ISHAM
INTERNATIONAL SOCIETY FOR
HUMAN AND ANIMAL MYCOLOGY

Brief Report

Genetic diversity and antifungal susceptibility of *Candida albicans* isolated from Iranian patients

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Abstract

A total of 105 independent *Candida albicans* strains isolated from patients in Iran were investigated. According to CLSI documents M27-A3 and M27-S4, the 24 h geometric mean MICs of caspofungin, itraconazole, and fluconazole were 0.27, 3.19, and 11.91 μg/ml, respectively. Microsatellites analysis of *CEF3, CAIII, LOC4* Loci identified 93 different allelic genotypes clustered apart into six different clades. Antifungal susceptibility was not linked with the source of isolation and the corresponding genotype of *C. albicans* strains.

Key words: *Candida albicans*, microsatellite genotyping, antifungal susceptibility, Iran.

Candidiasis is an opportunistic fungal infection associated with significant morbidity and mortality among hospitalized patients with serious underlying disease.[1–4] Despite changes in species distribution of *Candida* species over the last three decades, *C. albicans* is still the most frequent cause of candidiasis, followed by *C. glabrata* complex, *C. parapsilosis* complex, *C. tropicalis*, and *C. krusei*.[4] Several studies has shown that antifungal susceptibility of *C. albicans* strains may differ based on genotype and source of isolation.[5] For effective management of systemic candidiasis, it is therefore important to determine the susceptibility profiles of isolated *C. albicans*.[6] In addition, exploring

1

genetic diversity of *C. albicans* also plays a crucial role for clinical management of candidemia and superficial candidiasis.[7,8] Identification of the molecular genotypes of corresponding *Candida* species can uncover the presence of endemic genotypes,[9,10] in order to implement appropriate preventive strategies in hospitals and health care-associated institutes under risk.[11,12] Data on genetic diversity and antifungal susceptibility profiles of *C. albicans* in Iran are scarce. In the current study, we therefore investigated antifungal susceptibility profile and molecular genotyping of a large collection of *C. albicans* strains collected from patients in Iran.

A collection of 105 independent *C. albicans* strains were investigated. The isolates were isolated between 2013 and 2016 from patients in two University Hospital of Tehran, and Mashhad in Iran. Sixty-three samples were obtained from sputa and bronchoalveolar (BAL) fluids of intensive care unit (ICU) patients underlying hematological malignancies, lung cancer, and respiratory disorders. Twenty-six strains were isolated from recurrent vulvovaginal candidiasis and 16 from skin lesions. All the patient-related data were processed anonymously, and the ethics committee waived informed consent.

All samples were initially identified based on conventional morphology using *Candida* chrome agar and production of germ-tube. The identity of all the isolates were then confirmed by PCR-RFLP, as described previously.[13] Briefly, each sample was grown on sabouraouds dextrose agar (LAB M, Bury, UK) at 32°C for 48 h. DNA extraction was performed by glass bead disruption and phenol/chloroform Method, as described previously.[14] Using internal transcribed spacer1 (ITS1) (5′-TCCGTAGCTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATG-3′) primers of *C. albicans*, the ribosomal DNA ITS region was amplified to polymerase a ∼537 bp fragment. The MSPI enzyme were then employed to produce two different DNA fragments (∼239 bp, ∼298bp bands).[13]

