MOLECULAR CHARACTERIZATION OF AZOLE-SUSCEPTIBLE AND RESISTANT CANDIDA ALBICANS ISOLATED FROM CANCER PATIENTS

ABSTRACT:
In the present study, the genotypic characterizations of fluconazole and itraconazole (antifungal agents) susceptible and resistant C. albicans isolates were investigated. Thirteen C. albicans isolates were genotypically characterized using both restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) methods. Hind I restriction fragments revealed an obvious polymorphism among the samples yielding five different types of patterns for the C. albicans isolates. Pattern 1 contained C. albicans ATCC 90029 reference strain along with all the five fluconazole/itraconazole susceptible isolates and the two fluconazole susceptible/itraconazole resistant isolates, patterns 2, 3, and 4 each contained a single isolate of the fluconazole/itraconazole resistant C. albicans, and pattern 5 contained the remaining two fluconazole/itraconazole resistant isolates. The RAPD-PCR method on the other hand followed by phylogenetic analysis of the resulting patterns identified the formation of two original clonal type groups (A and B). In group A, C. albicans ATCC 90029 along with all five fluconazole/itraconazole susceptible isolates were further clustered into three subgroups (genotypes), and in group B, two fluconazole susceptible/itraconazole resistant and the remaining five fluconazole/itraconazole resistant isolates were further clustered into five subgroups (genotypes). In relation to RAPD-PCR analysis, most azole susceptible isolates were classified into group A and resistant isolates in group B demonstrating that azole drugs may affect the genotypic behaviour of C. albicans depending on their susceptibility to these drugs.

INTRODUCTION:
Candida species especially Candida albicans (C. albicans) are part of the commensal flora, which can be isolated from healthy individuals with different frequency (Qi et al., 2005). At the same time, C. albicans is one of the most pervasive opportunistic pathogens, which can cause endogenous infection ranging from superficial to seriously deep-seated mycoses known as candidiasis, under the altered host conditions, such as the malignant cancer patients (Sun et al., 2009). Also anticancer treatment including chemotherapy or/and radiotherapy is still associated with several side effects, including oral candidiasis as one major source of illness, thereby resulting in treatment delays and necessitating dose reduction (Mead, 2002). Furthermore some studies have shown its potential importance in the development of systemic candidiasis which would be fatal for these patients (Xu et al., 2002).

C. albicans may be found in infected tissue as unicellular budding yeasts, hyphae, or pseudohyphae, this dimorphism facilitates tissue invasion, increases tissue viability, and allows it to escape from macrophage and neutrophil engulfment (Watamoto et al., 2009).

Treatment for C. albicans infections generally relies upon topical, oral, or intravenous administration of triazole antifungics, with fluconazole and itraconazole being the oldest and most widely prescribed members of this class (Da-Costa et al., 2009), and like all widely prescribed antimicrobials, fluconazole and itraconazole are susceptible to the development of drug resistance by its target pathogens, this problem poses a rapidly increasing challenge especially with the growing numbers of immunocompromised patients worldwide (Kontoyiannis and Lewis, 2002, Pfaffer and Diekema, 2007, Enwuru et al., 2008).

Genetic analysis of Candida isolates reveals that virulence attributes may be intimately related to genotypic profiles. Molecular methods have been used for such analysis because of their sensitivity and specificity, allowing intra-specific identification of isolates as well as detection of small differences in nucleic acid content.
among species and ancestries of the same species (Samaranayake et al., 2003b; Enache-Soare et al., 2009). Molecular technique with high discriminatory power like restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) has provided an alternative fast, relatively simple to perform and reliable methods for the genotyping of pathogenic yeast fungi including C. albicans, 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 (Ribeiro et al., 2005; Mijiti et al., 2010; Santos et al., 2010; Shokohi et al., 2010).

The emergence of fluconazole and itraconazole resistance may be associated with continued exposure of C. albicans population to this drug, leading to a gradual elevation of the minimal inhibitory concentration (MIC) of some isolates (Kontoyiannis and Lewis 2002; Enwuru et al., 2008). Evidence shows that azole-resistant isolates, especially fluconazole and itraconazole, result from genomic alterations (Dassanayake et al., 2002; Sanglard and Odds, 2002). However, there is little information about the genomic diversity of resistant isolates to antifungal drugs compared with those that are susceptible to the drugs.

