INTRODUCTION

Candida species are considered as one of the most important causes of human infections. Candidiasis range from mild infection such as onychomycosis or perlial to potentially fatal systemic candidiasis. Among the causative agents of bloodstream infections, Candida ranks fourth in the United States and seventh in Europe. Until recently, Candida albicans was, by far, the predominant species in most of the countries, causing up to two-thirds of all cases of invasive candidiasis. However, other species of Candida including Candida dubliniensis and Candida glabrata have gained more attention nowadays due to rapid development of resistance to antifungal drugs. Amphotericin B, a polyene fungicidal agent, has been the standard treatment for candidal infections for decades, but the toxicity of its conventional form and the costs of its lipid forms limit its use. More recently, azole antifungal compounds, with lower cytotoxicity and perfect efficacies, have emerged as the principal drugs used in treatment of candidal infections. However, prolonged use of azoles has led to the development of drug resistance in C. albicans and other species. Among the factors contributing to development of resistance to azoles, the selection of intrinsically less susceptible organisms, such as C. glabrata and Candida krusei, and the acquisition of resistance by previously susceptible strains of C. albicans following long-term azoles exposure have been documented. To manage the patients with candidiasis, antifungal susceptibility testing has become an important step in guiding physicians in the selection of proper antifungal therapy. Among antifungal susceptibility tests, disk diffusion has served as rapid, simple and cost-effective method for screening the susceptibility pattern of the yeasts. To standardize the disk diffusion test, CLSI subcommittee on antifungal susceptibility tests has developed recommendations in M44-A document. In the present study, we determined the susceptibility profiles of clinically isolates of Candida species against...
four antifungal drugs, including amphotericin B (AMP), ketoconazole (KET), fluconazole (FLU) and itraconazole (ITR).

**MATERIALS AND METHODS**

**Isolation and identification of the Candida isolates**

The study was conducted on a total of 206 clinical isolates of *Candida*. Samples were collected from two laboratories in Shiraz and Esfahan, Iran, between January 2009 and November 2010. Isolates were from different sites of the body including oral cavity ($n=118$, 57.6%), blood ($n=64$, 30.7%), genital tract ($n=17$, 8.3%) and respiratory tract ($n=7$, 3.4%). The predisposing factors were using intravenous catheters and antibiotic administration ($n=36$, 17.5%), malignancy and organ transplantation ($n=11$, 5.3%), pulmonary diseases ($n=7$, 3.4%), having denture ($n=120$, 59.3%), vaginitis ($n=16$, 7.8%), surgery ($n=9$, 4.4%) and others ($n=9$, 4.4%).

The isolates were identified by physiological methods such as chlamydoconidia formation in corn meal agar, germ tube production in the serum and also molecular methods, PCR-RFLP, as originally described by Mirhendi et al.[15,16] Briefly, genomic DNA was extracted and purified using glass bead.[17] A set of universal primers (ITS1, 5'-TCCGTAGGTGAACCTGCGG and ITS4, 5'-TCCTCCGCTTATTGATATGC) (Metabion International, Martinsried, Germany), were used to amplify the specific pattern. Moreover, restriction endonuclease, to achieve the best species-specific pattern. Amplified PCR products were digested with MspI restriction endonuclease, to achieve the best species-specific pattern. Moreover, *C. dubliniensis* was differentiated from *C. albicans* by using additional enzyme (BfuI (-AvrII)). Digestion was performed by incubating a 21.5µl of aliquot of PCR product with 10U of the enzyme in a final reaction volume of 25 µl at 37°C for 2.5 h. Restriction fragments were separated by 2% agarose gel electrophoresis in TBE buffer for approximately 1 h at 100V and stained with ethidium bromide.

