Original article

Susceptibility of *Candida albicans* to common and novel antifungal drugs, and relationship between the mating type locus and resistance, in Lebanese hospital isolates

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Summary

The incidence of antifungal resistance is on the increase worldwide and novel drugs are constantly being developed to counter this trend. One hundred and sixteen clinical isolates of *Candida albicans* were collected from Lebanese hospitals in order to first determine the degree of resistance of Lebanese isolates to four common azoles: fluconazole (FL), itraconazole (IT), ketoconazole (KE), and voriconazole (VO), in addition to amphotericin B (AP) and caspofungin (CS) through the Epsilometer test method and second, determine any relationship between the allelic compositions of the mating type loci (*MTLa*, *MTL* α, *MTLa*/α) with drug resistance. Results showed that resistance, among *C. albicans* isolates, was the highest with 12% for IT, followed by 7.7% for VO, 6% for KE, 5% for FL, 1.7% for AP and 0% for CS. Three isolates (2.6%) were resistant to all azoles tested, including one that was resistant to all drugs used except CS. Eleven isolates were homozygous at the *MTL* locus (9.5%), five of which (45%) were resistant to at least one antifungal drug whereas 14 of the 105 heterozygous strains (13%) exhibited similar resistance ($P = 0.02$), indicating a strong correlation between *MTL* locus homozygosity and resistance.

Key words: *Candida albicans*, azoles, caspofungin, *MTL* locus, homozygosity, cross-resistance.

Introduction

Candida albicans is a dimorphic opportunistic pathogen that exists commensally in warm-blooded animals including humans. It colonises mucosal surfaces of the oral cavity, digestive tract or genitourinary system of healthy individuals and is capable of causing a variety of infections depending on the nature of the defect in the host.[1,2] Candidiasis due to *C. albicans* is the most common mycosis associated with human immunodeficiency virus infection, occurring in up to 90% of patients.[3] *Candida* infections have dramatically increased in the last decade because of immunosuppressive treatments, long-term catheterisation, prolonged use of broad-spectrum antibiotics and longer survival of immunologically compromised individuals.[4] *Candida* species are the fourth most common cause of nosocomial bloodstream infections in the United States, with *C. albicans* the most commonly encountered species.[1,5] However, statistical data on the ranking of *C. albicans* infections in Lebanon are absent.

When compared to the number of antibacterial drugs, antifungal drugs are very limited[1,6–8] because many potential antifungal drug targets have homologues of similar function and susceptibility to inhibition in humans and the toxic side effects dramatically reduce the number of antifungal agents that can be used therapeutically.[1] There are five major classes of systemic antifungal compounds currently in clinical use: polyenes, azoles, allylamines, fluoropyrimidines, and echinocandins and pneumocandins. The polyenes, mainly amphotericin B (AP) are fungicidal and have the broadest spectrum of activity of any clinically useful antifungal drug but can cause nephrotoxicity.[9] The azoles are fungistatic and have a broad spectrum of activity with few side effects with fluconazole (FL), itraconazole (IT), and voriconazole (VO), being the most

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frequently utilised.[1,8,10,11] Fluconazole is the preferred azole because of its high oral bioavailability, good safety profile and broad efficacy against most pathogenic yeast.[3,12] Itraconazole and some newer azoles like VO are also active against non-yeast fungal infections. Up to a third of all AIDS patients retain an azole-resistant *C. albicans* isolate orally.[13,14] The allylamines are fungicidal, have a broad spectrum of *in vitro* activity and limited clinical efficacy because of poor pharmacokinetics, with terbinafine being the only systemic antifungal in this class in clinical use.[1,9] The fluoropyrimidines, such as 5-fluorocytosine are fungicidal with a limited spectrum of activity.[1,6] Finally, the fifth and the most recent class, the echinocandins and pneumocandins, is fungicidal. Caspofungin (CS) (MK-0991), one of only three echinocandins licensed for use, is available as intravenous injections and cause minimal host toxicities.[1,10,15] Caspofungin, has been approved for the treatment of oropharyngeal and oesophageal candidiasis,[16] and invasive aspergillosis in patients not responding or intolerant to other antifungal drugs.[17,18] Recently, Laverdière *et al.* [19] reported, for the first time, the progressive loss of activity of echinocandin drugs against clonally related *C. albicans* isolates following long-term clinical exposure to this new class of antifungal agents.

