International Journal of Medical and Health Sciences Research
2014 Vol.1 No.10, pp.119-125
ISSN(e): 2313-2752
ISSN(p): 2313-7746

GENETIC EVALUATION AND ACTIVITY OF ANTIFUNGAL AGAINST CLINICAL ISOLATES *CANDIDA ALBICANS* BIOFILMS

Jaqueline Derissi Braz[1] --- Nayla De Souza Pitangui[2] --- Caetano Toshiro Yamashita[3] --- Ana Marisa Fusco-Almeida[4] --- Maria José Soares Mendes-Giannini[5] --- Janaina De Cássia Orlandi Sardi[6]

[1,2,3,4,5,6]Department of Clinical Analysis, Laboratory of Clinical Mycology, Faculty of Pharmaceutical Sciences, UNESP, Araraquara, Brazil

ABSTRACT

Candida yeasts are common in the oral cavity and can cause candidosis in the presence of predisposing factors, especially diabetes. The manifestation of the disease is related to this set of local factors such as the presence of dental prostheses, salivary pH, salivary flow and tobacco and the ability to form biofilms. Biofilms are specific and organized communities of cells under the control of signaling molecules rather than random accumulations of cells resulting from cell division and frequently are drugs resistance. Aim: The objectives of this study were to determine the genetic patterns of these C. albicans isolates and to evaluate the in vitro activity amphotericin B and caspofungin against C. albicans biofilms. Methods: Microbial samples were collected from subgingival sites and seeded in CHROMagar for subsequent identification of C. albicans by PCR. Genotypes were defined based on the identification of the transposable introns in the 25S rDNA by PCR. Results: In this study, 6 strains were identified as C. albicans and of these, 3 strains were genotype A and 3 were genotype B. The results showed that both amphotericin B and caspofungin exhibited strong antifungal activities against C. albicans biofilm formation and inhibiting the biofilm formation ranging from 70.8 – 95.3% and 77.7 - 88.7%, respectively. The antifungals studied had low inhibitory effect on preformed biofims, ranging from 39.5 - 50.8% for amphotericin B and from 23.1 - 36.9% for caspofungin at the same concentration. The activity of the two drugs was most effective in inhibit biofilm formation.

Keywords: *C. albicans*, Identification, Biofilm, Caspofungin, Amphotericin B, Resistance.

Contribution/ Originality

This study contributes in existent in literature, demonstrating that clinical isolates that deserve special attention in connection with the difficulty of treatment.

1. INTRODUCTION

Candida yeasts inhabit various ecosystems, including the oral cavity and participate as normal commensal microbiota without harming the host [1, 2]. However, systemic diseases like diabetes and AIDS, physiological conditions such as pregnancy, infancy or old age, nutritional factors, treatment with broad-spectrum antibiotics, immunosuppressive corticosteroids, in addition to local factors such use of prosthetic devices are conditions that predispose to the development of infections by *Candida* sp. [3] The mucosa is considered the main reservoir, but studies have shown that *C. albicans* aggregate seems to be contributing to bacterial biofilms in the formation of these structure and hindering the penetration of certain antimicrobial drugs. This fact may be an important factor in the manifestations of candidiasis and the colonization process periodontal pockets [4]. The ability to form biofilm is considered a potent virulence factor and various models have been proposed for understanding the mechanism of formation of these structures [5]. One of the most important consequences of the biofilm mode of growth is the marked resistance to many antifungal agents [6]. Caspofungin act as inhibitors of β-(1,3)-glucan synthesis in the fungal cell wall and have a favorable pharmacological profile. This study evaluated if amphotericin B and caspofungin are effective in the prevention and treatment of *C. albicans* biofilm isolated from patients with periodontitis and diabetes.

2. MATERIAL AND METHODS

This research was approved by the ethical committee for research of the Piracicaba Dental School, State University of Campinas, SP, Brazil.

