Iranian Journal of Pharmaceutical Research (2008), 7 (1): 69-75
Received: June 2007
Accepted: November 2007

Original Article

Fluconazole Susceptibility Profile of *Candida* isolates Recovered from Patients Specimens Admitted to Yazd Central Laboratory

Abbas Ali Jafari-nodoushan[a*], Abdol-Hassan Kazemi[b], Farzanah Mirzaii[c] and Modjgan Dehghani[d]

[a]*Medical School, Shahid Sadoughi University of Yazd Medical Sciences and Health services, Yazd, Iran.* [b]*Immunology Department, Tabriz Medical University, Tabriz, Iran.* [c]*Paramedical school, Shahid Sadoughi University of Yazd Medical Sciences and Health services, Yazd, Iran.* [d]*Taft Shaheed beheshti Hospital, Taft, Iran.*

Abstract

The increasing incidence of candidiasis and antifungal resistance as well as the introducing of new antifungal has recently encouraged performing of fungal susceptibility testing. The aim of this study was to evaluate the susceptibility profile of *Candida* species isolated from patients admitted to Yazd central laboratory to fluconazole. We used the broth microdilution method to evaluate the susceptibility profile of a large collection of recent clinical *Candida* isolates, recovered from patients' specimens, against fluconazole. Totally 462 yeast isolates were analyzed, including the following species: 284 isolates of *Candida albicans*, 69 isolates of *C. tropicalis*, 41 isolates of *C. parapsilosis*, 31 isolates of *C. glabrata*, 14 isolates of *C. krusei*, 11 isolates of *C. guilliermondii* and 10 isolates of *Candida kefyr* and 2 other yeasts. Susceptibility ranking to fluconazole obtained by all tested yeasts was: *C. tropicalis>C. albicans>C. parapsilosi > C. kefyr>C. guilliermondii>C. glabrata>C.krusei*. The majority (81%) of all tested yeast isolates were susceptible to fluconazole. There were seen statistical significant differences between the MIC of all isolates ($p<0.05$). Isolates of *C. glabrata* and *C. krusei* showed the highest rate of broth microdilution resistance among all tested isolates, but these only species represented 11.6% of all yeasts isolated from specimens in Yazd central laboratory. In order to identify any changes in the susceptibility patterns of fluconazole with the increased use of this antifungal agent, careful periodical surveillance testing was needed.

Keywords: *Candida*; Fluconazole susceptibility; Antifungal resistance; Microdilution.

Introduction

Candida species are known the fourth most common causes of nosocomial bloodstream infection in the United States (1, 2). These infections bring about increased mortality (3) and longer hospital stays with associated higher health care costs (4, 5). The other clinical manifestation of candidiasis such as vulvovaginal infections is common in women, which infected 75% of women in their lifetime (6). Different studies showed the prevalence of 20 to 43 percent among Iranian symptomatic women for this infection (6, 7). The increasing development of antifungal resistance, as well as the introducing of new antifungal drugs, has caused a novel interest in antifungal susceptibility testing. However the *in vitro* antibacterial susceptibility testing has routinely been used as a guide for clinicians in the selection of appropriate therapy, reliable antifungal

* Corresponding author:
E-mail: Jafariabbas@yahoo.co.in

susceptibility testing is clearly decades behind that for antibacterial susceptibility testing and remain in evolution (8-11).

The 1992 National Committee for Clinical Laboratory Standards (NCCLS) guidelines for susceptibility testing of *Candida* species and *Cryptococcus neoformans* described a broth macrodilution method (M27-P) using semisynthetic RPMI 1640 medium at pH 7.0, with a spectrophotometrically standardized inoculums and fixed incubation and endpoint reading criteria (12). Since the NCCLS method for measuring the performance of antifungal drugs on a large scale was labor-intensive and time-consuming, therefore recent efforts have been focused on the developing more simple methods that produce results compatible with the NCCLS methodology (13-15). Based on these efforts, the NCCLS published a guideline for antifungal susceptibility testing and interpretative breakpoints for triazoles and 5-flucytosine. The proposed method is a broth dilution method that has good inter- and intralaboratory reproducibility for testing yeasts (14, 17).

