

## Comparative Evaluation of Alternative Methods for Broth Dilution Susceptibility Testing of Fluconazole against *Candida albicans*

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**A comparative evaluation of methods for broth macro- and microdilution susceptibility testing of fluconazole was conducted with 119 clinical isolates of *Candida albicans*. Macro- and microdilution testing were performed according to National Committee for Clinical Laboratory Standards recommendations. For reference macrodilution testing, an 80% inhibition endpoint (MIC 80%) was determined after 48 h of incubation in accordance with National Committee for Clinical Laboratory Standards proposed standard M27-P. Microdilution endpoints were scored as the first tube or well in which a prominent reduction in turbidity (score 2 out of a possible 4) was observed compared with the growth control (Micro MIC-2). Alternative endpoint criteria were assessed independently of the reference MIC 80% and Micro MIC-2 values and included a colorimetric microdilution endpoint determined by using an oxidation-reduction indicator (Alamar Blue; Alamar Biosciences Inc., Sacramento, Calif.). The MICs for the two microdilution test systems were read after 24 and 48 h of incubation. The percentage of fluconazole MICs within 2 doubling dilutions of the macrodilution reference values was 94% for both microdilution tests read at 24 h. Agreement was slightly lower at 48 h and ranged from 91 to 93%. Comparison of Micro MIC-2 and colorimetric microdilution MICs resulted in agreements of 97 and 93% at 24 and 48 h, respectively. These results show excellent agreement among alternative methods for fluconazole susceptibility testing.**

Broth dilution testing of *Candida albicans* susceptibility to azole antifungal agents has been difficult because of a number of poorly controlled sources of test variation. The major sources of susceptibility test variation for fluconazole and other azoles in vitro have been reported to be the pH, the composition of the test medium, the inoculum size, and the temperature and duration of incubation (2, 6, 8, 9, 11, 12, 14-18). These factors have been the focus of a series of collaborative studies by the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests (1, 3, 5, 7, 12, 16). As a result of these studies, consensus within the Subcommittee has been achieved and a standardized reference method for broth dilution antifungal susceptibility testing has been proposed (10).

While considerable progress in standardizing antifungal susceptibility testing has been made, the determination of endpoints for the azole antifungal agents is still not satisfactory. Partial inhibition of fungal growth in vitro often takes place over a range of azole concentrations, which can make endpoint determinations both difficult and subjective (3, 5, 8, 11, 13-17). In addressing this problem, a number of different methods of endpoint determination have been applied, including visual, turbidimetric, colorimetric, and radiometric methods; dry-weight determination; the use of ATP photometry; and the use of oxidation-reduction indicators (3, 6, 9, 11, 15, 17-19). In the NCCLS proposed standard, M27-P (10), endpoints are determined by visually grading turbidity and the MIC is judged to be the lowest concentration of an antifungal agent that substantially inhibits the growth of the organism. For azoles, such as fluconazole and ketoconazole,

a less stringently defined endpoint allowing for slight turbidity above the MIC is recommended (3, 5, 10). In determining the endpoint for the azoles, the turbidities in all tubes are estimated visually and the MIC is defined as the lowest drug concentration that reduces growth by 80% relative to the growth control (MIC 80%) (3, 10, 17). The MIC 80% endpoint may be estimated by diluting the drug-free growth control tube 1:5 with test medium. This approach to the determination of MIC endpoints has been proposed for broth macrodilution testing of fluconazole and other azoles (3, 10).

In the present study, we describe a comparative evaluation of methods for macro- and microdilution susceptibility testing of fluconazole. MICs obtained by using the proposed NCCLS reference macrodilution method (MIC 80% read at 48 h) were compared with those obtained with a microdilution method performed according to NCCLS guidelines and a colorimetric microdilution method with an oxidation-reduction indicator (Alamar Blue; Alamar Biosciences Inc., Sacramento, Calif.) for 119 clinical isolates of *C. albicans*. The purpose of this study was to (i) provide additional data comparing microdilution testing of fluconazole with the reference macrodilution method and (ii) compare a novel colorimetric oxidation-reduction method of microdilution endpoint determination (Alamar) with the macro- and microdilution methods performed according to NCCLS guidelines.

