Susceptibility Testing of *Cryptococcus neoformans*: a Microdilution Technique

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Received 26 May 1992/Accepted 28 July 1992

We studied a series of test conditions in a microtiter system to define the optimal method for determining the susceptibility of *Cryptococcus neoformans* to antifungal agents. Twenty-one isolates of *C. neoformans* were grown for 24 or 48 h in four chemically defined media: yeast nitrogen base (BYNB 7); RPMI 1640; synthetic amino acid medium—fungal (SAAMF), buffered at pH 7.0 to select the medium that best supported growth of this fastidious yeast; and yeast nitrogen base, pH 5.4 (YNB 5.4). Maximum growth of *C. neoformans*, at 35°C, was obtained in YNB 5.4, with the next highest growth levels in BYNB 7, SAAMF, and RPMI. Growth at 24 h was uniformly poor in all media and lacked reproducibility. In contrast, incubation for 48 h gave adequate growth with low standard deviations, and 48 h was selected as the optimal incubation period for this study. Comparison of the relationship between growth kinetics and initial inoculum size for eight cryptococcal isolates showed that 10⁴ cells per ml yielded optimal growth in BYNB 7 and YNB 5.4, whereas 10⁵ cells per ml was optimal in RPMI and SAAMF. Furthermore, variation of inocula from 10³ to 10⁵ cells per ml showed small but significant inoculum effects in determining MICs of fluconazole, amphotericin B, and flucytosine for *C. neoformans*. Therefore, 10⁴ cells per ml was chosen as the optimal inoculum for susceptibility testing in this study. Mean MICs of fluconazole, amphotericin B, and flucytosine for 21 cryptococcal isolates in RPMI and BYNB 7 were low (for example, fluconazole had mean MICs of 1.2 and 1.3 µg/ml in RPMI and BYNB 7, respectively) and differed significantly from medium to medium. In contrast, the MICs obtained in SAAMF were significantly higher (e.g., fluconazole had a mean MIC of 2.2 µg/ml). Variance in MICs was large with fluconazole and flucytosine but small with amphotericin B, irrespective of the medium used. A microtiter system employing BYNB 7 as the medium, 48 h as the incubation period, and 10⁴ cells per ml as the final inoculum is a simple, accurate, and reproducible method for the testing of *C. neoformans* susceptibility to fluconazole, amphotericin B, and flucytosine.

Infection with *Cryptococcus neoformans* is an increasingly important problem in immunosuppressed patients, particularly those with AIDS (4), in whom this organism is the fourth most common (and leading fungal) cause of life-threatening infection (7, 19). Cryptococcal infection in patients having AIDS is considered incurable, and lifelong therapy is necessary to suppress the infection. The risk for relapse after a presumed successful course of therapy was greater than 50% in one series of AIDS patients (13); in addition, relapses have occurred when amphotericin B (Amph B) or fluconazole (FLU) was used for chronic suppression (20). Total doses of Amph B used in the therapy of cryptococcal meningitis in AIDS patients are higher than those necessary for nonimmunosuppressed patients (5). The selection of antifungal drugs, duration of therapy, and doses required for optimal anticryptococcal treatment are not well-defined. The development of methods for routine susceptibility testing of *C. neoformans* is important to facilitate making more appropriate choices among current and proposed antifungal agents and to provide a guide for selecting appropriate doses of these agents.

Most efforts toward developing a standardized reference method for general susceptibility testing of yeasts have

employed *Candida* species as the main test organisms (15, 17, 18). However, susceptibility testing methods developed for *Candida* spp. may not be readily extrapolated to *Cryptococcus* spp. For example, *C. neoformans* is a fastidious yeast that grows more slowly than *Candida* species (17), a problem which is particularly important in the broth microdilution method recommended for routine use in yeast susceptibility testing (17). Because of its slow growth, the 24-h incubation period suggested for determining the MICs of flucytosine (5FC) and azoles is not sufficiently long to determine the endpoints for inhibition of *Cryptococcus* spp., particularly with small inocula (8). Further evidence that *C. neoformans* should be treated separately comes from the work of Mazens et al. (14), who were unable to recommend a consistently reliable method for susceptibility testing of such yeasts. These authors obtained satisfactory correlations between micro- and macrodilution techniques with all yeast isolates except *C. neoformans*.

