Determination of Antifungal Susceptibility Patterns Among the Clinical Isolates of *Candida* Species

Kamiar Zomorodian[1,2], Mohammad Javad Rahimi[1], Kayvan Pakshir[1,2], Marjan Motamedi[1], Moosa Rahimi Ghiasi[1], Hasanein Rezashah[2]

[1]Departments of Medical Mycology and Parasitology, [2]Center of Basic Researches in Infectious Diseases, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Context: *Candida* species are opportunistic yeasts that cause infections ranging from simple dermatosis to potentially life-threatening fungemia. The emergence of resistance to antifungal drugs has been increased in the past two decades. **Aim:** the present study we determined to find out the susceptibility profiles of clinical isolates of *Candida* species against four antifungal drugs, including amphotericin B, ketoconazole, fluconazole and itraconazole. **Materials and Methods:** Antifungal susceptibility testing of the yeasts was done in accordance with the proposed guidelines for antifungal disk diffusion susceptibility testing of yeasts based on the CLSI document M44-A. **Results:** A total of 206 yeast isolates were assessed. Among the evaluated *Candida* species, the highest rates of resistance to ketoconazole were seen in *Candida glabrata* (16.6%) and *Candida albicans* (3.2%). Susceptibility and intermediate response to fluconazole were seen in 96.6% and 3.4% of the *Candida* isolates, respectively. A total of 19 (9.2%) yeast isolates showed petite phenomenon including 11 *C. glabrata*, 3 *C. albicans*, 2 *Candida dubliniensis* and one isolate of each *Candida krusei* and *Candida parapsilosis*. **Conclusion:** The high number of petite mutation in the isolated yeasts should be seriously considered since it may be one of the reasons of antifungal treatment failure.

Key words: *C. glabrata, Candida,* Disk diffusion, Petite mutation

INTRODUCTION

Candida species are considered as one of the most important causes of human infections.[1-3] Candidiasis range from mild infection such as onychomycosis or perlish to potentially fatal systemic candidiasis. Among the causative agents of bloodstream infections, *Candida* ranks fourth in the United States and seventh in Europe.[4,5] 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.[6] 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.[7] More recently, azole antifungal compounds, with lower cytotoxicity and perfect efficacies, have emerged as the principal drugs used in treatment of candidal infections.[8] However, prolonged use of azoles has led to the development of drug resistance in *C. albicans* and other species.[6,9-13] 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.[12,13] 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.[14] In the present study, we determined the susceptibility profiles of clinically isolates of *Candida* species against

Access this article online

Quick Response Code:

Website: www.jgid.org

DOI: 10.4103/0974-777X.91059

Address for correspondence:
Dr. Kamiar Zomorodian, E-mail: kzomorodian@gmail.com

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 allow the amplification of target ITS1-5.8s-ITS2 ribosomal DNA. PCR amplification was carried out in a final volume of 50 µl. Each reaction contained 1 µl of template DNA, 0.5 µM of each primer, 0.20 mM of each deoxynucleoside triphosphate (dNTPs), 5 µl of 10× PCR buffer, and 1.25 U of *Taq* polymerase (Roche Molecular Biochemicals, Mannheim, Germany). An initial denaturation step at 94°C for 5 min was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min, with a final extension step of 72°C for 7 min.

Amplified PCR products were digested with *Msp*I restriction endonuclease, to achieve the best species-specific pattern. Moreover, *C. dubliniensis* was differentiated from *C. albicans* by using additional enzyme (*B1n*I *(Avr*II*)*. 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 24h. 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*, 3 (1.5%) *C. krusei*, 2 (1%) *C. keyfer*, and a species of each of *C. lypolitica* (0.5%) and *C. guilliermondii* (0.5%).

Table 1 summarizes the interruptive data of the 206 *Candida* isolates based on their *in vitro* susceptibility to the studied

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

Species (%)	Antifungal Drugs			
	Ketoconazole	Itraconazole	Fluconazole	Amphotericin B
	S I R	S I R	S I R	S I R
C. albicans (93)	74 (79.6) 16 (17.2) 3 (3.2)	80 (86) 13 (14.0) 0 (0)	92 (46.5) 0 (0) 0 (0)	93 (100) 0 (0) 0 (0)
C. dubliniensis (13)	13 (100) 0 (0) 0 (0)	12 (92.3) 1 (7.7) 0 (0)	13 (6.6) 0 (0) 0 (0)	13 (100) 0 (0) 0 (0)
C. glabrata (42)	21 (50) 14 (33.3) 7 (16.7)	25 (59.5) 15 (35.7) 2 (4.8)	38 (90.5) 4 (9.5) 0 (0)	42 (100) 0 (0) 0 (0)
C. tropicalis (25)	20 (80) 4 (16) 1 (4.0)	16 (64) 9 (36.0) 0 (0)	24 (96.0) 1 (4.0) 0 (0)	25 (100) 0 (0) 0 (0)
C. parapsilosis (26)	23 (88.5) 2 (7.7) 1 (3.8)	24 (92.3) 2 (7.7) 0 (0)	25 (96.2) 1 (3.8) 0 (0)	26 (100) 0 (0) 0 (0)
C. krusei (3)	3 (100) 0 (0) 0 (0)	3 (100) 0 (0) 0 (0)	3 (100) 0 (0) 0 (0)	3 (100) 0 (0) 0 (0)
C.guilliermondii (1)	1 (100) 0 (0) 0 (0)	0 (100) 1 (100) 0 (0)	1 (100) 0 (0) 0 (0)	1 (100) 0 (0) 0 (0)
C. keyfer (2)	2 (100) 0 (0) 0 (0)	2 (100) 0 (0) 0 (0)	2 (100) 0 (0) 0 (0)	2 (100) 0 (0) 0 (0)
C. lipolytica (1)	1 (100) 0 (0) 0 (0)	1 (100) 0 (0) 0 (0)	0 (0) 1 (100) 0 (0)	1 (100) 0 (0) 0 (0)
Total (206)	158 (76.7) 36 (17.5) 12 (5.8)	163 (79.1) 41 (19.9) 2 (1)	198 (96.6) 7 (3.4) 0 (0)	205 (100) 0 (0) 0 (0)

