

## Fluconazole Disk Diffusion Test with Methylene Blue- and Glucose-Enriched Mueller-Hinton Agar for Determining Susceptibility of *Candida* Species

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**A 25- $\mu$ g fluconazole disk diffusion test using a Mueller-Hinton agar plate containing 2% glucose and 5  $\mu$ g of methylene blue/ml (GM-MH) was compared to the macrodilution reference method for 210 *Candida* species. The GM-MH agar plate was read at 24 h. The predictive values of disks with susceptible, intermediate, and resistant results on the GM-MH agar plate at 24 h were 97.1, 56.3, and 76.5%, respectively.**

Fungal infections are still an important cause of mortality and morbidity in immunocompromised patients (1, 7, 10). Resistance to antifungal agents is emerging and in vitro susceptibility data are required to guide the selection of antifungal chemotherapy (4, 6, 9, 15). A broth microdilution test derived from the standard reference test and the E test are simplified tests but are not cost-effective enough to be performed routinely for every yeast isolate in most clinical laboratories (3, 12, 14, 16). A simplified disk diffusion test using an RPMI 1640–glucose agar plate has been reported and was found to correlate well with the standard reference test (2, 11). We investigated the possibility and reliability of the fluconazole disk diffusion test using a less expensive medium.

Fluconazole testing powder was obtained from Pfizer Inc. America. RPMI broth was purchased from Sigma Chemical Company of America and was prepared with glutamine and buffered to pH 7.0 with morpholinepropanesulfonic acid (MOPS) organic buffer. Mueller-Hinton (MH) agar was purchased from Difco Laboratories and was solidified after the addition of 2% glucose and 5  $\mu$ g of methylene blue/ml (GM). Both glucose and methylene blue were obtained from Sigma Chemical Company. The isolates stored at  $-70^{\circ}\text{C}$  were subcultured onto a Sabouraud dextrose agar plate. After overnight culture, about five isolated colonies were then suspended in 5 ml of sterile saline. After adequate mixing with a vortex mixer, the turbidity of the suspension was adjusted to that of a McFarland 0.5 turbidity standard by addition of sufficient sterile saline to yield a suspension of  $1 \times 10^6$  to  $2 \times 10^6$  CFU/ml, which was confirmed by quantitative subculture (8). One-half milliliter of the McFarland 0.5 suspension was added to 4.5 ml of sterile saline. After adequate mixing, 1 ml of the resulting suspension was added to 4 ml of sterile saline to yield a final concentration of  $2 \times 10^4$  to  $4 \times 10^4$  CFU/ml, which was confirmed by quantitative subculture. Both a simple MH agar

plate and a GM-MH agar plate were inoculated with a swab moistened in the final inoculum suspension. One 25- $\mu$ g fluconazole disk (Pfizer Inc. America) was added to each inoculated plate. All tests were incubated in air at  $35^{\circ}\text{C}$ , and the results were read after 24 and 48 h of incubation. MICs were determined after 24 and 48 h by the NCCLS macrodilution method (8). For agar tests, inhibitory zones were measured at the point where there was a sharp decline in the amount of growth (approximately 80% inhibition). Two hundred ten isolates of *Candida* spp. were collected from the clinical laboratory of Chang Gung Memorial Hospital between January and December 1998, including 150 *Candida albicans*, 18 *C. glabrata*, 16 *C. tropicalis*, 14 *C. guilliermondii*, 9 *C. parapsilosis*, 1 *C. intermedia*, 1 *C. sake*, and 1 *C. utilis* isolate. Quality control strains included *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019, and *C. krusei* ATCC 6258.

Table 1 gives geometric mean MICs obtained by the NCCLS test for each species group. Table 2 compares the 48-h MIC category obtained by the NCCLS reference method with the results of fluconazole disk test using a GM-MH agar plate. Correlation between 24-h zone sizes on the GM-MH agar plate and 48-h MICs was excellent. Correlation between 48-h

TABLE 1. MICs of fluconazole against 210 *Candida* spp. according to NCCLS macrodilution method M27-A with RPMI 1640 broth

Species (no. of isolates) <sup>a</sup>	Geometric mean MIC <sup>b</sup> (range) at:	
	24 h	48 h
<i>C. albicans</i> (150)	1.99 (0.125–64)	6.71 (0.125–128)
<i>C. glabrata</i> (18)	5.91 (0.125–64)	18.43 (0.125–64)
<i>C. tropicalis</i> (16)	1.16 (0.125–4)	4.84 (0.125–32)
<i>C. guilliermondii</i> (14)	39.17 (0.125–64)	68.71 (1–128)
<i>C. parapsilosis</i> (9)	0.90 (0.125–2)	2.07 (0.125–8)
<i>C. intermedia</i> (1)	8	8
<i>C. sake</i> (1)	64	64
<i>C. utilis</i> (1)	0.125	1

<sup>a</sup> Numbers of isolates for which the MIC was  $\geq 8$   $\mu\text{g/ml}$  at 48 h are as follows: *C. albicans*, 15; *C. glabrata*, 8; *C. tropicalis*, 3; *C. guilliermondii*, 12; *C. parapsilosis*, 1; *C. intermedia*, 1; *C. sake*, 1.

