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Effect of Voriconazole on *Candida tropicalis* Biofilms: Relation with *ERG* Genes Expression

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Abstract *Candida tropicalis* has emerged as the third most prevalent fungal pathogens and its ability to form biofilms has been considered one of the most important virulence factors, since biofilms represent high tolerance to antifungal agents. However, the mechanisms of *C. tropicalis* biofilm resistance to antifungals remain poorly understood. Thus, the main aim of this work was to infer about the effect of voriconazole on the formation and control of *C. tropicalis* biofilms and disclose its relationship with *ERG* genes' expression. Planktonic cells tolerance of several *C. tropicalis* clinical isolates to voriconazole was determined through of antifungal susceptibility test, and the effect of this azole against *C. tropicalis* biofilm formation and pre-formed biofilms was evaluated by cultivable cells determination and total biomass quantification. *ERG* genes expression was analyzed by quantitative real-time polymerase chain reaction. This work showed that *C. tropicalis* resistance to voriconazole is strain dependent and that voriconazole was able to partially control biofilm formation, but was unable to eradicate *C. tropicalis* pre-formed biofilms. Moreover, *C. tropicalis* biofilms resistance to voriconazole seems to be associated with alterations of sterol content in the cell membrane, resulting in *ERG* genes overexpression. Voriconazole is unable to control *C. tropicalis* biofilms, and the overexpression of *ERG* genes is a possible mechanism of biofilm resistance.

Keywords *Candida tropicalis* · Biofilms · Resistance · Voriconazole · *ERG* genes

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Introduction

Invasive fungal infections, such as candidiasis, represent a public health problem of major importance, since these infections are among the leading causes of patients' morbidity and mortality [1–3]. The members of the genus *Candida* are the most frequently recovered from human fungal infections and are of particular importance in patients undergoing treatment for cancer, organ transplantation or receiving broad-spectrum antibiotics or antifungal drugs [4]. This type of infection has emerged due to the rise of the elderly population, to a high number of immunocompromised patients and to a more widespread use of indwelling medical devices [1, 5–7].

Candida albicans is the main cause of candidiasis and is responsible for the majority of mucosal and systemic infections [2, 4, 7]. However, in parallel with the overall increase in fungal infections, it has been observed that infections caused by non-*Candida*

albicans Candida (NCAC) species, namely *Candida tropicalis*, are also emerging [2, 8, 9]. Indeed, *C. tropicalis* has been reported as an important causative agent of the bloodstream and urinary tract infections in hospitalized patients [2]. This increased incidence of *C. tropicalis* may be attributed to its biofilm formation capability [2, 9]. Biofilms are known as surface-associated communities of microorganisms embedded in an extracellular matrix (ECM), which may promote infection and persistence in the host [1, 7, 10, 11]. Biofilms confer significant resistance to antifungal therapy and host immune responses, by limiting the penetration of substances through the ECM [1, 4, 12]. Moreover, *Candida* resistance to antifungal agents has been associated with alterations of sterol content in cell membrane, which should generally cause a defective ergosterol biosynthesis, resultant from mutations in the ergosterol synthesis-associated genes (*ERG* genes) [13–15].

For many decades, one of the most used antifungal agents, as a prophylactic or therapeutic response to candidiasis, was fluconazole (Flu). However, due to many Flu resistance case reported, another azole, voriconazole (Vcz), was developed in order to combat *Candida* infections [4].

Many previous studies have focused on *C. tropicalis* tolerance to Flu, whereas there is a lack of studies concerned Vcz [11, 12, 16]. Thus, the main goal of this work was to infer about the effects of Vcz on the formation and control of *C. tropicalis* biofilms and disclose its relationship with *ERG* genes' expression.