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

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*[18-20] and sometimes in other yeasts species such as *C. albicans*[21] and *Saccharomyces cervisiae*.[22] These petite mutants resulted from the loss of mitochondrial DNA or mutations in genomic DNA[18,23] which impair respiratory activity, and exhibited decreased susceptibility to antifungal drugs.[1,19,24]

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.[24] 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.*,[24] 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.[25,26] Furthermore, this species includes more than half of the isolated petite mutants. We also reported two petite phenomenons 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%.[27-29] 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.[30] Although among the *Candida* spp., *C. glabrata* exhibited the highest rate of resistance to FLU,[25] 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*,[12] all of the tested strains of this species were susceptible to FLU and this has been previously shown by Munoz *et al.* as well.[31]

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*[32] no AMP resistant was seen among the evaluated isolates in our study. This again is consistent with findings of Khosravi *et al.* study.[30] In the present study a mutant colony was found within the inhibition zone of AMP of a *C. albicans* isolate that showed completely to be resistant to AMP following isolation and testing against AMP. A study conducted by Badiee *et al.* revealed that 12 out of 142 isolates of *C. albicans* were resistant to ITR.[28] As has been shown in previous studies,[26] 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.[30]

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.

ACKNOWLEDGMENTS

We would like to thank Dr N. Shokrpour and Dr B. Sarkari for providing editorial assistance. This work is supported by Shiraz University of Medical Sciences, Shiraz, Iran. (Grant No.88-5063).

