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Full Length Research Paper

Molecular analysis and *in vitro* susceptibility profiling of some clinically important *Candida* spp. to the common antifungal drugs

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This study aimed to determine the *in vitro* susceptibility profiles of a group of *Candida* species to some of the common antifungal drugs in clinical use. Thirty nine *Candida* isolates were collected from the blood of immunocompromised patients with cancer. Isolates were cultivated on CHROMagar, identified using API20C kit and the ID was confirmed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. *Candida albicans* was found to be the most frequently isolated yeast (46%) in comparison to the other species *Candida krusei* (28%), *Candida tropicalis* (13%), *Candida glabrata* (10.4%) and *Candida parapsilosis* (2.6%). PCR based analysis of the transposable intron in the 25S rDNA of *C. albicans* isolates revealed that 72, 17 and 11% of the isolates belonged to genotype A, B and C, respectively. Susceptibility testing of the *Candida* isolates against five antifungal drugs (amphotericin B, 5-flucytosine, miconazole, itraconazole and fluconazole) was carried out using the broth microdilution method, and The MICs values for all five antifungal drugs was determined. The susceptibility profiles indicated that all isolates were susceptible to amphotericin B; most of the isolates were susceptible to 5-flucytosine and miconazole, while the susceptibility of the *Candida* isolates towards fluconazole and itraconazole varied depending on the species.

Key words: Clinical candidaisis, immunocompromised patients, susceptibility tests, polymerase chain reaction-restriction fragment length polymorphism assay, genotyping.

INTRODUCTION

Factors such as transplant surgery and concomitant immunosuppressive therapies, anti-cancer therapies, human immunodeficiency virus infection, diabetes, mellitus and others have contributed to the increased number of immunocompromised individuals. These immunodeficient individuals are at high risk of yeast infections, especially with the increase in the spectrum of offending species, which is due to the increase in the number of species that were considered to be saprophytic and are now becoming more commonly referred to as opportunists causing diseases is humans (Pincus et al., 2007).

The incidence of invasive systemic candidiasis has increased markedly among immunocompromised hosts, engendering excessive morbidity and mortality. This is thought to be the results of the increase in size of the population at risk due to the aforementioned reasons, the shortage of highly useful drugs with lower side effects, and the increased emergence of drug resistance at high rates (Hsueh et al., 2005; Perfect et al., 2003; Pfaller et al., 2005).

Although the majority of *Candida* infections are caused by *Candida albicans* (Girish Kumar et al., 2006; Hamza et al., 2008), none *albicans* species of *Candida* such as *Candida glabrata* and *Candida kruzei*, which are less susceptible to azole antifungal drugs especially fluconazole, have been reported with increasing

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frequency in the past decade (Bagg et al., 2003; Fadda et al., 2008; Pfaller et al., 2010). This increase in resistance is coinciding with the increase in the usage of azole antifungal derivatives, and is now a recognized problem (Brito et al., 2010; Capoor et al., 2005; Giusiano et al., 2006; Nucci and Colombo, 2002; Pfaller et al., 2003). Documentation of susceptibility to conventional antifungal drugs, investigation of newer agents and early diagnosis of invasive fungal infection is essential to reduce the mortality rates as well as for administrating effective antifungal therapy (Brito et al., 2010; Desnos-Ollivier et al., 2012; Hamza et al., 2008).

The conventional methods for the identification of yeast fungi including biochemical analysis, germ tube examination, chlamydospores formation, and the evolution of colonial morphologies on chromogenic agar, are reliable but can be time consuming; molecular technique with high discriminatory power like PCR-RFLP (restriction fragment length polymorphism), has provided an alternative fast, relatively simple to perform and reliable methods for diagnosis and identification of pathogenic yeast fungi including *Candida* species, which has proven to be useful especially in the epidemiological studies and to assess the transmission routes as well as to determine appropriate anti-fungal drugs (Mijiti et al., 2010; Mirhendi et al., 2005, 2006; Santos et al., 2010; Shokohi et al., 2010).

The PCR based method using primers designed to span the region that includes the site of the transposable group I intron of the 25S rRNA gene (rDNA) developed by McCullough et al. (1999), has shown to be useful in classifying *C. albicans*, based on the presence or absence of the self-splicing group I intron in the large subunit of rRNA genes. Strains can be differentiated into three genotypes: genotype A without the intron, genotype B harboring the intron, and genotype C possessing rDNA with and with-out the intron in a single genome.

The aim of this study was to determine the effectiveness of some classical antifungal drugs against 39 clinical isolates of *Candida* species using *in vitro* susceptibility tests. To achieve the goal of our study, a rapid PCR-based technique was performed using a one-enzyme restriction fragment length polymorphism (RFLP) for the identification of the *Candida* species, followed by another PCR for classifying *C. albicans* into its three genotypes, A to C. Finally, a screening for the antifungal susceptibility profiles of all *Candida* isolates was carried out, with the determination of the minimum inhibitory concentration (MIC) of the five tested antifungal drugs, using the broth microdilution method.

MATERIALS AND METHODS

Yeast isolates

A total of 39 *Candida* isolates were collected from the blood of immunocompromised patients with cancer being hospitalized at Al-Azhar Hussein University Hospital in Cairo. All isolates were cultured on Sabouraud's dextrose agar (SDA) slopes (Difco Laboratories, NJ, USA) supplemented with chloramphenicol (50 µg/ml) (Sigma Aldrich Chemical Co., St. Louis, USA), and slopes were incubated at 37°C for approximately 48 to 72 h prior to use. All clinical isolates were identified by inoculation on CHROM agar *Candida* (CHROM agar Company, Paris, France) for production of species-specific colors (Ghelardi et al., 2008; Sivakumar et al., 2009). Isolates were also inoculated into API20C AUX yeast identification test strips (bioMerieux SA, France) in order to determine the enzymatic activities (Smith et al., 1999).

