Detection of Fluconazole-Resistant Isolates of *Candida glabrata* by Using an Agar Screen Assay

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The ability of a fluconazole-containing agar screen assay to accurately detect isolates of *Candida glabrata* resistant to the azole antifungal agent fluconazole was evaluated on a collection of 100 clinical isolates of this organism. Results were correlated with the MIC of fluconazole for these isolates and compared with the results of a previously published disk diffusion-based fluconazole resistance screening test. Agar screen assay results were in categorical agreement with MIC-based determinations for 97% (97/100) of the isolates tested. This correlation was higher than that obtained with the disk diffusion technique, which categorized only 87% (87/100) of isolates correctly, and suggests that the agar screening approach can effectively expedite fluconazole susceptibility testing of *C. glabrata* isolates.

The relatively recent publication of approved procedures and interpretive categories for determining the susceptibility of clinical isolates of *Candida* spp. to antifungal agents (12, 18) provides a basis for susceptibility testing to become a valuable tool for the optimization of antifungal therapy for patients with invasive yeast infections (5, 7). Unfortunately, both the approved broth macrodilution method (12) and microdilution adaptations of this technique (6, 12) are relatively expensive and laborious and consequently are used in only a minority of clinical microbiology laboratories that routinely perform antibacterial susceptibility testing (3). The absence of institution-specific susceptibility information is unfortunate given concerns about the emergence of antifungal resistance in *Candida* spp. (17). *Candida glabrata* in particular has become a more frequent isolate in many laboratories (2, 16), and the fluconazole MIC at which 50% of tested isolates of this organism are inhibited is typically 8- to 16-fold higher than that seen for *C. albicans* (15, 16, 19). In addition, the typical fluconazole MICs for 10 to 15% of invasive isolates of *C. glabrata* place them in the resistant category, compared with less than 1% of *C. albicans* isolates (15, 20). Antifungal susceptibility testing of *C. glabrata* is, therefore, of greater clinical import than for many other *Candida* species, and techniques that facilitate the performance of fluconazole susceptibility testing of *C. glabrata* in routine clinical microbiology laboratories need to be developed. Previous studies have described the use of simple disk diffusion-based techniques for detecting resistance to fluconazole in *Candida* spp. (1, 8, 9, 10, 11, 13, 19), but with the notable exception of the study of Sandven (19), these studies have concentrated on testing *C. albicans* isolates. Use of fluconazole-containing agar plates has also been reported as a possible means of identifying fluconazole-resistant isolates of *Candida* spp. (15, 20), and Patterson et al. (13) reported an

excellent 97% correlation between agar screen testing and MIC determination for a small group of 30 isolates of *C. glabrata*. These results, and the relative lack of data evaluating fluconazole disk diffusion testing on *C. glabrata* isolates, suggested to us that it would be worthwhile to perform a comparative evaluation of a screening agar-based method and disk diffusion for the rapid detection of fluconazole resistance in a large collection of clinical isolates of *C. glabrata*.

All organisms used in the study were originally isolated from clinical specimens submitted for culture to the Microbiology Laboratory, Hennepin County Medical Center, between April 1998 and December 2000. The clinical sources of the isolates were as follows: urine (42 isolates), blood (31), surgically collected tissue or aspirate (11), abscess or drainage fluid (9), and wound (7). Using standard methods (4), isolates were identified as *C. glabrata*. The MICs of fluconazole for isolates were determined using the NCCLS reference broth microdilution method (12). Final fluconazole concentrations tested ranged from 0.125 to 64 µg/ml. Microtiter plates were incubated for 48 h at 35°C, and the MIC was read as the lowest concentration of fluconazole that effected a visually distinct decrease (>50%) in turbidity relative to the growth control. The fluconazole screen agar was formulated as described by Patterson et al. (13). To prepare the inoculum for the agar-screening assay, several isolated colonies of each isolate were picked and suspended in sterile saline (0.9%). The optical density of each suspension was then adjusted until it corresponded to a 0.5 McFarland standard, and a sterile calibrated loop (1 µl) was used to streak a set of three plates containing 0, 8, and 16 µg of fluconazole/ml. Inoculated plates were incubated at 30°C for a total of 48 h. Growth characteristics of individual isolates were recorded after 24 and 48 h of incubation by visually comparing the diameters of 15 to 20 colonies on the fluconazole-containing plates with those on the fluconazole-free plate. To provide for a more objective analysis of the data, results observed in the fluconazole-agar screen assay were divided into four categories: category I (colonies indistinguishable in size on media with and without fluconazole); category

