Antimicrobial Susceptibility Testing of Yeasts: a Turbidimetric Technique Independent of Inoculum Size

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The current development of potent antifungal drugs necessitates reliable methods of in vitro susceptibility testing of clinical isolates. Other laboratories have noted with some antimicrobials that conventional tube dilution visual end points for determining minimal inhibitory concentrations (MIC) vary markedly with the inoculum. With seven isolates (four species) we compared the conventional method with one developed with a spectrophotometer. Yeasts were diluted to inocula of 10^2 to 10^5 cells/ml and incubated at 37°C with 5-fluorocytosine or miconazole or without drug in a synthetic defined medium. At frequent intervals MICs were determined by the lowest drug concentration without visible turbidity. Concurrently, the percent transmission of each tube was measured and an inhibitory concentration ($IC_{1/2}$) was calculated from the turbidimetric measurements. MICs of both drugs for all strains varied with the inoculum. Intrastrain differences averaged 128-fold for miconazole and greater than 8-fold with 5-fluorocytosine. This variability in MIC occurred when readings were made after 12 to 48 h of incubation. In contrast, $IC_{1/2}$ values of all isolates varied less than two-fold with the same range of inocula. Most isolates showed this independence when measured at 48 h. Fast-growing yeasts showed greater $IC_{1/2}$ variability unless the turbidimetric measurements were taken earlier while the drug-free growth was still in a rapid growth phase. This method is reproducible, inoculum independent, rapid, free from subjectivity and observer variability, and suitable for adaptation to a clinical setting.

Fungal infections are an increasing problem with the growing use of immunosuppressive therapy, valve prostheses, and intravenous and urinary catheters and with the increased survival of patients with congenital and acquired immunodeficiencies (6, 9). Potent antifungal agents, such as amphotericin B and 5-fluorocytosine (5-FC), are currently available, but their administration carries the risk of serious toxic reactions (2). Newer agents are being developed, and they will require careful clinical evaluation. Reliable and practical in vitro sensitivity testing is necessary to select appropriate therapy.

There is no standardized method of testing the susceptibility of yeasts to antifungal agents. Most commonly, broth dilutions of the antifungal agent are performed. The lowest drug concentration that allows no visible growth of some inoculum is used as an end point (termed the minimal inhibitory concentration [MIC]). This method has the advantage of simplicity and can be performed in any clinical laboratory. However, it is subject to observer variability. Furthermore, 5-FC MICs for

cryptococci reportedly are influenced considerably by variations in the initial inoculum size (4). Inoculum size is not standardized between laboratories and often is not precisely standardized within the same laboratory (e.g., dilutions of an overnight broth culture).

We report here a method of susceptibility testing that employs turbidimetric techniques to measure growth inhibition. The lowest drug concentration that inhibits growth to a fraction of that in drug-free controls is determined. This method has the advantage of objective measurement, thus minimizing observer variability. In addition, it appears free from inoculum-dependent variability.

MATERIALS AND METHODS

Seven yeasts were studied: *Candida albicans* (two strains), *C. parapsilosis* (three strains), *C. tropicalis*, and *Torulopsis glabrata*. All isolates were from clinical specimens, except the *C. tropicalis* (laboratory reference strain). The yeasts were identified as to species by current standard methods (10) and were maintained on Sabouraud dextrose agar until tested.

All susceptibility testing was performed in a com-

pletely defined synthetic liquid medium (8), modified by the substitution of cysteine for cystine. This medium is free from known inhibitors of antifungal agents.

Miconazole (Janssen and Co., Beerse, Belgium) was stored as a solution, stable at room temperature. 5-FC (supplied as a powder by Hoffman-La Roche, Nutley, N.J.) was dissolved and frozen in portions until used.

Yeast inocula were prepared from 18- to 24-h cultures in liquid media. These were adjusted to 60% transmission (%T) at 540 nm (Coleman Junior spectrophotometer). A 1:100 dilution of this broth and subsequent 10-fold serial dilutions produced inocula of approximately 2 (range of 1.3 to 3.7) $\times 10^5$, 2×10^4 , 2×10^3 , and 2×10^2 cells/ml by hemocytometer count.