To date, and based on a one year survey which we conducted of five major Lebanese pharmacies located in Beirut, encompassing around 500 antifungal prescriptions, three categories of azole antifungal agents with diverse trademarks dominate all antifungal prescriptions. These are FL (82%), followed by ketoconazole (KE) (11%) and IT (7%).

Candida species have the ability to switch phenotypes and alternate between several different forms where each form expresses a unique set of genes and cell morphologies.[3,12] One such system, involves a reversible white-opaque transition.[20] The diploid yeast *C. albicans* possesses a single mating type-like (*MTL*) locus on chromosome 5, which is normally heterozygous *a*/α.[21] The *MTL* locus on one of the chromosome 5 homologues contains the *MTLa1* gene while the other homologue contains *MTL*α1 and *MTL*α2 genes.[20,22,23] The MTLa1p-MTLα2p complex, in *MTL*-heterozygotes forms a heterodimer that represses mating. In order to mate, *C. albicans* must undergo *MTL*-homozygosis to *a*/*a* or α/α.[23] *MTL*-zygosity regulates not only mating[24–26] but also phenotypic switching,[21] because only *MTL*-homozygotes can undergo the white-opaque transition. Lockhart [21] revealed that in nature, the majority of isolates are *MTL*-heterozygous and only a minority of clinical isolates are *MTL*-homozygous, with disease-associated isolates switching at higher rates than commensal isolates.[16] It has been demonstrated that switching can occur at sites of infection and between episodes of recurrent vaginitis, and that it may function to generate variability in commensal and infecting populations for adaptive reasons.[27] In a cutaneous mouse model, opaque cells are more virulent than white cells, while in a systemic mouse model white cells are more virulent than opaque cells.[20,28,29] To date, the findings on the relationship between *MTL* homozygosity and drug resistance remains contradictory. A study showed that homozygosity at the *C. albicans MTL* locus is associated with azole resistance,[30] whereas another study revealed that in *C. albicans*, drug resistance is not directly affected by mating type locus zygosity.[31]

This study involves the collection of a large number of *C. albicans* hospital isolates from different hospitals in Lebanon to test for their susceptibility against different antifungal drugs that are either currently in clinical use, or recently approved and released for clinical use using the Epsilometer test method. Our selection of antifungal drugs encompassed the most pharmaceutically prescribed antifungal drugs in Lebanon notably FL, KE and IT. Furthermore, imidazole, VO and the novel echinocandin, CS were chosen for this study as there are no published data of them being utilised in Lebanon so far, although their use by a small number of hospitals cannot be ruled out. This study will be performed in an effort to recommend their use by a wider number of hospitals if the antifungal susceptibility tests are deemed satisfactory. Moreover, as there are contradictory data on the correlation between *MTL* homozygosity and drug resistance, we will determine the *MTL* locus genotype of each isolate by amplifying the *a1*, α1, and α2 genes in an effort to determine a possible correlation between homozygosity at the *C. albicans MTL* locus and resistance to azole antifungal drugs.

Materials and methods

Hospital isolates

A total of 116 random clinical isolates labelled as *C. albicans* were collected from different hospitals in Beirut such as: Saint Georges Hospital (52 isolates), American University of Beirut Medical Center (49 isolates), Hotel-Dieu de France (seven isolates), Rizk Hospital (six isolates) and Sahel General Hospital (two isolates). The samples were recovered from a variety of niches as can been seen in Table 1. Although most hospitals refused to disclose clinical details on patients' history including possible antifungal drug therapy, their medical/health conditions varied from common

Table 1 Isolation frequency and location

Frequency	Location
35	Sputum
24	Stool
17	Urine
9	Deep tracheal aspirate
8	Vagina
8	Bronchial alveolar wash
5	Wound
2	Abdominal fluid
1	Tooth abscess
1	Pus
1	Penile swab
1	Peritoneal fluid
1	Nail
1	Blood
1	Jackson–Pratt drainage
1	Cerebrospinal fluid

Table shows the frequency of isolation and the location of all 116 *Candida albicans* samples. Note that sputum, stool and urine constitute around two-thirds of all isolates.

superficial infection to severe diseases such as cardiac arrest, cancer and AIDS (requiring antibiotic therapy).

