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ORIGINAL ARTICLE

# The effect of antifungal combination on transcripts of a subset of drug-resistance genes in clinical isolates of *Candida* species induced biofilms

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**Abstract** Biofilm formation is often associated with increased *Candida* resistance toward antifungal agents. Therefore, the current study aimed to assess the incidence of biofilm formation among *Candida* isolates and to investigate the effect of high doses of fluconazole {FLC}, voriconazole {VOC} and amphotericin B {AMB}, singly and in combination on mature biofilms. Moreover, it aimed to assess the expression of selected genes (CDR1, KRE1 and SKN1) responsible for *Candida* biofilm resistance. The study included 49 patients; samples were collected from the King Khalid Hospital, Riyadh, Saudi Arabia. Isolates were prepared for biofilm formation and quantification using 0.4% (w/v) crystal violet. Minimum Inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) were conducted by the broth microdilution method. Biofilm eradication was evaluated using counting, XTT stain intensity and observed under the inverted microscope. Selected genes were evaluated in *Candida* biofilms under the effect of antifungal exposure using QPCR. The major isolates were *Candida albicans* (65.3%) followed by *Candida tropicalis* and *Candida glabrata*. 77.6% of the strains were biofilm formers. AMB showed susceptibility in

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87.8% of isolates, followed by VOC (77.6%) and FLC (67.3%). MIC50 and MIC90 were (0.03, 0.125), (0.5, 8), (2, >128) μg/ml for AMB, VOC and FLC, respectively. 34.7% and 18.4% of the isolates were antagonistic to AMB/FLC and AMB/VOC, respectively. Mature biofilms of ten selected isolates were found resistant to FLC (1000 μg/ml). VOR and AMB concentration required to inhibit biofilm formation was 16–250 fold higher than the MIC for planktonic cells. Isolates showed significant reduction with antifungal combination when compared with the untreated controls ($p$ value $\leqslant 0.01$), or using fluconazole alone ($p$ value $\leqslant 0.05$). High doses of the antifungals were employed to assess the effect on the persisters' selected gene expression. Marked over expression of SKN1 and to a lesser extent KRE1 was noticed among the mature biofilms treated with AMB alone or in combination after 1 h of exposure, and SKN1 expression was even more sharply induced after 24 h. No statistically significant over expression of CDR1 was observed in biofilms after exposure to high doses of FLC, VOC or any of the combinations used.



## 1. Introduction

*Candida* species are the most common cause of fungal infections. *Candida* induced infections range from non-life-threatening mucocutaneous illnesses to invasive processes that may involve virtually any organ. The growing frequency of hospital acquired *Candida* especially bloodstream infections is due to the increased use of immunosuppressive therapy in cancer and transplant patients, which leads to breakdown of the barrier between the gut and bloodstream (Nucci and Anaissie, 2001).

*Candida* cells, as proven in many studies, are able to adhere to and colonize surfaces of medical devices, such as central venous catheters, orthopedic prostheses, intrauterine devices and prosthetic joints and valves, among others, resulting in the development of a biofilm (Douglas and Cobbs, 1992; Raad et al., 1993; Tunney et al., 1999). Infections due to the presence of fungal biofilms are a major clinical concern as these structures are characterized by increased resistance to antifungal therapy (Ramage et al., 2006).

Various antifungal agents are used to treat these infections, including azoles and polyenes (Pappas et al., 2004). Fluconazole (FLC) as well as voriconazole (VOC), approved in 2002, belong to the tiazoles, they interfere with ergosterol biosynthesis by binding to lanosterol 14-α demethylase (Richardson, 1990). The latter enzyme is crucial for ergosterol production, and inhibition of its activity which causes disruption of the cell membrane leading to growth inhibition of the fungus (Kelly et al., 1993). Amphotericin B (AMB) is a member of the polyene family (Warnock, 1991). This molecule binds to ergosterol and forms pores resulting in a disorganized membrane with increased permeability. In addition, AMB induces cell damage by generating lethal reactive oxygen species (Brajtburg et al., 1990).

The progression of drug resistance within *Candida* biofilms has been associated with a parallel increase in the maturation process (Sardi et al., 2011). Furthermore, some studies have shown that biofilms of *Candida* develop statically in the presence of a minimal matrix and exhibit the same level of resistance to antifungal treatment; as cells grown in shaker and exhibiting large amounts of matrix (Seneviratne et al., 2008; Sardi et al., 2011). However, several molecular mechanisms of resistance to antifungal agents in *Candida* have been described. In particular, these include the increased efflux of antifungal agents due to the overexpression of efflux genes, CDR1 and CDR2 (the family of ABC membrane transport proteins – the ATP binding cassette) (Sardi et al., 2011). Moreover, changes in β-1,6-glucan biosynthesis have also been proposed as a resistance mechanism against AMB (Gale, 1986). SKN1 and KRE1, two genes involved in β-1,6-glucan biosynthesis (Mio et al., 1997), were found to be differentially expressed after *in vitro* exposure to antifungal treatment (Liu et al., 2005).

A combined action of different mechanisms is believed to contribute to increased resistance, especially in the presence of persisters in the biofilm, which are able to tolerate high concentrations of antimycotics (Seneviratne et al., 2008). Interestingly, these persisters are not mutants but rather phenotypical variants of wild type cells (LaFleur et al., 2006). Until now, the molecular basis of persistence in *Candida* species biofilms is not fully understood (Lewis, 2010).

Most of the published studies examining the transcriptional expression of drug-resistance genes in *Candida* spp. have been confined to the planktonic mode of growth, and few data are available for the biofilm mode (Sanglard et al., 1995; White, 1997; Lopez-Ribot et al., 1998; Franz et al., 1998, 1999; Perea et al., 2001; Holmes et al., 2008). Therefore, the current study aimed to assess the incidence of biofilm formation among *Candida* clinical isolates and to investigate the effect of high doses of commonly used antifungal drugs (fluconazole, voriconazole and amphotericin B, singly and in combination) on *Candida* mature (48 h) biofilms. Moreover, it aimed to assess the expression of selected genes (CDR1, KRE1 and SKN1) of those known to be responsible for resistance in *Candida* biofilms under the effect of antifungal exposure.

## 2. Subjects and methods

### 2.1. Fungal isolates

The current study included 49 *Candida* isolates; they were collected from the Microbiology Laboratory, King Khalid Hospital, KSU, Riyadh, SA, between December 2010 and May 2012 from various clinical samples. Clinical data were collected including; sex, age, site of sample as well as departments involved. Further processing of the isolates was performed in the Microbiology laboratories of the College of Pharmacy, King Saud University.

### 2.2. Confirming the identity of the strains

All isolates of *Candida* spp. were identified using standard procedures based on the germ tube test, the formation of