PCR-RFLP and genotype analysis

Cellular DNA was extracted as previously described by Tamura et al. (2001). Briefly, two or three loop-full of yeast cells from the SDA slopes were suspended in 200 µl of TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA) in an Eppendorf tube (1.5 ml). 250 µl of GPT reagent (6 M guanidine thiocyanate in 50 mM Tris [pH 8.3]) and 450 µl of Tris (pH 8.0) – buffered phenol were added to the suspension of washed yeast cells. The mixture was boiled for 15 min. 250 µl of chloroform–isoamyl alcohol (24:1) was then added, and the aqueous phase was separated by centrifugation at 12,000 ×g, mixed with an equal amount of 100% (v/v) isopropanol and a 1/10 vol. of 3 M sodium acetate, and placed at 20°C for 1 h. Samples were centrifuged at 12,000 ×g for 20 min, and the obtained nucleic acid pellet was washed with ice-cold 70% ethanol, dried, and resuspended in sterile TE buffer at a concentration of 5 µg/ml.

The PCR amplification of the ITS1-5.8S-ITS2 rDNA regions was carried out in a final volume of 50 µl, each reaction contained 1.5 µl of template DNA, 0.2 µM of each forward (ITS1: 5'-TCC GTA GGT GAA CCT GCG G-'3) and reverse (ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-'3) primers, 2.5 U of Taq DNA polymerase (Fermentas Dream Taq PCR master mix, Thermo Fisher Scientific, USA), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and dNTP (each at 0.2 mM) in an automated DNA thermal cycler (Perkin-Elmer Applied Biosystems Inc., USA). The amplification cycle profile was as follows: an initial denaturation step at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 s; annealing at 56°C for 45 s, extension at 72°C for 1 min and a final extension step at 72°C for 7 min. After 25 cycles of amplification, the amplified PCR products were separated in a 1.5% (w/v) agarose gel by electrophoresis in Tris borate EDTA (TBE) buffer (0.09 M Tris, 0.09 M boric acid, and 20 mM EDTA, pH 8.3), stained with ethidium bromide (0.5 µg/ml) and photographed.

The RFLP was carried out as previously described by Mirhendi et al. (2006) and Shokohi et al. (2010). Digestion was performed by incubating a 20 µl aliquot of PCR product with 10 U of *Msp* I restriction enzyme (Fermentas Fast Digest, Thermo Fisher Scientific, USA) in final reaction volume of 25 µl at 37°C for 2 h. Then the restriction fragments were separated on 1.8% agarose gel electrophoresis in TBE buffer for approximately 45 min at 100 V and visualized by staining with ethidium bromide.

For the genotyping of *C. albicans* isolates, another PCR amplification was carried out using the forward (5'-ATA AGG GAA GTC GGC AAA ATA GAT CCG TAA-'3) and reverse (5'-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-'3) primer pairs spanning the site of the transposable intron in the 25rDNA as previously described by McCullough et al. (1999). The PCR amplification parameters were as follows: denaturation for 3 min at 94°C prior to 30 cycles of 94°C for 1 min, 65°C for 1 min, 72°C for 2.5 min, and a final extension at 72°C for 10 min.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed by broth microdilution method using RPMI 1640 medium (Difco Laboratories,

Table 1. Species distribution among the 39 clinical isolates of *Candida*.

Species	Number	%
C. albicans	18	46
C. krusei	11	28
C. tropicalis	5	13
C. glabrata	4	10.4
C. parapsilosis	1	2.6
Total	39	100

NJ, USA) with L-glutamine, without sodium bicarbonate, supplemented with 0.165 M 3-(N-morpholine)- propane sulfonic acid (MOPS) at pH 7.0. The following commercially available antifungal drugs were used in this test, Amphotericin-B (Sigma Aldrich Chemical Co., St. Louis, USA), 5-Flucytosine (Hoffman-La Roche Ltd., Basel, Switzerland), Miconazole (Janssen Pharmaceutica, Beerse, Belgium), Fluconazole and Itraconazole (Pfizer Pharmaceuticals, NY, USA). The antifungal agents and yeast inocula were prepared in accordance with the recommendations of the M27-A3 protocol of the Clinical and Laboratory Standards Institute (CLSI, 2008). Minimal inhibitory concentration (MIC) values were determined visually in comparison with diluted growth control after 48 h of incubation at 37°C. The final concentrations of all tested antifungal agents ranged from 0.0156 to 64 µg/ml. Interpretive criteria for 5-flucytosine (≥32 µg/ml), fluconazole (≥64 µg/ml) and itraconazole (≥1 µg/ml) were those published by the CLSI (2008). Isolates were considered resistant to amphotericin B with MIC (≥2 µg/ml) and miconazole with ≥8 µg/ml (Espinel-Ingroff et al., 2005 a, b; Hamza et al., 2008; Pfaller et al., 2005).

Ethical issues

The Ethics Committees of the Ain Shams University and Al-Azhar Hussein University Hospital Cairo, Egypt approved the study. All patient information and test results were kept confidential.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (IBM Corp, USA). Data were expressed using cross-tabulation as both number and percentage for the categorized data.