Microsatellite genotyping was performed on all *C. albicans* isolates, with a panel of three different short-nucleotide repeat fragments, using fluorescently labeled primers {*CAIII* (5′- Tamra -TTGGAATCACTTCACCAGGA-3′; 5′-TTTCCGTGGCATCAGTATCA-3′), *CEF3* (5′- Hex-TTTCCTCTTCCTTTCATATAGAA-3′; 5′- GGATTCAC TAGCAGCAGACA-3′), and *LOC4* (5′- FAM -GTAATG ATTACGGCAATGAC-3′; 5′-AGAACGACGTGTACTAT TGG-3′)}, as described previously.[15] These loci were selected because of high discriminatory power (DP) reported (DP = 0.87–0.97). A multiplex polymerase chain reaction (PCR) was performed in 10 µl reaction volumes containing 5 µl of Qiaqen Multiplex PCR (2 ×, Lot 148031955), 0.25 µl of each primer (F+R), 3 µl of ddH2O, and 1 µl of genomic DNA. PCR amplifications were performed in a thermocycler (BOECO, TC-Pro, Germany) operated with a temperature-cycling program that consisted of an initial denaturing step at 95 °C for 15 min, followed by 35 cycles of 30 s at 94°C, 90 s at 57°C, and 60 s at 72°C. The final extension step was for 10 min at 72°C. The sizes of the fragments were determined by addition of the GeneScan LIZ [500] marker and subsequent analysis of the fragments on the Applied Biosystems 3730 DNA analyzer. Assignment of repeat numbers in each marker was determined from the GeneScan data by using the Peak Scanner version 1.0 software (Applied Biosystems, Foster City, CA, USA). The sizes of the fragments were determined based on the LIZ500 marker, and the repeat numbers of these isolates were compared to each other. Allele-sharing distance matrices were generated from the tandem repeat numbers and were used as input to the Neighbor program of the PHYLIP version 3.6 software package to produce dendrograms.[16,17]

Isolates were tested for *in vitro* susceptibility to fluconazole (FLC: Pfizer Central Research Sandwich, Tadworth, Surrey, UK), itraconazole (ITC: Janssen Research Foundation, Beerse, Belgium), and caspofungin (CAS: Merck Sharp & Dohme BV, Haarlem, the Netherlands), using the clinical and laboratory standard institute (CLSI) M27-A3 guideline.[18] The minimum inhibitory concentration (MIC) values were interpreted according to CLSI document M27-S4 clinical breakpoints for fluconazole (S: susceptible $\leq$2; SDD: susceptible dose dependent = 4; R: resistant $\geq$8 µg/ml), itraconazole (S $\leq$ 0.12; SDD = 0.25–0.5; R $\geq$1 µg/ml), and caspofungin (S $\leq$ 0.25; intermediate = 0.5; R $\geq$1 µg/ml).[6,19] The final concentrations of the itraconazole and caspofungin ranged from 0.016 to 16 µg/ml. Fluconazole was assessed over a 2-fold concentration range, from 0.064 to 64 µg/ml. *C. parapsilosis* (ATCC 22019), and *C. krusei* (ATCC 6258) were used for quality controls in all experiments. All experiments on each strain were performed using three independent replicates on different days. Obtained data were analyzed using GraphPad Prism, version 7.0, for Mac (GraphPad Software, San Diego, CA, USA). Genotyping diversity and MIC distributions between the isolates originating from different locations were compared using Student t test and the Mann–Whitney–Wilcoxon test; differences were considered statistically significant at a P value of $\leq$.05 (two-tailed).

For all isolates, PCR of ribosomal DNA ITS region and further digestion with MSPI enzyme yielded DNA fragment corresponding to *C. albicans*. Table 1 shows the geometric MICs, the MIC ranges, the MIC_{50}, and MIC_{90} distributions of tested antifungals against 105 *C. albicans* strains. The MIC_{90s} of the antifungals across all isolates were the following (in increasing order): caspofungin (0.5 µg/ml), itraconazole (16 µg/ml), and fluconazole (64 µg/ml). No statistically significant differences in the susceptibility

Table 1. Geometric mean MIC, MIC ranges, and MIC_{50} and MIC_{90} values of 105 *Candida albicans* strains to tree antifungals, according to CLSI M27-A3 and M24-S4 guidelines.

	FLC	ITC	CAS
Geometric mean	11.91	3.19	0.27
Range	0.125 – >64	0.016 – >16	0.008–8
MIC_{50}	1	0.062	0.125
MIC_{90}	64	16	0.5

CAS: Caspofungin; FLC, fluconazole; ITC, itraconazole; MIC, minimum inhibitory concentration.

profiles of *C. albicans* were detected between the isolates from different sources investigated. MICs for quality control reference strains were within accepted limits for all antifungals tested. Microsatellite genotyping of three loci showed considerable diversity among 105 *C. albicans* strains, and 93 different allelic combinations were identified. The combined discriminatory power of the 3-locus (*CAIII, CEF3*, and *LOC4*) typing method was 0.94. Comparing the genetic relatedness by generating dendrograms of the genetic profiles showed that these strains were distinct from each other and clustered apart into 6 different clades (Fig. 1). Cluster analysis of similarities between allelic genotype combinations and antifungal susceptibility to fluconazole, itraconazole and caspofungin showed no link between tested *C. albicans* strains.

