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Evaluation of the NCCLS M44-P Disk Diffusion Method for Determining Susceptibilities of 276 Clinical Isolates of *Cryptococcus neoformans* to Fluconazole

M. A. Pfaller,[1,2]* S. A. Messer,[1] L. Boyken,[1] C. Rice,[1] S. Tendolkar,[1] R. J. Hollis,[1] and D. J. Diekema[1,3]

Departments of Pathology[1] and Medicine,[3] Roy J. and Lucille A. Carver College of Medicine, and Department of Epidemiology, College of Public Health,[2] University of Iowa, Iowa City, Iowa 52242

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We evaluated the NCCLS M44-P fluconazole disk diffusion method in comparison with the NCCLS M27-A2 broth microdilution method for determining the susceptibility of 276 isolates of *Cryptococcus neoformans*. Disk diffusion testing was performed using Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg of methylene blue/ml. Among the 276 isolates, 259 (93.8%) were susceptible, 16 (5.8%) were susceptible—dose dependent, and 1 (0.4%) was resistant to fluconazole as determined by the NCCLS broth microdilution method. The overall categorical agreement between the two methods was 86%, with 0% very major errors, 2% major errors, and 12% minor errors. The disk diffusion method using Mueller-Hinton agar supplemented with glucose and methylene blue appears to be a useful approach for determining the fluconazole susceptibility of C. *neoformans*.

Cryptococcus neoformans remains an important cause of opportunistic mycosis in both immunocompromised and immune-intact individuals (8, 10, 12–14, 18, 19). Treatment of cryptococcal meningitis includes the administration of amphotericin B with or without flucytosine as first-line agents, often followed by fluconazole for maintenance or consolidation therapy (19, 24). Given that in certain patient groups fluconazole therapy may be required for extended periods of time (24, 25), there is a concern about the development of resistance to this agent (6, 7). Performance of antifungal susceptibility testing on the infecting isolate has recently been suggested as an aid in the management of these difficult patients (23).

In vitro susceptibility testing of fluconazole may be performed using either broth-based or agar-based methods (1, 11, 20, 26). Recently the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Testing has proposed an agar disk diffusion method, M44-P, for testing of fluconazole against yeasts (2, 3, 5, 17). This method has been shown to be accurate and precise and correlates well with the NCCLS broth microdilution (BMD) MIC method when testing *Candida* (5, 21). Zone diameter interpretive criteria have been developed along with reference MIC correlates for the categories of susceptible (S) (19 mm [≤8 μg/ml]), susceptible—dose dependent (SDD) (15 to 18 mm [16 to 32 μg/ml]), and resistant (R) (≤14 mm [≥64 μg/ml]) (17). Although the NCCLS BMD method has been used to determine the susceptibility of *C. neoformans* to fluconazole (6, 20, 26), comparable studies of the NCCLS proposed disk diffusion method have not been published. As with *Candida*, disk diffusion testing may provide a faster, simpler method for determining the in vitro susceptibility of *C. neoformans* to fluconazole (5).

The purpose of this study was to evaluate the M44-P disk test (17) for determining the in vitro susceptibility of *C. neoformans* to fluconazole. The disk diffusion zone diameters obtained for each isolate were compared to the MICs determined by the M27-A2 BMD method (16).

A total of 276 clinical isolates of *C. neoformans* were obtained from 60 different medical centers worldwide during 2001. All were incident clinical isolates obtained from cultures of cerebrospinal fluid or blood from 276 different patients with cryptococcosis. Isolates were identified by Vitek and API yeast identification systems (bioMerieux, INC., Hazelwood, Mo.), and identification tests with these systems were supplemented by conventional methods as needed (9). Isolates were stored as water suspensions until used in the study. Prior to testing, each

TABLE 1. Overall interpretive agreement between results of the fluconazole 48-h disk diffusion susceptibility test and of the standard 72-h BMD reference MIC test for 276 *C. neoformans* isolates

Method[a]	% of isolates by category[b]			% of discrepant results[c]			% Categorical agreement[d]
	S	S-DD	R	Minor	Major	Very major	
BMD	93.8	5.8	0.4				
Disk	87.3	8.3	4.4	12.0	2.0	0	86

[a] The BMD method was used according to the guidelines for the NCCLS M27-A2 method (16). The disk method was used according to the guidelines for the NCCLS M44-P method (17).

[b] Percentage of isolates classified in the different susceptibility categories. See Materials and Methods for definitions.

[c] Percentage of test results with minor, major, or very major discrepancies compared to the results of the reference BMD method at 72 h. See Materials and Methods for definitions.

[d] Agreement rates reflect the percentage of isolates classified in the same category by both the disk and the reference BMD methods.

