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Prevalence and Fluconazole Susceptibility Profile of *Candida* spp. Clinical Isolates in a Brazilian Tertiary Hospital in Minas Gerais, Brazil

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ABSTRACT

Candidiasis has become an important concern for clinical practice, especially with the increasing incidence of immunocompromised patients. In this scenario, the development resistance to fluconazole presents a challenge for treating these opportunistic infections. The aim of this study was to evaluate some epidemiology features of *Candida* infections in a Brazilian University Hospital using data, previously unavailable. We observed that 44% of the 93 clinical isolates tested, belonged to *Candida albicans* species and 56% belonged to *non-Candida albicans* species (mainly *Candida tropicalis* and *Candida glabrata*). Most strains were isolated from urine samples where *C. albicans* was predominantly detected. 29 strains presented a fluconazole resistance phenotype and of these, 22 were chemosensitised by FK506, a classical inhibitor of ABC transporters related to azoles resistance. These data suggest the probable role of efflux pumps in this resistance phenotype. Our study highlights the need for developing effective control measures for fungal infections, rational use of antifungal drugs and development of new molecules able to abrogate the active transport of antifungals.

Key words: *Candida* spp., clinical isolates, epidemiology, fluconazole, resistance.

INTRODUCTION

Candida spp. infections are recognized as a major challenge in public health, commonly associated with high morbidity and mortality since its diagnosis and treatment present difficulties and high healthcare costs (Colombo et al. 2008, Gudlaugsson et al. 2003, Arnold et al. 2010). In Latin America, mortality rates are usually higher than those observed in the Northern Hemisphere; however, it is worth mentioning that epidemiology of candidemia is poorly studied in this region in comparison to the United States and Europe (Nucci et al. 2013, Colombo et al. 2008, Santolaya et al. 2014).

In Brazil, studies demonstrated that incidence rates of *Candida* infections are heterogeneous in different regions (Colombo et al. 2006, Nucci et al. 2010). There are several studies that focus

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specifically on the Southeast (Colombo et al. 1999, 2006, Costa et al. 2000, Resende et al. 2002, de Resende et al. 2006, Aleva et al. 2007, Ribeiro et al. 2010, Oliveira et al. 2014, Moretti et al. 2013), Midwest (Leite Junior et al. 2011, Hoffmann-Santos et al. 2013, Akeme Yamamoto et al. 2012), North-east (Mascarenhas et al. 2012, Araujo Paulo de Medeiros et al. 2014, Nascimento Mdo et al. 2014) and South of Brazil (Antunes et al. 2004, Pasqualotto et al. 2008, da Costa et al. 2014).

Fluconazole (FCZ) is the first option for prophylaxis and treatment, due to its good tolerance, few side effects and low costs (Li et al. 2014). However, the widespread and prolonged use of antifungal agents induces tolerance development as well as collateral resistance to other drugs (Prasad and Rawal 2014). Some species, such as *Candida glabrata* and *Candida krusei*, already present decreased susceptibility or resistance to FCZ (Wingard et al. 1991, Marr et al. 2000). Preventing the intracellular accumulation of a drug by rapid extrusion of the antifungal agent is a known mechanism of resistance (Prasad et al. 2002). The overexpression of efflux pumps, such as ATP-binding cassette (ABC) family or major facilitator superfamily (MFS), has been shown to be the cause of resistance in some fungi (Cannon et al. 2009).

The aim of this study is to evaluate the frequency of yeast species isolated from urine, faeces, catheter, blood and other secretions, analyze the susceptibility to FCZ as well as investigate the possible mechanism of resistance of clinical strains obtained from the University Hospital of the Federal University of Juiz de Fora, Minas Gerais, Brazil, over a period of two years (2012-2014).

MATERIALS AND METHODS

Yeast Strains and Patients

Ninety-three *Candida* spp. isolates were obtained from patients at the University Hospital of Universidade Federal de Juiz de Fora, Minas Gerais, Brazil, during the period of 2012 to 2014. Isolates were collected from different clinical material: urine, faeces, catheter, blood and other secretions, from patients treated in ICU and/or ambulatory units. *Candida* ATCC strains were used as control for species identification: *C. albicans* 10231, *C. tropicalis* 750*, C. parapsilosis* 90018, *C. glabrata* 2001, *C. krusei* 34135, *C. dubliniensis* MYA-646, *C. guilliermondii* 7350. This project was approved by Instituto de Estudos em Saúde Coletiva – IESC/UFRJ – Protocol Nº 030/2001.