2.1. Fungal Isolate

A total of 6 *Candida* spp. isolates were obtained from the subgingival biofilm of patients with chronic periodontitis and controlled insulin-dependent type 2 diabetes mellitus from the Faculty Clinic. Pooled biofilms from each site were separated in Eppendorf® microtubes containing 1mL of reduced transport fluid (RTF). Immediately after collecting, the samples from each site were diluted and plated onto a Sabouraud Dextrose Agar (SDA) with Chloramphenicol and chromogenic medium (CHROMagar Candida®, Biomerieux, Paris, France) and incubated at 37°C for 48 h in aerobic condition. The green colonies grown on the agar plate were randomly selected and cultures were stored in glycerol stock at -20°C for later identification by PCR.

2.2. PCR (Polymerase Chain Reaction)

DNA from the *Candida* isolates was extracted using a protocol described by Nascimento, et al. [7] and quantified in a spectrophotometer at 260 nm (Genesys 10UV, Rochester, NY, USA) to obtain a standard concentration of 100 ng/mL and was stored at – 20°C for subsequent PCR reactions. DNA samples were identified by PCR using specific primers for the portion corresponding to the gene AAT1a (ID 3643468) (F: 5'ACT GCT CAA ACC ATC TCT GG -3' and R: 5' CAC AAG GCA AAT GAA GGA AT – 3' with fragment size of 472bp) of *C. albicans*. Sardi, et al. [8]. Purified DNA from *C. albicans* (ATCC 90028) was used as a positive control.

This primer was designed specifically for *C. albicans*. The molecular mass ladder (100 bp DNA ladder, Gibco, Grand Island, NY, USA) was included for running in the agarose gel. PCR amplification was performed with a GeneAmp PCR system 2400 (Perkin-Elmer-Applied Biosystems) under the following thermal conditions: 72º C for 5 min, 38 cycles of 95º C for 30 s, 55ºC for 45s and 72º C for 30 s and extension at 72ºC for 5 min. The PCR products were separated by electrophoresis in 2% agarose gels and Tris-borate-EDTA running buffer (pH.8.0). The DNA was stained with 0.5ug ethidium bromide/mL and visualized under UV illumination (Pharmacia LKB-MacroVue, San Gabriel, CA, USA).

2.3. Genotyping

Ribosomal sequences are extensively used for genotyping of many fungal pathogens. The method developed by McCullough, et al. [9] uses a pair of primers designed to span the region that includes the site of the 1 intron transposable group of the 25S rRNA gene (rDNA), to classify *C. albicans* strains into three genotypes, according to the size of the PCR products: genotype A (approximately 450 bp), genotype B (approximately 840 bp), and genotype C (two products: approximately 450 and 840 bp). DNA of the yeasts previously extracted and spectrophotometrically quantified (100 ng/mL) was submitted to PCR reactions. PCR was performed using the primers CA-INT-L (5' ATA AAG GGA AGT CGG CAA ATA GAT CCG TAA – 3') and CA – INT- R (5' CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT – 3'). The products were analyzed by electrophoresis through 3% (wt/vol) agarose gel. Bands were visualized by UV transllumination after ethidium bromide staining.

2.4. Biofilm Tests and Determination of Antifungal Activity on the Inhibition of Biofilms and On Pre-Formed Biofilms

The biofilm assays were performed according to Pierce, et al. [10] with some modifications. Yeasts were incubated in 20 ml of YPD medium at 30°C for 18 hours. Then we proceeded to wash the cells in phosphate buffered saline - (PBS). Subsequently, the cells were adjusted at a concentration of 1 x 10^7/mL in RPMI. They were then dispensed 100 µL of this suspension in the wells of 96 microtiter wells. The plates were incubated in a bacteriological incubator, shaking at 37°C for 24 hours. Subsequently, the medium was aspirated and the supernatant plate was gently washed with PBS. The quantification of the biofilm assay was performed using the colorimetric method 2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) to see if there was the formation of biofilm. To determine the antifungal activity of caspofungin and amphotericin B, after the formation of biofilms were placed antifungal solutions (100 µL solution of antifungal agent) in serial dilution incubated at 37°C for 24 hours. The tests were performed in duplicate. After the incubation time, aspired to the drug and each well was washed three times with sterile PBS. Subsequently, placed in each well 100 µL of the dye XTT cell viability and the plates were incubated for 4 hours at 37 ° C. The reading was performed and quantified by ELISA (Enzyme-linked immunosorbent assay) to 490nm. The concentration of caspofungin ranged from 64 µg/mL to 0.0625 µg/mL and amphotericin B 16 µg/mL to 0.0312 µg/mL. For the inhibition