### MATERIALS AND METHODS

**Antifungal drugs.** Fluconazole powder was obtained from Pfizer Inc. (New York, N.Y.). Upon receipt, a concentrated stock solution (12,800 µg/ml) was prepared in dimethyl formamide and frozen at -60°C until used.

**Test organisms.** One hundred nineteen strains of *C. albicans* were selected for testing. The strains were all recent

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clinical isolates from individuals with oropharyngeal candidiasis. Oropharyngeal swabs were plated onto Sabouraud dextrose agar plates (BBL), and the plates were incubated for 48 h at 25°C. Colonies of *C. albicans* were streaked for isolation onto Sabouraud dextrose agar and identified by standard methods (20). Single colonies were picked and were stored as suspensions in water at ambient temperature until used in the study. Prior to testing, each isolate was passaged at least twice on Sabouraud dextrose agar (Difco, Detroit, Mich.) to ensure optimal growth characteristics.

**Antifungal susceptibility test methods.** Each isolate was tested by the reference macrodilution method and by two microdilution methods. Care was taken to follow NCCLS guidelines for inoculum preparation, medium formulation, and incubation (10). Quality control was performed with a strain of *Candida parapsilosis* (ATCC 90018) recommended for this purpose in NCCLS proposed standard M27-P (10).

(i) **Macrodilution reference method.** Broth macrodilution testing was performed according to NCCLS guidelines. Briefly, testing was performed with twofold drug dilutions in RPMI 1640 medium (Sigma, St. Louis, Mo.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma). The stock solution of fluconazole was diluted by previously described methods recommended for minimizing systematic pipetting errors (10). Final fluconazole concentrations were 0.125 to 64 µg/ml.

Yeast inocula were prepared as described in proposed standard M27-P (10). Yeast cells were grown on Sabouraud dextrose agar for 48 h at 30°C, and the inoculum suspension was prepared by picking five colonies of at least 1 mm in diameter and suspending the material in 5 ml of sterile 0.85% saline. The resulting suspension was vortexed for 15 s, and the cell density was adjusted with a spectrophotometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard at a 530-nm wavelength. The final inoculum suspension was made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension with RPMI 1640 broth medium, which resulted in  $0.5 \times 10^3$  to  $2.5 \times 10^3$  cells per ml. In each case, the inoculum size was verified by enumeration of CFU obtained by subculture on Sabouraud dextrose agar.

Yeast inocula (0.9 ml) were added to sterile plastic tubes (12 by 75 mm) containing 0.1-ml aliquots of fluconazole solution (10× final concentration) by using sterile individual 1-ml pipettes. The contents of the tubes were mixed by inversion. The tubes were incubated in air at 35°C and were inspected after 48 h of incubation. Drug-free and yeast-free controls were included.

(ii) **Reference MIC endpoint reading.** Each tube was flicked immediately prior to being scored, and a MIC 80% was determined after 48 h of incubation by defining the MIC as the lowest drug concentration which resulted in a visual turbidity less than or equal to that of a 1:5 dilution of the growth control (0.2 ml of growth control plus 0.8 ml of uninoculated RPMI) (3, 10).

(iii) **Microdilution methods.** In order to assess microdilution methods for fluconazole susceptibility testing, each isolate was also tested by two microdilution methods. Both microdilution methods were performed according to the following NCCLS guidelines: use of the spectrophotometric method of inoculum preparation, an inoculum concentration of  $0.5$  to  $2.5 \times 10^3$  cells per ml, RPMI 1640 medium buffered to pH 7.0 with MOPS, and an additive twofold drug dilution procedure (10). Yeast inocula (100 µl) were added to each well of microdilution trays containing 100 µl of fluconazole solution (2× final concentration). The trays were incubated

TABLE 1. In vitro susceptibility of 119 *C. albicans* isolates to fluconazole by macro- and microbroth dilution methods

Test method	Incubation time (h)	MIC (µg/ml)		
		Range	50% <sup>a</sup>	90% <sup>a</sup>
Reference macrodilution <sup>b</sup>	48	≤0.12–>64	0.5	16
Micro MIC-2	24	≤0.12–64	0.25	8.0
	48	≤0.12–>64	0.5	8.0
Colorimetric microdilution	24	≤0.12–>64	0.5	16
	48	≤0.12–>64	1.0	64

<sup>a</sup> 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

<sup>b</sup> Macrodilution method performed according to NCCLS proposed standard M27-P (MIC 80% read at 48 h) (10).

in air at 35°C and were inspected after 24 and 48 h of incubation. Drug-free and yeast-free controls were included.

