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# Comparative Evaluation of National Committee for Clinical Laboratory Standards Broth Macrodilution and Agar Dilution Screening Methods for Testing Fluconazole Susceptibility of *Cryptococcus neoformans*

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**A simple screening method for fluconazole susceptibility of *Cryptococcus neoformans* using 2% dextrose Sabouraud dextrose agar (SabDex) with fluconazole was compared to the National Committee for Clinical Laboratory Standards (NCCLS) broth macrodilution method. By this method, fluconazole-susceptible *C. neoformans* isolates are significantly smaller on medium with fluconazole than on fluconazole-free medium. Isolates with decreased susceptibility have normal-size colonies on medium containing fluconazole. The 48-h NCCLS broth macrodilution MICs (NCCLS MICs) for isolates with normal-size colonies on 8- or 16-μg/ml fluconazole plates were predicted to be ≥8 or ≥16 μg/ml, respectively. On medium with 16 μg of fluconazole per ml, all strains (84 of 84) for which the NCCLS MICs were <16 μg/ml were correctly predicted, as were all isolates (7 of 7) for which the MICs were ≥16 μg/ml. Agar dilution appears to be an effective screening method for fluconazole resistance in *C. neoformans*.**

Serious fungal infections in immunocompromised patients are increasing in frequency (3–5, 10). Cryptococcal meningitis, caused by *Cryptococcus neoformans*, remains incurable in the population with AIDS (2, 4, 13). In this setting, the necessary long-term suppressive therapy with antifungal agents may lead to selection of resistant isolates (4, 6). Clinical resistance in *Candida* spp. is becoming a serious problem (7, 11, 21). While antifungal resistance in *Cryptococcus* is uncommon, the MICs for some isolates are elevated (4, 6). A rapid, reproducible method for detecting fluconazole resistance would be useful in determining the epidemiology of and optimal treatment for resistant isolates (9, 18, 20).

Recently, a standardized broth macrodilution technique for yeast susceptibility testing has been accepted (9, 12). This technique requires considerable time and expense and is not easily applicable for screening purposes, even with microdilution modifications (1). The National Committee for Clinical Laboratory Standards (NCCLS) method for fluconazole susceptibility testing of invasive yeasts includes both *Candida* spp. and *C. neoformans* (19) yet establishes susceptibility breakpoints for only *Candida* spp. (12). The utility of susceptibility testing of *C. neoformans* remains controversial (10, 12, 17, 22). We have developed a susceptibility screening method by adding fluconazole to 2% dextrose Sabouraud dextrose agar (2% SabDex) which allows detection of yeasts with decreased fluconazole susceptibility (14, 15). In this study, agar screening was compared with the NCCLS macrodilution method for determining fluconazole susceptibility of *C. neoformans*.

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## MATERIALS AND METHODS

**Clinical isolates.** Ninety-one clinical *C. neoformans* isolates that were submitted to the University of Texas Health Science Center Fungus Testing Laboratory (San Antonio, Tex.) and Yale-New Haven Hospital (New Haven, Conn.) for MIC determination by NCCLS methodology (12) were subcultured and evaluated blindly by the agar dilution method.

**2 and 4% SabDex.** Two-percent dextrose Sabouraud liquid broth modified antibiotic medium 13 (BBL, Cockeysville, Md.), which contains a final concentration of 20 g of dextrose per liter, was prepared from a powdered medium as suggested by the manufacturer. Bacto Agar (15 g/liter; Difco Laboratories, Detroit, Mich.) was added to a 1.5% final concentration. In addition, 4% SabDex (BBL), which contains a final dextrose concentration of 40 g/liter and agar at 15 g/liter, was prepared from a powdered medium as suggested by the manufacturer. Each medium was brought to a boil in sterile water for 15 to 30 s to dissolve the powdered medium and the agar and was cooled to 45°C in a water bath. Fluconazole intravenous solution (2 mg/ml; Pfizer-Roerig, New York, N.Y.) was added to the media at 45°C, with thorough stirring, to give final concentrations of 8 and 16 μg of fluconazole per ml. These solutions were maintained at 45°C and thoroughly stirred. Approximately 20 ml was poured into sterile 100-mm-diameter petri plates and allowed to cool and harden before use. Hardened plates were stored at 4°C for up to 1 week prior to use.

**CHROMagar Candida.** CHROMagar Candida (CHROMagar, Paris, France) was prepared from powdered medium according to the manufacturer's instructions, with the addition of fluconazole to give 8- and 16-μg/ml concentrations. The prepared medium, which contains chloramphenicol (0.5 g/liter) and agar (15 g/liter), was dispensed (20 ml) into plates and stored as described above.

