

Phenotypic and genotypic evaluation of adherence and biofilm development in *Candida albicans* respiratory tract isolates from hospitalized patients

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Abstract

In recent years, a significant number of epidemiological variations have been observed for fungal infections. In immunocompromised patients, *Candida albicans* is crucially involved in invasive infections, mostly originating in respiratory tract colonization. The global rise in candidiasis has led researchers to investigate possible correlations between fungal strains virulence profiles and their pathogenic potential, among the most investigated genes being those involved in adherence and biofilm development. In this study, we established the adherence gene profiles of *C. albicans* strains isolated from respiratory tract secretions in patients hospitalized for cardiovascular diseases and correlated them with the ability of the respective strains to colonize the epithelial cells and form biofilms on the inert substratum. The strains isolated from the lower respiratory tract exhibited the highest adherence capacity and were intensive biofilm producers. The *SAP9*, *ALS3*, *ALS5*, and *ALS6* genes were the most frequently detected. There was a significant association between the presence of *ALS 3* gene and the cellular substrate colonizing potential of the harboring strains. We also found that the strains expressing *SAP9* were more virulent in the phenotypic assays. Detecting the presence of adherence genes from different clinical isolates is a cost-effective tool that would allow researchers to predict the virulence of a certain strain and estimate its potential to adhere to host cells and develop biofilms.

Keywords: *Candida albicans*, adherence genes, adherence to cellular substratum, biofilm

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Introduction

Candida albicans is generally a widespread fungal pathogen that is involved either in superficial or blood-disseminated infections (1,2). Oral *Candida* spp. is the most widely recognized

contaminant found in the elderly or hospitalized patients, causing systemic or mucosal diseases (3-5). The oral colonization with *Candida* spp. in the elderly is favored by prostheses wearing, poor oral/denture cleaning and low salivation stream (6-9).

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The ability of *C. albicans* to form biofilms on both cellular and prosthetic device surfaces increases the severity of the infection. Biofilms are complex, three-dimensional microbial structures encased in an extracellular matrix that are connected to either a biotic or an abiotic surface (10–14). Biofilms decrease the host immune system by an increased microbial resistance to antimicrobial agents (10, 13–15) and thus play a significant role in virulence. The *C. albicans* biofilms consist of yeast and hyphae, both of which being indispensable to fungal adherence and subsequent biofilm development (16–19). The increased prevalence of candidiasis worldwide has led to investigations of the possible correlations between adherence genes profiles and the adherence and biofilm development capacity, assessed phenotypically. Recently, the detection of a variety of regulators and effectors that included biofilm formation in *C. albicans* has increased the understanding of the complex mechanisms concerned with this process [reviewed in (20)]. Various cell wall proteins, including Bcr1, a transcription factor that controls gene expression of cell wall proteins, have been revealed to play important roles in biofilm formation. In addition, Agglutinin-like sequences (*ALSI*, *ALS3*), and the hyphal wall protein 1 (*HWPI*) contribute to biofilm formation and maturation (21–23).

Also, a direct correlation between secreted aspartyl proteinase (SAP) production and *C. albicans* adherence capacity has been highlighted. It was observed that proteolytic *C. albicans* isolates exhibited a higher capacity of *in vitro* adherence to human oral epithelial cells compared with isolates that synthesized less proteinase (24). In mice, an isolate that produced higher levels of proteinases also exhibited increased *in vivo* tissue colonization capacity of the liver, kidneys, and spleen, and led to the death of the mice (25). The majority of the existing studies investigating the *C. albicans* biofilm production have focused on SAPs. In the anaerobic environments of the

root canal systems and periodontal pockets, SAP secretion increases. This observation suggests that the virulence of *C. albicans* is significantly influenced by the concentration of oxygen in the infection site (26). It has been demonstrated that pepstatin, an inhibitor of SAP2 (and probably of SAP1 and SAP3) decreased *C. albicans* biofilm development capacity with variable efficiency (27). Pepstatin treatment decreased *C. albicans* adhesion and invasion of human epidermal cells and mucosa (28, 29, 30).