Thus, this study genotypically characterizes fluconazole and itraconazole susceptible and resistant isolates. This research may help us to understand the antifungal drug susceptibility and genotypic characteristics of Candida albicans isolated from cancer patients in Cairo, and would assist in getting the accurate susceptibility test in clinical practice.

MATERIAL AND METHODS:

Yeast Isolates:
A total of twelve C. albicans isolates were evaluated in this study (all belonged to genotype A), all of which were clinical isolates recovered during a previous investigational study from the blood of immunocompromised patients with cancer being hospitalized at Al-Azhar Hussein University Hospital in Cairo, seven of these isolates were characterized as susceptible to fluconazole and five were resistant, the same five isolates that showed resistance to fluconazole along with two other isolates were resistant to itraconazole and the remaining five were susceptible (Hanafy and Morsy, 2012). In addition, a single fluconazole and itraconazole susceptible culture collection strain (C. albicans ATCC 90029) was obtained and included as a reference strain. The 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.

Genotypic characterization
Genotypic differentiation using the RFLP and RAPD-PCR techniques was applied to the C. albicans reference strain, and to all of the previously mentioned twelve C. albicans isolates.

Extraction of genomic DNA:
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 grown overnight at 30°C 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 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. DNA concentrations and A260/A280 ratios were determined using a spectrophotometer Lambda 1A (Perkin-Elmer, USA). An A260/A280 ratio of 1.8-2.1 was considered acceptable.

Restriction Enzyme Analysis:
RFLP digestion of the genomic DNA was performed for each of the 13 C. albicans strains individually by incubating 10 μl of genomic DNA aliquots (5 to 10 μg) overnight at 37°C with restriction endonuclease Hind I (2 U/μg of DNA; Fermentas Fast Digest, Thermo Fisher Scientific, USA) in a final reaction volume of 25 μl. Digestion was halted with a “stop” buffer and RFLPs relatedness were detected based on visual examination of the number and the positions of the major bands.

Random Amplified Polymorphic DNA Analysis:
Amplification reactions using primer RSD6 (5’-GGCATCCCCCA-3’) was performed as described by (Samaranayake et al., 2003a). The reactions were performed in volume of 25 μl, including about 20 ng of DNA template, 10 mM of Tris-HCl, pH 8.3, 50 mM of KCl, 1.5 mM of MgCl2, 0.2 mM of each dNTP, and 2.5 U of Taq DNA polymerase (Fermentas Dream Taq, Thermo Fisher Scientific, USA), added with 1.5 μl of RSD6. Amplification was performed in an automated DNA thermal cycler (Perkin-Elmer Applied Biosystems Inc., USA) programmed as...
follows: The first five cycles included denaturation at 94°C for 30s, annealing at 27°C for 2 min, and primer extension at 72°C for 2 min, followed by 45 denaturation cycles at 94°C for 30 s, annealing at 32°C for 2 min, and extension at 72°C for 2 min, with a final extension held at this temperature for 15 min.

**Electrophoresis and Data Analysis:**

DNA products were electrophoresed on agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) at 100 V for 45-120 min, in a gel composed of 1.5% and 2% for RAPD-PCR and RFLP products respectively. All gels were stained with 0.5 µg/ml of ethidium bromide in distilled water for 20 min and then de-stained in distilled water for 10min. The DNA bands were visualized with a UV trans-illuminator, the sizes of the resulting DNA fragments were determined by comparison with 1kb plus and 100 bp DNA ladder standard molecular weight markers (Invitrogen Life Technologies, Carlsbad, CA, USA) and photographed using gel documentation system (GelDoc 2000, Bio-Rad, UK). Analysis of the RFLP/RAPD-PCR DNA band patterns was done with Gelcompar II software (Applied Maths, Kortrijk, Belgium).

Highly related strains (groups) were defined as those having at least 90% homology on the basis of banding patterns.

**RESULTS:**

**DNA typing by RFLP pattern analysis:**

The *Hinf I* digests displayed a complex pattern of bands in which each sample yielded eight to nine major fragments ranging from 300 bp to 8 kb. Fragments, in the size range 2 to 8 kb, allowed the differentiation of specific pattern types and revealed an obvious polymorphism among the samples.