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TABLE 1. Distribution of 48-h broth microdilution fluconazole MIC for *C. glabrata* isolates evaluated in the present study

Total no. of isolates tested	No. of isolates with fluconazole MIC (µg/ml) of:							
	0.5	1	2	4	8	16	32	≥64
100	1	2	9	26	31	14	4	13

II (colonies visually smaller on fluconazole-containing media but with a typical colony diameter >50% of the diameter seen on fluconazole-free media); category III (colonies significantly smaller on fluconazole-containing media and typical colony diameter <50% of the diameter seen on fluconazole-free media); and category IV (no growth or only pinpoint colonies on fluconazole-containing media). Fluconazole disk diffusion testing was performed as described by Barry and Brown (1). Zone diameters were measured after 48 h of incubation at 35°C in ambient air, and breakpoints of ≥19 mm (susceptible), 15 to 18 mm (intermediate), and ≤14 mm (resistant) were used, corresponding (according to previously published data) to fluconazole MICs of ≤8, 16 to 32, and ≥64 ug/ml, respectively (1).

The fluconazole MICs for the 100 isolates of *C. glabrata* evaluated in this study are shown in Table 1. Significant clustering of isolates around the susceptible breakpoint of 8 µg/ml was observed, with fluconazole MICs either at or within one doubling dilution of this value for 71% (71/100) of the isolates. The fluconazole MICs for a total of 31 isolates were >8 µg/ml, with 18 (58%) of these showing fluconazole MICs in the NCCLS susceptible–dose-dependent (S-DD) range and 13 (42%) testing in the resistant (R) range (≥64 µg/ml). The results of the agar screen assay are shown in Table 2. The use of 8 µg of fluconazole/ml and an incubation time of 24 h enabled the most accurate differentiation of fluconazole-susceptible *C. glabrata* isolates (MIC ≤ 8 µg/ml) from those in the S-DD and R categories. Under these conditions, all 31 isolates with fluconazole MICs of >8 µg/ml were placed in category I or II (less than 50% decrease in mean colony diameter), with only 3 of the 69 isolates (4.3%) with fluconazole MICs of ≤8 µg/ml being similarly designated. Using 8 µg of fluconazole/ml but prolonging the incubation period to 48 h resulted in a marginal improvement in sensitivity but greatly decreased the specificity of the assay (Table 2). The use of 16 µg of fluconazole/ml did not improve differentiation of isolates with fluconazole MICs of 8 µg/ml or less from those with fluconazole MICs of 16 µg/ml or above, as had been reported in a previous

TABLE 3. Correlation between results obtained by disk diffusion and MIC broth microdilution testing of fluconazole susceptibility of *C. glabrata* isolates

MIC (µg/ml)[a] (no. of isolates)	No. of isolates with disk diffusion zone sizes (mm) and interpretation[a] of:		
	≥19 (susceptible)	15–18 (intermediate)	≤14 (resistant)
≤8 (69)	56	6	7
16–32 (18)	0	6	12
≥64 (13)	0	0	13

[a] Categories: susceptible (≤8 µg/ml), susceptible-dose dependent (16 to 32 µg/ml), resistant (≥64 µg/ml).

study (14). Indeed, a majority of isolates with fluconazole MICs of 16 or 32 µg/ml (13 of 18 [72%] at 24 h and 15 of 18 [83%] at 48 h) were designated susceptible (category III or IV) when 16 µg of fluconazole/ml was added to the medium. All isolates determined to be in the R category by the reference MIC method were designated category I (little or no decrease in mean colony diameter) in the fluconazole agar screen assay irrespective of the concentration of fluconazole used or length of incubation. Unfortunately, under none of the tested conditions were isolates in the S-DD category able to be accurately differentiated from those isolates demonstrating outright resistance to fluconazole (Table 2). The results of disk diffusion testing are shown in Table 3. All 31 isolates placed in the R or S-DD category by MIC testing were identified as either intermediate or resistant by disk diffusion testing. A total of 13 of the 69 (18.9%) isolates in the S category were also classified as resistant (7 isolates) or intermediate (6 isolates) by disk diffusion, resulting in an overall accuracy of 87% (87/100).

