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BIOFILM FORMATION AND ANTIFUNGAL SENSITIVITY OF *CANDIDA ALBICANS*, *CANDIDA TROPICALIS*, *CANDIDA GLABRATA* E *CANDIDA AURIS*

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Abstract: In recent years, the incidence of opportunistic fungi has increased significantly, with candidiasis being the most common mycosis. Recently, non-albicans Candida species increased considerably, being more frequent than Candida albicans, the species: *Candida tropicalis*, *Candida glabrata, Candida dubliniensis*, *Candida auris* among others, which can cause anything from a superficial infection to a systemic infection; However, currently: *C. albicans* seems to have regained dominance as the etiological agent of this opportunistic mycosis. On the other hand, the increase in strains resistant to antifungals has become a public health problem, with fatal consequences for the patient. Candidiasis can represent a significant burden of infections in the hospital population because this mycosis is associated with the formation of biofilms, these constitute layers of the microorganism adhered to biotic or abiotic surfaces that complicate the access of drugs and thus its elimination, becoming a focus of dissemination of the infection, increasing hospital stay and, therefore, care costs and mortality. In this chapter we will describe the formation of biofilms and sensitivity to antifungals using as a model: *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. auris*.
Keywords: Candidiasis, *Candida* spp., antifungals, biofilms.

INTRODUCTION

Of all the Candida species, fifteen have been found to be pathogenic for humans, and especially five have represented 90% of invasive candidiasis: C. albicans, C. glabrata, C. parapsilosis, C. krusei, and C. tropicalis, while species such as C. dubliniensis, C. guilliermondii, C. inconspicua, C. lusitaniae, C. norvegensis, C. kefyr, C. fameta and C. rugosa represent a group of occasionally isolated species. C. albicans is the most studied species in terms of pathogenicity and is the main causal agent of superficial and systemic candidiasis (Pappas et al., 2018; Silva et al., 2017; Yapar, 2014).

Candida auris is an emerging pathogenic yeast that causes infections and outbreaks associated with a high rate of morbidity and mortality in hospitalized patients. It presents resistance to various antifungal drugs and a high capacity to spread in hospital environments (Ahmad et al., 2021).

It can cause infections such as candidemia, pericarditis, infections in the respiratory tract, urinary tract, central nervous system, abdomen, bones, skin and soft tissues. In most cases, these infections occur in patients who are critically ill or who undergo invasive procedures (Lockhart et al., 2019).

Invasive candidiasis is the fungal infection with the highest incidence among hospitalized patients; its mortality rate reaches 40% even after antifungal treatment has been given. Candidemia has been associated with a mortality of up to 47% (Pappas et al., 2018). Among yeasts, C. albicans was the predominant species responsible for 35% to 60% of the isolates. However, infections caused by C. parapsilosis, C. tropicalis, C. glabrata, C. krusei, and C. auris, which are the most common non-albicans Candida species identified in cultures, are increasingly being documented. The majority of C. glabrata and C. krusei present greater resistance to treatment with azoles (Howard et al., 2020) [(Mora Carpio et al., 2021).

CANDIDA ALBICANS

It is a round or oval yeast measuring 3-8 by 2-7 microns. The various morphological forms of C. albicans have been associated with the change of commensal or pathogenic states; it can form blastoconidia, pseudohyphae and true hyphae depending on temperature, pH and nutrients. The switch from yeast to hyphae is thought to aid cell adhesion and facilitate

tissue infection, macrophage evasion, and biofilm development (Verma-Gaur et al., 2016). The C. albicans genome is made up of eight pairs of homologous chromosomes whose total size is 16 Mb (McManus et al., 2014). C. albicans ferments glucose, galactose and maltose with the formation of acids and carbon dioxide. C. albicans is frequently found as part of the normal microbial flora of humans: mouth, digestive tract, genitourinary tract, which facilitates its encounter with most implanted biomaterials and host surfaces. Macroscopically, on Sabouraud agar the colonies are fast growing, circular, smooth, white or creamy, pasty and soft, with precise edges, a slightly prominent center. In chromogenic medium (CHROMagar® Candida) the C. albicans colonies turn light to medium green.