Five types of patterns were seen for the *C. albicans* isolates (Fig. 1a & b). Pattern 1 contained *C. albicans* ATCC 90029 reference strain along with all the five isolates that were susceptible to both fluconazole and itraconazole antifungal drugs (Ca2, Ca3, Ca4, Ca7, and Ca8) and the two isolates that were susceptible to fluconazole only (Ca1 and Ca11), patterns 2, 3 and 4 each contained a single isolate of the fluconazole and itraconazole resistant *C. albicans* Ca5 and Ca12, respectively (Fig. 1a & b).

![Fig. 1. DNA from the *C. albicans* isolates digested with *Hinf I*, and electrophoresed on 2% agarose gel. Lanes M: DNA molecular size marker (1 kb plus DNA ladder). Lane CaR: *C. albicans* reference strain (ATCC 90029), lanes (Ca2, Ca3, Ca4, Ca7 and Ca8): fluconazole/itraconazole susceptible *C. albicans* isolates, lanes (Ca1, Ca5, Ca6, Ca9 and Ca12): fluconazole/itraconazole resistant *C. albicans* isolates and lanes (Ca10 & Ca11): fluconazole susceptible/itraconazole resistant *C. albicans* isolates.](image-url)
In pattern 5 slight differences in the RFLP patterns were noted in the two remaining fluconazole and itraconazole resistant *C. albicans* isolates \((Ca_5 & Ca_9)\) having a double instead of a single band in the range of 4 to 5 kb. Also the positions of a double of band seen between 4 and 5 kb were slightly lower for isolate \(Ca_5\) compared with the positions of the bands for isolates \(Ca_9\) (Fig. 1a & b). However, these small variations were not taken into account and the patterns of isolates \(Ca_5\) and \(Ca_9\) were interpreted as being similar and were thus kept in the same group.

**Genotypic characterization using RAPD-PCR:**

*C. albicans* isolates showed a number of major DNA bands (ranging from 200 bp to over 3 kb) generated by primer RSD6 using the RAPD-PCR method. Data interpretation criteria were based on differences in formation and/or position of the bands. Eight RAPD profiles (designated RAPD1–8) were obtained allowing the identification of eight different genotypes among the 13 fluconazole and itraconazole-resistant or susceptible *C. albicans* isolates (Fig. 2a & b).

**Fig. 2.** Random amplified polymorphic DNA fingerprinting electrophoresed on 1.5% agarose gel, of fluconazole/itraconazole susceptible *Candida albicans* isolates (Ca2, Ca3, Ca4, Ca7 and Ca8) panel (a), fluconazole/itraconazole resistant isolates (Ca1, Ca5, Ca9, Ca6 and Ca12) and fluconazole susceptible/itraconazole resistant isolates (Ca10 and Ca11) panel (b), obtained using primer RSD6. Lane CaR: *C. albicans* reference strain (ATCC 90029), Lanes M: DNA molecular size marker (100bp DNA ladder).

RAPD types 1, 2, and 4 could easily be recognized based on their unique profiles. RAPD types 2, 3, and 5 were differentiated based on a slight difference in DNA fragments position at approximately 1 kb for RAPD type A3 and a single DNA fragment generated in the profiles at approximately 1.2 kb for RAPD type 5. RAPD types 1 and 6 were
differentiated based on a slight difference in DNA fragments position at approximately 2 kb (Fig. 2a & b).

**Heterogeneity among antifungal susceptible/resistant *C. albicans* isolates genotypes:**

The variations in fluconazole and itraconazole-resistance/susceptibility and the RAPD genotypes of the *C. albicans* isolates were analyzed with Gelcompar II software (Applied Maths, Kortrijk, Belgium). In agreement with the dendrogram of genetic similarity using Dice similarity coefficient index (a probabilistic measure based on presence-absence data), all 13 *C. albicans* isolates were classified as groups A and B. The UPGMA had a high Cophenetic correlation of 0.5, suggesting that the dendrogram preserves the pair wise distances between the original un-modeled data points. Group A included fluconazole/itraconazole susceptible isolates CaA, Ca2, Ca3, Ca4, Ca5, and Ca8. Group B included fluconazole susceptible/itraconazole resistant isolates CaA0 and CaA1 and fluconazole/itraconazole resistant isolates Ca1, Ca3, Ca6, Ca9, and Ca12, as it is shown in figure 3.