In one set of microdilution plates, the MIC endpoints were assessed after 24 and 48 h of incubation by the MIC-2 approach described by Espinel-Ingroff et al. (3). The broth microdilution wells were scored with the aid of a reading mirror; the growth in each well was compared with that in the growth control (drug-free) well. A numerical score, which ranged from 0 to 4, was given to each well by using the following scale: 0, optically clear; 1, slightly hazy; 2, prominent decrease in turbidity; 3, slight reduction in turbidity; and 4, no reduction in turbidity. The MIC was defined as the lowest concentration of fluconazole with which a score of 2 (prominent decrease in turbidity) was observed (Micro MIC-2). In the second set of microdilution plates, the MIC was determined colorimetrically by adding an oxidation-reduction indicator (Alamar Blue; Alamar Biosciences Inc.) to each well at the time of inoculation (25 µl of Alamar Blue per well). The plates were then incubated at 35°C and read at 24 and 48 h. Growth in each well was indicated by a color change from dark blue to red. The colorimetric microdilution MIC was defined as the lowest concentration of fluconazole preventing the development of a red color (first blue well).

**Analysis of results.** All microdilution MICs (Micro MIC-2 and colorimetric MICs read at 24 and 48 h) were compared with the NCCLS reference macrodilution MICs (MIC 80% read at 48 h). Both on-scale and off-scale results were included in the analysis. The high off-scale MICs (>64 µg/ml) were converted to the next highest concentration (128 µg/ml), and the low off-scale MICs (≤0.125 µg/ml) were left unchanged. When skips (uneven patterns) were present, the MIC endpoint was the higher drug concentration. Discrepancies among MIC endpoints of no more than 2 dilutions (two tubes or wells) were used to calculate the percent agreement.

## RESULTS

Table 1 summarizes the in vitro susceptibility of 119 *C. albicans* isolates to fluconazole as judged by the three test methods. Overall, a total of 595 MICs were evaluated. The data are reported as the concentrations of fluconazole necessary to inhibit 50% (MIC<sub>50</sub>) and 90% (MIC<sub>90</sub>) of the isolates tested. The MIC<sub>50</sub>s were comparable for all test methods and ranged from 0.25 to 1.0 µg/ml. The MIC<sub>90</sub>s were considerably higher and ranged from 8.0 to 16 µg/ml at 24 h and from 8.0 to 64 µg/ml at 48 h of incubation. The values obtained with the reference macrodilution and the colorimetric microdilution methods tended to be higher than

TABLE 2. Percent agreement of two microdilution test methods with the macrodilution reference method for testing fluconazole<sup>a</sup>

Test method	Incubation time (h)	% Agreement with reference MIC <sup>b</sup>
Micro MIC-2	24	94
	48	93
Colorimetric microdilution	24	94
	48	91

<sup>a</sup> Percent fluconazole MICs within fourfold of those obtained by the macrodilution reference method for 119 isolates of *C. albicans*.

<sup>b</sup> The reference macrodilution method was performed according to NCCLS proposed standard M27-P (MIC 80% read at 48 h) (10).

those obtained with the Micro MIC-2 method. This was especially true for the colorimetric method at the 48-h readings.

The percentage of fluconazole MICs within fourfold of the macrodilution reference values for the two microdilution methods is shown in Table 2. The agreement with the macrodilution reference values was 94% for each of the microdilution approaches at 24 h. The variation in the 48-h microdilution MICs was only slightly greater, and the level of agreement with the reference method ranged from 91 to 93%.