Cell Growth and Culture Conditions

The yeast strains grown in YPD medium (2% glucose, 2% peptone, 1% yeast extract) at 37 °C under agitation, were harvested in the exponential phase of growth and stored at 4 °C.

Strain Identification

Isolate identification was performed by MALDI Microflex LT (Bruker Daltonics, Bremen, Germany) measurement, according to manufacturer formic acid extraction procedure. Briefly, in this identification method, a single colony of each strain grown overnight on YPD agar was suspended in 300µL of de-ionized water and 900µL of absolute ethanol and centrifuged at 14,462 xg for 2 min. The supernatant was discarded and the pellet was air-dried. 1:1 v 70% formic acid and 100% acetonitrile were added to the pellet and vortexed. The samples were centrifuged at 14,462 xg for 2 min, and 1µLof the supernatant was spotted in duplicate onto a steel target and air-dried at room temperature. Before identification, each spot was overlaid with 1µL of HCCA (α-Cyano-4-hydroxycinnamic acid, Bruker) matrix solution saturated with organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid) and air dried completely. The spectra were externally calibrated using standard ATCC *Escherichia coli* 25922, before plate identification. Raw spectra were processed using MALDI BIOTYPER Realtime Classification software version 3.1 (Bruker Daltonik MALDI Biotyper). Strains with

score values ≥ 2 were indicated as reliable species identification.

Antifungal Susceptibility Tests

In vitro susceptibility tests were performed using the broth microdiluition assay according to the protocol of the Clinical and Laboratory Standards Institute (CLSI) M27-A2 protocol. According to CLSI, FCZ MIC_{50} (Minimal Inhibitory Concentration) end points ≤ 8µg/mL were categorized as susceptible. MIC_{50} end points between 16 and 32µg/mL were classified as susceptible dose-dependent (SDD), and resistant strains obtained MIC_{50} end points ≥ 64µg/mL. Cell growth was analyzed in a microplate reader at 600 nm (Fluostar Optima, BMG Labtech, Offenburg, Germany).

Evaluation of Fluconazole Resistance Reversion by FK506

Since FK506 is a classical ABC transporter inhibitor (Egner et al. 2000), it was used as an identifier for resistance mediated by these transporters. The "spot test" was used as a measure of growth as previously described by (Reis de Sa et al. 2014).

TABLE I
Number (n) and percent (%) of strains collected from different clinical sources. The isolates were collected mainly in urine samples.

Clinical sources	Number of strains (n)	Percent (%)
Urine	71	76.3
Blood	8	8.6
Catheter	5	5.4
Tracheal secretion	4	4.3
Ascitic fluid	2	2.2
Faeces	1	1.1
Abdominal secretions	1	1.1
Gastrostomy secretion	1	1.1
Total	93	100

Flow Cytometry Assay

The experiment was performed using eight representative resistant *Candida* spp. isolates. The cells were incubated overnight in 20 ml of YPD medium 1 x 10^3 cells/mL at 37 °C under agitation. After incubation, with a value of 1.0 to 3.0 OD, the cells were centrifuged (5000 x *g* / 5 min) and washed four times with deionized water. After washing, the strains were maintained on ice for 2h. A concentration of 6 x 10^5 cells/mL were incubated with Rhodamine 6G (R6G) (5µM), an ABC transporter fluorescent substrate (Maesaki et al. 1999), in the presence or absence of glucose (2%) for 1h, at 37 °C under agitation. The cells were centrifuged (9000 x *g* / 2 min) and washed with PBS buffer (3 X) before analysis in duplicate by BD Accuri™ C6 Flow Cytometer, in order to measure of R6G efflux by strains.

Data Analyses

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS©) software (version 20).

RESULTS

Clinical Source Analysis and Species Identification

We analyzed a total of 93 *Candida* spp. isolates in a period of two years (2012-2014). The isolates were collected from different clinical samples as summarized in Table I. Most *Candida* spp. isolates were detected in urine samples (n=71, 76%). The other samples presented a fewer number of isolates: blood (n=8, 8.6%), catheter (n=5, 5.4%), tracheal secretions (n=4, 4.3%), faeces (n=1, 1.1%), ascetic fluid (n=2, 2.2%), abdominal (n=1, 1.1%) and gastrostomy secretions (n=1, 1.1%).