**Susceptibility testing**

The Neo-Sensitabs tablet assay was performed according to the manufacturer’s instructions (Neo-Sensitabs user’s guide; Rosco Diagnostica, Taastrup, Denmark) and M44-A guidelines.[14] Briefly, the isolated *Candida* spp. were cultured on Sabouraud Dextrose Agar at 35°C for 24 h. Then, the yeasts were suspended in 5 mL of sterile physiological serum and thoroughly vortexed to achieve a smooth suspension. The optical density (OD) of the suspensions was adjusted to 0.08 to 0.1 at a wavelength of 625 nm to yield turbidity equal to 0.5 McFarland standards. A sterile cotton swab moistened with the inoculums suspension was used and applied to a 90-mm diameter plate, containing Mueller-Hinton agar supplemented with 2% glucose (to support the growth) and 0.5 µg/ml methylene blue (to improve the zone edge definition). The plates were allowed to dry for 3-10 minutes. To determine the antifungal susceptibility patterns of the isolates, a Neo-Sensitabs disk of each antifungal drugs (Rosco Diagnostica), including FLU (25 µg/disk), amphotericin B (10 µg/disk), ITR (8 µg/disk) and ketoconazole (15 µg/disk) was dispensed onto the inoculated plates. Zones of inhibition around the disk were measured following incubation of the plates for 18-24 hours at 35-37°C. When insufficient growth was encountered at the 24-hour reading, the plates were re-evaluated after a further 24 hours. The susceptibility of *Candida* spp. was evaluated based on the zone interpretive criteria of the manufacturer (Rosco Diagnostica). Quality control was censured by testing the Neo-Sensitabs user’s guide and CLSI recommended control strains *C. parapsilosis* ATCC 22019 (AMP:24-28mm, KET: 30-33mm, ITR:23-26mm, FLU: 27-30mm) and *C. krusei* ATCC 6258 (AMP:19-22mm, KET: 22-24mm, ITR:17-20mm, FLU: 9-12mm).[14] All control strains were included in each series of tests. In the case of the presence of resistance colonies within the inhibition zone around the azoles disk, they were isolated and sub-cultured in new plates and rechecked by disk diffusion method. These yeasts were considered as a resistant mutant so called petite isolate when they were grown completely around the disks.

**RESULTS**

The study was conducted on a total of 206 yeast isolates including 93 (45.1%) *C. albicans*, 42 (20.4%) *C. glabrata*, 26 (12.6%) *C. parapsilosis*, 25 (12.1%) *C. tropicalis*, 13 (6.3%) *C. dubliniensis*, 8 (4%) *C. krusei*, 2 (1%) *C. keyfer*, and a species of each of *C. hyalina* (0.5%) and *C. guillermondii* (0.5%).

Table 1 summarizes the interruptive data of the 206 *Candida* isolates based on their in vitro susceptibility to the studied
antifungal drugs. Of the whole isolates, 158 (76.7%) were susceptible to KET, 36 (17.5%) were dose-dependent susceptible, and the remaining isolates were found to be resistant to the aforementioned drugs. The highest rate of resistance to KET were seen in C. glabrata (16.6%) and C. albicans (3.2%). The two ITR-resistant species (1%) were C. glabrata which consist 4% of this species. Fluconazole susceptible and intermediate were seen in 96.6% and 3.4% of the Candida isolates, respectively. All the evaluated Candida species were susceptible to AMP. A total of 19 (9.2%) yeast isolates showed petite phenomenon including 11 C. glabrata, 3 C. albicans, 2 C. dubliniensis and one isolate of each of C. krusei and C. parapsilosis.

**DISCUSSION**

The petite mutants produce small colonies around the inhibition zone of azole disks. These petite positive phenomenon have been frequently reported in C. glabrata and sometimes in other yeasts species such as C. albicans and Saccharomyces cerevisiae. These petite mutants resulted from the loss of mitochondrial DNA or mutations in genomic DNA which impair respiratory activity, and exhibited decreased susceptibility to antifungal drugs. In the present study, we documented the petite phenomenon in almost one tenth of clinically isolated Candida species. This might be resulted from excessive and uncontrolled use of azole derivatives drugs in the past decade. Brun et al., demonstrated that all of the mutant colonies are resistant to the tested azoles. In our study, C. glabrata showed the highest rate of intermediate susceptibility to the examined azoles and this is consistent with previous studies. Furthermore, this species includes more than half of the isolated petite mutants. We also reported two petite phenophenons in two C. dubliniensis for the first time.

In Iran the rate of resistance to FLU among Candida species have been reported to be from null to 15%. In our study no FLU-resistant Candida spp. was found within the examined isolates and this is in keeping with findings of Khosravi et al. study. Although among the Candida spp., C. glabrata exhibited the highest rate of resistance to FLU only 4 (9.5%) of the isolated C. glabrata showed intermediate susceptibility to this azole and the rest were all susceptible. In spite of high rate of resistance to FLU among C. krusei, all of the tested strains of this species were susceptible to FLU and this has been previously shown by Munoz et al. as well.

Amphotericin B, one of the most potent and rapidly acting antifungal agents, is considered as the first line of treatment for many systemic mycoses. Although it has been reported that Candida lusitaniae tends to be absolutely resistant to AMP, as happen in about 7% of clinical isolates of C. albicans no AMP resistant was seen among the studied strains. As has been shown in previous studies, in the current study 1% of Candida species (2 isolates of C. glabrata) were resistant to ITR and 19.9% were dose-dependent susceptible although in one study all of the Candida have been susceptible to ITR.

