Fluconazole Susceptibility of Brazilian Candida Isolates Assessed by a Disk Diffusion Method

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The increasing magnitude of antifungal resistance as well as the advent of new antifungal drugs has generated a renewed interest in fungal susceptibility testing. We used a previously described disk diffusion method to evaluate the susceptibility profile of a large collection of recent clinical *Candida* spp. isolates against fluconazole. A total of 1,784 yeast isolates were tested, including the following species: *Candida albicans* (1,036), *C. tropicalis* (279), *C. parapsilosis* (202), *C. glabrata* (119), *C. guilliermondii* (90), *C. krusei* (32), *C. lusitaniae* (7), *Candida* spp. (14) and other yeasts (5). Susceptibility ranking to fluconazole obtained with all yeasts tested was: *C. parapsilosis* ≅ *C. tropicalis* ≅ *C. guilliermondii* > *C. glabrata* > *C. krusei*. The majority (94%) of all yeast isolates tested were susceptible to fluconazole. Isolates of *C. glabrata* and *C. krusei* exhibited the highest rate of DDS/resistance among all isolates tested but they represented only 9% of all yeasts routinely sent to our lab. Careful periodical surveillance is needed in order to identify any changes in the susceptibility patterns of fluconazole with the increased use of this antifungal agent in Brazilian tertiary care hospitals.

Key Words: Candida, fluconazole, antifungal resistance, disk diffusion method.

The increasing magnitude of antifungal resistance, as well as the advent of new antifungal drugs, has generated a renewed interest in antifungal susceptibility testing. Unlike *in vitro* antibacterial susceptibility testing, which has been routinely used as a guide for clinicians in the selection of appropriate therapy, antifungal susceptibility assays remain in evolution [1,2,3,4,5].

Given the need to standardize methods for testing major agents of mycoses, the National Committee for Clinical Laboratory Standards (NCCLS) established a subcommittee on antifungal susceptibility testing. Several multicenter studies were conducted by the NCCLS to address all key technical problems affecting the interlaboratory reproducibility of testing [3,5]. 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 yeast [6,7].

Measuring the performance of antifungal drugs on a large scale by susceptibility tests with the NCCLS method is difficult because it is time consuming and labor intensive. Recent efforts have been devoted to developing more simple agar-based methods that generate results compatible with the NCCLS methodology, including Etest and disk diffusion tests [6,8,9,10].

Fluconazole is a well-tolerated and safe triazole antifungal agent that has good clinical activity against *Cryptococcus neoformans* and most *Candida* spp. isolates. The increased use of this drug has given rise to the development of resistance among *Candida* spp, mainly *C. glabrata* and *C. krusei* isolates [2,4]. A

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simple disk diffusion test was developed for testing the susceptibility of *Candida* spp. isolates against fluconazole. Despite some limitations for identifying truly resistant fluconazole clinical isolates on an individual basis, the fluconazole disk diffusion method may be a useful tool for the evaluation of epidemiological trends in fluconazole sensibility [6,9,10].

We used a disk diffusion method to evaluate the susceptibility profile of a large collection of recent clinical *Candida* spp. isolates to fluconazole.

Materials and Methods

Microorganisms: From January 1998 to December 2000 we selected all recent clinical yeast isolates that were sent to our laboratory from cultures made at two public institutions: Hospital São Paulo and Hospital e Maternidade Santa Marcelina. These cultures were originated from various types of biological material, including oral cavity swabs, urine, blood, and bronchoalveolar lavage. The yeasts were identified at the species level by standard methods, and stored as water suspensions at room temperature [11].

Antifungal susceptibility testing: We used a previously described disk diffusion assay to test all yeast isolates [10,12,13]. The test disks contained 25µg/mL. fluconazole (Becton Dickinson, Sparks, MD), kindly supplied by Pfizer Inc. They were stored in a desiccator at 4°C. Initially, a yeast inoculum suspension adjusted to match a 0.5 McFarland density standard was prepared. A sterile cotton swab moistened with the inoculum suspension was used to applied to a 90 mm diameter plate containing Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue. The plates were allowed to dry for 5-15 minutes before a 25µg/mL fluconazole disk was placed in the center of the agar. The plates were incubated for 18-24 hours at 35-37° and the slowly-growing isolates could be read after 48 hours incubation. A quality control (QC) strain, *C. albicans* ATCC 90028 with a recommended acceptable performance range of 32-43 mm, was tested once a week. Test results with an out-of-range QC were excluded from analysis.