The MALDI-TOF mass spectrometry method enabled *Candida* spp. strain identification in eight species: *Candida albicans* (n=41, 44.1%), *Candida tropicalis* (n=26, 28%), *Candida glabrata* (n=14, 15.1%), *Candida parapsilosis* (n=7, 7.5%), *Candida kefyr* (n=2, 2.2%), *Candida metapsilosis*

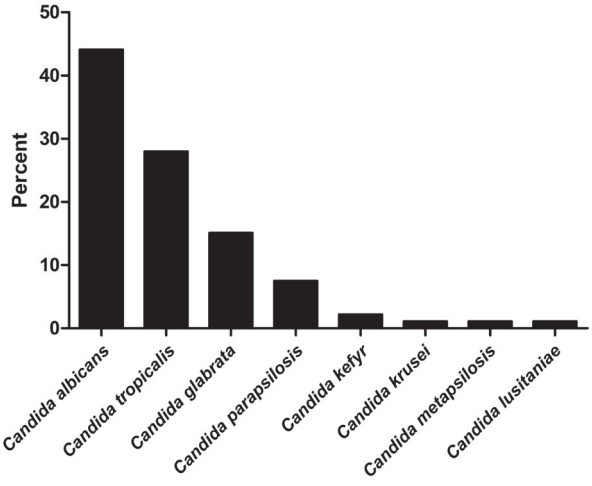

Figure 1 - Percentage of species identified by MALDI-TOF mass spectrometry. *C. albicans* (44.1%), *C. tropicalis* (28%), *C. glabrata* (15.1%), *C. parapsilosis* (7.5%), *C. kefyr* (2.2%), *C. krusei* (1.1%), *C. metapsilosis* (1.1%), *C. lusitaniae* (1.1%).

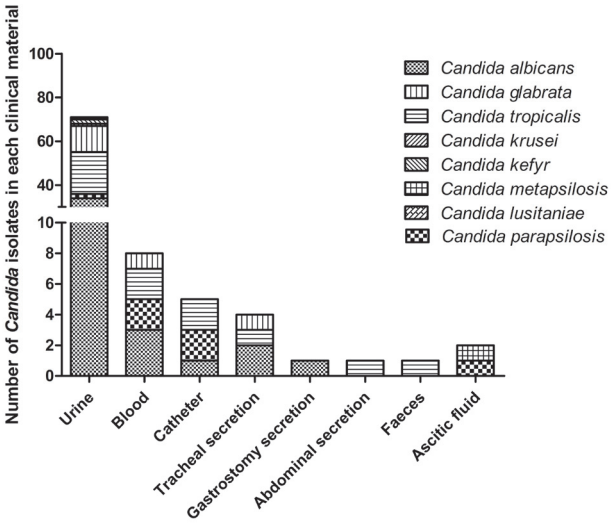

Figure 2 - Number of species *Candida* isolated from each clinical source. The majority of clinical isolates were *C. albicans* in urine samples.

TABLE II
Fluconazole susceptibility profile of *Candida* spp. isolates.

	(n)	Susceptible (%)	Susceptible - dependent dose (%)	Resistant (%)
Candida albicans	41	27 (46.6)	3 (50)	11 (38)
Candida tropicalis	26	12 (20.7)	1 (16.7)	13 (45)
Candida glabrata	14	10 (17.2)	0 (0)	4 (13.8)
Candida parapsilosis	7	7 (12)	0 (0)	0 (0)
Candida krusei	1	0 (0)	0 (0)	1 (3.4)
Candida kefyr	2	1 (1.7)	1 (16.7)	0 (0)
Candida metapsilosis	1	1 (1.7)	0 (0)	0 (0)
Candida lusitaniae	1	0 (0)	1 (16.7)	0 (0)
Total	93	58	6	29

(n=1, 1.1%), *Candida krusei* (n=1, 1.1%) and *Candida lusitaniae* (n=1, 1.1%) (Fig. 1).

Correlating clinical sources and species identification, *C. albicans* was the most prevalent specie in urine samples (n=34, 48%), followed by *C. tropicalis* (n=19, 27%) and *C. glabrata* (n=12, 17%). *C. albicans* was also prevalent in blood samples (n=3, 37.5%), gastrostomy secretion (n=1, 100%) and tracheal secretions (n=2, 50%) (Fig. 2).