RESULTS

The 39 clinical *Candida* isolates from immunocompromised patients were identified on the basis of species specific color by cultivation on CHROM agar and tested for enzymatic activity with API20C kit assay. *C. albicans* was the most frequently isolated species, followed by *C. krusei*, *C. tropicalis*, *C. glabrata* and *C. parapsilosis*, respectively (Table 1).

The preliminary identification using CHROM agar and the API 20C (kit) was confirmed by PCR–RFLP assay. Fungus-specific universal primer pair (ITS1 and ITS4) were successfully able to amplify the ITS1-5.8S-ITS2 rDNA region of all tested yeast isolates, providing a single PCR product of approximately 510 to 870 bp (Figure 1). PCR amplicons were then digested with *Msp* I as previously described; the products of digestion are shown in Figure 2. The digestion of the ITS1-5.8S-ITS2 rDNA region of *Candida* species by *Msp* I enzyme generated 2 bands for each of *C. albicans*, *C. krusei*, *C. glabrata* and *C. tropicalis*, while there was no recognition site for the *Msp* I enzyme within the ITS region of *C. parapsilosis*. The RFLP pattern produced for each *Candida* species was specific and none of the examined species was mistaken for another (Figure 2). The results of the PCR-RFLP assay for the identification of the clinical *Candida* isolates, were similar to those obtained using CHROM agar *Candida* and the API 20C kit.

The genotypes of all *C. albicans* isolates were analyzed by PCR amplification as previously described, the results showed that of the 18 tested isolates, 13 (72%) belonged to genotype A (450 bp product), 3 (17%) belonged to genotype B (840 bp product) and 2 (11%) were of genotype C (450 and 840 bp products) (Figure 3).

The *in vitro* antifungal susceptibility testing was performed using the CLSI broth microdilution method against five antifungal agents (amphotericin B, 5-flucytosine, miconazole, itraconazole and fluconazole) with the MIC values of all 39 *Candida* species isolates summarized in Table 2.

The antifungal susceptibility testing showed that among the five tested antifungal, amphotericin B was the most effective. All *Candida* isolates showed susceptibility towards amphotericin B with recorded MIC values ranging between 1 and 0.0313 µg/ml (Table 3). Only 3 (7.7%) *C. krusei* isolates were resistant to miconazole, while the remaining 36 (92.3%) *Candida* spp. isolates were susceptible, the MIC values recorded for miconazole ranged between 8 and 0.0313 µg/ml (Table 3).

The same three *C. krusei* isolates that showed resistance to miconazole along with four other isolates were resistant to 5-flucytosine (Table 2), while the remaining 32 (82.1%) isolates were susceptible (Table 3).

The highest resistance rate (64.1%) among *Candida* isolates was observed against fluconazole and itraconazole, *C. krusei* isolates were found to be the least susceptible against the two previous drugs, while the susceptibility of the remaining *Candida* spp. isolates towards the same drugs varied depending on the species (Table 3).

The single isolate of *C. parapsilosis* along with one of the *C. tropicalis* isolates were susceptible to all five tested antifungal agents (Table 3).

Relationship between *C. albicans* genotype and resistance to antifungal drugs was analyzed (Table 4). *C. albicans* isolates showing resistance to fluconazole and itraconazole belonged to genotypes A, B and C, while *C. albicans* isolates showing resistance to 5-flucytosine were either genotype A or genotype B.

Table 2. Minimum inhibitory concentration profiles of 39 *Candida* isolates to various antifungal agents.

Candida spp.	No.	MIC (µg/ml)				
		5-FC	FLCZ	ITCZ	MICZ	AMPH-B
C.albicans	a1	0.0625	≥64	≥32	0.5	1
	a2	0.0625	1	0.0313	0.0313	0.125
	a3	0.0625	0.5	0.0313	0.0313	0.25
	a4	0.0625	≥64	≥32	1	0.25
	a5	0.0625	≥64	≥32	0.0313	1
	a6	0.0625	≥64	≥32	1	1
	a7	≥64	≥64	≥32	2	0.5
	a8	0.0625	≥64	≥32	1	0.25
	a9	0.0625	≥64	≥32	0.0313	0.5
	a10	0.0625	0.5	0.0313	1	0.125
	a11	≥64	≥64	≥32	4	0.5
	a12	≥64	≥64	≥32	0.5	0.5
	a13	0.0625	≥64	≥32	0.0313	0.5
	a14	0.0625	16	≥32	1	0.0625
	a15	0.125	0.5	0.0313	0.0313	0.0313
	a16	0.0625	0.5	0.0313	1	0.0313
	a17	0.0625	32	≥32	0.0625	0.125
	a18	0.0625	16	0.125	0.0625	0.0313
C. krusei	k1	4	≥64	2	2	0.5
	k2	8	≥64	2	2	0.5
	k3	4	≥64	2	2	0.25
	k4	4	≥64	2	2	0.5
	k5	32	≥64	2	8	0.5
	k6	32	≥64	1	8	0.5
	k7	64	≥64	1	8	0.5
	k8	8	≥64	1	4	0.25
	k9	16	≥64	0.5	4	0.25
	k10	8	≥64	0.5	4	0.25
	k11	16	≥64	0.5	4	0.125
C. tropicalis	t1	0.0313	4	0.0313	0.0625	0.0313
	t2	0.0313	≥64	0.5	0.0625	0.0625
	t3	0.0313	≥64	≥32	0.125	0.0313
	t4	0.0313	≥64	8	0.25	0.0313
	t5	. 1	1	0.0313	0.0313	0.0313
C. glabrata	g1	0.0625	8	1	0.0313	0.5
	g2	4	64	4	0.5	0.125
	g3	0.125	16	1	0.0313	0.5
	g4	64	0.5	0.0313	0.0313	0.0313
C. parapsilosis	p1	0.0313	0.25	0.0313	0.0313	0.125

5-FC: 5-flucytosine, FLCZ: fluconazole, ITCZ: itraconazole, MICZ: miconazole, AMPH-B: amphotericin B.