Materials and Methods

Organisms and Growth Conditions

Three different *C. tropicalis* clinical isolates were used during this work: one oral strain (AG1) isolated from a patient of the Clinic Dentistry, Congregados, Braga, Portugal; one urinary tract strain (519468) recovered from a patient of the Hospital of S. Marcos, Braga, Portugal; and one vaginal strain (12) belonging to archive collection of the University of Maringá, Brazil. The strains identification of all isolates was confirmed using CHROMagar *Candida* medium (CHROMagar, France) and by polymerase chain reaction (PCR)-based sequencing using specific primers (ITS1 and ITS4) against the 5.8S subunit gene reference. Genomic deoxyribonucleic acid (DNA) was extracted following previously described procedures [17]. The PCR products were sequenced using the ABI-PRISM Big Dye terminator cycle sequencing kit (PerkinElmer, Applied Biosystems, Warrington, UK). For all experiments, *C. tropicalis* strains were subcultured on Sabouraud dextrose agar medium (SDA; Merck, Germany) for 48 h at 37 °C.

Antifungal Susceptibility Tests

Minimum inhibitory concentrations (MICs) for Voriconazole (Vcz; Pfizer, New York, USA) were determined, using the microdilution method, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [18]. Vcz was tested in concentrations of 0.5, 0.75, 1, 2, 3, 4, 6 and 10 mg/L. To confirm the MICs values, the cell suspension of each strain was recovered to a new well and serial dilutions were plated in SDA at 37 °C. After 48 h of incubation, the total colony-forming units (CFUs) were determined and the results presented as Log_{10} CFUs per milliliter (Log_{10} CFUs/mL). Assessments of the response profiles with antifungal agent were performed in triplicate and on three different occasions.

Biofilm Formation

Biofilm formation was performed as described previously by Silva et al. [1]. An inoculum of each yeast, obtained from SDA plates, was resuspended in 30 mL of Sabouraud dextrose broth medium (SDB; Merck, Germany) and incubated for 16–18 h at 37 °C, under agitation at 120 rpm. Then, cells were harvested by centrifugation at 5000*g*, for 10 min at 4 °C and washed twice in phosphate buffer saline (PBS), pH 7.0, 0.1 M. The cell pellets were resuspended in PBS and the cellular density adjusted to 2×10^5 cells/mL in Roswell Park Memorial Institute 1640 medium (RPMI; Sigma, St. Louis, USA) using a Neubauer counting chamber, to be used in each experiment. The experiments were repeated in triplicate on three different occasions.

Voriconazole Effect on Biofilms

In order to study the effect of Vcz on biofilm formation, different concentrations of antifungal agent (5, 10, 50 and 100 mg/L) were prepared in RPMI 1640 medium and added at the beginning of the process of

biofilm formation. The different concentrations of Vcz used were selected in accordance with MICs previously determined. For that, 100 µL of each *Candida* suspension containing 2×10^5 cells/mL and 100 µL of antifungal agent were added to the respective 96-well microplates (Orange Scientific, Braine-l' Alleud, Belgium). The microplates were incubated for 48 h at 37 °C and at 120 rpm, the same period of the treatment applied during planktonic cells assays.

In order to infer about the effect of Vcz against *C. tropicalis* pre-formed biofilms, biofilms were previously formed during 24 h at 37 °C and at 120 rpm, in RPMI 1640 medium. A volume of 100 µL of each *Candida* suspension was added to the respective 96-well microplates containing 100 µL of RPMI 1640 medium. After this, the medium was aspirated and different concentrations of Vcz (10, 50, 100 and 200 mg/L) prepared in RPMI 1640, were added to the specific wells to treat the pre-formed biofilms and incubated for additional 48 h at 37 °C and at 120 rpm. Controls were performed with *Candida* cells without antifungal agent.

Biofilm Analyses

Biofilm Biomass Quantification

Biofilm total biomass was quantified by crystal violet (CV) staining [1]. For that, after biofilm formation the medium was totally aspirated and non-adherent cells were removed by washing once with 200 µL of PBS. The biofilm was fixed with 200 µL of methanol (100 % v/v), which was removed after 15 min of contact. The microplates were allowed to dry at room temperature, and 200 µL of CV (1 % v/v) was added to each well and incubated for 5 min. The wells were then gently washed twice with 200 µL of sterile ultrapure water, and 200 µL of acetic acid (33 % v/v) was added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a microplate reader at 570 nm. The results were presented as absorbance per unit area (Abs_{570nm}/cm^2).