FLUCONAZOLE SUSCEPTIBILITY

In order to classify susceptible (S), susceptible dose-dependent (SDD) and resistant strains (R), we performed a FCZ susceptibility assay. 58 isolates presented a susceptible profile (62.5%), six were SDD (6.5%) and 29 were selected as resistant to FCZ (31%).

The majority of resistant strains belonged to the *C. tropicalis* species (n=13, 45%), while *C. albicans*

TABLE III
Minimal inhibitory concentration (MIC_{50}) of resistant to FCZ *Candida* spp. isolates according to CLSI M27-A2 protocol.

Code	Species	MIC_{50} FCZ (μg/mL)
1002	*Candida tropicalis*	>1000
1016	*Candida albicans*	>1000
1050	*Candida tropicalis*	>1000
107	*Candida glabrata*	>1000
109	*Candida glabrata*	>1000
1114	*Candida albicans*	>1000
24i	*Candida tropicalis*	>1000
1027	*Candida tropicalis*	>500
154i	*Candida albicans*	>500
224A	*Candida albicans*	>500
338i	*Candida tropicalis*	>250
424i	*Candida tropicalis*	>250
451A	*Candida tropicalis*	>250
541i	*Candida albicans*	>250
211i	*Candida tropicalis*	>250
330i	*Candida tropicalis*	>125
44i	*Candida tropicalis*	>125
124i	*Candida krusei*	>64
148A	*Candida tropicalis*	>64
14A	*Candida albicans*	>64
163A	*Candida glabrata*	>64
218i	*Candida glabrata*	>64
242A	*Candida tropicalis*	>64
250i	*Candida albicans*	>64
25i	*Candida tropicalis*	>64
326A	*Candida albicans*	>64
337i	*Candida albicans*	>64
479i	*Candida albicans*	>64
389A	*Candida albicans*	>64

was the most prevalent susceptible (n=27, 46.6%) and SDD (n=3, 50%) isolate (Table II). The MIC_{50} values of each resistant strain are summarized in Table III. Seven *Candida* isolates presented MIC_{50} higher than the maximum tested concentration: three of these strains were *C. tropicalis,* two *C. albicans* and two *C. glabrata*. Three isolates were resistant to concentrations higher than 500μg/ml, five for 250μg/ml, two for 125μg/ml and eleven for 64μg/ml.

Reversion of Fluconazole Resistance

After the screening of clinical strains resistant to FCZ, we performed a chemosensitization assay using FK506 in order to evaluate the possible resistance mechanism of these *Candida* spp. isolates. The resistance phenotype of 22 clinical strains reverted upon treatment with the ABC transporter inhibitor, while the other seven (1016, 250i, 14A, 163A, 1027, 124i, 1114) isolates remained resistant, even in the presence of FK506 (Fig. 3). Among the 22 resistant *Candida* strains chemosentitized by FK506, we observed that 11 were *C. tropicalis,* seven were *C. albicans*, 4 *C. glabrata*.

Flow Cytometry

Flow cytometry assay was performed in order to check the capability to extrude Rhodamine 6G (R6G), a fluorescent substrate pumped by ABC transporters, by resistant strains. For this test, we used four representative strains reverted by FK506 (Fig. 4a) and four that remained resistant even in the presence of FK506 (Fig. 4b). The data revealed that strains reverted by FK506 were able to pump out R6G in the presence of glucose whereas strains that displayed high level of resistance to FCZ, even in the presence of FK506, were not able to efflux the fluorescent probe in the presence or absence of glucose. These results confirm those obtained by chemosensitization assay (Fig. 3) since reverted strains also presented an efflux of R6G.

