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Multicenter Evaluation of Broth Microdilution Method for Susceptibility Testing of *Cryptococcus neoformans* against Fluconazole

HOMAYOON SANATI,[1] SHAWN A. MESSER,[2] MICHAEL PFALLER,[2] MALLORY WITT,[3] ROBERT LARSEN,[4,5] ANA ESPINEL-INGROFF,[6] AND MAHMOUD GHANNOUM[1,7]*

Division of Infectious Diseases[1] and Division of Allergy and Immunology,[3] Department of Medicine, Harbor-UCLA Medical Center, Torrance, California; Department of Pathology, University of Iowa College of Medicine, Iowa City, Iowa[2]; Section of Communicable Diseases, Department of Medicine, University of Southern California School of Medicine, Los Angeles County-USC Medical Center,[4] and UCLA School of Medicine,[7] Los Angeles, California; California Collaborative Treatment Group, University of California, San Diego, La Jolla, California[5]; and Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia[6]

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We have developed a microdilution method for measuring the susceptibility of *Cryptococcus neoformans* to fluconazole. The present study evaluated the interlaboratory agreement of the results for the microdilution method obtained at three different sites and compared this method with the National Committee for Clinical Laboratory Standards M27-P reference method. Excellent interlaboratory agreement among the results obtained at the three sites was achieved with this method (83 and 96% agreement within 1 and 2 $\log_2$ dilutions, respectively). An overall agreement of 90% between the microdilution method and the M27-P method was observed, demonstrating the comparability of the two methods. However, there are inherent problems with the M27-P method in relation to measuring *C. neoformans* susceptibility, including suboptimal growth of the organism in RPMI 1640, a longer incubation period, and a narrow range of MICs. On the basis of these data, the microdilution method tested in this study is recommended for inclusion in the National Committee for Laboratory Standards method for testing the antifungal susceptibility of *C. neoformans*.

Cryptococcus neoformans is the cause of the most common life-threatening fungal infection in patients with AIDS. Depending on the study, estimates of the frequency of cryptococcosis among AIDS patients range from 5% to slightly greater than 10% (1, 3, 15). Given the high incidence of relapse after initial antifungal therapy, the current management of *C. neoformans* infections includes lifelong suppressive therapy with antifungal agents. In recent years, fluconazole (FLU) has become the drug of choice for suppressive therapy because it is effective, can be given orally, and has few side effects (7, 10, 14, 16). Chronic use of FLU for long-term suppressive therapy in AIDS patients may become a factor in the selection of cryptococcal isolates that are more resistant to azole therapy (2). Consequently, there is a greater need for a reproducible in vitro susceptibility testing method as a guide for selecting and monitoring antifungal therapy.

Despite advances represented by the recent standardization of a broth macrodilution procedure (M27-P) by the National Committee for Clinical Laboratory Standards (NCCLS), additional efforts toward the development of a method that is appropriate for *C. neoformans* are necessary. To this end, Ghannoum et al. (5) developed a broth microdilution method for measuring cryptococcal susceptibility to FLU. Using this technique, they demonstrated a significant range in the susceptibilities of clinical isolates of *C. neoformans* to FLU (5). In separate studies, it was found that cryptococcal susceptibility to FLU as determined by this microdilution method is an important predictor of treatment success in patients with acute AIDS-associated cryptococcal meningitis (17).

The present collaborative (three-center) study was performed to determine whether there was interlaboratory agreement of the results obtained by this technique and whether these results were in agreement with those obtained by the NCCLS reference method as a prelude to incorporating the microdilution procedure in a modification of the NCCLS method (M27-T; tentative).

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Antifungal agents. FLU was provided as a powder by Roerig-Pfizer (New York, N.Y.). A concentrated stock solution (1 mg/ml) was prepared in distilled water and stored at −70°C for use on the day of susceptibility testing.