* Corresponding author. Mailing address: Medical Microbiology Division, C606 GH, Department of Pathology, University of Iowa College of Medicine, Iowa City, Iowa 52242. Phone: (319) 384-9566. Fax: (319) 356-4916. E-mail: michael-pfaller@uiowa.edu.

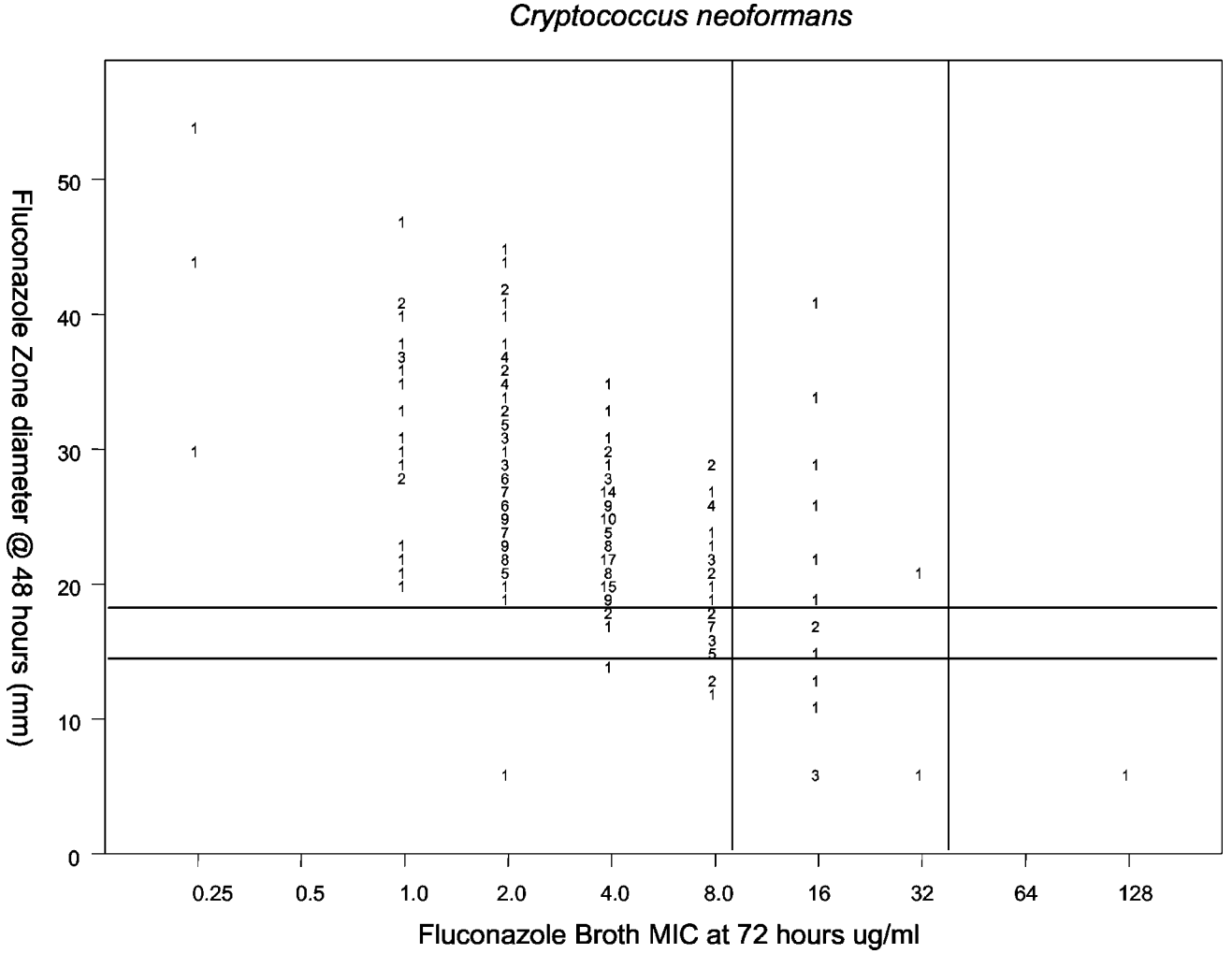

FIG. 1. Zones of inhibition around 25-μg fluconazole disks on Mueller-Hinton methylene blue agar plotted against the 72-h MICs determined by the reference BMD method for 276 isolates of *C. neoformans*. The least-squares method was used to calculate a regression line ($y = 67 - 3.9x$; $r = 0.6$). The horizontal lines indicate the S (≥19 mm) and R (≤14 mm) zone diameter breakpoints for the fluconazole disk test. The vertical lines indicate the S (≤8 μg/ml) and R (≥64 μg/ml) MIC breakpoints for fluconazole. The numbers inside the graph indicate numbers of isolates.

isolate was passaged on potato dextrose agar (Remel, Lenexa, Kans.) and CHROMagar (Hardy Laboratories, Santa Maria, Calif.) to ensure purity and viability.

