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NOTES

Comparison of Microdilution and Broth Dilution Techniques for the Susceptibility Testing of Yeasts to 5-Fluorocytosine and Amphotericin B

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Microdilution and broth dilution techniques were compared for the susceptibility testing of 50 clinical yeast isolates to 5-fluorocytosine and amphotericin B. Good correlation between methods was obtained with all isolates except *Cryptococcus neoformans*.

Several broth dilution techniques for the susceptibility testing of yeasts to 5-fluorocytosine (5-FC) and amphotericin B (AmB) have been described elsewhere (5, 9, 11-13). These methods are cumbersome to perform, and the development of more rapid and simple procedures is desirable.

This report describes the comparison of a microdilution and broth dilution technique for the susceptibility testing of yeasts to both 5-FC and AmB. Comparisons were made between tests incubated for 24 and 48 h at 25 and 35°C. Stability of the drugs at −70°C is also discussed.

Organisms. Fifty yeast isolates were obtained from clinical specimens in the mycology laboratory at Hartford Hospital and identified by standard methods (2-4). These included 14 strains of *Cryptococcus neoformans*, 22 strains of *Candida albicans*, 15 *Candida* species not *albicans*, 4 strains of *Torulopsis glabrata*, and 1 strain each of *Geotrichum candidum* and *Trichosporon* species. The yeasts were maintained at room temperature on Sabouraud dextrose agar slants.

Drugs. 5-FC was supplied as a pure powder by Hoffman-LaRoche, Inc., Nutley, N.J. The drug was dissolved in sterile distilled water with the aid of gentle heat from a bunsen burner, sterilized through a 0.45-μm filter, and frozen at −70°C in 1.5-ml portions of 1,000 μg/ml. AmB (Fungizone) was obtained from E.R. Squibb and Sons, Inc., Princeton, N.J., rehydrated with 100% dimethyl sulfoxide, and frozen at −70°C in 1.5-ml portions of 1,000 μg/ml.

Methods. The broth dilution technique for 5-FC was performed in yeast nitrogen broth as described by Shadomy (11, 12). AmB was tested in Sabouraud dextrose broth (5, 7, 9). Duplicate twofold dilutions of each drug were prepared in 1-ml volumes on the day of testing and inoculated with 50 μl of a 10^5-cells-per-ml yeast suspension in the appropriate liquid medium. The final density was 5×10^3 cells per ml. One set of tubes was incubated at 35°C, and another was incubated at 25°C. The microdilution technique was performed in microtiter U-bottom trays (Flow Laboratories, Rockville, Md.) with a semiautomatic pipetter and diluter (Cooke Laboratory Products, Alexandria, Va.) to prepare the twofold serial dilutions. The trays were individually wrapped in plastic and stored at −70°C. Trays were removed from the freezer 0.5 h before use and thawed at room temperature. The same suspension as used for broth dilution was used for microdilution, yielding a final inoculum density of 10^4 cells per ml. Duplicate trays were sealed with tape; one tray was incubated at 25°C and another was incubated at 35°C. The minimum inhibitory concentration (MIC) was read at 24 and 48 h as the least amount of drug which inhibited visual growth. A *C. albicans* no. 6631 control was provided by Hoffman-LaRoche and run each day of testing with both 5-FC and AmB.

To test the accuracy and precision of the microdilution system, 30 duplicate tests to determine the MIC of 5-FC and 12 duplicate tests for measurement of the MIC of AmB were performed with the *C. albicans* control strain. Approximately 95% agreement between duplicate pairs within ±1 $\log_2$ was obtained with both drugs for all periods of incubation (range, 92 to 100%). Replicate studies with *C. albicans* no. 6631 over a 6-month period showed 100% agree-

ment between the microdilution and broth dilution within ±2 $\log_2$. Regression analyses showed good statistical correlation between methods with both 5-FC and AmB. There was no loss of activity of 5-FC or AmB when stored at −70°C in microdilution trays for 6 and 3 months, respectively.