DISCUSSION

At present, yeast infections are usually treated as a general fungal infection and agents such as polyene, amphotericin B, or azole drugs, are used to control a broad array of fungi (Hof, 2008).

Drug resistance is becoming a major problem in treating yeast infections, and many strains of *Candida* are

Table 3. Resistance and Susceptibility profiles of the 39 *Candida* isolates to various antifungal agents.

Antifungal drugs			*Candida* Isolates					Total
			C. albicans	*C. glabrata*	*C. kruzei*	*C. parapsilosis*	*C. tropicalis*	
5-FC	S	Count	15	3	8	1	5	32
		%	83.3	75.0	72.7	100.0	100.0	82.1
	R	Count	3	1	3	0	0	7
		%	16.7	25.0	27.3	0	0	17.9
FLCZ	S	Count	8	3	0	1	2	14
		%	44.4	75.0	0	100.0	40.0	35.9
	R	Count	10	1	11	0	3	25
		%	55.6	25.0	100.0	0	60.0	64.1
ITCZ	S	Count	6	1	3	1	3	14
		%	33.3	25.0	27.3	100.0	60.0	35.9
	R	Count	12	3	8	0	2	25
		%	66.7	75.0	72.7	0	40.0	64.1
MICZ	S	Count	18	4	8	1	5	36
		%	100.0	100.0	72.7	100.0	100.0	92.3
	R	Count	0	0	3	0	0	3
		%	0	0	27.3	0	0	7.7
AMPH- B	S	Count	18	4	11	1	5	39
		%	100.0	100.0	100.0	100.0	100.0	100.0
	R	Count	0	0	0	0	0	0
		%	0	0	0	0	0	0

S: indicates susceptible isolates, R: indicates resistant isolates.

Table 4. Distribution of antifungal resistance among the various genotypes of *Candida albicans*.

Candida albicans	No.	Amph. B MIC ≥2 µg/ml		5- Flucytosine MIC ≥32 µg/ml		Itraconazole MIC ≥1 µg/ml		Miconazole MIC ≥8 µg/ml		Fluconazole MIC ≥64 µg/ml	
		S	R	S	R	S	R	S	R	S	R
Genotype A	13	13	0	11	2	5	8	13	0	7	6
Genotype B	3	3	0	2	1	1	2	3	0	2	1
Genotype C	2	2	0	2	0	0	2	2	0	0	2

S: indicates the number of susceptible isolates, R: indicates the number of resistant isolates.

becoming resistant to some of the commonly used antifungal drugs, making the treatment of candidiasis difficult, especially in immunocompromised patients (Capoor et al., 2005; Fisher and Zaoutis, 2008), and in order to determine the effective antifungal therapy, it is very important to carry out the proper species identification and susceptibility profiles for clinical *Candida* isolates; this can also facilitate the control over hospital infections and could better aid in disease management.

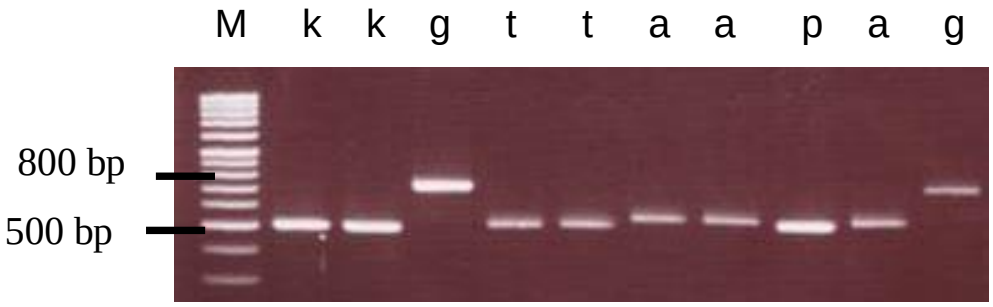

Figure 1. PCR product from ten randomly selected representative isolates of *Candida* species. Lane M: DNA molecular size marker (One step 100 bp ladder), Lanes k: *C. krusei*, Lanes g: *C. glabrata*, Lanes t: *C. tropicalis*, Lanes a: *C. albicans* and Lane p: *C. parapsilosis.*