CANDIDA TROPICALIS

It is a diploid yeast, round 3.5-7 C. tropicalis has become one of the most important Candida species, being considered the second most virulent species of that genus. It frequently affects leukemic and neutropenic patients, it has great invasive capacity and it is estimated that 50% to 60% of cases develop disseminated candidiasis, unlike C. albicans, which does so in 2 to 15% of cases. cases (Nucci et al., 2005). Studies have shown that the production of their biofilms is greater than in C. albicans. It can produce virulence factors such as adhesion to buccal epithelial cells as well as endothelial cells, secretion of lytic enzymes such as proteinases, phospholipases and hemolysins, morphogenesis (transition from buds to hyphae) and phenotypic switching (white to opaque state) (Seervai et al. al., 2013). The morphology of C. tropicalis colonies in SDA is white to cream in color, with a creamy texture and smooth appearance and may have slightly wrinkled edges (Zuza-Alves et al., 2017). In chromogenic medium (CHROMagar® Candida) the C. tropical colonies appear grayish blue to greenish blue or metallic blue.

CANDIDA GLABRATA

C. glabrata is a non-dimorphic yeast that exists as small blastoconidia under all environmental conditions as a pathogen. Its blastoconidia measure 1 to 4 µm (Fidel et al., 1999) and no pseudohyphae have been observed. The colonies are white or cream, pasty and smooth, with growth of 3 to 5 days on Sabouraud medium. It is important to note that the morphological change of yeast to the hyphal form has not been reported, although the appearance of pseudohyphae in response to the lack of nitrogen and exposure to carbon dioxide has been (Kumar et al., 2019).

A considerable number of strains may be resistant in vitro to triazole antifungals. The maximum growth temperature is 43-45ºC and the optimal temperature for strains of clinical origin is 35-37ºC. In chromogenic media such as CHROMagar, it presents colonies of variable color, from lilac to purple.

CANDIDA AURIS

C. auris was isolated and identified for the first time in Japan in 2009 from a sample of the external auditory canal of a patient admitted to a geriatric hospital in Tokyo (Spivak et al., 2018).

Within its genome it contains genes that code for various virulence mechanisms such as the expression of phospholipases, proteinases, hemolysins, adhesins, biofilm formation, resistance to antifungals and environmental stress. However, knowledge about its biology remains scarce and continues to be studied (Hernando-Ortiz et al., 2021). It has been described that unlike other species of the genus Candida, C. auris grows at temperatures of up to 42°C and tolerates high concentrations of NaCl (10%), which could

explain the morphological changes it presents as a form of adaptation to these conditions. stress conditions (Du et al., 2020; González-Durán et al., 2022).

It is a yeast-shaped fungus that can be observed individually or in aggregate with an oval, elongated and budding shape, with a size of 2.5-5.0 µm. C. auris rarely exhibits the formation of hyphae or pseudohyphae and does not have the ability to form germ tubes. Its optimal growth temperature is 35-37°C. On Sabouraud dextrose agar it forms soft, creamy white or cream colonies and on CHROMagar Candida, C. auris colonies can present different pigments ranging from pale pink to dark pink (Bentz et al., 2019).

BIOPILICS

Antoni van Leeuwenhoek first wrote about biofilms in 1683 for the Royal Society of London (Gulati et al., 2016). The fungal biofilm is a heterogeneous structure composed of hyphae, pseudohyphae and yeasts, it develops at the interface between an aqueous medium and a solid. Biofilms can be isolated from biotic and abiotic surfaces, some within the patient. In the last two decades, the increased use of medical implant devices has led to an increase in the rate of Candida infections (Sandai et al., 2016), showing an associated increase in mortality.

The most common substrates are catheters, dentures (abiotic), and mucosal cell surfaces (biotic) (Mayer et al., 2013). Cells within a biofilm show reduced susceptibility to the commonly used antifungal, observing that the cells are less sensitive to death by components of the immune system. Microorganisms that make up biofilms behave very differently from planktonic cells. The National Institute of Health (NIH) in the USA has indicated that pathogenic biofilms are directly or indirectly responsible for 80% of all microbial infections in humans (Jamal et al., 2018), and these range from superficial mucosal infections, dermatological to disseminated infections with a high mortality rate, reaching 50% in several cases (Pereira et al., 2021).

BIOFILMS OF *CANDIDA* SPP

Candida species produce adhesins, which are proteins responsible for specific adhesion prior to biofilm formation. Agglutinin-like sequence adhesins (Als) are a family of glycoproteins located on the surface of the yeast cell wall, which are known to be associated with pathogenicity; they are present in C. albicans and in non-albicans Candida species such as C. tropicalis (Chandra et al., 2015).

Studies related to biofilms of Candida spp. have been carried out mostly on C. albicans, so the process of formation of these structures in other species is still largely unknown. C. auris can form biofilms and aggregative phenotypes, promoting rapid transmission from person to person through direct or indirect contact (Ahmad et al., 2021), Figure 1 shows the process of biofilm formation for C. albicans, C tropicalis, C. glabrata and C. auris. Biofilm formation for C. albicans is a multifaceted process (Gulati et al., 2016).