Reading and interpretation of zone diameter measurements: all inhibition zone diameters generated by disk diffusion tests were read and recorded by using the BIOMIC Plate Reader System [14], a semi-automatic electronic image-analysis system. This system is connected to a high-resolution video camera able to capture each plate image and measure the inhibition zone diameter generated by the disk diffusion test. The zone diameters were measured at the transitional point where growth pattern abruptly decreased. The BIOMIC system has software that transforms inhibition zone diameter sizes into corresponding fluconazole MIC values by using a previously obtained standard regression analysis curve. The interpretive breakpoints used for fluconazole disk tests were based on zones that correlated with US-NCCLS recommended category breakpoints for the reference broth dilution method. Fluconazole breakpoints were: $S \leq 8$ µg/ml, SDD =16-32 µg/ml, and $R \geq 64$ µg/ml. In accordance with NCCLS indications, all *C. krusei* isolates were considered resistant to fluconazole [5,7].

Results

We were able to test 1,784 yeast isolates sequentially sent to our laboratory, including the following species: *Candida albicans* (1,036), *C. tropicalis* (279), *C. parapsilosis* (202), *C. glabrata* (119), *C. guilliermondii* (90), *C. krusei* (32), *C. lusitaniae* (7), *Candida* spp. (14) and other yeasts (5). Clinically significant yeast isolates were obtained from different body sites, as shown in Table 1. Except for the oral cavity specimens, all strains were isolated from hospitalized patients.

The average inhibition zone diameters of fluconazole disk diffusion tests obtained with the different species of *Candida* are shown in Table 2. Inhibition zone diameters were mostly dependent on the species of *Candida* tested, regardless of the source of the pathogen. Disk diffusion tests

performed with *C. glabrata* and *C. krusei* isolates generated smaller inhibition zones than in the assays of *C.albicans*, *C. parapsilosis* and *C. tropicalis* strains. *C. krusei* isolates generated the smallest inhibition zones.

The values of MIC-50% and MIC-90% for the isolates representative of the most frequently isolated species of *Candida* are shown in Table 3. The susceptibility ranking for fluconazole was: *C.parapsilosis* ≅ *C.tropicalis* ≅ *C. guilliermondii* > *C. albicans* > *C. glabrata* > *C.krusei*.

Fluconazole susceptibility categories are summarized by *Candida* species in Table 4. Overall, 94% of the 1,728 yeast isolates tested were susceptible to fluconazole. The incidence of isolates judged as DDS/resistant to fluconazole was 1% for *C. parapsilosis*, 2.1% for *C.tropicalis*, 4% for *C. albicans*, 4.4% for *C. guilliermondii*, 16% for *C.glabrata* and 100% for *C. krusei* isolates.

Discussion

Agar disk diffusion tests with antifungal drugs are not standardized and a direct correlation between disk diffusion test results and clinical outcome has not yet been reported. Consequently, antifungal disk diffusion tests should not be used as a guide for the selection and monitoring of antifungal therapy [6]. However, considering that disk-diffusion assays are simple to perform and inexpensive, they may be a useful tool in large-scale surveys of clinical isolates to identify population distribution patterns of susceptibility to fluconazole. The disk diffusion test using 25µg fluconazole disks on an MH agar plate containing 2% glucose and 5mg methylene blue/mL is sufficiently reproducible and accurate to be used as a screening test [9,10,12,13]. Recently, this methodology has been used by different investigators for global surveillance of fluconazole susceptibility [14,15].

In our study the fluconazole disk diffusion procedure was used to survey a clinical collection of 1,728 yeasts sent to our laboratory for further identification. The BIOMIC System image-analyses of plates provided consistent and objective inhibition zone endpoint readings. 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 Brazil.

We were able to test a significant number of isolates representative of the most clinically relevant species of *Candida*. The non-*albicans* species isolates accounted for as much as 42% of all yeasts tested. The appearance of non-*albicans* isolates among patients admitted to tertiary care hospitals has been reported by different centers, including medical institutions from Brazil [16-19]. Contrary to the United States and Europe, where *C. glabrata* is the second or third most commonly species of *Candida* isolated from patients with invasive infections [17,20], *C. glabrata* and *C. krusei* isolates together represented only 9% of all yeast isolates in our study in Brazil.

As antifungal drug resistance may become more prevalent, it is increasingly important to evaluate current susceptibility profiles and emerging trends [2-4,20]. 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 to 100% of the isolates tested, depending on the species of *Candida* considered for analysis. In agreement with data reported by other investigators [4,17,20,21], DDS/resistance to fluconazole was most commonly reported among *C. glabrata* and *C. krusei* strains.

