Mycopathologia (2015) 179:391–395
DOI 10.1007/s11046-015-9862-9

Detection and Quantification of Fluconazole Within *Candida glabrata* Biofilms

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Received: 20 July 2014 / Accepted: 12 January 2015 / Published online: 23 January 2015

Abstract *Candida* infections are often associated with biofilms and consequent high resistance to most common drugs (e.g. azoles). These resistance mechanisms are not only associated with the biofilm yeast physiology, but also with the presence of a diffusional barrier imposed by the biofilm matrix; however, the real biochemical role of the biofilm components remains very unclear. So, in order to further clarify this issue, we intend to determine, for the first time, fluconazole in biofilms within both supernatants and matrices. *Candida* biofilms were formed in the presence of fluconazole, and it was recovered from both supernatant and matrix cell-free fractions. Then, high-pressure liquid chromatography was used to identify and quantify the amount of drug that was present in the two fractions. Moreover, this study also showed that the presence of fluconazole in both fractions indicated that the drug administrated did not completely reach the cells, so this phenomena can easily be associated with lower biofilm susceptibility, since the drug administered did not completely reach the cells.

Keywords *Candida* · Biofilm matrix · Antifungal · Method · Resistance

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Introduction

The incidence of fungal infections has increased meaningfully in the last decades, especially in immunosuppressed and/or hospitalized patients, causing high levels of morbidity and mortality [1]. The rise in antimicrobial resistance and the restricted number of effective antifungal drugs, which still have many side effects, may be responsible for this event, specially related to infections caused by yeasts from the *Candida* genus [2]. In fact, *Candida* species are the third most common cause of nosocomial infections and the most common etiologic agent of fungal-related biofilm infections [3, 4]. Between the *Candida* species, *Candida glabrata* is the second most prevalent pathogenic fungal species in humans, after *Candida albicans* [2]. Even though *C. glabrata* is not capable of producing hyphae, it has a number of virulent factors, comprising secretion of hydrolytic enzymes, adhesion to host cells or to medical devices, and biofilm formation [3]. This last and very important capability can occur on the host mucosa and on the

medical indwelling surfaces devices, and it involves the production of an extracellular matrix that encloses yeasts' microcolonies [4]. Biofilms are biological communities with an extraordinary degree of organization, in which microorganisms form structured, coordinated, and functional communities, embedded in a self-created extracellular matrix. Biofilm production is also associated with a high level of antifungal resistance. The ability of *Candida* species to form drug-resistant biofilm is an important factor in their contribution to human disease [5–7]. In the widely held view of microbial biofilms, sessile cells within biofilms are less susceptible to antimicrobial agents than planktonic cells, since the development of drug resistance has been linked with an increase in the maturation process [8, 9]. Additionally, many authors have questioned whether biofilm matrices interfere in its resistance. Still, it is difficult to overcome this problem, since there is a lack of methodologies which allow the detection of the amount of xenobiotics able to diffuse into matrix and reach biofilm cells.

High-pressure liquid chromatography (HPLC) is a very sensitive technique used to separate, identify, and quantify the components in a mixture, and it is very useful in biomedical assays. Reverse-phase HPLC (RP-HPLC) is a particular model of HPLC which operates on the principle of hydrophobic interactions, allowing the measurement of these interactive forces [10]. As several drugs were already identified and quantified using this procedure [11], this methodology was used to detect fluconazole.

Thus, the present work proposes an adapted methodology for the detection and quantification of an antifungal agent (fluconazole) in the biofilm environment, by HPLC, in order to understand its diffusion within the matrix and to contribute to a better understanding of biofilms' tolerance to antifungal agent phenomena.

Methods

Candida Biofilm Formation

Fluconazole (Flu) was kindly provided by Pfizer, S.A., in its pure compound. Aliquots with a final concentration of 1,000 mg/L were prepared in dimethyl sulfoxide and the final dilutions in Sabouraud dextrose broth medium (SDB) (Merck, Darmstadt, Germany).