In addition to excellent agreement with the macrodilution reference method, a high level of agreement was observed between the two microdilution methods at 24 and 48 h. Comparison of the 24-h Micro MIC-2 and colorimetric microdilution MICs resulted in an agreement of 97%. A high level of agreement (93%) was also seen when the 48-h colorimetric microdilution MICs were compared with 48-h Micro MIC-2 values.

## DISCUSSION

The results of the present study confirm and extend those of previous comparisons of macro- and microdilution methods for testing fluconazole (3, 4). In an NCCLS collaborative study reported by Espinel-Ingroff et al. (3), excellent agreement between macro- and microdilution fluconazole MICs was obtained. Furthermore, the fluconazole macro- and microdilution MIC determinations showed good agreement among laboratories, with 93 and 89% interlaboratory agreement, respectively. In agreement with the study of Espinel-Ingroff et al. (3), we found a 94% agreement between the NCCLS reference macrodilution method and microdilution fluconazole susceptibility tests using the MIC-2 endpoint criteria read at 24 h. The agreement was only slightly lower (93%) at 48 h. Thus, the microdilution method for testing fluconazole is entirely comparable to the NCCLS reference macrodilution method and provides the potential clinical advantage of an earlier MIC determination. Our results with MIC-2 and 24 h of incubation further support the suggestion by Espinel-Ingroff et al. (3) that the microdilution test is an adequate tool for antifungal susceptibility testing when performed by following NCCLS standards for macrodilution susceptibility testing of yeasts.

The use of an oxidation-reduction indicator is a novel approach to defining the MIC endpoint for antifungal susceptibility testing. Previously, Tellier et al. (19) described a drug susceptibility assay based on the yeast reduction of a tetrazolium salt and quantitation of the resulting formazan solution. Their assay required the addition of an electron-coupling agent and a centrifugation step prior to reading the

tubes in a spectrophotometer. This method allowed the generation of dose-response curves that were a reliable indicator of the number of viable yeast cells present and allowed the determination of a metabolic MIC by precise criteria (90% reduction in optical density relative to the control). In the present study, we have adapted the microdilution assay which we performed according to NCCLS proposed standards to a colorimetric format by the simple addition of a commercially available oxidation-reduction indicator, Alamar Blue (Alamar Biosciences Inc.). The result is a colorimetric microdilution method which provides clear MIC endpoints for fluconazole that can be determined visually and which does not require the addition of electron-coupling agents or centrifugation. The agreement of the Alamar microdilution MIC readings with the standard macrodilution MIC 80% and Micro MIC-2 readings was excellent: 94 and 97%, respectively. The method is simple to perform and may provide a more objective and more reproducible endpoint than either the macrodilution MIC 80% or the Micro MIC-2 determination. As with the tetrazolium-based assay of Tellier et al. (19), this assay may be read spectrophotometrically and has the potential for quantitation and automation.

It should be noted that the present study was limited to clinical isolates of *C. albicans* tested against fluconazole. Studies to evaluate the applicability of these in vitro test methods for the testing of other antifungal agents, including other azoles, and other species of yeasts, including non-*C. albicans* *Candida* spp., *Torulopsis glabrata*, and *Cryptococcus neoformans*, are ongoing but not yet published. Likewise, preliminary data suggest that adaptation of the macrodilution and microdilution methods described for yeasts may be useful for testing molds such as *Aspergillus fumigatus* (15); however, further study is clearly indicated. Finally, it must be stated that the clinical role of these in vitro tests is not well documented, and it is not recommended that they be performed routinely (10, 15, 17). Interpretive breakpoints for antifungal agents obtained by using these methods have not been established and await clinical trials. The limited data regarding the correlation of in vitro testing with in vivo outcome have been reviewed recently by Rex et al. (17), and the interested reader is referred to that review for additional discussion of this important matter.

In summary, we have evaluated two microdilution methods for determination of MICs of fluconazole for *C. albicans*. We have confirmed the excellent agreement between macro- and microdilution methods performed according to NCCLS guidelines. Additionally, we have proposed the use of a colorimetric endpoint determined by using a commercially available oxidation-reduction indicator to provide an objective, easy-to-read MIC with a microdilution format. These studies demonstrate further progress in adapting the recently proposed NCCLS standard macrodilution method to a format that may be more practical and user-friendly in the clinical laboratory.

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