The *EAP1* gene (extracellular adhesion protein) encodes a putative cell wall adhesin (31) that contains a signal peptide and a glycosylphosphatidylinositol anchor site; *EAP1* gene is homologous to other yeast genes that code for cell wall proteins.

Cell surface hydrophobicity (CSH) plays a vital role in *C. albicans* pathogenesis. Hydrophobic cells displayed higher adherence to both epithelial and endothelial cells and to extracellular matrix proteins, as well as greater resistance to phagocytosis (32). CSH varied due to different environmental conditions and growth phases (33).

The aim of the current study was to establish the adherence gene profiles of *C. albicans* isolates from respiratory tract secretions in patients hospitalized for cardiovascular diseases and to correlate them with the ability of the respective isolates to colonize the epithelial cells and to form biofilms on the inert substratum.

Materials and Methods

Fungal strains

In this study, we analyzed twenty-nine *C. albicans* strains isolated from patients admitted for cardiovascular surgery, aged 25–89 years and diagnosed with respiratory tract infections. Nineteen specimens were isolated from sputum and 10 samples from bronchial secretions. None of the patients was dental prosthesis wearer. The *C. albicans* ATCC10231 was used as reference strain.

Sabouraud Dextrose Agar (SDA) medium was used to initially isolate the strains, which, subsequently, were biochemically identified using the VITEK II automatic analyzer (bioMérieux), after inoculating Yeast plastic cards (YPC) with the required inoculum suspension. The YBC cards were then incubated at 37°C for 24-48 h and submitted for reading.

Biofilm microtiter assay

A microtiter plate method was used to assess the ability of *C. albicans* isolates to attach to an inert substratum and to examine the subsequent biofilm production. *C. albicans* isolates were maintained overnight in Sabouraud broth at 37°C and then centrifuged at 150 rpm. The cells were collected, washed by using phosphate buffered saline (PBS), and standardized to a density of 1×10^7 yeast cells/ml. A volume of 100 μ l of the yeast cells suspension was added to the wells of a 96-well plate and incubated at 37°C for 96 h. After incubation, the liquid content of the wells was discarded, and the wells were washed three times with distilled water in order to remove the non-adherent cells. Then, the cells adherent on the plastic wells were fixed with cold methanol for 5 min and then stained with crystal violet (CV) solution for 15 min (34, 35). In order to evaluate the stained biofilms, first the wells were examined using an inverted microscope. Then, the biofilm formed on the plastic wells was re-suspended with a 33% acetic acid solution and the optical density of the biofilm cell suspension was determined by measuring the absorbance of the colored suspension at 490 nm using an enzyme linked immunosorbent assay (ELISA) plate reader, the results being interpreted according to Melo et al. (36). All tests were done in triplicate and biofilm production quantities were reported as the arithmetic mean of absorbance values of the three replicate test. The absorbance values of the negative controls (containing no cells) were subtracted from the values of the test

wells to minimize background interference. The biofilm forming intensity was quantified according to their cut-offs by OD value as none absent (unable to form a biofilm), low (when $< \sim \leq 2x$ ODC), moderate (when $< \sim \leq 4x$ ODC) or high ($> 4x$ ODC) (37).

Evaluation of the capacity of adherence to HeLa cells

An adaptation of the Cravioto method was used to determine adherence capacity of each isolate to HeLa cells. The assay was performed as previously described (38). HeLa cells were grown in 6-well plates for 24 h in Dulbecco's Modified Eagle's Medium (DMEM) involved with antibiotics; after this incubation, the cell monolayers reached 80% confluence. Wells were washed 3 times with PBS to remove the growth medium. To prepare the fungal suspension, yeast was grown in Sabouraud glucose broth supplemented with 3% glucose for 48 h at 37°C. Cells were centrifuged at 1500 rpm for 10 min, and the pellets were washed with PBS (pH ~ 7.2). The cell density in the final suspension was 10^7 colony forming units/ml (using a 0.5 McFarland standard). One ml of the final fungal suspension was added aseptically above each cellular monolayer. The plates were then incubated at 37°C for 2 h to allow the yeast cells to properly adhere to the cellular surface. Then, the cells were fixed with methanol for 5 min after washing with PBS three times. The wells were stained with a 1:10 Giemsa solution (Merck, Darmstadt, Germany) for 20 min, washed with tap water, used room temperature for drying, examined under microscope (2500 total magnification) with I.O., and then photographed with a Contax camera (supplied with Zeiss microscope). The adherence index (AIn) for each isolate was calculated as the percentage of HeLa cell showing adherent fungal cells to the total number of HeLa cells. The average number of fungal cells adhered to one HeLa cell was also calculated.