To delineate the variables influencing the susceptibility testing of *C. neoformans*, we used a microtiter method to investigate the effects of growth medium, inoculum size, and time of incubation on the MICs of Amph B, FLU, and 5FC for 21 cryptococcal isolates. On the basis of our data, we propose a modified microtiter method that appears to meet the requirements for standardized susceptibility testing of *C. neoformans*.

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MATERIALS AND METHODS

Antifungal agents. FLU was provided by Roerig-Pfizer (New York, N.Y.), Amph B (Fungizone) was provided by E. R. Squibb & Sons (Princeton, N.J.), and 5FC was purchased from Sigma Chemical Co. (St. Louis, Mo.). Concentrated stock solutions (FLU, 1 mg/ml; Amph B, 5 mg/ml; and 5FC, 1 mg/ml) were prepared according to the manufacturers' recommendations. Stock solutions were stored at -70°C until thawed for use the day of susceptibility testing.

Media. Three chemically defined media buffered to pH 7.0 with 0.05 M morpholinepropanesulfonic acid (MOPS) (GIBCO Laboratories, Grand Island, N.Y.) were used: yeast nitrogen base medium (BYNB 7; Difco Laboratories, Detroit, Mich.), RPMI 1640 medium (GIBCO Laboratories), and synthetic amino acid medium—fungal (SAAMF; American Biorganic, Niagara Falls, N.Y.). Since a large number of in vitro studies of *C. neoformans* are performed with yeast nitrogen base, pH 5.4 (YNB 5.4), this chemically defined medium was included for comparison.

Microtiter plates. Sterile, individually wrapped polystyrene round-bottom 96-well assay plates (well diameter, 6.4 mm) (Corning Glass Works, Corning, N.Y.) were used in this study. The stock solutions of antifungal agents were dispensed in assay medium to obtain appropriate concentrations in wells 2 through 11 in each row; drug-free medium was dispensed in wells 1 and 12. Well 1 served as a sterility control and blank for the spectrophotometric assays. Well 12 served as a growth control. The antifungal concentrations ranged from 0.117 to 60 $\mu\text{g/ml}$, obtained by 10 twofold serial dilutions. A representative microtiter plate was incubated at 35°C for 3 days and observed daily to ensure sterility. The plates were wrapped in aluminum foil to minimize dehydration during storage in a -70°C freezer. Thawing was accomplished the day of the in vitro testing as described previously (1).

Organisms. Twenty-one *C. neoformans* strains were tested to determine drug MICs in different media. Two of these were ATCC 36556 (serotype D, virulent encapsulated strain) and ATCC 52817 (acapsular mutant) (American Type Culture Collection, Rockville, Md.). The remaining strains were obtained from the Clinical Microbiology Laboratory at Harbor-UCLA Medical Center. Clinical strains were isolated from blood or cerebrospinal fluid. *Candida albicans* ATCC 36082 was included in these studies to compare its growth with that of *Cryptococcus* spp. All isolates were maintained at 4°C on Sabouraud dextrose agar slants (Difco).

Inocula. Starting inocula of *C. neoformans* were obtained by picking five colonies >1 mm in diameter from 48-h-old cultures. The yeast cells were suspended in 5 ml of sterile 0.85% saline. The resulting yeast suspension was vortexed for 15 s, and the turbidity was adjusted to 1×10^6 to 5×10^5 CFU/ml and was determined by using spectrophotometric methods recommended by Pfaller et al. (16). Initial inoculum sizes of 5×10^2 , 1×10^3 , 5×10^3 , 1×10^4 , 5×10^4 , and 1×10^5 yeast cells per ml were subsequently tested. Quantitative colony counts were used to confirm the number of CFU per milliliter for each inoculum used. We have shown in pilot studies that there is a close correlation between yeast colony counts obtained by quantitative culture and counts obtained by spectrophotometric techniques.