Media. Two chemically defined media were used: yeast nitrogen base (YNB) medium (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% (wt/vol) glucose and buffered to pH 7.0 with 0.05 M morpholinepropanesulfonic acid (MOPS; Fisher Scientific, St. Louis, Mo.), and RPMI 1640 medium (American Biorganics, Inc., Niagara Falls, N.Y.) with L-glutamine, buffered to pH 7.0 with MOPS buffer (165 mM).

Organisms. The 53 *C. neoformans* strains used in this study were obtained from the blood or cerebrospinal fluid of patients enrolled in two clinical trials conducted by members of the California Collaborative Treatment Group for the treatment of acute AIDS-associated cryptococcal meningitis (7, 8). These isolates were identified as *C. neoformans* var. *neoformans* by standard methods (6).

Susceptibility testing. FLU susceptibilities of the cryptococ-

* Corresponding author. Mailing address: Division of Infectious Diseases, Saint John's Cardiovascular Research Center, RB-2, Harbor-UCLA Medical Center, 1000 W. Carson St., Torrance, CA 90509. Phone: (310) 222-3813. Fax: (310) 782-2016. Electronic mail address: ghannoum@humc.edu.

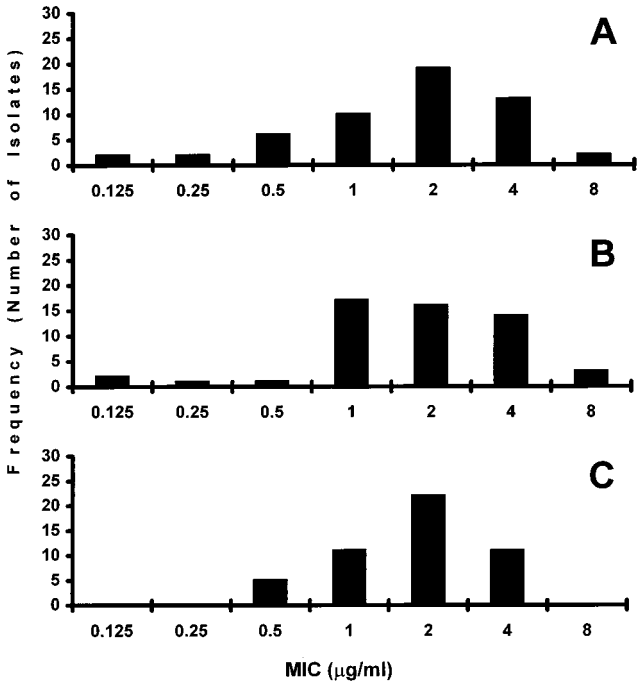

FIG. 1. MICs of FLU for *C. neoformans* isolates. Data were obtained by the YNB microdilution method at three different sites: the University of Iowa (A), the Harbor-UCLA Medical Center (B), and the Medical College of Virginia (C).

cal isolates were measured by the method of Ghannoum et al. (5). Briefly, the FLU stock solution was diluted in YNB medium to appropriate concentrations and dispensed into wells 2 through 11 of a microtiter assay plate (wells 2 and 11 with the highest and the lowest FLU concentrations, respectively). Drug-free YNB medium was dispensed into wells 1 and 12. Well 1 served as a sterility control and spectrophotometric blank. Well 12 served as a growth control. Wells 2 through 12 were inoculated with *C. neoformans* cells to 10^4 CFU/ml. The plates were incubated at 35°C for 24 or 48 h. The A_{420} of each well was measured spectrophotometrically, and the MIC at which 50% of the isolates of each strain were inhibited was determined. A different plate reader was used at each institution (a Microplate Reader 341 [Du Pont Instruments] was used at the Harbor-UCLA site, while a Whittaker Bioproducts Microplate Reader 2001 and a Kinetic Microplate Reader [Molecular Devices] were used at the Iowa and Virginia sites, respectively). The end point for the MIC at which 50% of the isolates were inhibited was defined as the lowest drug concentration exhibiting a 50% (or greater) reduction in optical density at 420 nm compared with the growth in the control well.