To validate the method, two strains of *C. glabrata* (534784 and 562123 from the Hospital of Braga, Portugal) were used. The identification of all isolates strains was confirmed using CHROMagar *Candida* (CHROMagar, Paris, France) and by PCR-based sequencing using specific primers (*ITS1* and *ITS4*) against the 5.8-s subunit gene [10]. The PCR products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (Perkin Elmer, Applied Biosystems, Warrington, UK). *C. glabrata* strains were subcultured on Sabouraud dextrose agar (SDA) (Merck, Darmstadt, Germany) for 24 h at 37 °C. Cells were then inoculated in SDB (Merck, Darmstadt, Germany) and incubated for 18 h at 37 °C under agitation at 120 rev/min. After incubation, the cells were harvested by centrifugation at 3,000*g* for 10 min at 4 °C and washed twice with phosphate-buffered saline (PBS 0.1 M, pH = 7.2). Pellets were then suspended in SDB, and the cellular density was adjusted to 1×10^5 cells/mL using a Neubauer counting chamber. Standardized cell suspensions (500 µL) were placed into selected wells of 24-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). As negative control, SDB was used without cells and antifungal agent. As positive control, only cell suspensions were tested without antifungal agent. At 24 h, 250 µL of cell suspension was removed and an equal volume of fresh SDB plus 250 µL of Flu (1,000 mg/L, 2× concentrated) were added. The plates were incubated at 37 °C for more 24 h, a total of 48 h at 120 rpm/min. After the biofilm formation, the medium was aspirated and non-adherent cells removed by washing the biofilms with sterile ultrapure water [11].

Extraction of the Antifungal Agent from Biofilms

After biofilm formation in the presence of the antifungal agent, each well of the 24-well plate was treated as one single biofilm microecosystem. A diagram summarizing the protocol steps is presented in Fig. 1. Firstly, the supernatant from the biofilms was collected (volume = 350 µL), centrifuged at 12,000*g* during 10 min, and filtered with a 0.22-µm filter to remove possible contaminant cells (supernatant fraction). In parallel, the biofilm remaining in the wells was scrapped with 150 µL of sterile water and the suspension was collected, sonicated (Ultrasonic Processor, Cole Parmer) during 10 s at 30 %, and centrifuged at 12,000*g* during 10 min [11]. Finally, the

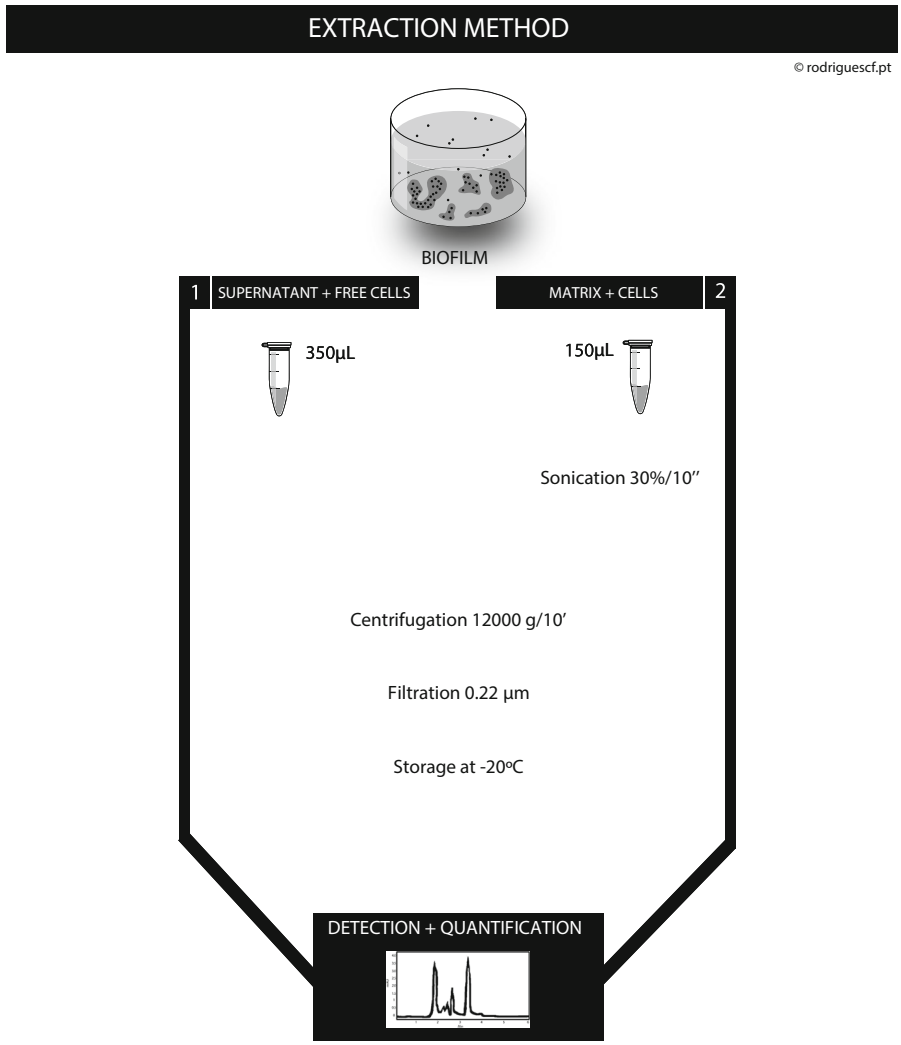

Fig. 1 Scheme of the method for detection and quantification of fluconazole within *Candida* biofilms

resultant supernatant was filtered with a 0.22-µm filter to remove cells (matrix). Sonication time was optimized for this procedure, making colonies formation units counts as controls. The samples were stored at −20 °C until the detection, and quantification method was executed. Each assay was performed with six samples, in three independent assays.