In our series, we had 4% *C.albicans* isolates considered DDS/resistant to fluconazole. Most of these isolates were obtained from the oral cavity of HIV-infected/AIDS patients (data not shown). Invasive infections due to fluconazole-resistant *C. albicans* isolates are still considered a rare phenomenon [2,3,17,20]. Most strains of *C. parapsilosis* and *C. tropicalis* tested were very susceptible to fluconazole.

Our data indicate that fluconazole has good *in vitro* activity against most clinically relevant strains of *Candida* spp. routinely isolated in public tertiary care hospitals. The clinical use of fluconazole in such Brazilian

Table1. Identification and sources of all 1,784 *Candida* spp. isolates

Species (number)	Blood	Urine	Oral cavity	BAL	Miscellaneous
C. albicans (1,036)	160 (15.5%)	262 (25.5%)	305 (29.5%)	48 (4.5%)	261 (25%)
C. tropicalis (279)	94 (33.5%)	105 (37.5%)	32 (11.5%)	1 (0.5%)	46 (17%)
C. parapsilosis (202)	99 (49%)	61 (30%)	27 (13.5%)	0 (0%)	15 (7.5%)
C. glabrata (119)	20 (17%)	68 (57%)	9 (7.5%)	0 (0%)	22 (18.5%)
C. guilliermondii (90)	78 (86.5%)	1 (1%)	4 (4.5%)	0 (0%)	7 (8%)
C. krusei (32)	6 (19%)	15 (47%)	3 (9%)	0 (0%)	8 (25%)
Candida spp. (27)	17 (62%)	3 (11%)	4 (15%)	1 (4%)	2 (8%)
Total (1,784)	**474 (26.5%)**	**515 (29%)**	**384 (21.5%)**	**50 (3%)**	**359 (20%)**

Table 2. Average Zone Diameters (mm) by Specimen Type and Species of *Candida* spp.

Species/number	Blood	BAL*	Oral cavity	Urine	Miscellaneous	Total/Specie
C. albicans (1,036)	38.9	34.2	39.7	37.2	38.1	37.4
C. parasilosis (202)	42.5	0.0	44.8	39.2	39.7	41.6
C. tropicalis (279)	35.8	27.0	37.7	35.6	34.1	35.8
C. glabrata (119)	25.0	0.0	21.7	29.3	28.1	27.5
C. krusei (32)	12.5	0.0	17.4	16.7	17.4	17.4
C. guilliermondii (90)	35.4	0.0	35.8	36.0	33.4	35.1
Other Yeasts	32.0	42.0	34.8	34.5	0.0	35.8

* BAL : bronchoalveolar lavage.

Table 3. Fluconazole MICs 50% and 90% obtained with the most frequently isolated species of *Candida* tested

	MIC (μg/ml)			
Organism	**MIC- 50%**	**MIC- 90%**	**N**	**% of all isolates**
Candida albicans	<0.25	4.6	1036	58
Candida tropicalis	0.3	1.6	279	16
Candida parapsilosis	<0.25	1.3	202	11
Candida glabrata	2.5	55.7	119	7
Candida guilliermondii	0.5	1.3	90	5
Candida krusei	16.0	>128.0	32	2

MIC-50%: MIC value able to inhibit 50% of all isolates tested.
MIC-90%: MIC value able to inhibit 90% of all isolates tested.
Breakpoints: Susceptible £ 8. Resistant ³64. Test Range <0.25 to >128 μg/ml).

Table 4. Fluconazole category of susceptibility in 1,784 yeast isolates

Organisms	S		S DD		R		Total
	N	%	N	%	N	%	N
Candida albicans	994	96	16	1.5	26	2.5	1,036
Candida tropicalis	273	97.8	4	1.4	2	0.7	279
Candida parapsilosis	200	99.0	1	0.5	1	0.5	202
Candida glabrata	100	84.0	6	5.0	13	11	119
Candida guilliermondii	86	95.6	4	4.4		90	
Candida krusei					32	100	32
Candida lusitaniae	7	100.0					7
Candida species	14	100.0					14
Other Yeast	4	80			1	20	5
Total	**1,678**	**94.1**	**31**	**1.7**	**75**	**4.2**	**1,784**

S: susceptible; SDD: susceptibility dose-dependent; R: Resistant.

public institutions has been limited due to cost considerations. Careful periodical surveillance is needed in order to identify potential changes in the susceptibility pattern of fungi to fluconazole, with the increased use of fluconazole in Brazil.

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