In general, biofilm formation in C. albicans takes place in 4 stages:

a) Cell wall adhesion mediated by a yeast protein to the cell surface.

b) Growth of the bound yeast within a thin layer of cells.

c) Maturation of the biofilm through the development of hyphae and pseudohyphae, as well as excretion of matrix material.

d) Dispersion of yeast from the biofilm possibly to allow colonization of distant locations.

BIOFILM DETECTION *IN VITRO*

There are different methods to detect the formation of biofilms, which can be qualitative and/or quantitative. Qualitative methods only reveal the formation of biofilms, which can be observed macroscopically and microscopically. In our group we have carried out the formation of biofilms with type strains and obtained from patients of C. albicans, C. tropicalis, C. glabrata and C. auris (Figure 1), using two techniques:

In the first technique, 15 mL Falcon tubes were used, containing 10 mL of glucose peptone yeast extract broth, they were inoculated with $1x10^6$ yeasts of each species, separately, incubating for 48 hours/37°C, subsequently 2 mL were added. of 0.5% crystal violet, to carry out the analysis of the biofilms formed, measuring absorbance in a spectrophotometer at 595 nm.

In the second technique, 200 µL of a suspension of 1 x 10^7 lev/mL were placed in triplicate in 96-well polystyrene plates, incubating at 30ºC with shaking at 90 RPM for 48 h, adding at 24 h the sufficient amount of Sabouraud glucose broth, or YPD for a final volume of 200 µL in each well. After 48 h, 40 µL of a 0.5% crystal violet solution was deposited to quantify the formation of biofilms in a spectrophotometer at 595 nm.

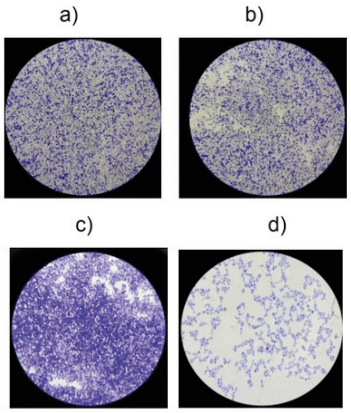

Figure 1: Biofilms of C. albicans, C. tropicalis, C. glabrata and C. auris at 48 hours of incubation stained with Gram staining.

ANTIFUNGAL AGENTS IN INFECTIONS CANDIDA

Candida generally proliferates as a community of adherent cells in an extracellular matrix, forming biofilms, which show innate resistance to multiple antifungals and are capable of increasing said resistance, more than those that only present planktonic cells. The antifungals available against Candida have minimal activity against the biofilms formed. In these cases, treatment is difficult and often critical for the patient's cure, consequently, the available antifungal therapies are not effective. If infections do not have adequate treatment, devastating complications can occur, becoming fatal (Taff et al., 2013).

The recommended treatment for Candida infections includes, in the first instance, the use of echinocandinases, amphotericin B, followed by oral therapies with azoles. Also, polyenes are another type of antifungals that have been used in this type of treatment (de Barros et al., 2020; McCarty et al., 2018; Silva et al., 2017).

Although there are different treatments, a definitive solution for its elimination has not yet been found, so other alternatives have been developed and tested, such as the combined use of echinocandins and the liposomal form of amphotericin B, which is still in studies. and is related to the production of nephrotoxicity (Adler-Moore et al., 2019).

The treatment to combat candidiasis can be topical or systemic depending on the type of infection. The most used antifungals are imidazole derivatives (fluconazole, itraconazole, ketoconazole, miconazole, etc.), however currently a decrease in the effectiveness of these agents is observed. These are mainly due to the emergence of resistant yeasts, the appearance of new pathogenic species, the irrational prescription of antifungals as prophylaxis and the increase in therapeutic doses (Arendrup et al., 2017).

Antifungals can be fungistatic or fungicidal depending on whether they inhibit the growth or cause lysis of the fungi. Imidazole derivatives inhibit oxidative enzymes associated with cytochrome P450 [CYP 3A4 and CYP 2C9] (lanosterol 14-α demethylase), blocking the conversion of lanosterol to ergosterol, which produces an alteration in the permeability of the membrane of fungal cells. In addition, they promote the accumulation of hydrogen peroxide, capable of damaging the structure of the intracellular organelles of the fungus (López et al., 2016).