A microdilution broth method, which is a miniaturization of the macrodilution broth (M27-P method), is equivalent to the macrodilution broth method and recommended as an alternative standard method by the NCCLS sub-committe on antifungal susceptibility testing (12, 14, 18).

Fluconazole is the most widely used drug and well tolerated azoles in the clinical setting that has good clinical activity against *C. neoformans* and most *Candida* species. The increased use of this drug has caused increasing rate of resistance among *Candida* spp, mainly *C. glabrata* and *C. krusei* isolates (15,16). The susceptibility patterns of *Candida* species to fluconazole may be changed following the increasing worldwide use of this antifungal, routine and precise surveillance testing as well as *in vitro* susceptibility testing is necessary to help clinicians in the selection of appropriate therapy (19).

The general purpose of the present study was to evaluate the *in vitro* susceptibility profile of a large recent clinical *Candida* isolates to fluconazole using the broth microdilution method.

Experimental

Microorganisms

From January 2006 to December 2006 we selected all recent clinical yeast isolates that were recovered from patients' specimens in Yazd central laboratory. Candida isolates were recovered from patients with both sexes and different ages, which referred for diagnosis of candidiasis infections such as cutaneous, vulvovaginal, oral, urinary tract candidiasis and onychomycosis. The yeast isolates, which recovered from patients with the history of antibiotic and immunosuppressive agents, were excluded from this study. These cultures were originated from various types of biological material, including oral cavity, vaginal discharge, and urine. The isolates of *Candida* species were then identified by sub-culturing on CHROMagar *Candida* (CHROMagar, France), performing the germ tube test, hyphae/pseudohyphae and chlamidospores growth, carbohydrate fermentation and assimilation, methods as described by Terai and Sandven (20, 21). A single colony from each isolate (24 hours culture) was re-suspended in 5 ml 0.85% sterile saline, and a density of 1-5×10^6 cell/ml prepared. The suspension was firstly diluted 1/50 in sterile saline followed by dilution 1/20 using sterile RPMI, and used for susceptibility analysis.

Antifungal susceptibility testing

Fluconazole MIC endpoints and checkerboard titrations was determined using broth microdilution tests according to the NCCLS document M27-A2 (14).

Fluconazole stock solutions (Sigma) prepared by dissolving 8192 µg fluconazole powder in 100 µl of Dimethyl Sulfoxide (Sigma), and stored at room temperature for 30 minutes. The drug solution were then diluted in two-fold increments RPMI 1640 broth (Sigma) medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (Sigma) to final concentration from 1024 to 0.5 µg/ml. The broth microdilution test was conducted using the sterile plastic microtitration plates containing 96 round-bottom wells, with their

Table 1. Frequency of *Candida* species isolated from various clinical specimens.

Species (number)	Urine	Oral cavity	Vaginal swab	Miscellaneous
C. albicans (284)	52 (18.3%)	119 (41.9%)	93 (32.4%)	21 (7.4%)
C. tropicalis (69)	17 (24.6%)	23 (33.3%)	25 (36.2%)	4 (5.8%)
C. parapsilosis (41)	6 (14.6%)	11 (26.8%)	9 (22%)	15 (36.6%)
C. glabrata (31)	19 (61.2%)	6 (19.4%)	6 (19.4%)	0 (0%)
C. krusei (14)	5 (35.7%)	3 (21.4%)	3 (21.4%)	1 (7.2%)
C. guilliermondii (11)	3 (27.3%)	2 (18.1%)	3 (27.3%)	3 (27.3%)
C. kefyr (10)	2 (20%)	2 (20%)	3 (30%)	3 (30%)
Total (460)	104 (22.6%)	168 (36.5%)	141 (30.6 %)	47 (10.2%)

Miscellaneous: isolated from nail, intertriginous candidiasis lesions, and pus specimens.

corresponding covers were as follows:

Microdilution plates were set up in accordance with the NCCLS reference method (14). Each microdilution well contained 100 µl of two-fold fluconazole concentration and 100 µl of inoculums (in RPMI medium). The total volume of all wells was therefore 200 µl, which gave final concentration of fluconazole from 512 to 0.25 µg/ml, and 0.5-2.5×10^3 cell/ml *Candida*. The microdilution plates were incubated 48 hours in 35 °C shaker adjusted at 150 rpm.