Species identification

All isolates were confirmed as *C. albicans* by *C. albicans* primer specific microsatellite DNA amplification (data not shown).

Quality control (QC) reference strain

The CLSI QC strains *Candida parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028[32–37] were used as reference strains in the antifungal susceptibility tests.

Growth and storage of isolates

All hospital isolates were initially cultured and purified on Tryptone Soy Agar (Himedia, Mumbai, India). Purified colonies were maintained at −80°C in Potato Dextrose Broth (Himedia), containing 20% glycerol for long term storage. Fresh inoculants from the −80°C stock were grown on Potato Dextrose Agar plates (Oxoid, Basingstoke, UK) at 30°C as needed.

Antifungal susceptibility media preparation

For azoles and AP, High Resolution Medium (Oxoid) was utilised, while RPMI1640 medium buffered with 165 mmol l^{-1} MOPS (Biowhittaker, Brussels, Belgium) was used for CS, as suggested by the manufacturer. Both media were prepared according to the manufacturer's instructions and poured on to 150 mm agar plates with a depth of 4 ± 0.5 mm.

Antifungal susceptibility testing

The antifungal susceptibility test was performed using the E-test method. E-test strips of FL, VO, KE, IT, AP, and CS were supplied by AB Biodisk, Solna, Sweden. Inoculum preparation, inoculation and strip application were performed according to the manufacturer's instructions. Minimal inhibitory concentration (MIC) readings were recorded after 48-h incubation at 35 °C. As recommended by the manufacturer, AP MICs were read as the lowest concentration on the E-test strip at which there was 100% inhibition i.e., at the point where the border of the elliptical inhibition zone intersected the strip, whereas CS and azole MICs were read as the least concentration at which there was 80% growth inhibition or where there was a significant decline in growth. Furthermore, all resistant isolates were re-tested utilising the Sensititre YeastOne with CS and posaconazole broth microdilution plates, according to the manufacturer's instructions (Trek Diagnostic Systems, Cleveland, OH, USA).

MIC interpretive breakpoints for antifungal agents

The MIC interpretive criteria for FL and IT were as follows: susceptible (S), ≤8 μg ml^{-1}; susceptible-dose dependent (S-DD), 16–32 μg ml^{-1}; and resistant (R), ≥64 μg ml^{-1} for FL, and (S), ≤0.125 μg ml^{-1}; R, ≥1 μg ml^{-1}; with intermediary MIC's considered as S-DD, for IT.[33,38] Although interpretive breakpoints have not yet been established for VO, KE, AP, and CS, we have selected the following criteria for purposes of comparison in our study: S, ≤1 μg ml^{-1}; R, ≥2 μg ml^{-1} for VO,[35,39] R, ≥0.38 μg ml^{-1} for AP,[40,41] and R, ≥1 μg ml^{-1} for KE, as its pharmacokinetics is similar to IT. For CS, no interpretive criteria have been established but an MIC value of >1 μg ml^{-1} is commonly used by most researchers to denote resistance.[42]

Quality control

The control limits for the *C. parapsilosis* ATCC 22019 strain were FL: 1–8 μg ml^{-1}; VO: 0.016–0.064 μg ml^{-1}; KE: 0.032–0.125 μg ml^{-1}; IT: 0.064–0.25 μg ml^{-1}; AP: 0.25–1 μg ml^{-1}; and CS: 0.25–2 μg ml^{-1} and for *C. albicans* ATCC 90028: FL: 0.125–0.5 μg ml^{-1}; VO: 0.004–0.016 μg ml^{-1}; KE: 0.008–0.032 μg ml^{-1}; IT: 0.064–0.25 μg ml^{-1}; AP: 0.125–0.5 μg ml^{-1}; and CS: 0.064–0.25 μg ml^{-1}. Our

MIC values were within the control limits for all antifungal drugs tested.