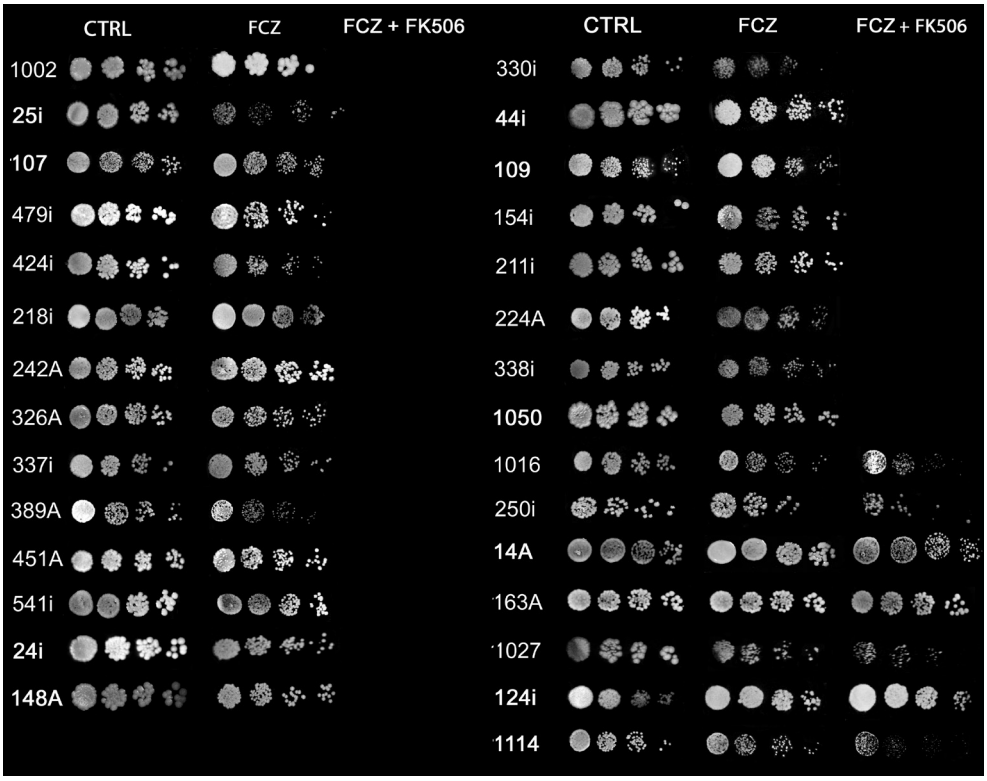

Figure 3 - Evaluation of FCZ reversion by FK506 on 29 resistant *Candida* spp. clinical strains. CTRL: yeast cells growth on Sabouraud solid medium in absence of FCZ for 48h. FCZ (+): yeast cell growth on Sabouraud solid medium containing 64µg/mL of FCZ. FCZ(+) and FK506: yeast growth in same conditions as CTRL plus FCZ (+) presenting FK506 10µM. Most clinical strains presented resistance reversion using FK506, a classic ABC transporter inhibitor. Only seven strains were still resistant to FCZ even in the presence of the inhibitor.

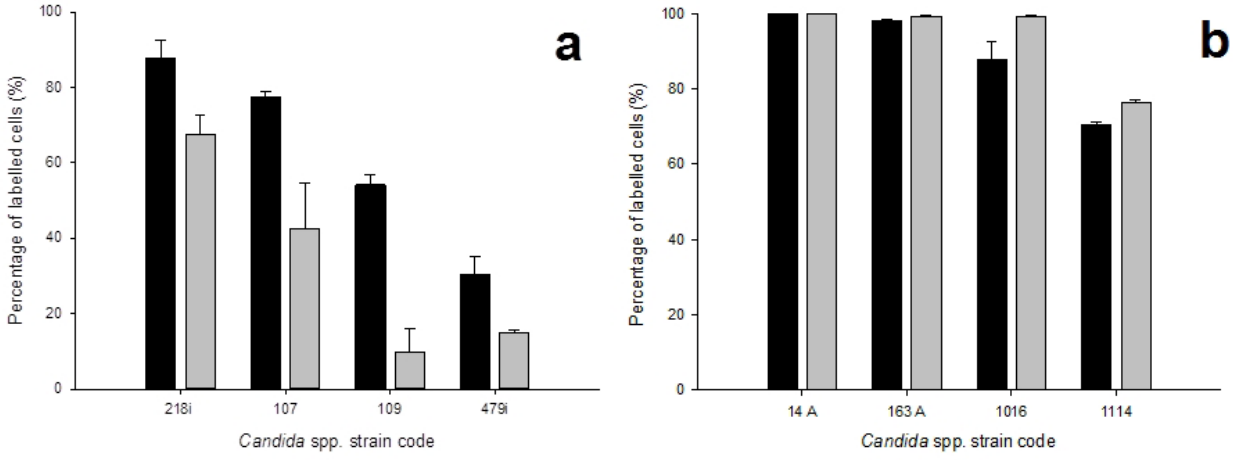

Figure 4 - Citometry efflux assay of *Candida* spp. strains using R6G as fluorescent substrate in the presence and absence of glucose. (**a**) Flow citometry conducted with clinical isolates that were chemosensitized by FK506. (**b**) Flow citometry assay of clinical isolates that retained resistance phenotype using FK506. Black bar = no glucose; Gray bar = with glucose. This experiment represents average of triplicate.