**Plating and interpretation: susceptibility testing.** Fluconazole was added to the media to differentiate resistant yeasts from susceptible yeasts. From each isolate stock (several isolated colonies placed in 3 ml of sterile, deionized $H_2O$), a sterile 10-μl loop was used to inoculate a set of three medium plates containing 0, 8, and 16 μg of fluconazole per ml. Samples were applied to one half of each plate. Plates were incubated at 30°C for 48 and 72 h prior to assessment of growth. Results from the fluconazole-containing medium were recorded as susceptible or mycologically resistant based on growth characteristics. Colonies that demonstrated growth on medium with fluconazole (usually visualized as pinpoint-sized colonies) that was suppressed compared to growth on medium without fluconazole were recorded as susceptible (Fig. 1). Colonies that demonstrated growth that was indistinguishable on medium with or without fluconazole were recorded as mycologically resistant. Results in this study were independently read by two laboratory personnel. Agar dilution susceptibility was tested

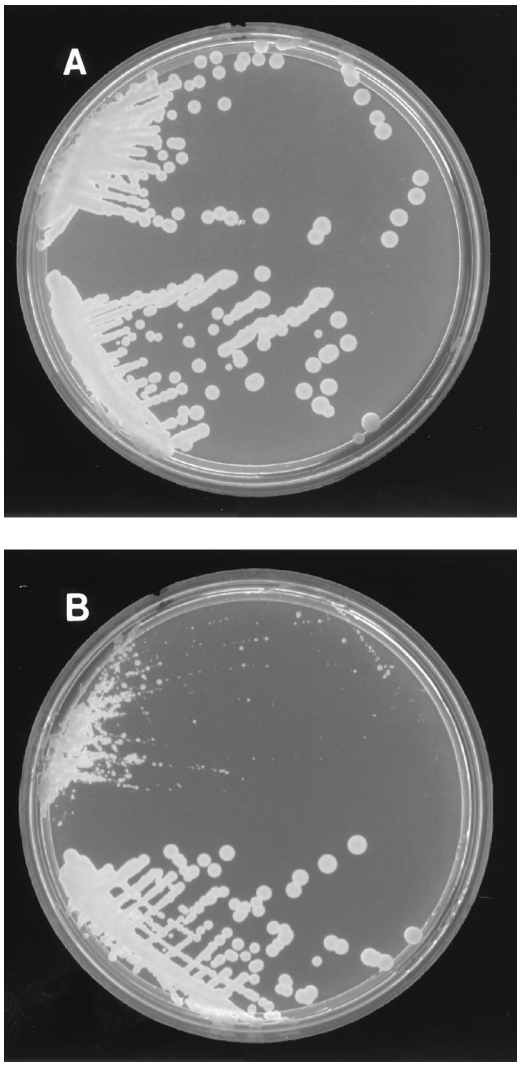


FIG. 1. Growth of susceptible *C. neoformans* (tops of plates) and *C. neoformans* with decreased fluconazole susceptibility (bottoms of plates) on 2% SabDex without fluconazole (A) and on 2% SabDex with 8 μg of fluconazole per ml (B).

on groups of 15 to 25 isolates. Control isolates with known susceptibilities were included for comparison with test samples.

## RESULTS

Ninety-one clinical isolates of *C. neoformans* were evaluated by broth macrodilution testing and by agar dilution. A wide range of fluconazole MICs (≤0.125 to >64 μg/ml) were detected, with 13 of 91 isolates (14%) inhibited by ≥8 μg/ml (48-h NCCLS broth macrodilution MICs [referred to hereafter in this work as NCCLS MICs]). Growth of susceptible *C. neoformans* isolates could be easily differentiated from resistant yeast isolates on fluconazole-containing agar by colony morphology. On 2% SabDex without fluconazole (Fig. 1A), growth characteristics of the susceptible *C. neoformans* (Fig. 1A, top) and *C. neoformans* with decreased fluconazole susceptibility (Fig. 1A, bottom) could not be distinguished. Fluconazole-impregnated medium allowed distinction of fluconazole-susceptible *C. neoformans* isolates (Fig. 1, tops of plates) from *C. neoformans* with decreased susceptibility (Fig. 1, bottoms of plates). The fluconazole-containing medium suppressed growth of the susceptible strain (Fig. 1B), seen as only pinpoint colonies (Fig. 1B, top), whereas colonies with decreased fluconazole susceptibility were seen to have normal growth characteristics (Fig. 1B, bottom).

TABLE 1. Correlation between NCCLS MICs and predicted MICs on 2% SabDex agar with 8 μg of fluconazole per ml

| Predicted 2% SabDex agar MIC (μg/ml) | No. (%) of cultures for which NCCLS MIC (μg/ml) was: | |
|---|---|---|
| | <8 ($n$ = 81) | ≥8 ($n$ = 10) |
| <8 | 72 (89) | 1[a] |
| ≥8 | 9[b] | 9 (90) |

[a] The NCCLS MIC for this *C. neoformans* isolate was 8 μg/ml.
[b] The NCCLS MICs for one, three, and five *C. neoformans* isolates were 1, 2, and 4 μg/ml, respectively. The NCCLS MICs for 78% of the *C. neoformans* isolates predicted to be mycologically resistant were ≥4 μg/ml.

