

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

^aMedical School, Shahid Sadoughi University of Yazd Medical Sciences and Health services, Yazd, Iran. ^bImmunology Department, Tabriz Medical University, Tabriz, Iran. ^cParamedical school, Shahid Sadoughi University of Yazd Medical Sciences and Health services, Yazd, Iran. ^dTaft 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 kefir* and 2 other yeasts. Susceptibility ranking to fluconazole obtained by all tested yeasts was: *C. tropicalis* > *C. albicans* > *C. parapsilosi* > *C. kefir* > *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 susceptibility testing is clearly decades behind that for antibacterial susceptibility testing and remain in evolution (8-11).

* Corresponding author:

E-mail: Jafariabbas@yahoo.co.in

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 inoculum 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-committee 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 chlamydospores 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 $1-5 \times 10^6$ 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 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 inoculum (in RPMI medium). The total volume of all wells was therefore 200 µl, which

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

Species (number)	Urine	Oral cavity	Vaginal swab	Miscellaneous
<i>C. albicans</i> (284)	52 (18.3%)	119 (41.9%)	92 (32.4%)	21 (7.4%)
<i>C. tropicalis</i> (69)	17 (24.6%)	23 (33.3%)	25 (36.3%)	4 (5.8%)
<i>C. parapsilosis</i> (41)	6 (14.6%)	11 (26.8%)	9 (22%)	15 (36.6%)
<i>C. glabrata</i> (31)	19 (61.2%)	6 (19.4%)	6 (19.4%)	0 (0%)
<i>C. krusei</i> (14)	5 (35.7%)	5 (35.7%)	3 (21.4%)	1 (7.2%)
<i>C. guilliermondii</i> (11)	3 (27.3%)	2 (18.1%)	3 (27.3%)	3 (27.3%)
<i>C. kefyr</i> (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.

gave final concentration of fluconazole from 512 to 0.25 µg/ml, and $0.5-2.5 \times 10^3$ cell/ml *Candida*. The microdilution plates were incubated 48 hours in 35 °C shaker adjusted at 150 rpm.

The MFC (minimum fungicidal concentration), MIC₉₀ (Minimum inhibition concentration at which 90% of the isolates are inhibited) and MIC₅₀ 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).

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, MIC₉₀, and MIC₅₀ of

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

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

MIC₅₀: Minimum inhibition concentration (MIC) at which 50% of the isolates are inhibited.

MIC₉₀: 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	%	
<i>C. albicans</i>	259	91.2	19	6.7	6	2.1	284
<i>C. tropicalis</i>	66	95.6	3	4.4	0	0	69
<i>C. parapsilosis</i>	35	85.4	4	9.7	2	4.9	41
<i>C. glabrata</i>	2	6.5	4	12.9	25	80.6	31
<i>C. krusei</i>	0	0	0	0	14	100	14
<i>C. guilliermondii</i>	2	18.2	5	45.4	4	36.4	11
<i>C. kefyr</i>	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

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. parapsilosis* > *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

Therapid 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 *Candida* 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.

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