DNA extraction

Pure genomic DNA was extracted from all 116 strains as described previously.[43]

MAT locus amplification

Fifty nanograms of pure genomic DNA was used to amplify the *a1*, α_1 and α_2, genes, by PCR, using the following primers (5′ → 3′): F-AAGAATGAAGACAAC GAGG3′, and R-CGTGTTTTTCTGCTATCAATTCC for *a1*; F-TACATTCTGGTCGCGATGCTC and R-GTAATCC AAAGCCTCGCATAA for α_1; and F-ATGAATTCACAT CTGGAGGC and R-CTGTTAATAGCAAAGCAGCC for α_2. (F denotes the forward primer and R the reverse primer). Fragment sizes obtained were 535, 423, and 615 bp respectively.[30] Reaction mixtures were typically heated for 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C. After a final incubation of 10 min at 72 °C, reaction mixtures were stored at 4 °C.

Statistical analysis

Chi-squared tests were performed on the data collected using SIGMASTAT 3.1 computer software (Systat Software Inc, Richmond, CA, USA) to assess the significance of any discrepancy.

Results

Antifungal resistance

All 116 isolates were tested against the six above-mentioned antifungal drugs. One isolate was recovered per patient with only two exceptions from Saint Georges Hospital, where two isolates were recovered from one patient and another three from a second patient. However, the drug susceptibility profiles for isolates retrieved from the same individual were different, suggesting that each isolate was unique and the patient did not suffer multiple infections from the same isolate, which could have skewed the data presented. A summary of their MICs, geometric mean MIC and MIC ranges, is presented in Tables 2 and 3. As far as azoles are concerned, and according to the MIC interpretive breakpoints for FL, 110 samples were S, ≤8 μg ml^{-1}; none of the samples were S-DD, 16–32 μg ml^{-1}; and six samples were R, ≥64 μg ml^{-1}. Similarly for IT, 90 samples were S, ≤0.125 μg ml^{-1}; 12 samples were S-DD, 0.25 to 0.75 μg ml^{-1}; 14 samples were (R ≥1 μg ml^{-1}). By using the putative breakpoints for VO of S with an MIC ≤1 μg ml^{-1} and R with an MIC ≥2 μg ml^{-1}, with no intermediate or S-DD category, it was found that for the 116 isolates tested, 107 were sensitive. Of the nine resistant *C. albicans* isolates, eight had MICs ≥32 μg ml^{-1} and one isolate had an MIC of 3 μg ml^{-1}. In the absence of interpretive criteria for KE and by utilising the MIC ≥1 μg ml^{-1} to be a breakpoint for resistance, it was noted that seven isolates were resistant, four of which possessed an MIC >32 μg ml^{-1} reflecting clear resistance. To confirm the azole resistance phenotype, isolates were re-tested by a second technique utilising the Sensititre Yeast One kit. Resistance patterns were comparable with no statistically significant discrepancies observed. By assuming that the AP breakpoint for resistance as MIC ≥0.38 μg ml^{-1}, it was noted that only two isolates were resistant.

As far as CS, none of the isolates displayed an MIC that could exceed the maximum drug concentration on the E-test strip to reflect clear resistance because all isolates exhibited MICs <1 μg ml^{-1} and were hence deemed sensitive.

Table 2 Antifungal MIC distribution vs. isolates

	Number of isolates with the following MIC range (μg ml^{-1})									
Antifungal	0.002–0.016	0.023–0.032	0.047–0.094	0.125–0.25	0.38–0.75	1–2	3–6	8–16	>32	>256
FL	0	0	9	26	47	20	7	1	0	6
VO	54	21	22	10	0	0	1	0	8	0
KE	67	21	12	9	0	2	1	0	4	0
IT	29	19	26	16	12	7	2	0	5	0
AP	4	9	45	56	1	1	0	0	0	0
CS	1	6	65	42	2	0	0	0	0	0

FL, fluconazole; VO, voriconazole; KE, ketoconazole; IT, itraconazole; AP, amphotericin B; CS, caspofungin; MIC, minimal inhibitory concentration.

For the sake of clarity, MIC values were categorised instead of showing the value for each individual isolate.