of biofilm, the drug was placed with the inoculum and after 24 hours, the reading was performed. The diluition of the drugs was carried out in accordance with the microdilution method described according to the M27-S3 of the CLSI 2008, with modifications.

3. RESULTS

3.1. Identification of *C. albicans* by PCR

In this current study, 6 *Candida* spp. strains were obtained from the subgingival biofilm of patients with chronic periodontitis and controlled insulin-dependent type 2 diabetes mellitus, and isolated in CHROMagar. All were identified as *Candida albicans* by PCR.

3.2. Genotyping

A total of 6 strains isolated from patients with chronic periodontitis diabetics were submitted to molecular typing by the method described above. 50% were genotype A and 50% were genotype B. Genotype C was not found in this present study.

3.3. Biofilm Tests and Determination of Antifungal Activity on the Inhibition of Biofilms and On Pre-Formed Biofilms

All isolates were able to form biofilm. The results showed that both amphotericin B and caspofungin exhibited strong antifungal activities against *C. albicans* biofilm formation and inhibiting the biofilm formation ranging from 70.8 – 95.3% and 77.7 – 88.7%, respectively at concentration of 2µg/mL, as showed in Figure 2A and B. The antifungals studied had low inhibitory effect on preformed biofilms, ranging from 39.5 - 50.8% for amphotericin B and from 23.1 - 36.9% for caspofungin at the same concentration, as showed in Figure 2C and D.

4. DISCUSSION

In this current study, 6 *Candida* spp. strains were obtained from the subgingival biofilm of patients with chronic periodontitis and controlled insulin-dependent type 2 diabetes mellitus, and isolated in CHROMagar and were identified as *Candida albicans* by PCR. This isolates were submitted to molecular typing by the method described above. 50% were genotype A and 50% were genotype B. Genotype C was not found in this present study. The ability of *C. albicans* to form biofilm is well established in literature [10, 11]. In our study, all isolates were able to form biofilm. In relation the action of the antifungal agents in biofilms, many studies have shown that biofilm formation impairs the action of conventional antifungals [5]. Our results showed that both amphotericin B and caspofungin exhibited strong antifungal activities against *C. albicans* biofilm formation and inhibiting the biofilm formation ranging from 70.8 – 95.3% and 77.7 – 88.7% respectively at concentration of 2µg/mL (Figure 2 A and B). The antifungals studied had low inhibitory effect on preformed biofilms, ranging from 39.5 to 50.8% for amphotericin B and from 23.1 to 36.9% for caspofungin at the same concentration (Figure 2C and D). There was no difference between the MICs of genotypes A and B. Tobudic, et al. [12] studied amphotericin B, and caspofungin in biofilms obtained MIC (minimum inhibitory concentration) of 4 µg/ml for

both antifungals. The ability of *Candida* spp. to form drug-resistant biofilms is an important factor in its contribution to human disease. Like the vast majority of microbial biofilms [13], sessile cells within a *C. albicans* biofilm are less susceptible to antimicrobial agents than are planktonic cells [14, 15]. The formation of biofilms causes clinical problems of concern because they increase resistance to antifungal therapies; the mechanism of biofilm resistance to antimicrobial agents is not fully known. One hypothesis that can explain this resistance is the presence of the matrix, which restricts the penetration of drugs through the formation of a diffusion barrier [16] and only the most superficial layers are in contact with lethal doses of antibiotics. Cateau, et al. [17] found that micafungin has activity inhibitory excellent adhesion of *C. albicans* to catheters silicone prevents the development of biofilms on this substrate and eradicates the biofilm developed on 12 h, keeping this effect for 48 h, allowing the utility to consider potential for sealing intravascular catheters. Antibiofilm activity of echinocandins has also been check in various animal models or cellular [18], without found significant advantages in this activity with the combination caspofungin with other antifungals such as voriconazole [19, 20].