Medium containing 8 μg of fluconazole per ml correctly detected 72 of 81 strains for which the NCCLS MICs were <8 μg/ml and 9 of 10 strains for which the NCCLS MICs were ≥8 μg/ml as well (Table 1). One isolate for which the NCCLS MIC was predicted to be <8 μg/ml was found to be inhibited by 8 μg/ml (NCCLS MIC), while nine isolates for which the NCCLS MICs were predicted to be ≥8 μg/ml were inhibited by 1 ($n$ = 1), 2 ($n$ = 3), and 4 ($n$ = 5) μg/ml (NCCLS MICs). Overall agreement on the 8-μg/ml fluconazole plates was within 1 $\log_2$ broth macrodilution for 87 of 91 isolates (96%). 2% SabDex agar containing 16 μg of fluconazole per ml correctly detected 84 of 84 strains for which the NCCLS MICs were <16 μg/ml as well as 7 of 7 strains for which the NCCLS MICs were ≥16 μg/ml (Table 2).

Studies using either 4% SabDex or CHROMagar medium were less successful in determining susceptibility. 4% SabDex or CHROMagar containing 8 μg of fluconazole per ml correctly detected 7 of 17 strains (41%) or 9 of 17 strains (53%), respectively, for which the NCCLS MICs were <8 μg/ml, and 6 of 6 strains (both media) for which the NCCLS MICs were ≥8 μg/ml. Examination of these isolates on either 4% SabDex or CHROMagar medium containing 16 μg of fluconazole per ml correctly detected 13 of 20 strains (65%) or 9 of 20 strains (45%), respectively, for which the NCCLS MICs were <16 μg/ml and 3 of 3 (both media) strains for which the NCCLS MICs were ≥16 μg/ml.

The sensitivities of correctly predicting yeasts with increased resistances by normal colony growth on 2% SabDex medium containing 8 or 16 μg of fluconazole per ml were 90 and 100%, respectively. The specificities of correctly predicting isolates to be fluconazole susceptible based on suppressed growth on 2% SabDex media containing fluconazole at either 8 or 16 μg/ml were 89 and 100%, respectively.

TABLE 2. Correlation between NCCLS MICs and predicted MICs on 2% SabDex agar with 16 μg of fluconazole per ml

| Predicted 2% SabDex agar MIC (μg/ml) | No. (%) of cultures for which NCCLS MIC (μg/ml) was: | |
|---|---|---|
| | <16 ($n$ = 84) | ≥16 ($n$ = 7) |
| <16 | 84 (100) | 0 |
| ≥16 | 0 | 7 (100) |

## DISCUSSION

Screening *C. neoformans* for susceptibility to antifungals has been difficult. In the present studies, 2% SabDex containing 8 μg of fluconazole per ml correctly detected 89% of the clinical *C. neoformans* isolates for which the NCCLS MICs were <8 μg/ml and 90% of the strains for which the NCCLS MICs were ≥8 μg/ml. Medium containing 16 μg of fluconazole per ml correctly detected all isolates for which the NCCLS MICs were <16 μg/ml as well as all isolates for which the NCCLS MICs were ≥16 μg/ml. Overall, agreement was within 1 $\log_2$ broth tube macrodilution for 87 of 91 isolates (96%) on the 8-μg/ml fluconazole medium and 91 of 91 isolates (100%) on the 16-μg/ml fluconazole medium. While this screening method correlates well with the NCCLS M27-A method, the clinical significance of *C. neoformans* susceptibility testing remains unclear (15).

The sensitivity of correctly predicting mycologically resistant yeasts by normal colony growth on medium containing 8- or 16-μg/ml fluconazole was 100% with either 4% SabDex or CHROMagar. However, the specificities of correctly predicting isolates to be fluconazole susceptible based on suppressed growth on medium containing fluconazole at either 8 or 16 μg/ml were 41 and 65%, respectively, on 4% SabDex medium and 53 and 45%, respectively, on CHROMagar medium containing fluconazole. Thus, 2% SabDex appeared superior to these other media for susceptibility screening. The use of Yeast Nitrogen Base agar could possibly have improved the growth of *C. neoformans* but was not tested in this study (10, 12, 22). Data obtained in these experiments supports previous observations that medium-specific differences pertaining to MIC determination exist (5, 8, 12, 16, 22).

The use of 2% SabDex medium with fluconazole appears to be a rapid, simple, and sensitive method for detection of fluconazole-resistant yeasts. Additional studies should be conducted to determine the utility of this method in screening clinical samples and in establishing the optimal management of resistant cryptococcal infections.

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