As standard controls, the susceptibilities of the cryptococcal isolates were measured by the NCCLS M27-P standard method (9).

MIC end point discrepancies of no more than two dilutions (two wells or tubes) were used to calculate the percent agreement.

The distribution of FLU MICs obtained for 53 isolates of *C. neoformans* in three laboratories is shown in Fig. 1. A broad range of MICs was observed for these clinical isolates in all three laboratories. Overall, the level of agreement among the three laboratories was good, with 83% of MICs agreeing within ± 1 $\log_2$ dilution and 96% agreeing within ± 2 $\log_2$ dilutions. This level of agreement is well within the bounds of what has been considered good agreement in similar studies (11, 12).

The distribution of FLU MICs obtained by the NCCLS M27-P reference method is shown in Fig. 2. The range of MICs was slightly narrower than that observed with the YNB microdilution method (0.5 to 8.0 μg/ml versus 0.125 to 8.0 μg/ml, respectively), and the modal MIC was slightly higher for the former (4.0 μg/ml versus 2.0 μg/ml, respectively); however, the overall agreement between the two methods was 90% (± 2 $\log_2$ dilutions).

The results of the present study confirm the previous observations of Ghannoum et al. (5) and provide new documentation of the interlaboratory reproducibility of and the agreement between the YNB microdilution method and the NCCLS reference method for testing the susceptibility of *C. neoformans* to FLU. The initial studies of Witt et al. documented that the microdilution method supported excellent growth of *C. neoformans*, was reproducible, and provided a range of FLU MICs that generally reflected the clinical response to this agent (17).

In the present study, we have established the excellent interlaboratory reproducibility of the YNB microdilution method. Similar MICs were obtained in all three laboratories with the microdilution method when MIC readings were taken after 48 h of incubation. The range of MICs for FLU was broad and in agreement with that reported previously by Ghannoum et al. (5). The use of a spectrophotometer provided an entirely objective means of determining MIC end points and undoubtedly contributed to the excellent interlaboratory agreement. It should be noted that this level of agreement is equal to or better than that observed when *Candida* isolates were tested by either micro- or macrodilution methods (4, 11, 13).

The level of agreement between the readings for the YNB microdilution method after 48 h of incubation and the M27-P reference method after 72 h of incubation was 90%. When data discrepancies between the reference method and the YNB microdilution method occurred, the MICs obtained by the YNB microdilution method were generally lower than those obtained by the reference method. These differences reflect, in part, the influence of trailing on visual MICs obtained by the reference method and underscore the usefulness of a spectrophotometer in clarifying MIC end points. Again, this level of agreement between the data obtained by these methods is equal to or better than that reported for *Candida* spp. (11, 12).

Given these results, it appears that the YNB microdilution method for FLU susceptibility testing of *C. neoformans* is a viable alternative to the NCCLS reference method. The improved growth of *C. neoformans*, ease of MIC determination, and potential for automation provided by the YNB microdilution method make this approach particularly attractive. Additional studies will be necessary to further standardize the YNB

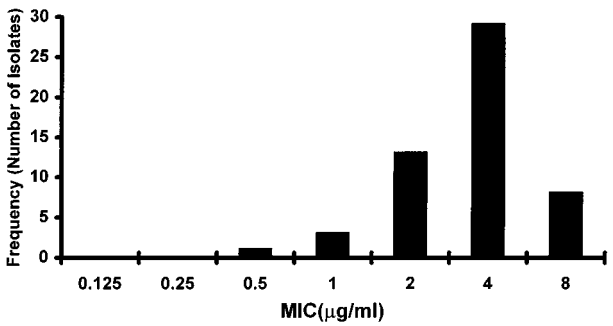

FIG. 2. MICs of FLU for *C. neoformans* isolates. Data were obtained at the University of Iowa by the M27-P method.

microdilution method so that the susceptibility of *C. neoformans* to other antifungal agents can be tested.

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