Microtiter plates were inoculated by using an Eppendorf pipettor programmed to dispense 10 μl into each well. The inoculum sequence proceeded from well 12 to well 2 (from the well containing the lowest drug concentration to that

TABLE 1. Mean growth of 21 isolates of *C. neoformans*^a

Growth medium	$A_{420} \pm \text{SD}$	Variance (% of mean)	No. of isolates with $A_{420} \leq 0.01$
BYNB 7	0.062 ± 0.03	42	1
YNB 5.4	0.072 ± 0.04	55	2
SAAMF	0.031 ± 0.01	39	0
RPMI	0.045 ± 0.02	49	3

^a Cells were grown for 48 h with an inoculum of 10^4 cells.

containing the highest). All susceptibility tests were performed in duplicate in each experiment.

Incubation times. Inoculated plates were incubated for 24 or 48 h at 35°C . Absorbance was determined spectrophotometrically (A_{420}) after agitation of the plates for 15 s with a vortex mixer (Vortex-Genie 2 mixer, Scientific Industries, Inc., Bohemia, N.Y.). This agitation formed a near-homogeneous suspension in the wells. Spectrophotometric readings were performed with an automatic microplate reader (Dupont Instruments, Wilmington, Del.) set at single beam, 199 calibration, and 199 threshold.

Endpoint criteria. The MIC endpoint was defined as the lowest drug concentration exhibiting approximately 50% (or more) reduction of growth compared with the that of control well. With the turbidity assay, this was defined as the lowest drug concentration at which turbidity in the well was $<50\%$ of that of the control (IC_{50}). IC_{30} and IC_{80} , defined as concentrations causing >30 or $>80\%$ inhibition, respectively, were also recorded to provide an indication of the slope of inhibition versus drug concentration curve through the inhibitory range. The advantages of choosing the spectrophotometric endpoint rather than the visual endpoint include objectivity, rapidity, and elimination of subjective judgments concerning minimal turbidity (9).

Growth studies. The growth rate of *C. neoformans* ATCC 36556 was compared with that of *C. albicans* ATCC 36082. Yeast cells were grown for 24 h at 27°C on a rotating drum in yeast nitrogen base broth with amino acids (Difco) and enriched with 0.5% dextrose (BYNB). After being harvested by centrifugation (5 min at $1,500 \times g$), the yeast cells were sonicated for 4 s (model 350 Sonifier; Branson, Danbury, Conn.), washed twice with 0.85% saline, and sonicated again. These cells were used to inoculate 50 ml of BYNB 7 at an initial concentration of 10^4 organisms per ml. The organisms were incubated at 35°C on a rotary shaker. At intervals, 2 ml of each suspension was withdrawn and mixed well, and the A_{420} was determined spectrophotometrically (10).

Eight of the *C. neoformans* isolates were randomly selected for studies to define optimum inoculum size and times of incubation in the microtiter plates for antifungal testing.

RESULTS

Twenty-one isolates of *C. neoformans* were grown in four chemically defined media to determine which medium may be best for susceptibility testing. Growth, measured by spectrophotometric absorbance after 48 h of incubation, showed that maximum yield was obtained in YNB 5.4. Growth in this medium was followed in order by growth in BYNB 7, RPMI, and SAAMF (Table 1). Table 1 also shows the variance in growth yield in each medium, expressed as a percentage of the mean, and the number of isolates that failed to grow or showed poor growth ($A_{420} < 0.01$) in each medium.

FIG. 1. Growth curves for *C. albicans* ATCC 36082 and *C. neoformans* ATCC 36556, each grown as a shake culture in 50 ml of BYNB 7 medium at 35°C.

Figure 1 shows that in BYNB 7, *C. albicans* ATCC 36082 reaches the late exponential phase within 10 h, while *C. neoformans* ATCC 36556 is just entering the exponential phase at this time. The generation times for the *C. albicans* and *C. neoformans* strains are 1.5 and 2.4 h, respectively. Analysis of the growth rates of eight additional cryptococcal isolates in microtiter plates showed only limited growth at 24 h of incubation on each of the four media tested. An accurate estimation of growth could not be made because of both the low growth yield and the large standard deviations (SDs) of the absorbance measurements. In contrast, after 48 h, the growth yield was substantially better, with lower SDs (Table 2).