The MFC (minimum fungicidal concentration), MIC_{90} (Minimum inhibition concentration at which 90% of the isolates are inhibited) and MIC_{50} endpoints (Minimum inhibition concentration at which 50% of the isolates are inhibited) were defined as the lowest concentration at which a prominent decrease in growth (approximately 90% and 50% relative to the growth of the control wells occurred after 48 h of incubation), as determined by plating out the wells suspension onto Sabouraud agar plates (Oxoid, UK) and performing a colony count (22). One way analysis of variance test (ANOVA) was used for the determination of statistical differences of MICs between *Candida* species. A quality control (QC) strain, *C. albicans* ATCC 10321, was tested periodically and the test results with an out-of-range QC were excluded from analysis (23). For each isolate test, two drug-free growth wells were included as control well. The breakpoints used for the definition of susceptibility categories are those proposed by the NCCLS and were as follows:

Fluconazole susceptible if MIC≤8.0 µg/ml, dose dependent susceptibility (DD-S) if MIC from 16.0 to 32.0 µg/ml, and resistant if MIC≥ 64.0 µg/ml (24).

Table 2. Susceptibility profile of 460 *Candida* spp. isolates to fluconazole.

Candida species	MIC Range	MIC (µg/ml)		MFC (µg/ml)
		MIC_{50}	MIC_{90}	
C. albicans	0.25-64	0.5	≥4	128
C. tropicalis	0.5-32	0.5	2	64
C. parapsilosis	0.25-128	1	8	256
C. glabrata	1-128	4	≥64	512
C. krusei	32-≥256	32	≥128	≥512
C. guilliermondii	4-256	4	32	≥256
C. kefyr	2-64	2	16	≥128

MIC_{50}: Minimum inhibition concentration (MIC) at which 50% of the isolates are inhibited.
MIC_{90}: MIC at which 90% of the isolates are inhibited.
MFC: Minimum fungicidal concentration.

Table 3. Fluconazole category of susceptibility in 460 *Candida* tested isolates.

Candida Species	S		DDS		R		Total
	N	%	N	%	N	%	
C. albicans	259	91.2	19	6.7	6	2.1	284
C. tropicalis	66	95.6	3	4.4	0	0	69
C. parapsilosis	35	85.4	4	9.7	2	4.9	41
C. glabrata	2	6.5	4	12.9	25	80.6	31
C. krusei	0	0	0	0	14	100	14
C. guilliermondii	2	18.2	5	45.4	4	36.4	11
C. kefyr	6	60	2	20	2	20	10
Total	373	81	34	7.4	53	11.6	460

S: Susceptible
DDS: Dose dependent-susceptible
R: Resistant

Results

We were able to test 462 yeast isolates sequentially recovered from specimens cultured in Yazd central laboratory, including the following species: 284 isolates of *C. albicans*, 69 isolates of *C. tropicalis*, 41 isolates of *C. parapsilosis*, 31 isolates of *C. glabrata*, 14 isolates of *C. krusei*, 11 isolates of *C. guilliermondii* and 10 isolates of *C. kefyr* and 2 other yeasts. Clinically significant yeast isolates were obtained from different body sites, were shown in Table 1.

The values of MFC, $MIC_{90,}$ and MIC_{50} of fluconazole broth microdilution tests obtained from the different species of *Candida* are shown in Table 2. Inhibition concentrations were mostly dependent on the species of *Candida* tested, regardless of the source of the pathogen. Broth microdilution susceptibility performed with *C. krusei* and *C. glabrata* isolates generated higher inhibition concentrations than *C. tropicalis*, *C. albicans,* and *C. parapsilosis*. *C. krusei* isolates showed the highest inhibition concentrations in current study.