This study investigated the genetic diversity and antifungal susceptibility profile of a large collection of *C. albicans* strains originating from patients in Iran. The antifungal susceptibility of *C. albicans* was not linked with the source of isolation and corresponding molecular genotype. For all tested *C. albicans* strains, caspofungin was the most active antifungal, and 99.4% of strains showed susceptible profile, whereas 16.1% and 21.9% of the isolates were resistant to fluconazole and itraconazole, respectively. Consistent with several previous studies, our finding agrees that resistance of *C. albicans* to echinocandins is rare.[5,20–22] Similarly, *Shokohi* et al. also reported that 2.7% and 5.4% of *C. albicans* isolated from cancer patient receiving azole prophylaxis were resistant to fluconazole and itraconazole.[23] Our results are also compatible with the study of Li et al. who reported 6.6% and 4.9% of *C. albicans* strains originating from candidemia patients were resistant to fluconazole and itraconazole, respectively.[24] More recently, Dagi et al. also studied the antifungal susceptibility of a collection of 95 *C. albicans* strains isolated from bloodstream infections in Konya, Turkey. Resistance to caspofungin and anidulafungin was not detected in any strain.[25] Of note, the MIC values reported in our study appeared to be slightly higher, which could be due to different methodology applied and exposure to different antifungals during prophylaxis strate-

gies. To better characterize the phenotype-genotype mapping for drug resistance, one might also consider the relationship between genotypes and antifungal profiles against *Candida* strains based on the presence or absence of drug prophylaxis. In the present study, however there was no association between patients' receipt of antifungal prophylaxis 3 months prior to presentation with diseases and the 3 microsatellite loci genotypes tested.

Our results demonstrated significant genetic diversity among *C. albicans* strains. Analysis of different allelic combinations of 3 microsatellite loci (*CAIII, CEF3*, and *LOC*) concluded that all *C. albicans* strains were unrelated, as they had unique microsatellite genotypes. This exclude highlighting the occurrence of an endogenous infection due to a common source of infection in the centers investigated. In a previous study by Garcia-Hermoso et al. using *EF3, CDC3, HIS3* loci, molecular genotyping of 50 *C. albicans* strains in a surgical intensive care unit provided 38 different genotypes.[26] Chávez-Galarza et al. employed CAI and *CEF3* genotyping on 116 *C. albicans* strains, including 78 unrelated clinical isolates from Portugal, 29 from the five known clades and nine atypical *C. albicans* strains from Angola and Madagascar and found 87 different genotypes clustered in five different clades.[27] In another study, Dalle et al. also demonstrated that bloodstream and non-bloodstream strains of *C. albicans* have a heterogeneous structure at the *CEF3* polymorphic microsatellite locus. Seven nondescribed combinations were observed, resulting in 15 and 11 distinct *CEF3* profiles in bloodstream and control strains, respectively.[11] Using a single CAI loci, 44 different genotype were obtained among 114 *C. albicans* strains isolated from several episodes of recurrent vulvovaginal infections.[28] Similarly, Guzel et al. also previously studied a collection of 216 vaginal *C. albicans* isolates and identified 20 allelic combination-based genotypes using the microsatellite marker analysis of *CEF3* gene.[15]

Importantly, *C. albicans* is a colonized organism in different body sites, with the possibility of being transmitted between different individuals.[29] *C. albicans* infections can be superficial, affecting the skin, and mucosal membranes of the gastrointestinal and urogenital tract. Dissemination of the fungus can also lead to candidemia or localized infection of internal organs.[3,4] Several studies also described that *C. albicans* strains undergo microevolutionary events in response to environmental stress conditions, such as exposure to antifungals.[27,29,30]