A comparison of average growth yield with initial inoculum size for eight cryptococcal isolates showed that 10^4 cells per ml led to maximal growth on BYNB 7 and YNB 5.4 for these media after 48 h of incubation; inoculation with 10^5 cells per ml gave no further increase in total absorbance at 48 h (Table 3). In contrast, in RPMI and SAAMF, the larger initial inocula (10^5 cells per ml) resulted in a higher ultimate growth yield. Initial inocula of less than 10^4 cells per ml yielded poor growth rates with higher SDs of absorbance

TABLE 2. Effect of incubation time on growth yield of eight *C. neoformans* isolates^a

Growth medium	Growth yield with incubation time of:	
	24 h	48 h
YNB 5.4	0.019 ± 0.013	0.224 ± 0.14
BYNB 7	0.016 ± 0.01	0.113 ± 0.09
RPMI	0.009 ± 0.006	0.041 ± 0.03
SAAMF	0.005 ± 0.004	0.058 ± 0.054

^a Average growth is defined as the sum of absorbance values (±SD) for all tests employing eight isolates divided by eight. The inoculum size was 10^4 cells.

TABLE 3. Effect of inoculum size on growth yield of eight *C. neoformans* isolates

Growth medium	Growth yield ^a with inoculum size of:	
	10^4 cells	10^5 cells
YNB 5.4	0.240 ± 0.14	0.201 ± 0.154
BYNB 7	0.113 ± 0.09	0.136 ± 0.085
RPMI	0.041 ± 0.03	0.103 ± 0.073
SAAMF	0.058 ± 0.05	0.080 ± 0.047

^a Measured as $A_{420} \pm$ SD at 48 h.

values at 48 h (data not shown). Figure 2 illustrates the growth yield of *C. neoformans* 18165 in each of the four media as a function of initial inoculum size after 48 h of incubation. These results are typical of those obtained for other isolates.

Because MICs have been reported to depend on inoculum size (2), we studied the effect of inoculum size of *C. neoformans* (10^3 to 10^4 cells per ml) on the three study drugs in MIC testing. With the assay techniques used, small but significant differences in IC_{30} , IC_{50} , and IC_{80} were observed for different inoculum sizes, suggesting a need for a constant inoculum size in MIC testing (Table 4). We therefore chose 10^4 cells per ml as the initial inoculum size and an incubation period of 48 h for all subsequent in vitro tests.

MICs of the three study antifungal agents for each of the 21 isolates of *C. neoformans* in BYNB 7, SAAMF, YNB 5.4, and RPMI were determined. The results are shown in Fig. 3. Arrowheads mark the mean MICs for all 21 isolates in each medium. In general, there is a heterogeneous distribution of MICs of each antifungal agent for the 21 strains in each medium tested. Mean MICs of Amph B and FLU are lowest in RPMI, slightly higher in BYNB 7, and significantly higher in SAAMF. However, MICs of 5FC are much lower for YNB 5.4 than for any other medium. The R values on Fig. 3 are calculated as the differences in concentrations of drug equal to the MIC plus and minus the SD for the 21 isolates divided by the mean value: $[R = (MIC + SD) - (MIC - SD)]/\text{mean}$. This formula provides an indication of the vari-

FIG. 2. Comparison of *C. neoformans* 18165 growth in microtiter plates containing 100 μ l of BYNB 7, YNB 5.4, SAAMF, or RPMI as a function of inoculum size. Plates were incubated for 48 h at 35°C. Microtiter plates were agitated for 15 s, and the A_{420} was determined spectrophotometrically.

TABLE 4. Change in inhibition resulting from changes in inoculum size^a

Antifungal agent	Change in inhibition		
	IC ₃₀	IC ₅₀	IC ₈₀
FLU	0.5	1.0	0.5
Amph B	None	None	None
5FC	0.75	1.0	1.25

^a Changes are expressed as the numbers of twofold-dilution differences for 10³ yeast cells versus 10⁴ cells in the initial inoculum. Values are the average for two tests on four isolates.

ance in MIC that is only minimally biased by the dilution procedures used in determining the MIC. The use of *R* values avoids the problem of higher concentrations always giving larger SDs in a dilution assay. Smaller *R* values indicate less variance in experimental measurement of MICs for the 21 isolates (Fig. 3).