Table 3 MIC ranges of antifungal agents on *Candida albicans* isolates

Antifungal agent	*n*	MIC range (μg ml^{-1})	Geometric mean MIC
Fluconazole	116	0.047–>256	0.70
Voriconazole	116	<0.002–>32	0.04
Ketoconazole	116	0.002–>32	0.03
Itraconazole	116	0.002–>32	0.08
Amphotericin B	116	0.008–1	0.1
Caspofungin	116	<0.002–0.5	0.09

MIC, minimal inhibitory concentration.

Note that fluconazole has the highest geometric mean MIC, while ketoconazole has the lowest.

Mating type-like loci

Polymerase chain reaction amplification at the *a1*, α1 and α2 loci was performed. Isolates that lacked either the *a1* or the α1 and α2 alleles were deemed homozygous. One hundred and five isolates were heterozygous and 11 were homozygous. Of the 11 homozygous isolates, 10 were *MTL*α and one was *MTLa*. Table 4 shows the distribution of *MTL* homozygous isolates with respect to the location of infection and azole drug resistance. Chi-squared analysis of these results generated a *P*-value of 0.02 indicating a strong correlation between resistance and *MTL* homozygosity.

Discussion

In this study, we determined the susceptibility of *C. albicans* and other yeast to five commonly used antifungal agents (FL, VO, KE, IT and AP) in addition to CS, which has been approved for clinical use relatively recently. E-test on solid media was utilised as an alternative test to the broth methods as it is easier to perform than the macro and microbroth methods, and more convenient and reliable in clinical diagnostic laboratory testing.[44]

Based on the defined MIC interpretive breakpoints for FL and IT, few resistant *C. albicans* isolates were found against FL (5.17%) than IT (12.06%). However, by utilising the putative interpretive breakpoints for VO, and AP, we found that 7.75% of the *C. albicans* isolates were resistant to VO and only 1.7% was resistant to AP.

Even though no MIC interpretive criteria have been defined for KE and CS, KE MICs of ≥1 were observed for seven isolates (6.03%); three strains had MICs of 1.5, 2 and 3 μg ml^{-1} and the remaining four had MICs >32 μg ml^{-1}, which reflects clear resistance. As for CS, most laboratories use an MIC >1 μg ml^{-1} to designate susceptibility and hence all our strains are susceptible. Thus the order of antifungal drug resistance against *C. albicans* is as follows: IT > VO > KE > FL > AP > CS.

In vitro studies have suggested that cross-resistance may occur with FL and other azole compounds.[45,46] The mechanism for such cross-resistance has been clearly demonstrated in a number of studies and most often involves the upregulation of genes encoding the ATP-binding cassette efflux transporters, or CDR pumps.[47–49] Cross-resistance to azole antifungal agents was noted for eight isolates including three that were completely resistant to all azoles tested. Interestingly, whenever resistance to FL was observed, resistance to VO was also seen. This could be attributed to the fact that these two triazoles are structurally related.[50] Thus, resistance to VO might be considered secondary

Table 4 Comparison of *MTL*-homozygosity to drug resistance

Isolate location	*MTL* genotype	MICs (μg ml^{-1}) of azoles			
		FL	VO	KE	IT
Tooth abscess	α	0.094 (S)	0.003 (S)	0.004 (S)	0.004 (S)
Urine	α	4 (S)	0.032 (S)	0.032 (S)	2 (R)
BAW	α	0.064 (S)	0.032 (S)	0.016 (S)	0.016 (S)
BAW	α	0.047 (S)	<0.002 (S)	0.002 (S)	0.002 (S)
Peritoneal fluid	α	6 (S)	0.125 (S)	0.25 (S)	0.75 (SDD)
Sputum	*a*	0.25 (S)	0.008 (S)	0.006 (S)	0.023 (S)
Stool	α	1 (S)	0.032 (S)	0.023 (S)	0.032 (S)
DTA	α	4 (S)	0.094 (S)	0.25 (S)	1 (R)
Urine	α	>256 (R)	0.094 (S)	0.32 (S)	0.25 (SDD)
DTA	α	>256 (R)	>32 (R)	>32 (R)	>32 (R)
Sputum	α	6 (S)	0.19 (S)	0.064 (S)	1 (R)

MIC, minimum inhibitory concentration; FL, fluconazole; VO, voriconazole; KE, ketoconazole; IT, itraconazole; (R), resistant; (S), susceptible; SDD, susceptible dose dependent; BAW, bronchial alveolar wash; DTA, deep tracheal aspirate.

resistance if the patient has been exposed to FL which has a similar structure implying possible cross-resistance. In our samples, out of the eight isolates that were VO resistant, four were FL resistant suggesting a possible secondary resistance through such a mechanism. However, primary resistance cannot be ruled out unless specific antifungal hospital treatment for each patient is analysed and correlated with resistance. Such an undertaking depends on the hospital's cooperation and is not always feasible.