Biofilm Cultivable Cells Quantification

The number of cultivable biofilm's cells was determined by the enumeration of CFUs. For this, the medium was aspirated and the biofilms washed once with 200 µL of PBS to remove non-adherent cells. Then, the biofilm was scraped from the 96-well microplates and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix [7]. Serial tenfold dilution in PBS was plated in SDA and incubated at 37 °C for 24 h. The results were presented as the total of CFUs per unit area ($\log_{10}$ CFUs/cm^2).

Gene Expression Analysis

Gene Selection and Primers Design for Quantitative Real-Time PCR

Five genes (*ERG1*, *ERG3*, *ERG6*, *ERG9* and *ERG11*) were selected to study their expression in biofilm cells, in the presence and absence of Vcz. *ACT1* gene was selected as a reference *Candida* housekeeping gene [19]. The gene sequences of interest were obtained from *Candida* Genome Database (CGD) and the primers for quantitative real-time PCR (qRT-PCR) were designed using Primer 3 (http://simgene.com/Primer3) Web-based software (Table 1). In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from *C. tropicalis* strains genomic DNA. Moreover, genomic DNA was used to infer about the primer efficiency and to determine the temperature of melting (Tm).

Biofilm Cells Preparation

Biofilms were pre-formed in 24-well microplates with a cellular density adjusted to 1×10^5 cells/mL in RPMI 1640 medium, using a Neubauer counting chamber. After 24 h, 1 mL of Vcz (at 100 mg/L) was added to each well and the plate incubated at 37 °C for additional 48 h at 120 rpm. After this, the medium was aspirated and non-adherent cells were removed by washing twice with 1 mL of PBS, pH 7.0, 0.1 M. Then, the biofilms were scraped from the 24-well microplates, resuspended with 1 mL of PBS, and the suspensions were vigorously vortexed for 2 min to disaggregate cells from the matrix. Cells were harvested by centrifugation at 5000*g* for 10 min at 4 °C.

RNA Extraction

Ribonucleic acid (RNA) extraction was performed using E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Norcross, USA). Prior to RNA extraction, the lysis

Table 1 Primers used for qRT-PCR, the respective melting temperature and product size

Genes	Primers	Sequences	Melting temperature (°C)	Product size (bp)
ACT1	Forward	GACCGAAGCTCCAATGAATC	57	181
	Reverse	AATTGGGACAACGTGGGTAA		
ERG1	Forward	GAGTTCGTGGTGTTGCTTTC	56	100
	Reverse	GTAACGGTTGCTTCAACAGC		
ERG3	Forward	GCAACTAGAGCCATTCCAGT	57	194
	Reverse	GGCCAATGTAACCATCTGTG		
ERG6	Forward	GAAACCAGGTGGTGTCTTTG	57	108
	Reverse	CCATCACCAACTTCGATACC		
ERG9	Forward	ACCATAAGTCACACCCAAGG	56	130
	Reverse	TCCGGAACAGTGGTCATAGT		
ERG11	Forward	CCAAGGCTAGTGCTGTTTCT	58	111
	Reverse	TGTGTCTACCACCACCGAAT		

buffer was prepared adding 1 % of β-mercaptoethanol. Then, 600 µL of lysis buffer and glass beads (425–600 µm diameter) were added to each pellet. These mixes were homogenized thrice for 30 s using a MP Biomedicals FastPrep™-24 Instrument (Thermo Fisher Scientific, Rockford, USA). After cells disruption, the E.Z.N.A.® Total RNA Kit I was used for total RNA extraction according to the manufacturer's recommended protocol. To avoid potential DNA contamination, samples were treated with RNase-free DNase I. The RNA extraction was performed in three different independents assays.

Synthesis of cDNA

To synthesize the complementary DNA (cDNA), the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, USA) was used according to the manufacturer's instructions. For each sample, 10 µL of the extracted RNA of defined concentration was used at a final volume of 20 µL of reaction, which was completed with the remaining components of the mastermix. cDNA synthesis was performed firstly for 10 min at 25 °C and then for 1 h at 37 °C. The reaction was stopped by heating for 5 min at 85 °C.