Table 1 shows the percent agreement between the microdilution and broth dilution techniques. There was insufficient growth of the yeasts after 24 h at 25°C to accurately assess the results. The best overall correlation was obtained with 48 h of incubation at 25°C. Under these conditions as much as fourfold higher MICs were occasionally observed with both drugs when tested by the microdilution technique. This usually occurred at low microgram-per-milliliter levels and did not change the interpretive category of susceptibility (12) to either antifungal agent. Incubation at a higher temperature resulted in lower 5-FC MICs when tested by a microdilution technique, as has also been observed by Marks and Eickhoff (8). In our study, there was less correlation between methods within ±1 $\log_2$ when tubes and microtitration trays were incubated at 35°C.

In an attempt to provide a more rapid antifungal susceptibility test, a comparison was made between microdilution tests incubated at 35°C for 24 h and broth dilution tests incubated at 25°C for 48 h to determine whether a shorter incubation period at a higher temperature would yield the same results as the conventional 48-h incubation at a lower temperature (Fig. 1). Microdilution MICs were consistently lower than those obtained by broth dilution. This did not change the interpretive category of AmB results, but did produce 12% false susceptibility with 5-FC by the microdilution method. Fisher and Armstrong (6) used a higher inoculum in a 24-h colorimetric microdilution method for testing 5-FC and obtained good correlation with a standard 48-h broth dilution technique. Several investigators have observed that the inoculum size

TABLE 1. *Percent agreement between microdilution and broth dilution methods*

Drug	$\log_2$	% Agreement at:		
		24 h, 35°C[a]	48 h, 35°C	48 h, 25°C
5-FC[b]	±1	91	81	86
	±2	93	98	98
AmB[c]	±1	72	76	88
	±2	96	100	100

[a] Duration and temperature of incubation.
[b] 5-FC tested with 40 yeast strains.
[c] AmB tested with 25 yeast strains.

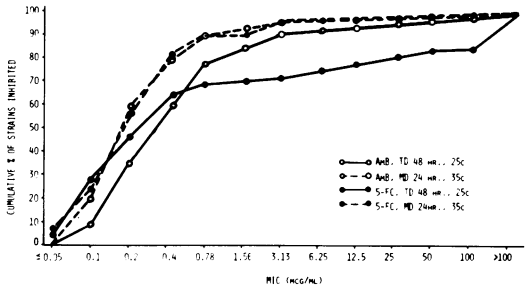

FIG. 1. *Comparison of microdilution (MD) trays with broth dilutions (TD) incubated for 24 h at 35°C and for 48 h at 25°C, respectively.*

used in the susceptibility testing of yeasts produces considerable variability in test results (1, 10, 13). Perhaps a larger inoculum in our microdilution technique would have allowed a shorter period of incubation without loss of correlation with the standard dilution procedure.

It was apparent early in the investigation that *C. neoformans* results could not be interpreted by microdilution. Buttons of cells appeared in the bottom of all inoculated wells precluding the precise measurement of an end point of inhibition by visual turbidity. The *C. neoformans* strains tested were all mucoid, although to varying degrees, and all produced buttons of cells in the microtitration wells. Additional studies undertaken to try to resolve the problem included the use of a 10^2 inoculum, the addition of a color indicator to the microtitration wells after incubation, and attempts to shake the trays during incubation. The problem remained unresolved, and the test data with *C. neoformans* were excluded from our statistical analyses.

The microdilution technique, with incubation of trays at room temperature (25 to 30°C) for 48 h, is recommended as an economical and reliable method for susceptibility testing of yeasts, except *C. neoformans*, to 5-FC and AmB. The microdilution trays are easy to prepare, and there is no loss of 5-FC and AmB activity when trays are stored at −70°C for several months. Microdilution is not recommended for testing *C. neoformans* because of difficulty in establishing a clear end point of inhibition.

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