![Dendrogram and gel band patterns of RAPD fingerprints of 13 *C. albicans* isolates typed with primer RSD6. The scale at the top represents percent similarity. The isolate numbers and genotypes types are listed to the right of the figure and correspond to those in table 1.](image)

**DISCUSSION:**

Antifungal drug resistance is considered as an important virulence factor of *Candida* species, which facilitate the establishment of infection (Yang, 2003). Although Azole antifungal drugs such as fluconazole and itraconazole are the most widely used antifungal agent for the treatment of infections caused by *C. albicans*, an increased number of resistant isolates have been observed (Ribeiro et al., 2005; Enwuru et al., 2008). Therefore, detection of resistance among isolates is considered of great importance.

Yeast genotypes may vary according to drug susceptibility. The genetic diversity among Azole resistant *C. albicans* isolates has revealed similar results. Genotypic characterization of fluconazole resistant isolates into groups has been defined by Mijiti et al. (2010) and Costa et al. (2011) who observed that resistant strains to this drug were genetically more similar to each other than they were to susceptible strains.

Similar to previous studies (Qi et al., 2005; Sun et al., 2009), the DNA-typing methods used in the present study exhibited the ability to group the fluconazole/itraconazole susceptible isolates recovered and also differentiate them from fluconazole/itraconazole resistant ones, both visually and with the aid of computer-assisted procedures. These DNA-typing techniques were also able to discriminate differences in highly related isolates.

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<th><em>Candida albicans</em> types</th>
<th>Isolates</th>
<th>Molecular profile</th>
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<tr>
<td>Fluconazole/itraconazole Resistant</td>
<td>CaA</td>
<td>B1</td>
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<tr>
<td>Fluconazole/itraconazole susceptible</td>
<td>Ca5</td>
<td>B2</td>
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<tr>
<td>Fluconazole susceptible / itraconazole Resistant</td>
<td>Ca10</td>
<td>B5</td>
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<tr>
<td><em>C. albicans</em> reference strain (ATCC 90029)</td>
<td>CaA0</td>
<td>A1</td>
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In general, RAPD-PCR technique employed the primer RSD6 in this study for it has been successful in sub-grouping the yeast strains (Teanpaisan et al., 2008; Sun et al., 2009; Costa et al., 2011). The RAPD-PCR using RSD6 primer resulted in the most complex banding patterns and provided increased levels of clustering and discriminatory power compared to RFLP analysis with Hinf I, this was found to be particularly important in revealing the presence of several genotypic variations among the two fluconazole susceptible /itraconazole resistant tested isolates, which led to group them under the fluconazole/itraconazole resistant cluster (B), similar results reported the superior clustering and discriminatory power of RAPD-PCR compared to RFLP based techniques in the genetic diversity studies of Candida albicans (López-Ribot et al., 2000; Dassanayake and Samaranayakel, 2003).

In the present study, fingerprinting generated by RSD6 was further evaluated using a well-proven computer-assisted data analysis system, to define related strains on the basis of banding patterns, thus showing diversity of genotypes. The molecular profile found in this work using the primer RSD6 by RAPD-PCR followed by computer-assisted data analysis system, classified all 13 C. albicans isolates into two major groups of original clonal types A and B, each group was further divided into three and five sub groups (genotypes) respectively. All susceptible isolates were classified in group A and resistant isolates in group B demonstrated that RAPD-PCR typing method presented a similar standard between the two groups, suggesting that, by this technique, a strong correlation between genotypes and fluconazole/itraconazole-resistant samples may be found. These results confirmed previous reports obtained in experiment with primer RSD6 on the discriminatory power of DNA-based methods for strain delineation of azole resistant Candida albicans (Teanpaisan et al., 2008; Sun et al., 2009; Mijiti et al., 2010; Costa et al., 2011).

In conclusion, the current study provided data on the relation between RAPD genotypes profiles and antifungal resistance/susceptibility of 13 C. albicans isolates recovered from cancer patients living in Cairo. A total of 2 major clonal types and 8 clusters of genotypes were found. The results of the genotyping suggested high heterogeneity among C. albicans isolates. Such findings may be useful as baseline information on systemic C. albicans colonization in the Egyptian population living in Cairo.

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REFERENCES:


Hanafy AM., Candida albicans, Azole resistance, genotyping, RAPD-PCR, RFLP.


Samaranayake YH, Samaranayake LP, Yau JYY, Dassanayake RS, Tsangw K, Cheung BPK, Yeung KW S. 2003b. 'Genotypic shuffling' of azole resistance, genotyping, RAPD-PCR, RFLP.