Table 5 shows the difference between IC₃₀ and IC₈₀ levels for each drug in the four media. This is a measure of the slope of the inhibition response curve, an important consideration when drug combinations are to be used. Values are given in terms of the average number of dilutions in a standard dilution test required to go from 80 to 30% inhibition. The sharpest response (i.e., minimum concentration change between 30 and 80% inhibition) was in YNB 5.4, with less-intense responses in RPMI, BYNB 7, and SAAMF. Note also in Table 5 that with these experimental conditions,

the concentration range between 30 and 80% inhibition is smallest for Amph B, then FLU, and then 5FC.

DISCUSSION

A standardized yeast susceptibility testing technique should feature both an ideal growth medium and an ideal protocol with reference to initial inoculum, incubation time, and MIC endpoint determination which is applicable to all yeasts (18). Three current protocols are used widely in susceptibility testing: macro- and microdilution and agar dilution techniques. Current general susceptibility testing for yeasts is not well suited to studies of *Cryptococcus* species because of the fastidious nature of these yeasts (6, 14). This is clear by the difference in generation times between *C. albicans* and *C. neoformans* which was observed in this investigation. Therefore, we have attempted to define an improved method for susceptibility testing using the microtiter system. Since future testing of *C. neoformans* for drug susceptibility will certainly involve testing of multiple drug combinations, it is essential that the methods employed allow rapid examination of large numbers of samples in multifactorial analysis. Currently, the use of microtiter assays and automated sample reading offer the only available means for accomplishing such analyses. The microtiter technique is both efficient and economical (8); it allows test solutions to be made in advance and frozen until used (6), and it yields endpoint determinations that are accurate and precise, particularly with automated plate-reading technology. Moreover, the equipment needed to perform the micro-

FIG. 3. MICs of Amph B (A), FLU (B), and 5FC (C) for 21 isolates of *C. neoformans* grown in the four test media for 48 h at 35°C. The initial inoculum size was 10⁴ cells per ml. Arrowheads mark the average MIC for all isolates. *R* values are a measure of the variance in MICs (see text).

TABLE 5. Difference between IC₃₀ and IC₈₀ levels for each drug in the four media^a

Antifungal agent	Difference between IC ₃₀ and IC ₈₀ in growth medium:				Agent avg
	RPMI	SAAMF	BYNB 7	YNB 5.4	
Amph B	0.76	2.4	1.4	0.57	1.28
5FC	2.9	3.1	3.5	1.2	2.7
FLU	1.9	2.3	1.4	1.6	1.8
Medium avg	1.9	2.6	2.1	1.1	

^a Mean values for 21 isolates, expressed as the number of twofold dilutions of drugs between IC₃₀ and IC₈₀ levels required to go from 80 to 30% inhibition of growth. Large number indicates a shallow slope of the drug concentration versus growth inhibition curve.

titer method in this study is an enzyme-linked immunosorbent assay plate reader and a vortex, both of which are basic equipment in most microbiology laboratories.

Two studies have shown that MICs determined by the microdilution method are higher than MICs determined by the microdilution method (3, 8). Cook et al. (3) hypothesized that there may be a decreased ability to detect turbidity and differences in the absolute number of yeast cells within the wells of a microdilution tray than within macrodilution tubes. They found that conditions similar to those used in this study (incubation temperature of 35°C and yeast inoculum of less than 10⁴ cells per ml) improve the agreement between these two methods. In addition, Espinel-Ingroff et al. (8) have previously shown that *C. neoformans* grew better in microtiter wells than in macrotubes. Anaissie et al. (1) reported that microtiter plate agitation yielded reproducible visual endpoints for *C. albicans* that were independent of inoculum size, temperature of incubation, and time of reading and correlated well with the degree of fungal inhibition as determined spectrophotometrically.

Previous attempts to use microtiter plates for susceptibility testing of *C. neoformans* have not been successful (6, 14). Failure of the microtiter system for *Cryptococcus* spp. was attributed in the first study to slow growth, while in the second, failure was ascribed to the buttonlike growth of *C. neoformans* in the wells, which prevented the precise determination of endpoints. The results reported here overcome these difficulties because the cells were grown for longer periods and the plates were agitated before the spectrophotometric assay was performed.