Detection of adherence genes

The total DNA was isolated using the thermolysis method according to the protocol of Zang et al (39). All purified strains were grown on SDA medium at 37°C for 24 h. Subsequently, isolated fungal colonies were routinely subcultured in Yeast Nitrogen Base (YNB; BD Diagnostics, Cowley, UK) medium enhanced with glucose (0.5% w/v) overnight at 37 °C, under stirring at 120 rev/min. Cells were harvested by centrifugation at 3000 g for 10 min at 4°C and the pellet was washed 2 times with 10 mL PBS (pH 7). The cellular density for each strain was adjusted to 1×10^7 cells/ml. Pooled biofilms from each collection site were carefully transferred to Eppendorf tubes that contained 1 mL of reduced transport fluid (RTF). Immediately after collection, the samples from each site were diluted and plated onto SDA medium with chloramphenicol (bioMérieux, Paris, France). The plates were incubated in a reduced oxygen atmosphere (10% CO₂ and 90% air) for 48 h at 37°C. DNA was extracted with slight modifications from a protocol by Nascimento et al. (40). The yeast colonies were suspended in 1 mL of a solution containing 1 M sorbitol and 125 M ethylenediaminetetraacetic acid (EDTA). This suspension was centrifuged at 13000 g for 10 min at 25 °C. The pellet was resuspended in 0.5 mL lysis solution [1 M TrisHCl, pH 8.0, with 250 mM EDTA and 5% sodium dodecyl sulfate (SDS)] with 10 mL of proteinase K (Invitrogen), and incubated for 1 h at 65°C. Subsequently, 500 µl of 5 M potassium acetate was added to the mixture and incubated on ice for 2 h. The mixture was centrifuged at 13000 g for 10 min at 25 °C (40). The supernatant was transferred to an Eppendorf tube containing 1 mL absolute ethanol. The tubes were mixed by inversion centrifuged at 13000 g for 10 min at 4°C. The pellet was washed with 500 µl of ice cold 70% ethanol and then centrifuged at 13000 g for 10 min at 4°C. The final pellet was

re-suspended in 0.5 mL of sterile MilliQ water. The DNA concentration was determined at 260 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). These data were used to prepare a standard with of 100 ng/ml concentration. The standard was stored for the next PCR reactions at 20°C. Simplex and multiplex PCR reactions using specific primers were used to identify *C. albicans* adherence genes (41). A positive control was performed using DNA purified from the *C. albicans* ATCC 10231 reference strain. The GeneAmp PCR system 2400 (Perkin-Elmer-Applied Biosystems) was employed for PCR amplification using the following thermal conditions: 94°C for 5 min, 35 cycles of 94 °C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. Electrophoresis was used to separate PCR products on 2% agarose gels using Tris-borate-EDTA running buffer (pH 8.0). The DNA, including the molecular mass ladder (1000 bp DNA ladder), was stained using a 0.5 µg/ml solution of ethidium bromide. The stained gel was visualized with UV illumination (Pharmacia LKB-MacroVue, San Gabriel, CA, USA).