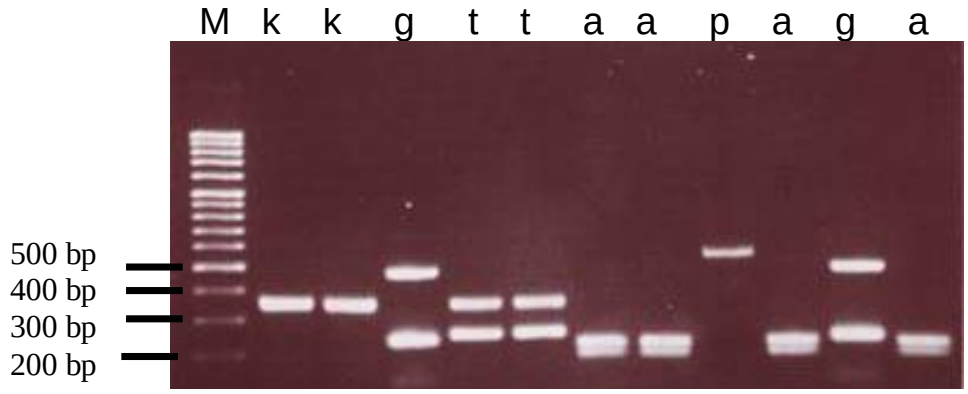

Figure 2. Restriction digestion patterns of the (ITS1-ITS4) PCR products with *Msp* I enzyme from eleven randomly selected representative isolates of *Candida* species. Lane M: DNA molecular size marker (One step 100 bp ladder), Lanes k: *C. krusei*, Lanes g: *C. glabrata*, Lanes t: *C. tropicalis*, Lanes a: *C. albicans* and Lane p: *C. parapsilosis*.

In this study, a PCR-RFLP method was applied to identify the medically important *Candida* spp., using ITS1 and ITS4 universal primers to amplify the ITS1, ITS2 and 5.8S regions of the rDNA gene of several *Candida* spp. followed by RFLP analysis of the PCR product using single enzyme digestion.

In accordance to the previous reports (Mirhendi et al., 2006; Shokohi et al., 2010), similar results were obtained by CHROMagar, API20C AUX and PCR-RFLP method in detecting different *Candida* spp., confirming that the used molecular method for the identification of medically important *Candida* spp. can be applied in clinical laboratories, PCR-RFLP has an advantage of being easy, fast and more reliable in comparison with conventional methods.

In agreement with previous reports (Borg-von Zepelin et al., 2007; Fleck et al., 2007; Hajjeh et al., 2004; Kiraz and Oz, 2011; Lass-Flörl et al., 2008; Panizo et al., 2009), *C. albicans* was the most frequently isolated species in this study, the same previous reports also identified both *C. glabrata* and *C. tropicalis* as the most prevalent pathogenic yeast species of the non *albican Candida* group; however, unlike previous reports, this study identified *C. krusei* as the most prevalent pathogenic yeast species of the *Candida* non-*albican* group, also unlike previous reports (Hajjeh et al., 2004; Pfaller et al., 2003), *C. parapsilosis* was not frequently isolated, and the only isolate in our study was susceptible to all the tested antifungal drugs.

In this study, CLSI microbroth dilution method was used as a screening tool to assess the yeast susceptibility to commonly used antifungal drugs. The test revealed that all the tested isolates (39 isolates) were susceptible to amphotericin B, similar to what have been previously reported that amphotericin B was active against the majority of the tested yeast and filamentous fungi, including species known to cause rare and difficult to treat infections, and therefore this agent plays an important role in the management of invasive fungal infection (Borg-von Zepelin et al., 2007; Pfaller et al., 2005).

Oral drugs such as fluconazole, miconazole or

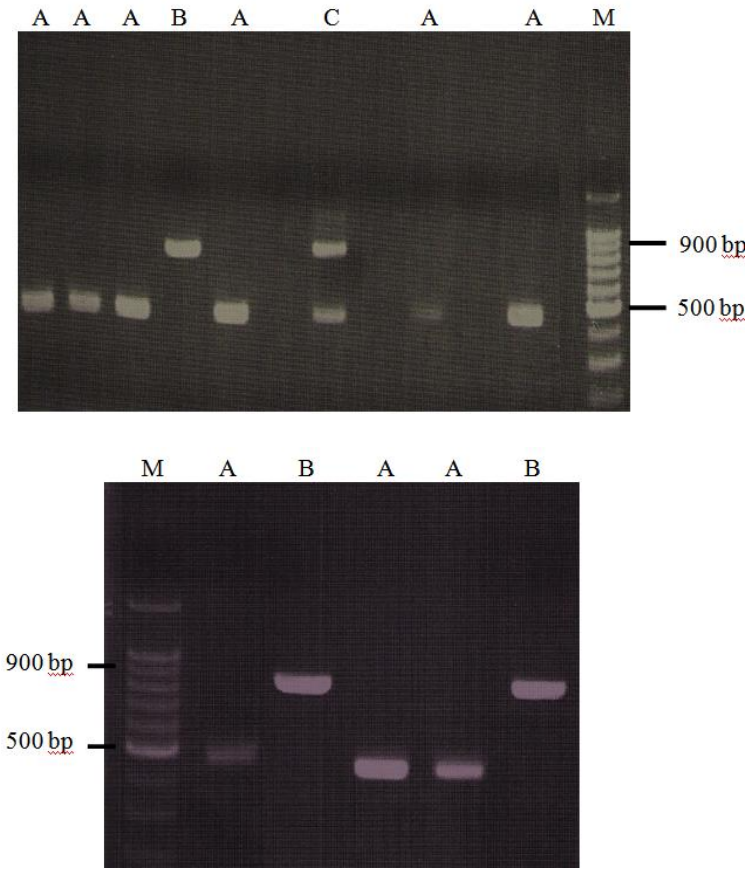

Figure 3. Genotype profiles of the *Candida albicans* isolates: genotype A (450 bp), genotype B (840 bp) and genotype C (450 and 840 bp). Lane M: DNA molecular size marker (one step 100 bp ladder).

itraconazole are the antifungal agents currently used in the treatment of severe fungal infections (Brito et al., 2010; Hamza et al., 2008). Azole antifungals target the ergosterol biosynthetic enzyme, and they are the most widely applied class of antifungal agents due to their broad therapeutic window, wide spectrum of activity and low toxicity (Pfaller et al., 2003). In the current study, the susceptibility of the isolated *Candida* species was variable towards azole drugs, only 8% of the isolates (all belonging to *C. krusei*) were resistant to miconazole, the remaining 92% were susceptible indicating that recent clinical isolates remain susceptible to this antifungal drug and as previously reported miconazole could be used as first-line in treatment of candidiasis (Isham and Ghannoum, 2010).