REFERENCES

1. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Nosocomial bloodstream infections in United States hospitals: A three-year analysis. Clin Infect Dis 1999;29:239-44.
2. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 2004;39:309-17.
3. Pfaller MA. Editorial Response: The Epidemiology of invasive mycoses-narrowing the gap. Clin Infect Dis 1998;27:1148-50.
4. Horn DL, Fishman JA, Steinbach WJ, Anaissie EJ, Marr KA, Olyaei AJ, *et al.* Presentation of the PATH Alliance registry for prospective data collection and analysis of the epidemiology, therapy, and outcomes of invasive fungal infections. Diagn Microbiol Infect Dis 2007;59:407-14.
5. Kullberg BJ, Oude Lashof AM. Epidemiology of opportunistic invasive mycoses. Eur J Med Res 2002;7:183-91.
6. Ostrosky-Zeichner L, Pappas PG. Invasive candidiasis in the intensive care unit. Crit Care Med 2006;34:857-63.
7. Marchetti O, Moreillon P, Entenza JM, Vouillamoz J, Glauser MP, Bille J, *et al.* Fungicidal synergism of FLU and cyclosporine in *Candida albicans* is not dependent on multidrug efflux transporters encoded by the CDR1, CDR2, CaMDR1, and FLU1 genes. Antimicrob Agents Chemother 2003;47:1565-70.
8. Georgopapadakou NH, Tkacz JS. The fungal cell wall as a drug target. Trends Microbiol 1995;3:98-104.
9. Montravers P, Jabbour K. Clinical consequences of resistant *Candida* infections in intensive care. Int J Antimicrob Agents 2006;27:1-6.
10. Borg-von Zepelin M, Kunz L, Ruchel R, Reichard U, Weig M, Gross U. Epidemiology and antifungal susceptibilities of *Candida* spp. to six antifungal agents: Results from a surveillance study on fungaemia in Germany from July 2004 to August 2005. J Antimicrob Chemother 2007;60:424-8.
11. Pfaller MA, Messer SA, Gee S, Joly S, Pujol C, Sullivan DJ, *et al. In vitro* susceptibilities of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. J Clin Microbiol 1999;37:870-2.
12. Snydman DR. Shifting patterns in the epidemiology of nosocomial *Candida* infections. Chest 2003;123:500S-3S.
13. Defontaine A, Bouchara JP, Declerk P, Planchenault C, Chabasse D, Hallet JN. *In-vitro* resistance to azoles associated with mitochondrial DNA deficiency in *Candida glabrata*. J Med Microbiol 1999;48:663-70.
14. Clinical and Laboratory Standards Institute. Method for antifungal disk diffusion susceptibility testing of yeasts: approved standard. Wayne, PA: Clinical and Laboratory Standards Institute; CLSI M44-A; 2006.
15. Mirhendi H, Makimura K, Zomorodian K, Maeda N, Ohshima T, Yamaguchi H. Differentiation of *Candida albicans* and *Candida dubliniensis* using a single-enzyme PCR-RFLP method. Jpn J Infect Dis 2005;58:235-7.
16. Mirhendi H, Makimura K, Khoramizadeh M, Yamaguchi H. A one-enzyme PCR-RFLP assay for identification of six medically important *Candida* species. Nippon Ishinkin Gakkai Zasshi 2006;47:225-9.
17. Yamada Y, Makimura K, Merhendi H, Ueda K, Nishiyama Y, Yamaguchi H, *et al.* Comparison of different methods for extraction of mitochondrial DNA from human pathogenic yeasts. Jpn J Infect Dis 2002;55:122-5.
18. Sanglard D, Ischer F, Bille J. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. Antimicrob Agents Chemother 2001;45:1174-83.
19. Brun S, Berges T, Poupard P, Vauzelle-Moreau C, Renier G, Chabasse D, *et al.* Mechanisms of azole resistance in petite mutants of *Candida glabrata*. Antimicrob Agents Chemother 2004;48:1788-96.
20. Chen XJ, Clark-Walker GD. The petite mutation in yeasts: 50 years on. Int Rev Cytol 2000;194:197-238.
21. Cheng S, Clancy CJ, Nguyen KT, Clapp W, Nguyen MH. A *Candida albicans* petite mutant strain with uncoupled oxidative phosphorylation overexpresses MDR1 and has diminished susceptibility to fluconazole and voriconazole. Antimicrob Agents Chemother 2007;51:1855-8.
22. Kontoyiannis DP. Modulation of fluconazole sensitivity by the interaction of mitochondria and erg3p in *Saccharomyces cerevisiae*. J Antimicrob Chemother 2000;46:191-7.
23. Vandeputte P, Tronchin G, Rocher F, Renier G, Berges T, Chabasse D, *et al.* Hypersusceptibility to azole antifungals in a clinical isolate of *Candida glabrata* with reduced aerobic growth. Antimicrob Agents Chemother 2009;53:3034-41.
24. Brun S, Aubry C, Lima O, Filmon R, Berges T, Chabasse D, *et al.* Relationships between respiration and susceptibility to azole antifungals in *Candida glabrata*. Antimicrob Agents Chemother 2003;47:847-53.
25. Yoo JI, Choi CW, Lee KM, Kim YK, Kim TU, Kim EC, *et al.* National surveillance of antifungal susceptibility of *Candida* species in South Korean hospitals. Med Mycol 2009;47:554-8.
26. Silva V, Diaz MC, Febre N. Chilean Invasive Fungal Infections Group. Invasive fungal infections in Chile: A multicenter study of fungal prevalence and susceptibility during a 1-year period. Med Mycol 2004;42:333-9.
27. Nemati SL, Shams-Ghhfarokhi M, Yadegari MH. Evaluation of disk diffusion and broth microdilution methods for fluconazole susceptibility testing in one group of *Candida* spp. in Tehran. Daneshvar Med 2008;15:51-8.
28. Badiee P, Alborzi A, Shakiba E, Ziyaeyan M, Rasuli M. Molecular identification and *in-vitro* susceptibility of *Candida albican*s and *C. dubliniensis* isolated from immunocompromised patients. Iran Red Crescent Med J 2009;11:391-7.
29. Jafari-nodoushan AA, Kazemi A, Mirzaii F, Dehghani M. Fluconazole susceptibility profile of *Candida* isolates recovered from patients specimens admitted to Yazd Central Laboratory. Iran J Pharm Res 2008;7:69-75.
30. Khosravi AR, Shokria H, Mansourib P, Katiraeea F, Ziglaria T. *Candida* species isolated from nails and their *in vitro* susceptibility to antifungal drugs in the department of dermatology (University of Tehran, Iran). Med Mycol J 2008;18:210-5.
31. Munoz P, Sanchez-Somolinos M, Alcala L, Rodriguez-Creixems M, Pelaez T, Bouza E. *Candida krusei* fungaemia: Antifungal susceptibility and clinical presentation of an uncommon entity during 15 years in a single general hospital. J Antimicrob Chemother 2005;55:188-93.
32. Brajtburg J, Powderly WG, Kobayashi GS, Medoff G. Amphotericin B: Current understanding of mechanisms of action. Antimicrob Agents Chemother 1990;34:183-8.

How to cite this article: Zomorodian K, Rahimi MJ, Pakshir K, Motamedi M, Ghiasi MR, Rezashah H. Determination of antifungal susceptibility patterns among the clinical isolates of *Candida* species. J Global Infect Dis 2011;3:357-60.

Source of Support: Shiraz University of Medical Sciences, Shiraz, Iran. (Grant No.88-5063). **Conflict of Interest:** None declared.