Statistical analysis

Statistical analysis was carried out using SPSS version 16.0. For all statistical tests, $P < 0.05$ was considered significant and $P < 0.01$ highly significant. Inferential data analysis was performed to test. The contingency coefficients (C.C.) test was used to determine the correlation of the cause to association tables. The null hypothesis under Ho says that redistribution of the observed frequencies in the created association table through applying the new suggested method using stem-leaf technique are randomly distributed (meaningless relationship) versus the alternative hypothesis which says that the contingency coefficient of the association table is meaningful. In addition, under and upper redistribution cutoff

points were used to create 2 x 2 rank association tables and these were applied to the Steam-Leaf plotting technique.

Results

In this study, we analyzed 29 *C. albicans* isolates recently isolated from patients aged 25 to 89 years. The majority of the isolates (65%) were isolated from lower respiratory tract (bronchial) secretions. We observed no significant difference in isolation rates based on gender (male to female ratio was 1.08, $P>0.05$) (Table 1).

Most of the *C. albicans* isolates from the bronchial secretions exhibited a localized adherence pattern, while the vast majority of sputum strains exhibited an aggregative adherence pattern (Figure 1, Table 1). We observed that the isolated *C. albicans* strains adhered to HeLa cellular substratum with different intensities as revealed by the adherence index values (AIn) that ranged from 70 to 87.7% (Table 1). Bronchial-derived strains had higher AIn compared to strains isolated from sputum (Table 1).

The majority of isolates from both bronchial and sputum samples developed biofilms on the inert

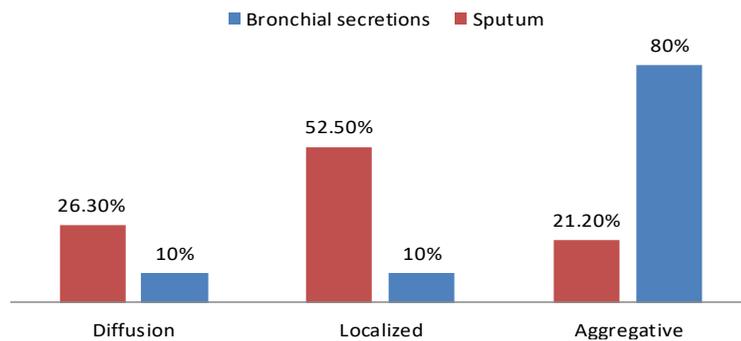


Fig. 1. Adherence patterns (%) of *C. albicans* strains isolated from sputum and bronchial secretions

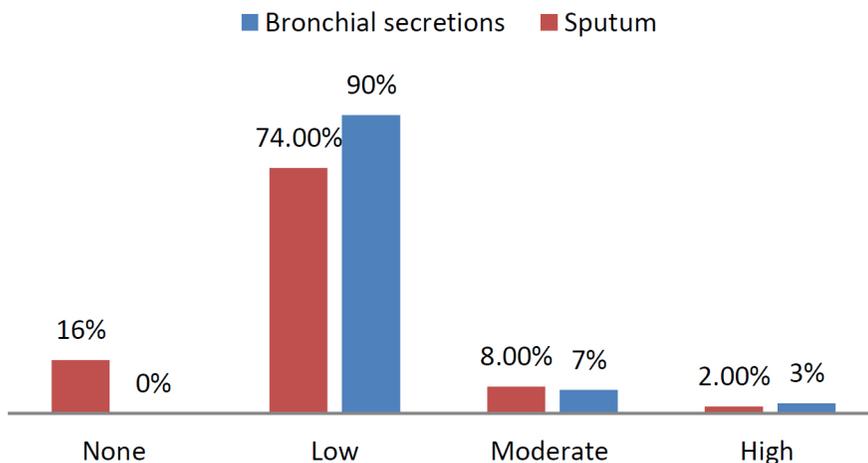


Fig. 2: Distribution of *C. albicans* strains according to source and capacity to develop a biofilm on an inert substratum. The ability to form a biofilm was classified as absent (unable to form a biofilm), low, moderate, or high.