Overall, our results indicated that caspofungin showed the greatest *in vitro* activity among systemic antifungals tested. No statistically significant differences in the susceptibility profiles of *C. albicans* were detected between the isolates from different sources. The pattern of investigated genotypes was diverse, and there were no relationships
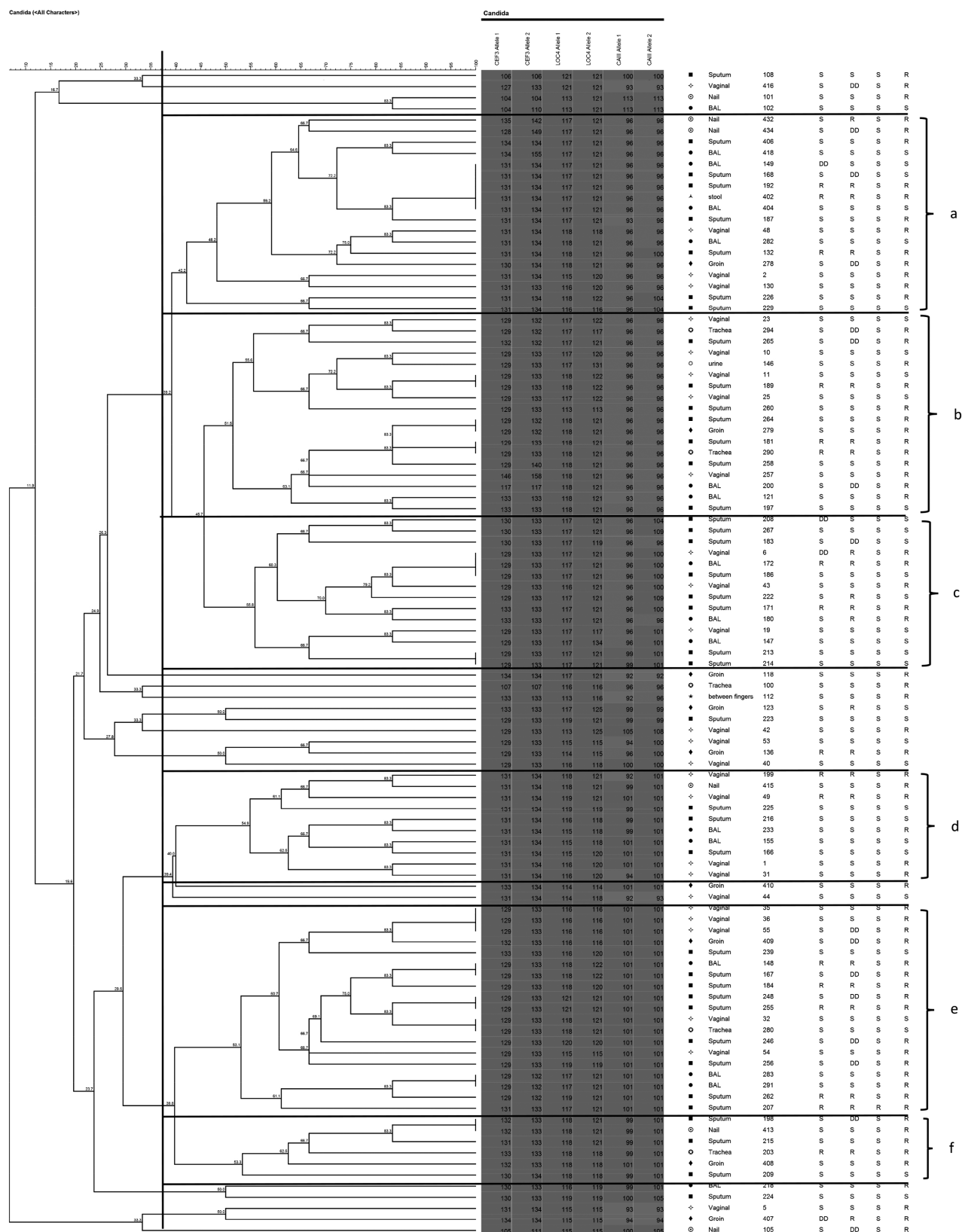
Figure 1. Dendrogram showing the genetic diversity, and grouping of 105 *Candida albicans* isolated from Iranian patients into six different clades. The dendrogram is generated based on a categorical analysis of *CEF3, CAIII, LOC4* microsatellite markers in combination with UPGMA clustering.

between susceptibility to each compound within the different genotypes studied.

Declaration of interest

The authors declare no conflict of interests related to this publication.

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