It was reported that fluconazole is the first line of therapy for management of candidiasis (Kourkoumpetis et al., 2010; Maida et al., 2007), this agrees with data from *in vitro* assays which revealed that fluconazole gave good activity against the majority of *Candida* spp.; however, this study showed that all *C. krusei* isolates in addition to most of *C. albicans* and *C. tropicalis* isolates, were resistant to fluconazole, thus fluconazole resistant *Candida* isolates are posing an emerging challenge in everyday clinical practice. The *in vitro* resistance of *C. krusei* to fluconazole was detected in all of the isolates, this finding is similar to previous reports (Fleck et al., 2007; Pfaller et al., 2005), indicating that *C. krusei* may be inherently resistant to fluconazole.

In the present study, 60% of the *C. tropicalis* and 75% of the *C. glabrata* isolates were found to be resistant to fluconazole and itraconazole respectively, which agrees with what have been previously reported that, infections due to *C. tropicalis* and *C. glabrata* has increased dramatically on a global scale, with increasing resistance to fluconazole and itraconazole in both developed and undeveloped countries (Borg-von Zepelin et al., 2007; Hajjeh et al., 2004; Kiraz and Oz, 2011; Kothavade et al., 2010), cross-resistance to more than one azole antifungal especially between fluconazole and itraconazole (Panizo et al., 2009) has also been observed in our study, indicating the importance of development and usage of newer agents with varying target sites and modes of action.

In addition to antifungal susceptibility screening, the present study evaluated the distribution of different

genotypes among *C. albicans* isolates using PCR amplification of the transposable intron in the 25S rDNA, which has revealed the prevalence of genotype A. Higher incidence of genotype A among *C. albicans* genotypes has been reported earlier (Girish Kumar et al., 2006; Nawrot et al., 2010; Tamura et al., 2001). No specific association was found between the genotype status of the *C. albicans* isolates and the resistance to antifungal drugs. Azole resistance was recorded among the three genotypes (A, B and C), while 5-flucytosine resistance was exhibited by genotypes A and B only.

Demonstration of the *Candida* spp. resistance to more than one azole antifungal indicates the importance to develop newer, safer and more effective antifungal agents with varying target sites and modes of action that can circumvent both innate and acquired drug-resistance mechanisms (Desnos-Ollivier et al., 2012; Ikeda et al., 2009) coupled with further investigating the possible synergistic effect between azole-type antifungals and other antifungal drugs that exhibit different action mechanisms against fungi (Hanafy et al., 2007).

It is now clear that *C. albicans* and non *albicans Candida* species pathogenic for man are becoming resistant to antifungal agents, in particular azole compounds, the clinical consequences of antifungal resistance to azoles can be seen either in treatment failures in patients and changes in the prevalence of *Candida* species causing disease or in standardized susceptibility testing methods and definitions of a resistant fungal isolate (Brito et al., 2010; Desnos-Ollivier et al., 2012; Hamza et al., 2008).

The establishment of the definitive etiological diagnosis, allows the prediction of some species potential pathogenicity, and guides the selection of proper antifungal therapy. PCR-RFLP can be considered to be an “old tool” for PCR product analysis, but it is still being used for characterization studies of microorganism because of its simplicity, reliability, easy adaptation for identifying several genera or species, and in addition, it does not require expensive materials or equipments. These characteristics are important in cost-effective studies and indicate that the use of this technique will probably become routinely accepted in clinical laboratories.

It is also important to point out that, one of the limitations in this study, was the small number of the total isolates, but despite the limited number of tested isolates, the resistance observed in this study against conventional antifungal agents should be viewed with concern and necessitates continuous monitoring through surveillance studies.

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REFERENCES

Bagg J, Sweeney MP, Lewis MA, Jackson MS, Coleman D, Al MA, Baxter W, McEndrick S, McHugh S (2003). High prevalence of non-*albicans* yeasts and detection of anti-fungal resistance in the oral flora of patients with advanced cancer. Palliat. Med. 17:477-481.

Borg-von Zepelin M, Kunz L, Rüchel R, Reichard U, Weig M, Gross U (2007). Epidemiology and antifungal susceptibilities of *Candida* spp. to six antifungal agents: results from a surveillance study on fungaemia in Germany from July 2004 – August 2005. J. Antimicrob. Chemother. 60:424-428.

Brito GN, Inocêncio AC, Querido SM, Jorge AO, Koga-Ito CY (2010). *In vitro* antifungal susceptibility of *Candida* spp. oral isolates from HIV-positive patients and control individuals. Braz. Oral. Res. 25:28-33.

Capoor MR, Nair D, Deb M, Verma, PK, Srivastava L, Aggarwal P (2005). Emergence of non-*albicans Candida* species and antifungal resistance in a tertiary care hospital. Jpn. J. Infect. Dis. 58:344-348.

Clinical and Laboratory Standards Institute (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, Approved Standard. CLSI document M27-A3. Pennsylvania: Clinical and Laboratory Standards Institute (CLSI) pp. 221-227.