The high-pressure liquid chromatography (HPLC—Varian 9002/Pro-Star) method was performed using a C18 column (YMC, Inc). For that, a slightly modified method from Sadasivudu et al. [12] was performed. The mobile phase was acetonitrile:water (50:50) (Fisher Chemicals) with a flow of 1 mL/min, during 6 min for each sample, read at 260 nm. Standards of Flu used for the calibration curve were prepared with the mobile phase in the following range: 1.0, 2.0, 4.0, 8.0, 20.0, 40.0, and 100.0 mg/L. The obtained equation was Area = (0.091 × [Flu]) + 0.007, $r^2 = 0.996$.

Results and Discussion

In the last years, biofilms are gaining much more importance not only in the research field, but also on the clinical practice. As it is known, they confer significant resistance to antifungal therapy, by limiting the penetration of the xenobiotic through the matrix, thus protecting cells [2, 9, 14]. However, the knowledge on this ground is still scarce, especially in *Candida* biofilms. The fact that biofilms are very complex structures, due to their environment and matrix, make the studies more difficult to execute. Also, it is known

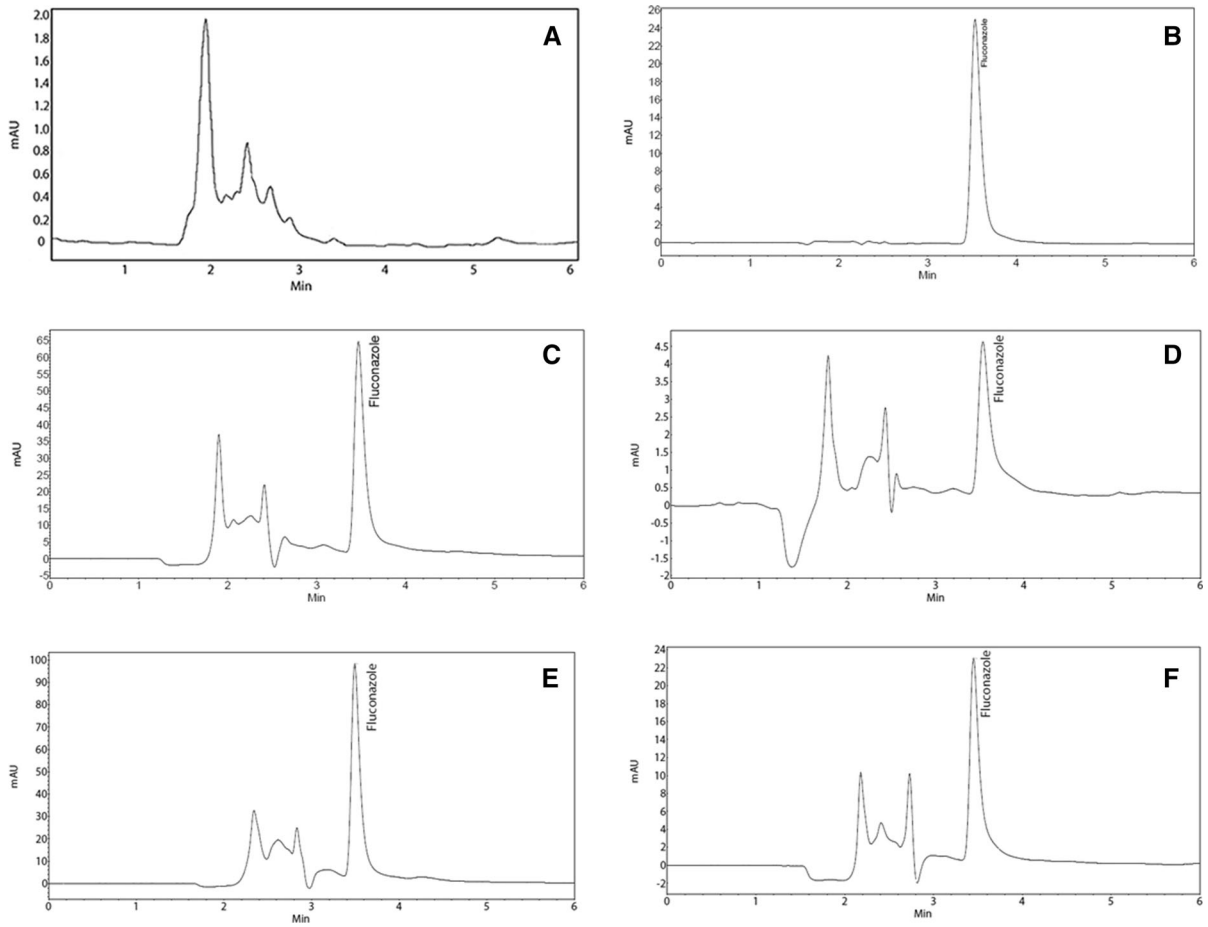

Fig. 2 Chromatograms of the detection and quantification of fluconazole within *C. glabrata*'s biofilms. **a** Control 0 mg/L; **b** fluconazole's standard 40 mg/L; **c** *C. glabrata* 562123 matrix; **d** *C. glabrata* 562123 supernatant; **e** *C. glabrata* 534784 matrix; **f** *C. glabrata* 534784 supernatant

Table 1 Quantification of fluconazole (Flu) present in *C. glabrata* strains biofilm supernatant (S) and matrix (M) fractions

Biofilm strains	Average [Flu] mg/L ± SD	
C. glabrata 562123	S	551.96 ± 44.22
	M	60.81 ± 1.43
C. glabrata 534784	S	707.29 ± 34.97
	M	67.96 ± 2.54

SD standard deviation

that antifungal agents' action in biofilms is poor, and one of the main responsible for that inactivity the extracellular matrix [14]. The mechanisms involved in this phenomena and the pathway taken by the antifungal compounds or the amount of drug that reaches the cells, crossing the biofilm matrix, are still unknown [14–16]. So, it is of major importance to verify if the antifungal agents are still in the biofilm environment (supernatant fraction) or if they are retained in the extracellular matrix (matrix fraction).

Therefore, the aim of this work was to detect and quantify the presence of the Flu on *C. glabrata*'s biofilms, after its isolation from supernatants and matrices (Fig. 1). The HPLC methodology proposed by Sadasivudu et al. was slightly modified in order to quantify this drug, recovered from both fractions. As observed in Fig. 2, Flu was easily detected in *C. glabrata*'s biofilm fractions and presented a peak at the retention time of 3.5 min, at 260 nm. In addition, the calibration curve obtained (Area = 0.091 [Flu] + 0.007) and confirmed that HPLC is a good methodology to detect Flu, with a correlation factor of 0.996. The

