal treatment and can provide a permanent source of persistent infection. The presence of medical devices such as central venous catheters (CVC's) are known to be important risk factors [22] suggesting that biofilm formation is a key feature in the pathogenesis of yeast related infections. *Candida* spp. produces biofilms on synthetic materials, which facilitates adhesion of the organisms to devices and renders them relatively refractory to medical therapy [23].

C. albicans strains exhibited a heterogeneous biofilm formation when grown in Sabouraud broth at 37^{0}C. Sabouraud broth was shown to support the optimal growth of *C. albicans* over the time span of the investigated periods, respectively 24, 48 and 72 h. Isolates were categorized as low biofilm formers (LBF), moderate biofilm formers (MBF) and high biofilm formers (HBF) depending on the absorbance value at 490 nm of the microbial biofilms colored with violet crystal. The HBF strains formed a biofilm which could be observed macroscopically after staining with violet crystal. In contrast, minimal staining was retained on isolates classified as LBF. The strains with the highest biofilm production capacity were those isolated from respiratory tract secretions and urine cultures (fig. 5).The strains isolated from urine cultures and wound secretions exhibited the highest capacity to form biofilms. Other studies have shown that biofilm production was more frequent in bloodstream isolates while in urine, both biofilm-negative and intense biofilm producer populations have been noticed [24].

Conclusions

These results could suggest the implication of common fungal structures in the colonization of inert and cellular substrata, while the elucidation of the molecular mechanisms involved in these processes could bring an important benefit to the appropriate management of fungal infections, depending on the isolation source.

Acknowledgments: This work has been supported by the national research projects PN II/PCCA 94/2012 and PN-III-P2-2.1-BG-20160369.

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Manuscript received: 3.07.2017