<sup>b</sup> In micrograms per milliliter.

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TABLE 2. Comparisons of 24- and 48-h disk diffusion susceptibility tests using the GM-MH agar plate with the NCCLS macrodilution test

MIC 48-h category with reference method <sup>a</sup> (MIC <sup>b</sup> )	No. of isolates classified as indicated by fluconazole disk test on GM-MH agar plate at:					
	24 h			48 h		
	≥19 mm (susceptible)	15–18 mm (intermediate)	≤14 mm (resistant)	≥19 mm (susceptible)	15–18 mm (intermediate)	≤14 mm (resistant)
Susceptible (≤8.0)	172	1	1	140	13	21
S-DD <sup>c</sup> (16)	2	5	0	1	1	5
S-DD (32)	2	4	3	0	0	9
Resistant (≥64)	1	6	13	1	3	16

<sup>a</sup> NCCLS broth dilution. In comparison with the NCCLS test, the overall predictive values of susceptible, intermediate, and resistant disk test results were 97.1, 56.3, and 76.5% at 24 h and 98.6, 5.9, and 31.4% at 48 h, respectively; predictive values for *C. albicans* were 97.8, 50, and 85.7% at 24 h and 99.3, 100, and 58.3% at 48 h, respectively; the predictive values for other *Candida* species were 94.7, 58.3, and 77.8% at 24 h and 80.0, 0, and 23.1% at 48 h, respectively.

<sup>b</sup> In micrograms per milliliter.

<sup>c</sup> S-DD, susceptible, dose-dependent.

zone sizes on the GM-MH agar plate and 48-h MICs was inferior to that of 24-h zone sizes (Table 2). Correlations between zone sizes at 24 and 48 h on a simple MH agar plate and 48-h MICs were also inferior to that between 24-h zone sizes on a GM-MH agar plate and 48-h MICs. The MICs for control strains were within the range proposed by NCCLS in document M27-A (8, 13). All disk tests on the GM-MH agar plate at 24 h gave zones within the following ranges for the control strains: for *C. albicans* ATCC 90028, 32 to 43 mm; for *C. parapsilosis* ATCC 22019, 26 to 37 mm; for *C. krusei* ATCC 6258, 6 to 17 mm. These results are consistent with fluconazole zone sizes obtained for these control strains on an RPMI 1640 agar plate in a prior study (2).

The predictive values of this method we propose at 24 h are not inferior to the predictive values of the disk diffusion method using an RPMI-glucose agar plate at 48 h. Barry and Brown reported that the subjectivity of zone size measurements added an important source of variability to the test with RPMI-glucose agar due to the trailing phenomenon around zone edges (2). We also found this problem with the fluconazole disk diffusion test using a simple MH agar plate. However, trailing phenomena around the zone margin were infrequent and minimal on the GM-MH agar plate, unlike the situation with simple MH agar. Zone edges with this method were frequently definite and clear, facilitating the measurement of zone sizes and minimizing subjectivity in zone size measurements. The occurrence of macrocolonies near the center of the clear zone was also infrequent with this method. The addition of methylene blue to the GM-MH agar plate made the GM-MH agar plate slightly blue so that it could easily be identified and differentiated from the simple MH agar plate. Moreover, the methylene blue in the GM-MH agar plate also stained the *Candida* colony on the plate slightly blue so that it could be identified more easily than on a simple MH agar plate. In this study, we found that using a *Candida* suspension with a higher concentration of  $2 \times 10^4$  to  $4 \times 10^4$  CFU/ml for inoculation on the GM-MH agar plate produced a good layer of growth for interpretation of results at 24 h of incubation, unlike Barry and Brown's study, which used  $0.5 \times 10^3$  to  $2.5 \times 10^3$  CFU/ml for inoculation on an RPMI-glucose agar plate and 48 h of incubation for interpretation of results.

Our study also showed that the disk test with GM-MH agar did not adequately separate fully resistant strains from those with dose-dependent susceptibilities, consistent with the disk test with an RPMI 1640 agar plate (2). This problem is not

unique to disk tests for antifungal agents. Disk tests for antibacterial agents may also have this problem. Since the predictive value of a susceptible disk result with a GM-MH agar plate at 24 h is 97.1% in our study, any *Candida* isolate screened and found susceptible by this disk test can be reported susceptible without further testing. Of our isolates, 82.9% (174 of 210) fell into this category. The other isolates that were not susceptible by the disk test should undergo more-precise procedures such as NCCLS macrodilution tests or microdilution tests (5). We conclude that fluconazole disk tests on an MH agar plate containing 2% glucose and 5  $\mu$ g of methylene blue/ml at 24 h can be used as a routine screening procedure for susceptibility of *Candida* species to fluconazole in clinical laboratories. This improved simple method is sufficiently accurate and cost-effective for routine testing.

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