The susceptibility ranking for fluconazole was: *C. tropicalis* > *C. albicans* > *C. parapsilosi* > *C. kefyr* > *C. guilliermondii* > *C. glabrata* > *C.krusei*. There were seen the statistical significant differences (one way ANOVA) between the MIC and MFC of all *Candida* isolates ($p<0.01$).

Fluconazole susceptibility categories are summarized by *Candida* species in Table 3. Overall, 81% of the 460 *Candida* isolates tested were susceptible to fluconazole. The incidence of isolates judged as resistant to fluconazole was 4.9% for *C. parapsilosis*, 20% for *C. kefyr*, 2.1% for *C. albicans*, 36.4% for *C. guilliermondii*, 80.6% for *C. glabrata* and 100% for *C. krusei* isolates.

Discussion

The rapid development and spread of antifungal resistance for treatment of systemic candidiasis has become an increasingly serious public health problem in a wide range of infectious diseases. Failure in treatment following the development of azoles resistant Candia strains seems to be becoming more common after long-term azole's treatments. This new phenomenon has increased the efforts towards developing a standardized reference method for general susceptibility testing of yeasts (25).

The major source of susceptibility test variation for azoles is probably the fact that endpoints are less sharp compared with those of other antifungal (e.g., amphotericin B). This lack of sharp endpoints is more frequently seen with *C. albicans*. Broth microdilution with agitation of the plates before reading (24) resulted in clear-cut visual endpoints, but visual reading is a subjective operation, particularly problematic with azoles. Probably, that is the cause of the modest agreement (50 to 75%) observed with 5 of 10 strains tested for fluconazole MICs in a recent international collaborative study

(25). More recently (27), variations such as less dense inoculate and second-day reading have demonstrated better interlaboratory agreement with the broth microdilution test for amphotericin B, fluconazole, and flucytosine than other previous collaborative studies (27). We suspect that the lack of reproducibility with azoles is due mainly to the visual reading of MICs.

In our study the fluconazole broth microdilution procedure was used to analyze the susceptibility of a clinical collection of 462 yeast isolates recovered from positive specimens sent to Yazd central laboratory. The plating out and culturing of the test results onto Sabouraud agar plates and performing the colony count provided consistent and objective values for endpoint readings. The broth microtiter technique, however is economical and less time-consuming than macrodilution tests, in addition, it was able to eliminate uncontrolled results as it facilitated data analysis. This is the largest surveillance study of fungal susceptibility to fluconazole performed in Yazd.

We were able to test a significant number of isolates representing the most clinically relevant species of *Candida*. The non-*albicans* species isolates consisted as much as 38.3% of all yeasts tested. The isolation of non-*albicans* isolates in present study was according to other reports by different centers (18, 28). In present study *C. tropicalis* was known as the second most commonly species of *Candida* isolated and recovered from clinical specimens, just in contrast to the United States and Europe, where *C. glabrata* was reported as the second or third most common isolated species from patients with invasive infections (29, 30). *C. glabrata* and *C. krusei* isolates together represented only 9.7% of all yeast isolates in our study in Yazd.

Since antifungal drug resistance may become more prevalent, it is increasingly important to evaluate current susceptibility profiles and emerging of resistance trends for *Candida* species (9, 11, 30). In our study most of the clinical yeast isolates were susceptible to fluconazole. However, it is clear that there are some species-specific differences in susceptibility to this antifungal agent. Notably, resistance rates to fluconazole ranged from 0 (*C. tropicalis*) to 100% (*C. krusei*) of the tested isolates, depending on the species of *Candida* considered for analysis. Resistance to fluconazole was most commonly seen among *C. glabrata* and *C. krusei* isolates in current study, just similar to data reported by other investigators (11, 29-31). Invasive infections due to fluconazole-resistant *C. albicans* isolates are still considered a rare phenomenon (9, 10, 29, 30). Most isolates of *C. tropicalis, C. albicans* and *C. parapsilosis* tested were very susceptible to fluconazole. There were seen statistical significant differences (one way ANOVA) between the fluconazole MFC and MIC in all *Candida* isolates ($p \leq 0.01$). Based on the results obtained in current study, it is important to determine the susceptibility of *Candida* isolates to fluconazole before beginning of treatment to reduce the prevalence of resistant *Candida* species and improve the treatment outcome.