Jain, et al. [21] who found that biofilms of isolates obtained from urinary catheters showed a resistance to caspofungin and fluconazole, although they were inhibited by MIC50 < 1 ug/ml amphotericin B.

In this study, the activity of the two drugs was most effective in inhibited biofilm formation because it shows that the selected isolates have different contents of lipids and glucans in biofilm formation, probably showing that caspofungin in the case of the glucan synthase may be a key to the structure of biofilms.

5. CONCLUSION

In conclusion, our results showed that both antifungal agents can inhibit biofilm formation however in preformed biofilms of the same action is lower.

REFERENCES

[1] P. Eggimann, J. Garbino, and D. Pittet, "Epidemiology of Candida species infections in critically ill non-immunosuppressed patients," *Lancet Infect Dis.*, vol. 3, pp. 685-702, 2003.

[2] L. C. Shao, C. Q. Sheng, and W. N. Zhang, "Recent advances in the study of antifungal lead compounds with new chemical scaffolds," *Yao Xue Xue Bao.*, vol. 42, pp. 1129-1136, 2007.

[3] CLSI, "Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard-second edition. CLSI document M38-A2," *Clinica and Laboratory Standards Institute (formely NCCLS), 940 West Valley Road, Wayne, Pa*, 2008.

[4] J. Sardi, C. Duque, J. Höfling, and R. Gonçalves, "Genetic and phenotypic evaluation of Candida albicans strains isolated from subgingival biofilm of diabetic patients with chronic periodontitis," *Med Mycol.*, vol. 50, pp. 467-75, 2012.

[5] J. Chandra, D. Kuhn, P. Mukherjee, L. Hoyer, T. McCormick, and M. Ghannoum, "Biofilm formation by the fungal pathogen Candida albicans: Development, architecture, and drug resistance," *J Bacteriol. Sep.*, vol. 183, pp. 5385-94, 2001.

[6] F. Růzicka, V. Holá, M. Votava, and R. Tejkalová, "Importance of biofilm in Candida parapsilosis and evaluation of its susceptibility to antifungal agents by colorimetric method," *Folia Microbiol (Praha)*, vol. 52, pp. 209-14, 2007.

[7] E. Nascimento, R. Martinez, and A. Lopes, "Detection and selection of microsatellites in the genome of paracoccidioides brasiliensis as molecular markers for clinical and epidemiological studies," *J Clin Microbiol.*, vol. 42, pp. 5007-5014, 2004.

[8] J. Sardi, A. Fusco-Almeida, and M. Mendes-Giannini, "New antimicrobial therapies used against fungi present in subgingival sites – A brief review," *Arch Oral Biol.*, vol. 56, pp. 951-9, 2011.

[9] M. McCullough, K. Clemons, and D. Stevens, "Molecular and phenotypic characterization of genotypic Candida albicans subgroups and comparison with Candida dubliniensis and Candida stellatoidea," *J Clin Microbiol.*, vol. 37, pp. 417-421, 1999.

[10] C. Pierce, P. Uppuluri, A. Tristan, F. J. Wormley, E. Mowat, G. Ramage, and J. Lopez-Ribot, "A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing," *Nat Protoc.*, vol. 3, pp. 1494-500, 2008.

[11] G. Ramage, E. Mowat, B. Jones, C. Williams, and J. Lopez-Ribot, "Our current understanding of fungal biofilms," *Crit Rev Microbiol.*, vol. 35, pp. 340-55, 2009.