DISCUSSION

Regional differences in the incidence pattern of antifungal resistance and patient suceptibility can be found when monitoring fungal infections. Knowledge of the epidemiological characteristics of a certain community has both local and global importance, considering the constant flow of individuals within a country and around the world (Nucci et al. 2010). Since *Candida* spp. are the most common fungal nosocomial pathogens, contributing significantly to morbidity and mortality (Wisplinghoff et al. 2014, Pfaller et al. 2014), we decided to evaluate the prevalence of *Candida* infections at a Brazilian University Hospital located in a Southeast region, where no epidemiologic data relating to candidiasis had been previously collected. We also determined the susceptibility profile of *Candida* spp. strains - isolated from clinical material - as well as the possible role of ABC transporters in the FCZ resistance.

Our results revealed that the *Candida* species most commonly isolated were *C. albicans* (44.4%), *C. tropicalis* (28%) and *C. glabrata* (14%) (Fig. 1). These data were consistant with other Brazilian and international studies concerning nosocomial fungal infections, which show *C. albicans* as the most prevalent species isolated (Wille et al. 2013, da Costa et al. 2014, Corzo-Leon et al. 2014).

Furthermore, we observed a higher proportion of *non-albicans* species (56%) compared with *Candida albicans* (44%), a trend which has also been observed in other studies. Oliveira and colleagues evaluated cases of fungemia from 2007 to 2010 and observed a higher prevalence of candidemia caused by *Candida* species other than *C. albicans* during a period of four years (Oliveira et al. 2014). Moretti and colleagues observed an increasing incidence of candidemia caused by *C. tropicalis* and *C. glabrata* between 2006 and 2010 (Moretti et al. 2013). Pfaller et al. 2014 also pointed to the emergence of invasive fungal infections caused by non-albicans species of *Candida* in a study comprising 23 medical centers in the United States and two in Canada (Pfaller et al. 2014).

Urinary tract infections caused by *Candida* spp. are commonly observed in hospitalized patients, especially those in intensive care units (Kauffman 2014). The tendency of hospitalized patients to be predisposed to urinary tract infections caused by *Candida* spp is explained by factors such as instrumentation of the urinary tract, prolonged hospitalization, use of broad spectrum antibiotics and indwelling urinary tract devices (Sobel et al. 2011). The evaluation of bacteriuria and candiduria in an intensive care unit (ICU), conducted by Aubron and colleagues over a period of six years (2006-2011), demonstrated that *Candida* spp. represented 55% of pathogens isolated from positive urine cultures (Aubron et al. 2015). We also observed this high *Candida* spp. prevalence in urinary tract infections, with 71 of the 93 isolates tested, coming from urine samples (Table I). Of these, the three most prevalent species were *C. albicans* (34 / 71 - 48%), *C. tropicalis* (19 / 71 - 27%) and *C. glabrata* (12 / 71 - 17%) (Fig. 2). These data are confirmed by Sobel and colleagues who identify these three species as the main cause of candiduria (Sobel et al. 2011).

Azoles, specially FCZ, are by far the most commonly used antifungals in clinical practice and the emergence of the resistance to this chemotherapeutic is a concern in clinical practice (Vandeputte et al. 2012). When assessing the susceptibility profile to FCZ, we observed the resistance phenotype in 32.1% (29/93) of studied *Candida* strains (Table II). Our results differ from those obtained by many other studies, which detected a lower incidence of FCZ resistance (Wille et al. 2013, Tortorano et al. 2012, Lockhart et al. 2011, Pfaller et al. 2011, da Matta et al. 2007). These studies evaluated a higher number of samples compared to our study, which focused on a more specific population. This could explain the differences concerning the prevalence of resistance to FCZ. However, our results are consistent with other studies (Puig-Asensio et al. 2014, Tortorano

et al. 2012) when it comes to emergence of FCZ resistance in strains of *Candida tropicalis* and *Candida glabrata*. We observed that the most prevalent FCZ resistant species were *C.albicans* (11/29), *C.tropicalis* (13/29), *C. glabrata* (4/29) and *C. krusei* (1/29).