Table 1. Phenotypic and genotypic features of *C. albicans* isolates

<i>C. albicans</i> strain code	Isolation source	Age	Sex	AIn	Average no. of yeast cells/ HeLa cell	Adherence pattern	Biofilm to inert substratum	Adherence genes profile
256	Sputum	89	M	73.82%	8.77	Aggregative	None	ALS 2, 3, 4, 5, 6, 7, 8, SAP 4, 5, 9, 10, EAP 1
67	Sputum	62	M	66.66%	5	Localized	Low	ALS 1, 3, 5, 6, 8, SAP 9, 10
154	Sputum	39	M	73.98%	5.12	Aggregative	None	ALS 1, 3, 4, 6, 7, 8, SAP 5, 6, 9, 10
175	Sputum	85	M	65.07%	5.8	Aggregative	Low	ALS 1, 2, 3, SAP 2, 4, 5, 9, 10
489	Sputum	56	M	31.31%	2.28	Localized	Low	ALS 1, 2, 3, 5, 6, 7, 8, SAP 4, 6, 9, 10,
772	Sputum	46	F	32.73%	7.42	Diffusion	Low	ALS 3, 5, 6, SAP 6, 9
31	Sputum	38	F	41.29%	7.87	Localized	Low	ALS 1, 2, 3, 4, 5, 6, 7, 8, SAP 4, 5, 6, 9, 10, EAP 1
8159	Sputum	49	M	28.57%	0.7	Localized	Low	ALS 2, 4, 5, 6, 7, SAP 5, 6, 9, 10
47	Sputum	53	F	43.80 %	3.2	Localized	Low	ALS 1, 2, 3, 4, 5, 6, 7, 8, SAP 4, 5, 6, 9, 10, EAP 1
67	Sputum	44	M	33.76%	5.1	Diffusion	Low	ALS 3, 5, 7, SAP 6, 9, 10
29	Sputum	51	M	41.17 %	3.5	Localized	None	ALS 1, 2, 3, 4, 5, 6, 7, 8, SAP 2, 5, 6, 9, 10, EAP 1
1166	Sputum	60	M	48.27 %	5.6	Diffusion	low	ALS 2, 3, 4, 5, 6, 7, SAP 2, 4, 5, 9, 10
149	Sputum	33	F	46.92 %	4.8	Diffusion	low	ALS 1, 2, 3, 4, 5, 6, 7, SAP 2, 5, 6, 9, 10
58	Sputum	31	F	44.71%	2.62	localized	low	ALS 1, 4, 5, 6, 7, 8, SAP 4, 5, 6, 9, 10
81	Sputum	64	F	45.30%	3.76	localized	low	ALS 1, 2, 3, 4, 6, SAP 4, 5, 6, 9, 10
103	Sputum	50	F	44.64%	3.17	localized	low	ALS 1, 2, 3, 4, 5, 6, 7, 8, SAP 4, 5, 9, 10,
105	Sputum	44	M	47.29%	5.3	localized	low	ALS 3, 7, 8, SAP 9
2693	Sputum	41	F	40.0 %	3.4	Diffusion	Low	ALS 1, 2, 4, 5, 6, 7, 8, SAP 5, 9,
255	Sputum	37	M	82.79 %	5.92	Aggregative	Low	ALS 2, 3, 4, 5, 6, 7, 8, SAP 4, 5, 9, 10, EAP 1