Some of the most commonly used antifungals and their mechanisms of action are mentioned below (Howard et al., 2020; Quiles-Melero et al., 2021)

- Azoles (fluconazole, posaconazole, voriconazole). They block the synthesis of ergosterol, inhibiting the enzyme 14α-lanosterol demethylase, responsible for the synthesis of ergosterol in the cell membrane, thus inhibiting fungal growth and replication.
- Polyenes (amphotericin B and nystatin). They intercalate into ergosterol-containing membranes, creating pores that destroy the proton gradient, resulting in leakage of cytoplasm and other cellular contents.
- Echinocandins (caspofungin, micafungin and anidulafungin). They interrupt the synthesis of the enzyme β1,3-D-glucan-synthetase, a component of the cell wall of Candida species.
- Amphotericin B. Binds to ergosterol in the fungal membrane, creating pores that allow ions to diffuse through the membrane. Due to its hydrophobicity and poor gastrointestinal absorption, it is administered intravenously (ODDS, 2003). Its nephrotoxicity has been minimized in recent years with lipid formulations that have better solubility.

Recent studies have documented increasing rates of resistance to fluconazole, especially in C. *auris, C.* glabrata, C. krusei, C. tropicalis and C. parapsilosis (Duxbury et al., 2020; Pemán et al., 2016).

ANTIFUNGAL SENSITIVITY TEST

To perform the sensitivity test against antifungals, strains of *C. albicans*, *C. tropicalis*, *C. glabrata* y *C. auris*, they were cultured in YPD medium, from which a small portion was taken and placed in a tube with sterile physiological solution until a concentration of 1 x 10^6 Lev/ml was achieved using Mc Farland tube #5, with the help of an impregnated swab. of the previous solution was placed on Mueller Hinton agar plates, and massive streaking was performed, waiting 5 to 10 minutes until drying, and sensidisks of Fluconazole (FCA) BIO RAD 50 µg, Ketoconazole (KET) BIO RAD 50 were placed. µg, Amphotericin B (AB) BIO RAD 100 µg with tweezers previously sterilized with alcohol, a control was also placed with filter paper plus physiological solution, finally the discs were pressed lightly on the agar to ensure contact and the plates were incubated inverted for 48 to 72 hours until reading to observe defined halos according to the insert. Table 1 shows the results obtained.

CONCLUSIONS

All cultures of C. glabrata, C. tropicalis, C. albicans and C. auris formed biofilms.

All cultures of C. glabrata, C. tropicalis and C. albicans were sensitive to amphotericin B, the majority of isolates were sensitive to ketoconazole, fluconazole and/or clotrimazole.

The 3 strains of C. auris showed resistance to the antifungals ketoconazole, clotrimazole and fluconazole, intermediate resistance to amphotericin B and susceptibility to caspofungin.

Candida albicans													
Sensitivity or Resistance Profile													
Antifungal Show	CAF2	56 27P	44 19CI	43 19CD	46 19P	2641	43	46	2572	21P	27CD1	F1	F3
Ket	Rs	Rs	Rs	Se	Se	Rs	Se	Rs	In	Rs	In	Se	Se
Fca	Rs	Rs	Rs	Rs	Rs	Rs	Rs	Rs	Rs	Rs	Rs	Rs	Rs
Ctr	Se	Se	Se	Se	Se	Se	Se	Rs	Rs	Se	Rs	In	In
AB	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se
Candida tropicalis													
Sensitivity or Resistance Profile													
Antifungal Show	MYA 3404	2130	7809	7806	71	2131	2129	2132	358-03	163	28-04	261-03	216-07
Ket	Se	Se	Se	Se	Se	Rs	Se	Se	Se	In	Se	Se	Se
Fca	Se	Rs	Rs	Rs	Rs	Rs	Se	Se	Se	Se	Se	Rs	Rs
Ctr	Se	Se	Se	Se	Se	Se	Se	In	In	In	Se	Rs	Rs
AB	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se	Se
Candida glabrata													
Sensitivity or Resistance Profile													
Antifungal Show	ATCC 2001	20	190	152-2	131-2	219-2	36	112	274	308	130440	86(2)	E02
Ket	Rs	Se	Rs	Rs	In	Rs	Se	Se	Rs	Se	Se	In	Se
Fca	Rs	Rs	In	In	Se	In	Se	Se	Rs	Se	Se	Rs	Se
Ctr	Se	Se	Se	Se	Se	Se	Se	In	Rs	Se	Se	In	In
AB	Se	Se	Se	Se	Se	Se	Se	Se	Se	Rs	Se	Se	Se
Candida auris													
Sensitivity or resistance profile													
Sample Antifungal	Ca1					Ca2					Ca3		
KET	Rs					Rs					Rs		
CTR	Rs					Rs					Rs		
FCA	Rs					Rs					Rs		
AB	In					In					In		
CAS	Se					Se					Se		

Table 1: Antifungal sensitivity results of strains of *Candida* spp.

Rs: resistance, In: intermediate resistance, Se: sensitivity

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