The active transport of azoles out of the cell, mediated by membrane ABC transporters is an important mechanism of resistance in *Candida* species (Sanglard et al. 2009). Therefore, we decided to evaluate the role of ABC transporters in the FCZ resistance expressed by the 29 *Candida* spp. strains. For this, we used the chemosinsitization experiment with FK506 (tacrolimus), a classic inhibitor of *Candida* ABC transporters (Cannon et al. 2009). We observed that in 75.8% (22/29) (Fig. 3) of these strains, FK506 could reverse the FCZ resistance, revealing a probable contribution of ABC transporters to this phenotype of resistance. Among the strains chemosensitized by FK506, we identified 11 *C.tropicalis*, seven *C. albicans* and four *C. glabrata* (Table III). Other studies also observed the participation of efflux pumps in FCZ resistance in *Candida* species. White et al. (2002) assessed the mechanism of resistance in *C. albicans* strains isolated from clinical material and showed that out of 13 strains with a FCZ MIC_{50} for > 64µg/mL, six presented overexpression of ABC transporter genes related to resistance to azoles and CaCdr1/CaCdr2 (White et al. 2002). Evaluating the FCZ resistance mechanism of 20 strains of *C. glabrata*, Sanguinetti et al. (2005) noted that all FCZ resistant strains showed overexpression of at least one ABC transporter related to antifungal efflux (CgCdr1, CgCdr2 and CgSNQ), and that FK506 could chemosensitize all strains (Sanguinetti et al. 2005). Barchiesi et al. (2000) demonstrated that prolonged exposure of *C. tropicalis* to FCZ induced the expression of an ABC transporter that promotes the active efflux of this antifungal (Barchiesi et al. 2000). It is worth mentioning that seven of the clinical isolates did not present reversion of resistance phenotype using FK506. This data suggest that probably these strains possess other mechanism of resistance. Besides ABC transporters, *Candida* species can present an overexpression of major facilitator superfamily (MFS) proteins, another efflux pump that actively extrudes substrates using energy from proton gradient across membrane (Cannon et al. 2009). Additionally, resistance can be developed through different molecular mechanisms concerning: overexpression and/or mutation of ERG11 as well as alterations in ergosterol biosynthetic pathway (Lamping et al. 2010). In order to check and reinforce the possible involvement of ABC transporters on resistance phenotype, we also performed an efflux assay using R6G. Clinical isolates reverted by FK506 also extruded part of R6G, while the opposite was observed on strains that maintained resistance even in the presence of the inhibitor. These data corroborate the possible involvement of ABC transporters on resistant profile of those *Candida* spp. isolates reverted by FK506.

The data obtained so far, reinforce the emergence of FCZ resistant strains of *Candida* spp. in the hospital environment, as well as the involvement of active drug transporters in resistance phenotype. This phenomenon highlights the need for developing more effective control measures of fungal infections, rational use of antifungal drugs and development of new molecules able to abrogate the active transport of antifungals.

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RESUMO

A candidíase tem se tornado uma importante preocupação para a prática clínica, especialmente com o aumento da incidência de pacientes imunocomprometidos. Neste cenário, o desenvolvimento da resistência ao fluconazol se apresenta como um desafio ao tratamento dessas infecções oportunistas. O objetivo deste estudo consistiu em avaliar alguns aspectos epidemiológicos das infecções por *Candida* em um hospital universitário brasileiro, utilizando dados, anteriormente, indisponíveis. Nós observamos que 44% dos 93 isolados clínicos testados pertenciam à espécie *Candida albicans* e 56%, a espécies *Candida* não-albicans (principalmente *Candida tropicalis* e *Candida glabrata*). A maioria das cepas foi isolada de amostras de urina, onde *C. albicans* foi predominantemente detectada. 29 cepas apresentaram um fenótipo de resistência ao fluconazol e destas, 22 foram quimiosensibilizadas pelo FK506, um inibidor clássico dos transportadores ABC, envolvidos na resistência aos azóis. Estes dados sugerem a provável participação das bombas de efluxo nesse fenótipo de resistência. Nosso estudo ressalta a necessidade do desenvolvimento de medidas de controle efetivas para as infecções fúngicas, o uso racional de antifúngicos e o desenvolvimento de novas moléculas capazes de inibir o transporte ativo de antifúngicos.

Palavras-chave: *Candida* spp., isolados clínicos, epidemiologia, fluconazol, resistência.

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