Acknowledgements

We would like to thank Dr. Hekmati-Moghadam for his support and Ms. Mandegari for her contribution.

References

(1) Pfaller MA, Jones RN, Doern GV, Sader HS, Hollis RJ and Messer SA. International surveillance of bloodstream infections due to Candida species: frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the United States, Canada, and South America for the SENTRY Program. The SENTRY Participant Group. *J. Clin. Microbiol.* (1998) 36: 1886-9

(2) Pfaller MA, Jones RN, Doern GV, Sader HS, Messer SA, Houston A, Coffman S, Hollis RJ. Bloodstream infections due to Candida species: SENTRY antimicrobial surveillance program in North America and Latin America, 1997-1998. *Antimicrob. Agents Chemother.* (2000) 44: 747-51

(3) Maesaki S, Hossain MA, Miyazaki Y Tomono K, Tashiro T and Kohno S. Efficacy of FK463, a [1,3]-D-glucan synthase inhibitor, in disseminated azole-resistant *Candida albicans* infection in mice. *Antimicrob. Agents Chemother.* (2000) 44: 1728-1730

(4) Edmond M, Wallace S, McClish D, Pfaller M, Jones R and Wenzel R. Nosocomial bloodstream infections in United States hospitals: a three year analysis. *Clin. Infect. Dis.* (1999) 29: 239-244