[12] S. Tobudic, C. Kratzer, A. Lassnigg, W. Graninger, and E. Presterl, "In vitro activity of antifungal combinations against Candida albicans biofilms," *J Antimicrob Chemother., Feb). Epub 2009 Dec 8. PubMed PMID: 19996142*, vol. 65, pp. 271-4, 2010.

[13] R. Rajendran, D. P. Robertson, P. J. Hodge, D. F. Lappin, and G. Ramage, "Hydrolytic enzyme production is associated with Candida albicans biofilm formation from patients with type 1 diabetes," *Mycopathologia*, vol. 170, pp. 229-235, 2010.

[14] D. M. Kuhn and M. A. Ghannoum, "Candida biofilms: Antifungal resistance and emerging therapeutic options," *Curr Opin Investig Drugs*, vol. 5, pp. 186-197, 2004.

[15] R. Rautemaa and G. Ramage, "Oral candidosis--clinical challenges of a biofilm disease," *Crit Rev Microbiol.*, vol. 37, pp. 328-36, 2011.

[16] P. Gilbert, D. G. Allison, and A. J. McBain, "Biofilms in vitro and in Vivo: Do singular mechanisms imply cross-resistance?," *Symp Ser Soc Appl Microbiol.*, vol. 31, pp. 98-110, 2002.

[17] E. Cateau, M. Rodier, and C. Imbert, "In vitro efficacies of caspofungin or micafungin catheter lock solutions on Candida albicans biofilm growth," *J Antimicrob Chemother*, vol. 62, pp. 153-155, 2008.

[18] J. Shuford, K. Piper, J. Steckelberg, and R. Patel, "In vitro biofilm characterization and activity of antifungal agents alone and in combination against sessile and planktonic clinical Candida albicans isolates," *Diagn Microbiol Infect Dis.*, vol. 57, pp. 277-281, 2007.

[19] J. Shuford, M. Rouse, K. Piper, J. Steckelberg, and R. Patel, "Evaluation of caspofungin and amphotericin B deoxycholate against Candida albicans biofilms in an experimental intravascular catheter infection model," *J Infect Dis.*, vol. 194, pp. 710- 713, 2006.

[20] Q. Guillermo, y. María Villar-Vidal, and E. Elena, "Actividad de la micafungina contra las biopelículas de Candida," *Rev Iberoam Micol.*, vol. 26, pp. 49-55, 2009.

[21] N. Jain, R. Kohli, E. Cook, P. Gialanella, T. Chang, and B. Fries, "Biofilm formation by and antifungal susceptibility of Candida isolates from urine," *Appl Environ Microbiol*., vol. 73, pp. 1697-1703, 2007.

Figure-1. *C. albicans* biofilms observed in optical microscope 40x.

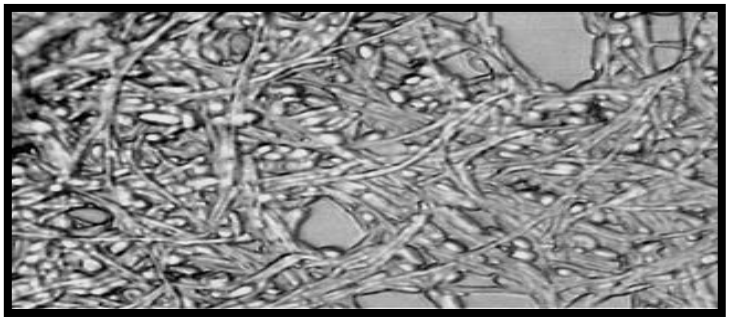

Figure-2. Activity of antifungal against clinical isolates *C. albicans* biofilms. Activity of amphotericin B **(A)** and caspofungin **(B)** in inhibiting the biofilm formation quantified by XTT. Activity of amphotericin B **(C)** and caspofungin **(D)** in preformed biofilm quantified by XTT.