Quantitative Real-Time PCR

The qRT-PCR (CF X96 Real-Time PCR System; Bio-Rad, Berkeley, USA) was used to determine the relative levels of *ERG1*, *ERG3*, *ERG6*, *ERG9* and *ERG11* messenger RNA (mRNA) transcripts in the RNA samples, using *ACT1* as reference *Candida* housekeeping gene. Each reaction mixture consisted of a working concentration of SsoFast EvaGreen Supermix (Bio-Rad, Berkeley, USA), 10 µM forward and reverse primers, and 4 µL cDNA, in a final reaction volume of 20 µL. Negative controls (water), as well as non-transcriptase reverse (NRT) RNA controls, were included in each run. The relative quantification of genes expression was performed by the ΔC_t method [20], which was normalized to the housekeeping gene (C_t average = 21.86 ± 0.84). Each reaction was performed in triplicate, and mean values of relative expression were determined for each gene.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6 software for Windows. The results were compared using a two-way ANOVA with the Bonferroni's multiple comparisons test. The variation factors assessed were always between the absence and presence of Vcz. All tests were performed with a 95 % confidence level.

Results

Candida tropicalis Voriconazole Minimum Inhibitory Concentrations

MICs of *C. tropicalis* were determined for Vcz using the microdilution method and CFU counts (Table 2).

Table 2 *Candida tropicalis* minimum inhibitory concentrations obtained for voriconazole

Candida tropicalis strains	Minimum inhibitory concentrations (mg/L)
AG1	>10
12	>10
519468	0.75–1

The results revealed that *C. tropicalis* AG1 and 12 (>10 mg/L) were resistant to Vcz according to the MIC breakpoint from CLSI (>1 mg/L; *C. tropicalis* ATCC 750) [18] and that *C. tropicalis* 519468 was sensible with the lowest value of MIC (0.75–1 mg/L).

Effect of the Voriconazole on *Candida tropicalis* Biofilms

Influence of Antifungal Agent on Biofilm Formation

In order to determine whether Vcz could prevent *C. tropicalis* biofilm formation, biofilms were formed during 48 h in the presence of different concentrations of antifungal agent (Fig. 1). The data showed that all Vcz concentrations were able to cause a significant reduction in the number of cultivable cells comparatively to the control ($p < 0.0001$). The same results were obtained in terms of the total biomass reduction ($p < 0.0001$), however, in lower extension for the clinical isolate *C. tropicalis* AG1 ($p < 0.0001$ only for 50 and 100 mg/L of Vcz).

Influence of Antifungal Agent on Pre-formed Biofilms

To determine the effect of Vcz on pre-formed *C. tropicalis* biofilms, 24-h-old biofilms were treated during 48 h with different concentrations of antifungal agent (Fig. 2). The results revealed that Vcz was unable to reduce *C. tropicalis* pre-formed biofilms, either in terms of the number of cultivable cells (Fig. 2a) and total biomass (Fig. 2b). Moreover, it was possible to observe for *C. tropicalis* 12 biofilms a significant increase in terms of total biomass at all Vcz concentrations ($p < 0.0001$ at concentrations of 10, 50 and 100 mg/L and $p < 0.05$ at concentrations of 200 mg/L). The total biomass also increased for *C. tropicalis* 519468, however, only for the lowest concentration of Vcz tested (10 mg/L) ($p < 0.01$) (Fig. 2b).

Effect of the Voriconazole on ERG Genes Expression

Figure 3 presents the mean *n*-fold expression levels of *ERG1*, *ERG3*, *ERG6*, *ERG9* and *ERG11* genes of the *C. tropicalis* cells (from pre-formed biofilms) treated with Vcz (100 mg/L), in comparison with the control (absence of antifungal agent). This concentration of