(5) Takakura S, Fujihara N, Saito T, Kudo T, Iinuma Y and Ichiyama S. National surveillance of species distribution in blood isolates of *Candida* species in Japan and their susceptibility to six antifungal agents including voriconazole and micafungin. *Antimicrob. Agents Chemother*. (2004) 53: 283-289
(6) Walker CK.Reproductive Tract Infections: sexually transmitted diseases. In: Larry J. Copeland, editors. Textbook of Gynecology, 2nd ed. Philadelphia: W.B.Saunders.(2000): 869-90
(7) Shanaz Ali B and Tohidi A. Prevalence of *Candida* vaginitis among the symptomatic patients in Kerman. *J. Qazvin Univ. Med. Sci.* (2000) 13: 42-47
(8) Fati AM, Tavasoli F, Mosavi SH and Boshralamin SA. The therapeutic effect of clotrimazole, nystatin, and povidion iodin in treatment of vaginal candidiasis. *Med. J. Mashhad Univ. Med. Sci*. (2007) 49: 375-378
(9) Neely MN and Ghannoum MA. The exciting future of antifungal therapy. *Eur. J. Clin. Microbiol. Infect. Dis*. (2000) 19: 897-914
(10) Rex J, Rinaldi MG and Pfaller MA. Resistance of *Candida* species to fluconazole. *Antimicrob. Agents Chemother.* (1995) 39: 1-8
(11) Espinel-Ingroff A. Clinical relevance of antifungal resistance. *Infect. Dis. Clin. North Am.* (1997) 30: 19-28
(12) Cormican MG and Pfaller MA. Review standardization of antifungal susceptibility testing. *Antimicrob. Agents Chemother.* (1996) 38: 561-578
(13) Rex JH, Pfaller MA, Galgiani JN, Bartlett MS, Espinel-Ingroff A, Ghannoum MA, Lancaster M, Odds FC, Rinaldi MG, Walsh TJ and Barry AL. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of *in vitro-in vivo* correlation data for fluconazole, itraconazole, and *Candida* infections. Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards. *Clin. Infect. Dis.* (1997) 24: 235-47
(14) Espinel-Ingroff A, Brachiesi F and Hazen KC. Standardization of antifungal susceptibility testing and clinical relevance. *Med. Mycol.* (1998) 36: 68-78
(15) National Committee for Clinical Laboratory Standards. *Reference Method for Broth Microdilution Antifungal Susceptibility Testing of Yeast, Approved Standard M27-A2*. National Committee for Clinical Laboratory Standards, Wayne Pa. (2002) (pages range)
(16) Messer SA, Diekema DJ, Boyken L, Tendolkar S, Hollis RJ and Pfaller MA. Activities of micafungin against 315 invasive clinical isolates of fluconazole-resistant *Candida* spp. *J. Clin. Microbiol.* (2006) 44: 324-326
(17) Guinea J, Sanchez-Somolinos M, Cuevas O, Pelaez T and Bouza E. Fluconazole resistance mechanisms in *Candida krusei*: The contribution of efflux-pumps. *Med. Mycol.* (2006) 44: 575-578
(18) Juliana C, De Resende P and De Resende MA. *In vitro* antifungal susceptibility of clinical isolates of *Candida* spp. from hospitalized patients. *Mycoses* (1992) 42: 641-644
(19) Rex JH, Pfaller MA, Barry AL, Nelson PW and Webb CD. Antifungal susceptibility testing of isolates from a randomized multicenter trial of fluconazole versus amphotericin B as treatment of non-neutropenic patients with candidemia. *Antimicrob. Agents Chemother.* (1995) 39: 40-44
(20) Majoros C, Kardos LG, Szabo B, Kova M and Mara A. Fluconazole susceptibility testing of *Candida inconspicua* clinical isolates: comparison of four methods. *Antimicrob. Agents. Chemother* (2005) 55: 275-276
(21) Terai H and Shimahara M. Atrophic tongue associated with *Candida*. *J. Oral Pathol. Med.* (2005) 34: 397-400
(22) Sandven P. Laboratory identification and sensitivity testing of yeast isolates. *Acta Odontol. Scand.* (1990) 48: 27-36
(23) Magaldi S, Mata S, Hartung C, Verde G, Debis L and Marcano C. *In vitro* susceptibility of 137 *Candida* sp. isolates from HIV positive patients to several antifungal drugs. *Mycopathologia* (2000) 149: 63-68
(24) Barry AL, Pfaller MA, Brown SD, Espinel-Ingroff A, Ghannoum MA, Knapp C, Rennie RP, Rex JH and Rinaldi MG. Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. *J. Clin. Microbiol.* (2000) 38: 3457-3459
(25) Yang YL, Li SY, Cheng HH and Lo HJ. The trend of susceptibilities to amphotericin B and fluconazole of *Candida* species from 1999 to 2002 in Taiwan. *BMC Infect. Dise.* (2005) 5: 1-5
(26) Baddley JW and Moser SA. Emerging fungal resistance. *Clin. Lab. Med*. (2004) 24: 721-735
(27) Anaissie E, Paetznick V and Bodey GP. Fluconazole susceptibility testing of *Candida albicans*: microtiter method that is independent of inoculum size, temperature, and time of reading. *Antimicrob. Agents Chemother*. (1990) 35: 1641-1646
(28) Espinel-Ingroff, Kish CW, Kerkering TM, Fromtling RA, Bartizal K, Galgiani JN, Villareal K, Pfaller MA, Gerarden T, Rinaldi MG and Fothergili A. Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. *J. Clin. Microbiol.* (1992) 30: 3138-3145
(29) Paiva Martins CA, Koga-Ito CY and Cardoso AO. Presence of *staphylococcus* spp. and *Candida* spp. in the human oral cavity. *Braz. J. Microbiol.* (2002) 33: 236-240
(30) Pfaller MA, Jones RN and Doern GV. Bloodstream infections due to *Candida* species: SENTRY Antimicrobial surveillance program in North America and Latin America, 1997-1998. *Antimicrob. Agents Chemother.* (2000) 44: 747-51
(31) Sanguinetti M, Posteraro B, Fiori B, Ranno S, Torelli R and Fadda G. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. *Antimicrob. Agents Chemother*. (2005) 49: 668-679

(32) Pfaller MA, Diekema DJ, Jones RN, Sader HS, Fluit AC, Hollis RJ and Messer SA. International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and *in vitro* susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY Antimicrobial Surveillance Program. *J. Clin.*

This article is available online at http://www.ijpr-online.com