detection of Flu, present in biofilm supernatants (Fig. 2d–f) and in their matrices (Fig. 2c–e), was obtained without any interference, presenting a clear peak with the same retention time. In order to confirm the reliability of the methodology, two biofilms of *C. glabrata* strains were assessed, since they showed significant different biofilm susceptibilities profiles to Flu ($P < 0.0001$): *C. glabrata*'s 562123 biofilm is more susceptible (20 % of biofilm biomass inhibition, using Crystal Violet assay [13]) than *C. glabrata*'s 534784 biofilm (with 13 % of inhibition, from crystal violet assay [13]). The quantification of Flu present on both biofilm supernatants and matrices is presented in Table 1.

Interestingly, in samples of *C. glabrata* 562123, which is the less resistant, we noticed a lower amount of Flu in both fractions, which means that it is likely that there was a higher proportion of the antifungal reaching the cells and, consequently, causing the greater inhibition. In opposition, *C. glabrata* 534784 had a high amount of antifungal in the medium, probably showing the capacity of the strain to avoid the antifungal entering in its cells. It is important to address that the method optimized, has the advantage of detecting low quantities of the drug, is easy and low time-consuming. Additionally, this methodology can also be used to study of the dynamic of interaction of other compounds (e.g., hormones, nutrients, and other chemical compounds) within the biofilm matrices of any other microorganism (yeast and bacteria).

In summary, we are proposing a methodology that will allow a better understanding of biofilms and drugs, regarding the amount of a compound present in the supernatant of matrices of biofilm, which will be an important step to understand the problematic of biofilm resistance and the higher number of diseases associated with them.

Acknowledgments This work was supported by the Programa Operacional, Fatores de competitividade—COMPETE and by national funds through FCT—Fundação para a Ciência e a Tecnologia on the scope of the projects FCT PTDC/SAU-MIC/119069/2010, RECI/EBB-EBI/0179/2012, PEst-OE/EQB/LA0023/2013 and Célia F. Rodrigue's SFRH/BD/93078/2013 PhD grant. The authors thank the Project "BioHealth—Biotechnology and Bioengineering approaches to improve health quality," Ref. NORTE-07-0124-FEDER-000027, co-funded by the Programa Operacional Regional do Norte (ON.2—O Novo Norte), QREN, FEDER. We also would like to acknowledge Pfizer®, S.A., for the kindly donation of fluconazole.

Conflict of interest The authors declare no competing financial interest.

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