Desnos-Ollivier M, Robert V, Raoux-Barbot D, Groenewald M, Dromer F (2012). Antifungal susceptibility profiles of 1698 yeast reference strains revealing potential emerging human pathogens. PLoS. One., 7:e32278.

Espinel-Ingroff A, Barchiesi F, Cuenca-Estrella M, Pfaller MA, Rinaldi M, Rodriguez-Tudela JL, Verweij PE (2005). 158 International and multicenter comparison of EUCAST and CLSI M27-A2 broth microdilution methods for testing susceptibilities of *Candida* spp. to fluconazole, itraconazole, posaconazole, and voriconazole. J. Clin. Microbiol. 43:3884-3889.

Espinel-Ingroff A, Barchiesi F, Cuenca-Estrella M, Fothergill A, Pfaller MA, Rinaldi M, Rodriguez-Tudela JL, Verweij PE (2005). Comparison of visual 24-hour and spectrophotometric 48-hour MICs to CLSI reference microdilution MICs of fluconazole, itraconazole, posaconazole, and voriconazole for *Candida* spp.: A collaborative study. J. Clin. Microbiol. 43:4535-4540.

Fadda ME, Podda GS, Pisano, MB, Deplano M, Cosentino S (2008). Prevalence of *Candida* species in different hospital wards and their susceptibility to antifungal agents: results of a three year survey. J. Prev. Med. Hyg. 49:69-74.

Fisher BT, Zaoutis TE (2008). Treatment of invasive candidiasis in immunocompromised pediatric patients. Paediatr. Drugs 10:281-98.

Fleck R, Dietz A, Hof H (2007). *In vitro* susceptibility of *Candida* species to five antifungal agents in a German university hospital assessed by the reference broth microdilution method and E test. J. Antimicrob. Chemother. 59:767-771.

Ghelardi E, Pichierri G, Castagna B, Barnini S, Tavanti A, Campa M (2008). Efficacy of Chromogenic *Candida* gar for isolation and presumptive identification of pathogenic yeast species. Clin. Microbiol. Infect. 14:141-147.

Girish Kumar CP, Hanafy AM, Katsu M, Mikami Y, Menon T (2006). Molecular analysis and susceptibility profiling of *Candida albicans* isolates from immunocompromised patients in South India. Mycopathologia 161:153-159.

Giusiano G, Mangiaterra M, Garcia Saito V, Rojas F, Gómez V, Díaz, MC (2006). Fluconazole and itraconazole resistance of yeasts isolated from the bloodstream and catheters of hospitalized pediatric patients. Chemotherapy 52:254-259.

Hajjeh RA, Sofair AN, Harrison LH, Lyon GM, Arthington-Skaggs BA, Mirza SA, Phelan M, Morgan J, Lee-Yang W, Ciblak MA,

Benjamin LE, Sanza LT, Huie S, Yeo SF, Brandt ME, Warnock DW (2004). Incidence of bloodstream infections due to *Candida* species and *in vitro* susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. J. Clin. Microbiol. 42:519-527.

Hamza OJ, Matee MI, Moshi MJ, Simon EN, Mugusi F, Mikx F.H, Helderman WH, Rijs AJ, van der Ven AJ, Verweij PE (2008). Species distribution and *in vitro* antifungal susceptibility of oral yeast isolates from Tanzanian HIV-infected patients with primary and recurrent oropharyngeal candidiasis. BMC Microbiol. 8:135.

Hanafy A, Uno J, Mitani H, Kang Y, Mikami Y (2007). *In-vitro* antifungal activities of sulfa drugs against clinical isolates of *Aspergillus* and *Cryptococcus* species. Nippon Ishinkin Gakkai Zasshi. 48:47-50.

Hof H (2008). Developments in the epidemiology of invasive fungal infections-implications for the empiric and targeted antifungal therapy. Mycoses 51:1-6.

Hsueh PR, Lau YJ, Chuang YC, Wan JH, Huang WK, Shyr JM, Yan JJ, Yu KW, Wu JJ, Ko WC, Yang YC, Liu YC, Teng LJ, Liu CY, Luh KT (2005). Antifungal susceptibilities of clinical isolates of *Candida* species, *Cryptococcus neoformans*, and *Aspergillus* species from Taiwan: surveillance of multicenter antimicrobial resistance in Taiwan program data from 2003. Antimicrob. Agents Chemother. 49:507-512.

Ikeda F, Saika T, Sato Y, Suzuki M, Hasegawa M, Mikawa T, Kobayashi I, Tsuji A (2009). Antifungal activity of micafungin against *Candida* and *Aspergillus* spp. isolated from pediatric patients in Japan. Med. Mycol. 47:145-148.

Isham N, Ghannoum MA (2010). Antifungal activity of miconazole against recent *Candida* strains. Mycoses 53:434-437.

Kiraz N, Oz Y (2011). Species distribution and *in vitro* antifungal susceptibility of clinical *Candida* isolates from a university hospital in Turkey over a 5-year period. Med. Mycol. 49:126-131.

Kothavade RJ, Kura MM, Valand AG, Panthaki MH (2010). *Candida tropicalis*: its prevalence, pathogenicity and increasing resistance to fluconazole. J. Med. Microbiol. 59:873-880.

Kourkoumpetis TK, Velmahos GC, Ziakas PD, Tampakakis E, Manolakaki D, Coleman JJ, Mylonakis E (2010). The Effect of Cumulative Length of Hospital Stay on the Antifungal Resistance of *Candida* Strains Isolated from Critically ill Surgical Patients. Mycopathologia 171:85-91.