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<i>C. albicans</i> strain code	Isolation source	Age	Sex	AIIn	Average no. of yeast cells/ HeLa cell	Adherence pattern	Biofilm to inert substratum	Adherece genes profile
1776	Bronchial Secretions	64	F	28.30%	2.14	Localized	Low	<i>ALS 2, 3, 5, 6, 8, SAP 6, 9, 10,</i>
1135	Bronchial Secretions	51	M	30.44 %	2	Diffusion	Low	<i>ALS 1, 3, 5, 6, SAP 2, 5, 6, 9, 10,</i>
373	Bronchial Secretions	62	F	68.10 %	6	Aggregative	Low	<i>ALS 2, 3, 4, 6, SAP 9, 10</i>
305	Bronchial Secretions	86	F	68.50 %	6.8	Aggregative	Low	<i>ALS 1, 5, 6, 8, SAP 6</i>
1995	Bronchial Secretions	60	F	82.09%	8.62	Aggregative	Low	<i>ALS 1, 2, 3, 4, 5, 6, 7, 8, SAP 9, 10, EAP 1</i>
1942	Bronchial Secretions	64	M	71.81 %	7.9	Aggregative	Low	<i>ALS 1, 3, 4, 5, 6, SAP 6, 9, 10</i>
1946	Bronchial Secretions	64	F	69.04 %	5.8	Aggregative	Low	<i>ALS 5, 6, SAP 9, 10</i>
1935	Bronchial Secretions	59	M	75.10 %	7.18	Aggregative	Low	<i>ALS 3, 5, 6, 7, 8, SAP 6, 9</i>
1675	Bronchial Secretions	25	M	81.67%	6.15	Aggregative	Low	<i>ALS 1, 2, 3, 4, 5, 6, 7, 8, SAP 4, 5, 6, 9, 10, EAP 1</i>
1676	Bronchial Secretions	31	M	74.42 %	4.57	Aggregative	Low	<i>ALS 3, SAP 6, 9, 10, EAP 1</i>

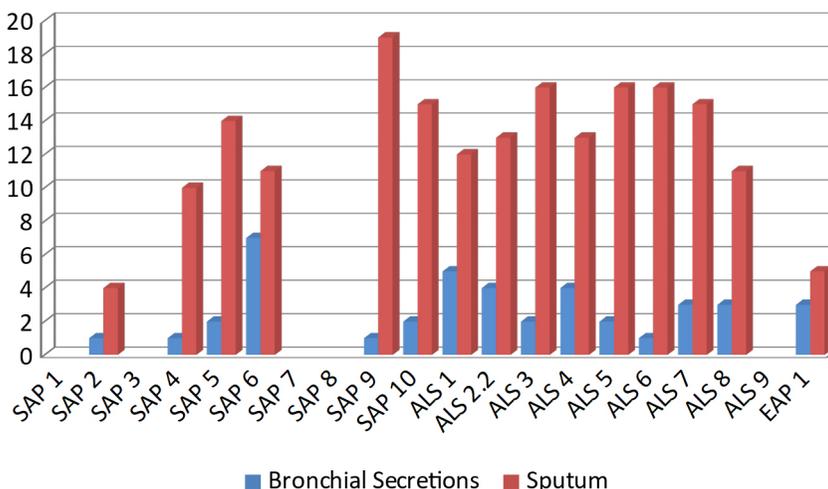


Fig. 3. Distribution of *C. albicans* adhesion genes in strains isolated from sputum and bronchial secretions

substrate (Fig. 2). Overall, isolates from the upper respiratory tract were higher biofilm producers, this feature being correlated with a high AIn (Fig. 2 insertion). Previous research studies have shown that strains isolated from invasive infections tend to produce biofilms more intensely in comparison with isolates from non-invasive infections (42).

Our results indicated a generally non-statistically significant ($P > 0.05$) correlation between the presence of *ALS* and *SAP* genes and AIn for *C. albicans* isolates that were isolated from sputum and bronchial secretions (Fig. 3, Table 1). There were, however, some exceptions. The *ALS3* gene presence in strains isolated from sputum and of *SAP2* in those isolated from bronchial secretions were significantly correlated with a high AIn ($P < 0.05$). However, the only significant associations between adherence genes and biofilm formation capacity were *ALS1* in tracheal and *ALS8* in sputum isolates ($P < 0.05$). The *ALS3* polymorphism led to variable biofilm production in different species isolated from sources such as blood, vulvo-vaginal secretions, and urine cultures (43).

Discussion

Fungal infections due to *C. albicans* in critically ill hospitalized patients are a significant cause of morbidity and mortality worldwide. Peterson et al. (44) observed that 55% of hospitalized patients carried yeasts in their saliva. More specifically, *Candida* spp. strains were found in between 47-87% of in patients with advanced cancer, and in the oral mucosa of up to 80% of diabetic patients (40). In our study, *C. albicans* strains that are isolated from patients hospitalized for cardiovascular surgery were predominantly from bronchial secretions (65 %).