The results of our investigation of simple techniques for screening isolates of *C. glabrata* for susceptibility to fluconazole are in general agreement with those reported by previous investigators (1, 11, 13, 19). Both the agar screen method that we developed and the disk diffusion technique correctly designated resistant all 31 isolates with fluconazole MICs of >8 µg/ml. Neither methodology effectively discriminated between isolates that were determined to be in the S-DD category by MIC testing and those determined to be resistant outright to fluconazole. In the agar screen assay, 56% (10/18) of S-DD isolates appeared fully resistant to fluconazole, while only 33% (6/18) of S-DD isolates would have been classified as intermediate by the disk diffusion assay. These results strongly suggest

TABLE 2. Correlation between decrease in colony diameter observed on fluconazole-screening agar and NCCLS interpretive categorization of *C. glabrata* isolates based on broth microdilution fluconazole MICs

MIC (µg/ml)[a] (no. of isolates)	No. of isolates of indicated colony size category[b] in test conditions (µg) of fluconazole/incubation time (h) of:															
	8/24				8/48				16/24				16/48			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
≤8 (69)	0	3	35	31	0	1	52	16	1	1	1	68	0	0	14	53
16–32 (18)	10	8	0	0	2	8	8	0	3	1	2	12	2	1	8	7
≥64 (13)	13	0	0	0	13	0	0	0	13	0	0	0	13	0	0	0

[a] Categories: susceptible (≤8 µg/ml), susceptible-dose dependent (16 to 32 µg/ml), resistant (≥64 µg/ml).
[b] Definitions of colony size categories are as follows: category I (no discernible difference in colony size on fluconazole-containing media); category II (colonies smaller on fluconazole-containing media but typically >50% of the size of control colonies); category III (colonies significantly smaller on fluconazole-containing media and typically <50% of the size of control colonies); and category IV (no growth or only pinpoint colonies on fluconazole-containing media).

that qualitative test methodologies cannot be used to definitively determine the level of susceptibility of *C. glabrata* isolates to fluconazole and that MIC determination is necessary to classify isolates as either R or S-DD. The principal goal of using a simple, qualitative screening assay for resistance testing is to minimize the necessity for performing laborious and costly MIC testing on all isolates. Assuming that any isolate designated less than susceptible by the screening test would have a fluconazole MIC assay performed, use of the disk diffusion assay would have eliminated 66% (66/100) of the MIC tests performed on *C. glabrata* isolates. Since the fluconazole-screening agar was somewhat more successful than disk diffusion in identifying susceptible *C. glabrata* isolates (with only 3/69 [4.3%] isolates with fluconazole MICs of ≤8 μg/ml being misclassified using the optimal assay conditions), use of this screening method would have decreased MIC testing by 75%. An evaluation similar to that reported here was also performed on clinical isolates of *C. albicans*. Using the agar screening assay, 100% (47/47) of the susceptible isolates of *C. albicans* tested (fluconazole MIC range, 0.12 to 2.0 μg/ml) were correctly categorized using the same test conditions utilized for *C. glabrata* (data not shown). These results suggest that use of the agar screen assay can provide a highly accurate means of rapidly identifying fluconazole-susceptible isolates of *Candida* spp., with better performance than disk diffusion testing for *C. glabrata*. The routine use of such a screening agar can potentially decrease antifungal susceptibility testing costs in laboratories currently performing macro- or microdilution MIC determinations while simultaneously increasing the spectrum of laboratories able to offer at least a qualitative assessment of the susceptibility of *Candida* spp. to fluconazole.

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