Lass-Flörl C, Mayr A, Perkhofer S, Hinterberger G, Hausdorfer J, Speth C, Fille M (2008). Activities of antifungal agents against yeasts and filamentous fungi: assessment according to the methodology of the European Committee on Antimicrobial Susceptibility Testing. Antimicrob. Agents Chemother. 52:3637-3641.

Maida CM, Milici ME, Oliveri S (2007). *In vitro* activity of fluconazole, voriconazole and caspofungin against clinical yeast isolates. J. Chemother. 19:277-282.

McCullough MJ, Clemons KV, Stevens DA (1999). Molecular and phenotypic characterization of genotype *C. albicans* subgroups and comparison with *Candida dubliniensis* and *Candida stellatoidea*. J. Clin. Microbiol. 37:417-421.

Mijiti J, Pu XM, Erfan A, Yaguchi T, Chibana H, Tanaka R (2010). Genotyping of fluconazole-resistant *Candida albicans* isolated from Uighurian people in Xinjing (China) using ALTS/RFLP and micro-TGGE method. Nippon Ishinkin Gakkai Zasshi. 51:165-168.

Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi A (2006). One-enzyme PCR-RFLP assay for identification of six medically important *Candida* species. Nippon Ishinkin Gakkai Zasshi. 47:225-229.

Mirhendi H, Makimura K, Zomorodian K, Maeda N, Ohshima T, Yamaguchi H (2005). Differentiation of *Candida albicans* and *Candida dubliniensis* using a single-enzyme PCR-RFLP method. Jpn. J. Infect. Dis. 58:235-237.

Nawrot U, Pajaczkowska M, Włodarczyk K, Mecler I (2010). rDNA-based genotyping of clinical isolates of *Candida albicans*. Pol. J. Microbiol. 59:213-216.

Nucci M, Colombo AL (2002). Emergence of resistant *Candida* in neutropenic patients. Braz. J. Infect. Dis. 6:124-128.

Panizo MM, Reviákina V, Dolande M, Selgrad S (2009). *Candida* spp. *in vitro* susceptibility profile to four antifungal agents. Resistance surveillance study in Venezuelan strains. Med. Mycol. 47:137-143.

Perfect JR, Marr KA, Walsh TJ, Greenberg RN, DuPont B, de la Torre-Cisneros J, Just-Nubling G, Schlamm HT, Lutsar I, Espinel-Ingroff A, Johnson E (2003). Voriconazole treatment for less-common, emerging or refractory fungal infections. Clin. Infect. Dis. 36:1122-1131.

Pfaller MA, Boyken L, Hollis RJ, Messer SA, Tendolkar S, Diekema DJ (2005). *In vitro* susceptibilities of clinical isolates of *Candida* species, *Cryptococcus neoformans*, and *Aspergillus* species to itraconazole: global survey of 935 isolates tested by clinical and laboratory standards institute broth microdilution methods. J. Clin. Microbiol. 43:3807-3810.

Pfaller MA, Boyken L, Hollis RJ, Messer SA, Tendolkar S, Diekema DJ, (2005). *In vitro* activities of anidualafungin against more than 2500 clinical isolates of *Candida* spp., including 315 isolates resistant to fluconazole. J. Clin. Microbiol. 43:5425-5427.

Pfaller MA, Castanheira M, Messer SA, Moet GJ, Jones RN (2010). Variation in *Candida* spp. distribution and antifungal resistance rates among bloodstream infection isolates by patient age: report from the SENTRY Antimicrobial Surveillance Program (2008-2009). Diagn. Microbiol. Infect. Dis. 68:278-283.

Pfaller MA, Diekema DJ, Messer SA, Boyken L, Hollis RJ, Jones RN (2003). *In vitro* activities of voriconazole, posaconazole, and four licensed systemic antifungal agents against *Candida* species infrequently isolated from blood. International Fungal Surveillance Participant Group. J. Clin. Microbiol. 41:78-83.

Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ, Diekema DJ (2003). Caspofungin activity against clinical isolates of fluconazole-resistant *Candida*. J. Clin. Microbiol. 41:5729-5731.

Pincus DH, Orenga S, Chatelier S (2007). Yeast identification past, present and future methods. Med. Mycol. 45:97-121.

Santos MS, Souza ES, S Junior RM, Talhari S, Souza JV (2010). Identification of fungemia agents using the polymerase chain reaction and restriction fragment length polymorphism analysis. Braz. J. Med. Biol. Res. 43:712-716.

Shokohi T, Hashemi Soteh MB, Pouri ZS, Hedayati MT, Mayahi S (2010). Identification of *Candida* species using PCR-RFLP in cancer patients in Iran. Indian J. Med. Microbiol. 28:147-51.

Sivakumar VG, Shankar P, Nalina K, Menon T (2009). Use of CHROMagar in the differentiation of common species of *Candida*. Mycopathologia 167:47-49.

Smith BM, Dunklee D, Vu H, Woods LG (1999). Comparative performance of the RapID Yeast plus system and the API 20 C AUX Clinical Yeast System. J. Clin. Microbiol. 37:2697-2698.

Tamura M, Watnabe K, Mikami Y, Yazawa K, Nishimura K (2001). Molecular characterization of new clinical isolates of *Candida albicans* and *C. dubliniensis* in Japan: Analysis reveals a new genotype of *C. albicans* with group I intron. J. Clin. Microbiol. 39:4309-4315.