Caspofungin and resistance

The MIC range for CS obtained was the lowest compared to the other antifungal drugs tested. Furthermore, the MIC values of CS obtained were similar for the different isolates and ranged from <0.002 to 0.38 μg ml^{-1}. However, an MIC of 0.38 μg ml^{-1} remains questioned in the absence of interpretive breakpoints for CS. As this study was conducted in Lebanon without any information about the utilisation of CS by Lebanese hospitals and before the clinical release of VO, and because all the hospital isolates were susceptible to CS (MICs <1 μg ml^{-1}), we could, in part, either postulate that the rate of primary resistance to CS is very low, or eliminate the possibility of primary resistance in Lebanese isolates and thus any resistance that develops to this drug in the future could be considered as secondary resistance. It should be noted, however, that the lack of universally approved interpretive breakpoints for CS would limit our ability to confirm the presence of primary and secondary resistance. Our results reveal that resistance to azoles among Lebanese yeast clinical isolates is so far preliminary and it is in favour of azole therapy with continuous and cautious observation for the development of resistance. However, these isolates were overwhelmingly collected from sites of colonisation rather than invasive sites and thus the possibility remains that the susceptibility pattern among bloodstream invasive isolates might differ. Nevertheless, it should be noted that three samples were resistant to all azoles including one sample that was resistant to three out of four azoles used in this study and one sample, a deep tracheal aspirate sample isolated from a patient in cardiac arrest, was resistant to all azoles and AP. Bearing in mind that CS was the only drug on which this isolate showed no resistance, the use of CS in such cases may prove critical in treatment and should be recommended. However, CS treated patients should be monitored with care because *C. albicans* resistance has been reported in a patient with azole-refractory thrush-oesophagitis that initially responded to CS but where the treatment eventually failed and a correlation between *in vivo* failure of CS therapy and *in vitro* rise in CS MIC was recorded.[51]

Stevens [52] reported the reduced activity of CS against *C. albicans* clinical isolates at high drug concentrations, which might pose a hesitating prophylactic use of this drug if administered for long periods of time. Whether this will be the case in Lebanese isolates remains to be seen.

MTL homozygosity and drug resistance

Finally, the PCR screening of the *MTL* loci revealed that all the hospital isolates were heterozygous at the *MTL* locus (*a*/α) except for 11 isolates (9.5%) as shown in Table 4. Of these, only one isolate was *MTLa* and the remaining 10 were *MTL*α. Of the *MTL* homozygous isolates, five (45.5%) were resistant to at least one azole antifungal agent including one sample that was resistant to all the azoles tested. Three samples were resistant only to IT and the rest were susceptible. However, of the 105 *C. albicans* isolates heterozygous at the *MTL* locus, only 14 (13.3%) were resistant to at least one of the azole antifungal drugs. Our results reveal a strong correlation between homozygosity at the *MTL* locus and drug resistance ($P = 0.02$). Recently, Coste [53] showed that acquisition of homozygosity of *TAC1*, the transcriptional activator of the *CDR* pump, which is located 14 Kb from the *MTL* locus on chromosome 5, is accompanied by *MTL* homozygosity contributing to the azole resistant phenotype. However, it is the *TAC1* homozygosity at chromosome 5 that mediates antifungal resistance in *C. albicans* because hyperactive *TAC1* alleles are codominant with wild type alleles and thus cannot be expressed at high enough levels to confer azole resistance as heterozygotes, but necessitates the loss of the second, wild type allele. Our results support this study and suggest that, even though drug resistance might not be because of the *MTL* locus homozygosity, its closeness to the *TAC1* locus might make it a useful marker for future resistance studies.

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