Reference antifungal susceptibility testing of *C. neoformans* was performed according to the BMD method described by the NCCLS (16). The MIC endpoints were read visually following 72 h of incubation. Reference powder of fluconazole was obtained from Pfizer Pharmaceuticals (Groton, Conn.).

Disk diffusion testing of fluconazole was performed as described by Barry et al. (5) and in NCCLS document M44-P (17). Fluconazole (25-μg) disks were obtained from Becton Dickinson (Sparks, Md.) For disk diffusion testing, 150-mm-diameter plates containing Mueller-Hinton agar (Difco Laboratories) supplemented with 2% glucose and methylene blue (0.5 μg/ml) at a depth of 4.0 mm (67 to 70 ml) were used. The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to the turbidity of a 0.5 McFarland standard. The plates were incubated in air at 35°C and read at 48 h. Zone diameter endpoints were read at 80% growth inhibition by using the BIOMIC image analysis plate reader system (version 5.9; Giles Scientific, Santa Maria, Calif.) (21).

MIC interpretive criteria for fluconazole were those published by the NCCLS (16) and were as follows: S, MIC of ≤8 μg/ml; S-DD, MIC of 16 to 32 μg/ml; R, MIC of ≥64 μ/ml. The interpretive criteria for the fluconazole disk test were those published by Barry et al. (5) and the NCCLS (17): S, zone diameter of ≥19 mm; S-DD, zone diameter of 15 to 18 mm; R, zone diameter of ≤14 mm.

Quality control (QC) was performed for BMD in accordance with NCCLS document M27-A2 (16) by using *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 (4, 16). QC determinations made on each day of testing were within the control limits for fluconazole described by Barry et al. (4). QC for disk testing was performed by using *C. albicans* ATTC 90028 (28 to 39 mm) and *C. parapsilosis* ATTC 22019 (22 to 33 mm) (2, 17).

The diameters of the zones of inhibition (in millimeters) surrounding the fluconazole disk at 48 h of incubation were

plotted against their respective BMD MICs read at 72 h (5). The least-squares method was used to calculate a regression line for the comparison. The interpretive breakpoints described by Barry et al. (5) and the NCCLS (17) were used to determine the categorical agreement between the disk diffusion and BMD results for fluconazole. Major errors were identified as a classification of R by the disk test and S by BMD, very major errors were identified as a classification of S by the disk test and R by BMD, and minor errors occurred when the result of one of the tests was S or R and that of the other test was SDD (15).

Using the interpretive breakpoints developed for fluconazole BMD testing and *Candida* spp. (22), 259 of the 276 isolates of *C. neoformans* (93.8%) were classified as S, 16 (5.8%) were S-DD, and 1 (0.4%) was R (Table 1). Although precise MIC breakpoints for fluconazole susceptibility in the treatment of cryptococcosis have not been determined, it appears that MICs greater than 16 μg/ml may be seen with isolates from relapsed patients with prior exposure to fluconazole (1, 19). If isolates with fluconazole MICs of >16 μg/ml are considered R, the number of resistant strains in this collection would increase to three (1% of total). The supplemented Mueller-Hinton agar used for the disk test supported the growth of all 276 isolates and allowed results to be determined 24 h earlier than was required for BMD. Figure 1 shows the correlation of the 25-μg fluconazole disk zone diameters read at 48 h with the 72-h BMD MIC results. The regression statistics ($y = 67 - 3.9x$; $r = 0.6$) show a good level of agreement between the two methods. The overall categorical agreement by use of the interpretive criteria of Barry et al. (5) and the NCCLS (16, 17) was 86% with no very major errors, 2% major errors, and 12% minor errors (Table 1). If one considered MICs for fluconazole of ≥32 μg/ml to indicate R, and using the same zone diameter breakpoints, the overall agreement would increase slightly to 86.6% with one very major error, 2% major errors, and 11% minor errors (data not shown).

The results of this study expand the application of the fluconazole disk diffusion test to include the testing of *C. neoformans*. The study was limited by the small number of resistant isolates. Only one isolate was clearly resistant (MIC, ≥64 μg/ml), and two were possibly resistant (MIC = 32 μg/ml) (Fig. 1). However, the disk test provided a more conservative estimate of in vitro susceptibility to fluconazole, classifying more isolates as S-DD and R than the BMD test (Table 1). The vast majority of errors were minor, resulting from shifts between S-DD and S (Table 1). The disk diffusion method using Mueller-Hinton agar supplemented with glucose and methylene blue appears to be a useful approach for determining the susceptibility of *C. neoformans* to fluconazole.

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