The ability to colonize host tissues is considered one of the most significant factors for *C. albi-*

cans virulence (41). *Candida* spp. adherence to human tissues permits the fungus to reach the minimum infective threshold and colonize a specific niche environment. *Candida* has a specific feature which enables it to survive and develop within different circumstances of healthy human body and then to invade tissues. Thus, the colonized area provides the essential ground for *Candida* development, proliferation, invasion, and dissemination (45). However, any modification in the host immune system, or host environment, can lead to the switching *C. albicans* into a pathogenic form and then able of causing biofilm-associated infections were also noted to play a significant role in perpetuating these infections since these structures could allow the yeast to adhere to medical devices. However, there is limited information available as to the molecular mechanisms that mediate *C. albicans* adhesion to materials or mammalian cells. The relationship between the phenotype of a strain (e.g., the ability to adhere and/or form a biofilm) and genotype is also barely known. Our findings indicate that *C. albicans* isolated from bronchial secretions adhered better to cellular and inert substrata compared to sputum-isolated strains. These bronchial-derived strains also had a specific adherence gene profile. It has been previously described that *ALS* genes play an important role in *C. albicans* adherence and biofilm formation and growth, the *ALS1* protein product being associated with the capacity of *Candida* spp. strains to adhere to endothelial cells and form biofilms (46). We found that the presence of *ALS3*, *ALS5*, *ALS6*, and *SAP9* genes is correlated with *C. albicans* adhesion to polystyrene and human epithelial cells. The *SAP9* and *ALS3* adherence genes contribute to the overall virulence of *C. albicans* strains, being usually expressed in virulent strains isolated from symptomatic infections and enable attachment to cellular and inert substrata. Our results differed compared with a previous study (47) in which the authors report-

ed that other genes which were not identified in our study, respectively *ALS1* and *ALS9*, were detected in the clinical isolates. In our study, 90% of the analyzed strains were *SAP9* positive, the next most prevalent gene present in the strains being *SAP5*. We noticed that *C. albicans* isolates that expressed *SAP9* were more virulent in comparison with isolates that expressed *SAP5* and *SAP6*, and then the remaining analyzed genes, therefore *SAP9* plays a significant role in biofilm development, probably through favoring yeast to hyphal transition, as a response to environmental stimuli via a cyclic adenosine monophosphate (cAMP) dependent signaling pathway (48). Hyphal development contributes to *Candida* invasion of epithelial cells and leads to host cell damage.

Conclusions

Our work describes the relationship between different phenotypic features that are related to cell adherence and biofilm development and the presence of adherence genes. The analyzed *C. albicans* strains were isolated from the respiratory tract of patients undergoing cardiovascular surgery. The isolates were able to adhere to cellular and inert substrata and to form biofilms. The investigation of the adherence gene profiles in our *C. albicans* isolate revealed the presence of several *ALS* genes, correlated with the respective isolates ability to colonize the cellular substratum. The isolates expressing the *SAP9* were the most virulent, likely because *SAP9* favors the yeast to hyphal transition. Overall, our study demonstrates that the PCR detection of adherence genes could represent a simple and cost effective tool for the prediction of the virulence capacity and pathogenic potential of different *C. albicans* clinical isolates, replacing the more expensive and time-consuming phenotypic assays of adherence and biofilm development capacity.

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Author Contributions

OS, IG, GGP conceived and designed the experiments; IG, OS, AMH, LMD, IA, OA and DA performed the experiments; OB provided the *C. albicans* strains; IG, LMD, AMH and CC analyzed the data; OS, IG and MCC wrote and corrected the paper.

Abbreviations

ALS - Agglutinin Like Sequences
 SAP - Secreted Aspartyl Proteinase
 CSH - Cell Surface Hydrophobicity
 AIn - Adherence Index
 EAP - Extracellular Adhesion Proteins

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