Our studies show that YNB 5.4 produced the best average growth for the 21 cryptococcal isolates tested. Growth on the BYNB 7 medium was nearly equal to that on YNB 5.4, and the variance in growth of the 21 isolates was somewhat less. Growth levels in RPMI and SAAMF media were low and precluded the use of these media in the microtiter assays. All 21 isolates grew in SAAMF, and all but 1 grew in BYNB 7, while 2 failed to grow in YNB 5.4 and 3 failed to grow in RPMI. The inability to support general growth make RPMI and possibly YNB 5.4 questionable choices for general susceptibility testing.

In addition, unless the antifungal agents are stable in the test medium, the susceptibility testing procedure may give falsely high MICs because of the breakdown of the drug. For example, some antifungal agents (e.g., ketoconazole and, to a lesser extent, Amph B) have been shown to be unstable at pH 5.4 (11, 12). In our present study, definite differences in MICs for media with different pHs show that pH is an important criterion in selecting media for testing. Because of the pH instability of antifungal agents and because the

objective of susceptibility testing is to estimate the ability of drugs to kill yeasts under conditions close to the physiological pH of 7.4, we conclude that it is better to perform MIC testing for yeasts at or near this pH.

It is advantageous to perform yeast susceptibility tests with a medium for which the MICs of study drugs are only a function of differences in the strains, not an effect of the media used in determining the susceptibility. To test uniformly, we examined both the IC₅₀s and the variance in IC₅₀s for our 21 isolates on the four media. Testing of susceptibility to a drug in a given medium which gives a high IC₅₀ can show larger absolute values of variances than testing in a medium which gives a low IC₅₀, simply because of the magnitude of the concentration changes in twofold dilutions. We therefore compared variances by determining how many twofold dilutions in our experimental protocol were required to change drug concentrations over the range defined by the mean $\pm$ 1 SD IC₅₀. For example, if the mean IC₅₀ for the 21 isolates was 10 and the SD was $\pm$ 5, 2 dilutions are necessary to cover the range 5 to 15, and the range of variance can be reported as 2 dilutions. This method of calculation defines the variability in the experimentally measured IC₅₀, which is less biased by the absolute concentration of the drug. Comparison of values from susceptibility tests requires designation of a selected level of inhibition to be defined as the MIC. In this study, we measured drug concentrations causing 30, 50, and 80% inhibition for description of drug responses. Experimentally, IC₅₀s are generally the most accurately determined of these values since the slope of the absorbance versus drug concentration plot is steepest at this point (data not shown). We therefore propose using IC₅₀s for the MIC designation in the microtiter plate method of susceptibility testing.

Consideration of all the criteria we evaluated in the present study suggests that BYNB 7 is the optimal choice for standard susceptibility testing of *C. neoformans*. RPMI, SAAMF, and YNB 5.4 all had substantial flaws that would tend to eliminate them from consideration. In this respect, the study conducted by the National Committee for Clinical Laboratory Standards (17) indicated that there is no clear-cut choice of medium for antifungal susceptibility testing of yeasts but, rather, suggests that the use of RPMI 1640 medium, high-resolution antifungal assay medium, or BYNB may produce satisfactory results. Furthermore, their study concentrated on *Candida* spp. and in fact included only two strains of *C. neoformans*, and they recommended testing of a larger number of *Cryptococcus* isolates to determine the suitability of these media for susceptibility testing of these yeasts.

In conclusion, a broth microdilution method employing preagitation, using BYNB 7 and an initial inoculum size of 10⁴ yeast cells per ml with 48 h as the duration of incubation at 35°C is recommended as an efficient and reproducible method for susceptibility testing of *C. neoformans*. The reproducibility of this technique will need to be verified in a large blinded multicenter evaluation; such studies are being planned.

ACKNOWLEDGMENTS

This work was supported in part by grant AI 31696-01 from the National Institute of Allergy and Infectious Diseases and by grant TS-6933-01 from Roerig-Pfizer Pharmaceuticals.

We thank Laurie Mortara for reading the manuscript.

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