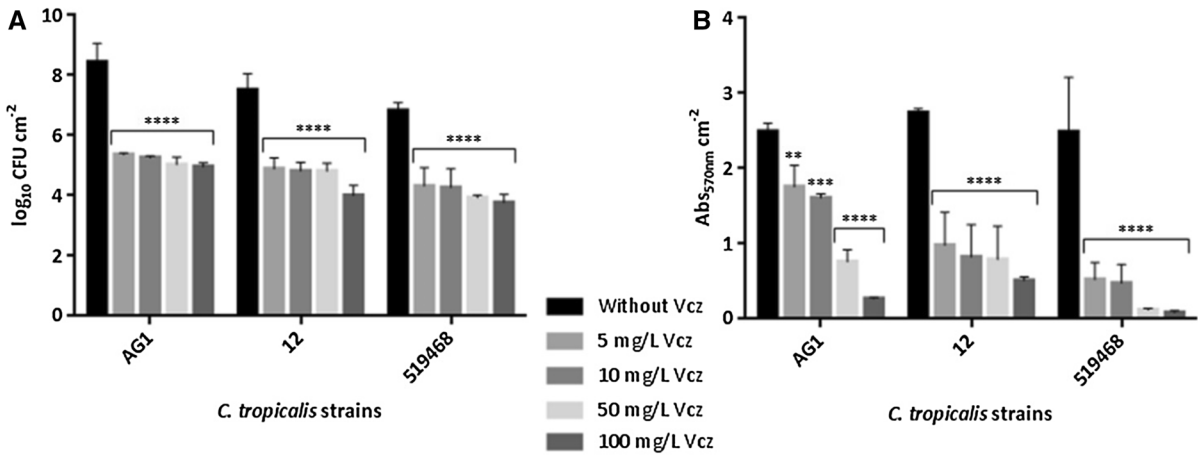


Fig. 1 Effect of voriconazole on *Candida tropicalis* biofilm formation after 48 h. **a** Mean values of the logarithm of CFUs normalized by unit of area ($\log_{10}$ CFU/cm^2); **b** mean values of the absorbance at 570 nm normalized by unit of area (Abs_{570nm}/cm^2). *Error bars* indicate the standard deviation. Statistically different compared to respective control. **, *** and ****correspond to $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively

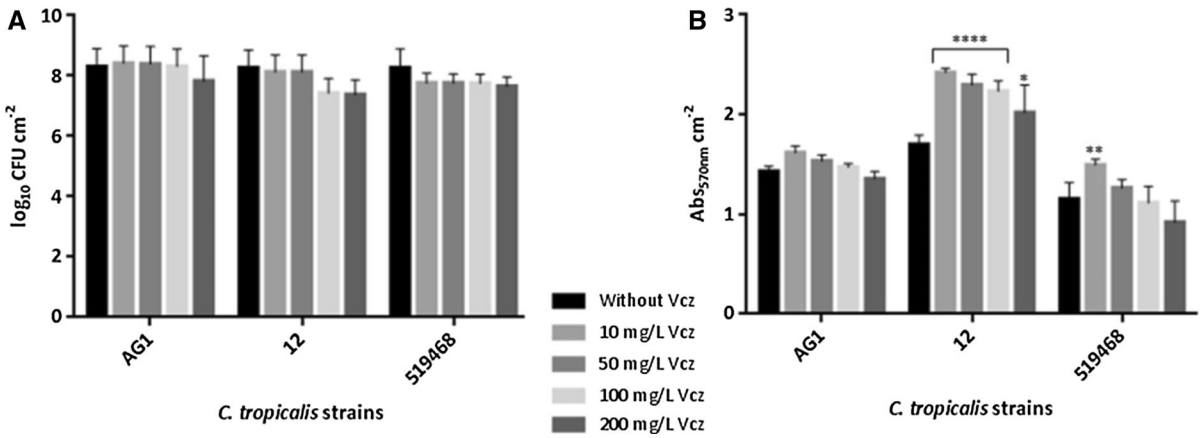


Fig. 2 Effect of voriconazole against 24 h pre-formed *Candida tropicalis* biofilms during 48 h. **a** Mean values of the logarithm of CFUs normalized by unit of area (log_{10} CFU/cm^2); **b** mean values of the absorbance at 570 nm normalized by unit of area (Abs_{570nm}/cm^2). *Error bars* indicate the standard deviation. Statistically different compared to respective control. *, **, *** and ****correspond to $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively

Vcz was selected once the main aim was to disclose the effect of the *ERG* genes on *C. tropicalis* biofilms resistance to azole.