**Table 1: In vitro antifungal activities of ketoconazole, itraconazole, fluconazole and amphotericin B against Candida species by using CLSI disk diffusion assay**

| Species (%) | Ketoconazole | Itraconazole | Fluconazole | Amphotericin B |
|-------------|--------------|--------------|-------------|---------------|
| C. albicans  | S            | S            | S           | S             |
| C. dubliniensis | I   | I            |             | R             |
| C. tropicalis | C. parapsilosis | C. krusei | C. guillermondii | C. kefyer | C. lipolytica | Total (206) |
| 74 (79.6) | 80 (86) | 92 (96.5) | 93 (100) |
| 16 (17.2) | 13 (14.0) | 0 (0)      | 0 (0)      |
| 3 (3.2)  | 0 (0)     | 0 (0)      | 0 (0)      |
| 13 (100) | 12 (92.3) | 13 (6.6)   | 13 (100)  |
| 0 (0)   | 1 (7.7)   | 0 (0)      | 0 (0)      |
| 0 (0)   | 0 (0)     | 0 (0)      | 0 (0)      |
| 21 (90)  | 25 (95.9) | 38 (90.5)  | 42 (100)   |
| 14 (53.3) | 25 (95.9) | 4 (9.5)    | 0 (0)      |
| 7 (25.7) | 2 (4.8)   | 0 (0)      | 0 (0)      |
| 20 (80)  | 16 (64)   | 24 (96.0)  | 25 (100)   |
| 4 (16)  | 9 (36.0)  | 1 (4.0)    | 0 (0)      |
| 1 (4.0)  | 0 (0)     | 0 (0)      | 0 (0)      |
| 23 (88.5) | 24 (92.3) | 25 (96.2)  | 26 (100)   |
| 2 (7.7) | 2 (7.7)   | 1 (3.8)    | 0 (0)      |
| 1 (3.8)  | 0 (0)     | 0 (0)      | 0 (0)      |
| 3 (100)  | 3 (100)   | 3 (100)    | 3 (100)    |
| 0 (0)   | 0 (0)     | 0 (0)      | 0 (0)      |
| 0 (0)   | 0 (0)     | 0 (0)      | 0 (0)      |
| 1 (100)  | 0 (0)     | 1 (100)    | 1 (100)    |
| 0 (0)   | 1 (100)   | 0 (0)      | 0 (0)      |
| 0 (0)   | 0 (0)     | 0 (0)      | 0 (0)      |
| 2 (100)  | 2 (100)   | 2 (100)    | 2 (100)    |
| 0 (0)   | 0 (0)     | 0 (0)      | 0 (0)      |
| 0 (0)   | 0 (0)     | 0 (0)      | 0 (0)      |
| 1 (100)  | 1 (100)   | 0 (0)      | 1 (100)    |
| 0 (0)   | 0 (0)     | 1 (100)    | 0 (0)      |
| 0 (0)   | 0 (0)     | 0 (0)      | 0 (0)      |
| 158 (76.7) | 153 (79.1) | 198 (96.6) | 205 (100)  |
| 36 (17.5) | 43 (21.9) | 7 (3.4)    | 0 (0)      |
| 2 (1.0)  | 2 (1.0)   | 0 (0)      | 0 (0)      |

S: susceptible; I: intermediate; R: resistant; Figures in parenthesis are in percentage

Despite in vitro induction of petite mutation by azole drugs, ethidium bromide or glycerol, these mutants have rarely been reported in clinical samples of patients who are undergoing antifungal therapies or prophylaxes. In the present study, we documented the petite phenomenon in almost one tenth of clinically isolated Candida species. This again is consistent with findings of Khosravi et al. study. Although among the Candida spp., C. glabrata showed the highest rate of intermediate susceptibility to the examined azoles and this is consistent with previous studies. Furthermore, this species includes more than half of the isolated petite mutants. We also reported two petite phenophenons in two C. dubliniensis for the first time.
CONCLUSIONS

In our study all of the tested yeasts were susceptible to FLU and AMP. Among the examined azoles, a high resistance rate in the isolated yeasts was found with KET. In this paper we also reported the petite phenomenons in two isolates of C. dubliniensis for the first time. Taken together, the high number of petite mutations (9%) in the isolated yeasts should be seriously considered as this might be one of the reasons of antifungal therapy failure.

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