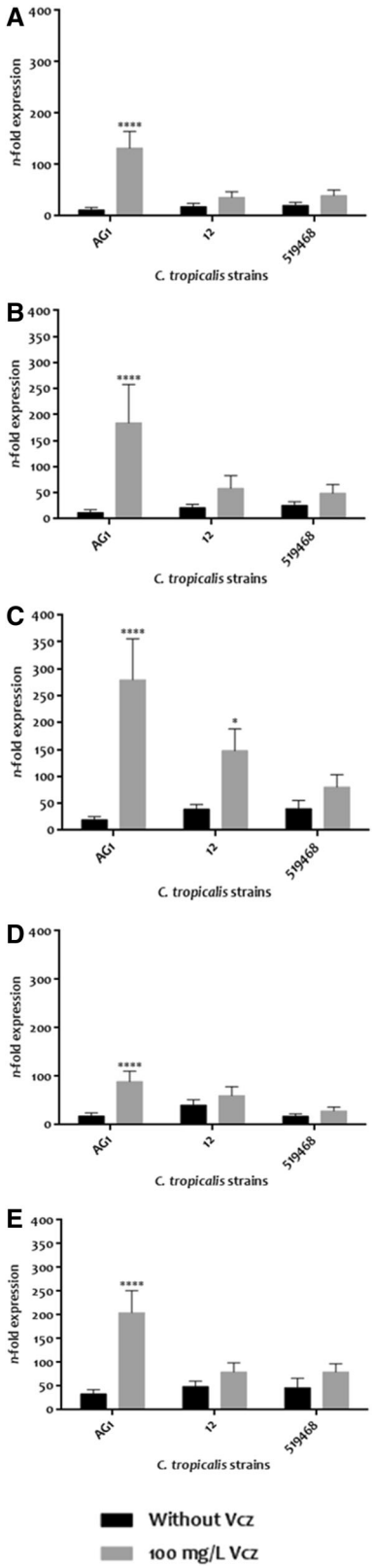
An overexpression of all *ERG* genes was evident for all *C. tropicalis* biofilm cells treated with Vcz (Fig. 3), although differences were observed depending on the strains and the *ERG* gene. *ERG6* presented the highest levels of expression for all strains (Fig. 3c), and *ERG1* (Fig. 3a) and *ERG9* (Fig. 3d) the lower levels for biofilm cells treated with Vcz. It should be noted that, in the presence of Vcz (Fig. 3), *C. tropicalis* AG1 biofilm cells showed the highest expression for all *ERG* genes with less significant differences for the other two strains.

Discussion

The main aim of this study was to determine the effect of the Vcz on *C. tropicalis* biofilms and to disclose its relationship with the *ERG* genes expression, and thus deepen the knowledge regarding *C. tropicalis* biofilms resistance mechanisms.

To reach the goal of this study, antifungal susceptibility tests were performed according to CLSI guidelines. Although the agent tested belong to the azoles' class, Vcz was selected due to its more recent use, which is related to a better efficacy in the combat of candidiasis and consequent lower resistance than other azoles [4]. So, as expected, not all clinical isolates were resistant to Vcz with MIC values strain dependent (Table 2). These differences highlight the strain susceptibility variations resultant from the different origins of the clinical isolates, which were recovered from patients submitted to different therapies [4, 8, 21, 22].

As it is known, biofilms have been considered the most prevalent growth form of microorganisms in natural environments [4, 23, 24]. Biofilms present a high versatility in adapting to a variety of different habitats, being described as surface-associated communities of microorganisms embedded within an extracellular matrix [1, 4, 7, 10]. All *C. tropicalis* clinical isolates assayed were able to form biofilms with higher resistant to Vcz than their planktonic cells, which is in agreement with other studies [4, 22, 25]. Additionally, this work revealed that Vcz possessed a significant effect on *C. tropicalis* biofilms' formation, as evidenced by the significant reductions in the number of cultivable cells and in total biomass values (Fig. 1), but without effect on pre-formed biofilms (Fig. 2). On the other hand, even the highest concentration of Vcz was unable to reduce/destroy *C. tropicalis* pre-formed biofilms (Fig. 2). It should also be emphasized that even the lowest concentration tested of Vcz (5 mg/L) was able to reduce the biofilm (Fig. 1), what is a very important issue regarding cytotoxicity and therefore patient's safety. As is now, pre-formed biofilms are mature biofilms, which subsequently possesses a complex extracellular matrix,



◀**Fig. 3** Effect of voriconazole on values of *n*-fold expression levels of *ERG1* (**a**), *ERG3* (**b**), *ERG6* (**c**), *ERG9* (**d**) and *ERG11* (**e**) genes in pre-formed *Candida tropicalis* biofilms. Biofilms were formed during 24 h and treated with 100 mg/L of voriconazole during 48 h. Comparisons were made with biofilm cells growth in the absence of antifungal. *Error bars* indicate the SDs. *, **, *** and **** correspond to $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively

that difficult the penetration of agents, presenting higher resistance to antifungals [4, 23, 26, 27]. Interestingly, it was observed an increase in total biomass when pre-formed biofilms were treated with intermediate concentrations of antifungal agent (Fig. 2b). This phenomenon is probably due to a response of *C. tropicalis* biofilm cells to the stress caused by the presence of the agent, which lead to an expansion of the biofilm matrices' production.

It is known that the antifungal agents from the azole class, with fungistatic activity, inhibit the biosynthesis of ergosterol by interfering with the fungal enzyme, lanosterol demethylase, which is a key enzyme in the biosynthesis of ergosterol [4, 23]. Several molecular mechanisms of azole antifungals' resistance have been reported for other *Candida* species, including alteration in the chemical structure of the demethylase enzyme, removal of the azole from the cell by multidrug transporter pumps and compensation by other sterol synthesis enzymes in membrane biosynthesis [23]. Despite some information related to other *Candida* species [13, 22, 23, 28], there is a lack of information concerning the involvement of well-known mechanisms of Vcz resistance in *C. tropicalis*. Thus, to disclose the effective role of expression of *ERG* genes (*ERG1*, *ERG3*, *ERG6*, *ERG9* and *ERG11*) on biofilms' resistance, their expression was measured in *C. tropicalis* biofilm cells treated with of Vcz, by qRT-PCR. This work was demonstrative that the expression of all *ERG* genes increased for biofilm cells treated with Vcz (Fig. 3). Thus, it can be concluded that the resistance of *C. tropicalis* biofilm cells to azole appears to be related to overexpression in *ERG11* gene, possibly due to qualitative modifications in enzyme lanosterol 14α-demethylase that convert lanosterol to ergosterol, leading to depletion of the sterol of cell membrane due to the inhibition of the biosynthesis of ergosterol. Moreover, *ERG6* gene was also over expressed in the presence of Vcz (Fig. 3c). *ERG6* gene is described as required for a normal

membrane function and is localized after the *ERG11* gene in ergosterol biosynthesis cascade. Thus, it can be speculated that after qualitative modifications in enzyme lanosterol 14α-demethylase, caused by Vcz due to the overexpression in *ERG11* gene, it could be reflected in an overexpression of *ERG6* gene and thus can affect the normal membrane function [4]. In contrast, the genes with lowest expression levels were the *ERG1* (Fig. 3a) and the *ERG9* (Fig. 3d). It should be pointed out that *ERG9* and *ERG1* appear before the *ERG11* gene in ergosterol biosynthesis, thus seeming to be possible that its response assumes less importance than from the other genes, in *C. tropicalis* biofilm cells resistance to Vcz. Mature *C. tropicalis* biofilms are constitutes by several layers of cells that make impossible the antifungal agents reach all cells and this may be an explanation for the absence statically differences between *C. tropicalis* 12 and 519468 levels of *ERG* genes expression.

Conclusions

In conclusion, this study confirmed that the pattern of resistance of *C. tropicalis* planktonic cells to Vcz is strain dependent. Moreover, it was also demonstrated that Vcz was able to control partly *C. tropicalis* biofilm formation but unable of eradicating *C. tropicalis* pre-formed biofilms. In addition, this work revealed for the first instance that *ERG* genes could be implicated in the mechanisms of *C. tropicalis* biofilm's cells resistance to Vcz.

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