For susceptibility studies, antifungal agents were diluted in media to 20 and 500 μ g of miconazole and 5-FC, respectively, per ml, and then serial twofold dilutions were made. A 0.9-ml amount of drug suspension or drug-free media was combined with an equal volume of fungal suspension in sterile, disposable plastic tubes (12 by 75 mm) and placed at 37°C for stationary incubation. At the onset of incubation all tubes were completely clear. At intervals the tubes were inspected for turbidity. Simultaneously the %T of each tube was measured against a tube containing only medium.

Susceptibility was estimated by both a visual and a turbidimetric method. The visual MIC was read as the lowest drug concentration that remained clear. The inhibitory concentration (IC_n) was then calculated from the turbidimetric data as the lowest drug concentration that met the criterion $\%T \geq (\%T_{\text{control}} + n[100 - \%T_{\text{control}}])$, where %T = percent transmission, control = drug-free tube, and n = a selected fraction less than 1 ($1/2$, $3/4$, or $7/8$). This formula was constructed to define a fraction of inhibition (set by the value of n) as a function of the turbidity in the drug-free control tubes.

RESULTS

Variation in MIC (visual) end point with inocula. Seven isolates were tested against 5-FC and six were tested against miconazole at inocula of 10^5 to 10^2 cells/ml (Fig. 1 and 2). All isolates except one (Me strain against 5-FC) showed inocula-dependent variation, with more resistant MICs for higher inocula. The one isolate not showing this effect was resistant to $>250 \mu$ g of 5-FC per ml at all inocula tested. The MIC of each of two isolates spanned the entire 11-tube range of miconazole concentrations tested (10 to 0.01 μ g/ml). The mean difference between the highest and the lowest for the range of inocula tested was 128-fold for all strains tested against miconazole. 5-FC MIC variation with inoculum size was not as great, but still averaged more than eightfold.

Independence of the IC (densitometric) end point from inocula effects. In contrast to

intrastrain MIC variability, there was remarkable intrastrain uniformity in ICs calculated from turbidimetric measurements. Values for the $IC_{1/2}$, $IC_{3/4}$, and $IC_{7/8}$ are shown compared with the concurrent MICs for several isolates against miconazole and 5-FC in Fig. 3 and 4, respectively. Although a variety of n values yield similarly uniform inoculum independence, we have chosen $IC_{1/2}$ values to be represented in further comparisons. For all strains tested against both drugs, variation in the $IC_{1/2}$ over the range of inocula was never greater than twofold.

Relationship of incubation time to MIC variability. The MICs shown in Fig. 1 and 2 were read at 48 h to allow the control growths with the smallest inocula to become turbid. However, repeated MIC determinations were performed on most isolates (four strains with 5-FC and five strains with miconazole) beginning at 12 h of incubation. The variability in MIC with inoculum size was observed at all observation times for each strain, with no apparent tendency toward convergence at either early or late readings. This is illustrated in the next section, which compares MIC as a function of increasing cell growth.

FIG. 1. MIC of 5-FC at different inoculum sizes for several strains of yeast. Symbols: $\blacklozenge$, *C. parapsilosis* (Me strain); $\circ$, *C. albicans* (St strain); $\triangle$, *C. albicans* (Ba strain); $\bullet$, *C. parapsilosis* (Ba strain); $\blacktriangle$, *C. parapsilosis* (Wi strain); $\square$, *C. tropicalis* (laboratory strain); $\blacksquare$, *T. glabrata* (Cu strain).

FIG. 2. The MIC of miconazole at different inoculum sizes for several strains of yeast. Symbols are the same as those in Fig. 1.

$IC_{1/2}$ dependence on control growth. During the course of our studies it was realized that faster-growing yeasts showed increased variation of $IC_{1/2}$ (i.e., four- to eightfold differences) over the inoculum range tested unless turbidimetric readings were taken earlier than 48 h. The time period during which $IC_{1/2}$ readings remained uniform was related to continued growth in the control tubes. Figure 5 demonstrates this relationship for one isolate whose growth in drug-free medium reached a "plateau" at 29%T (optical density, 0.53). The plateau is defined as the phase of the growth curve during which turbidity does not further increase with continued observation. As can be seen from this example, the $IC_{1/2}$ for various inocula showed no greater than twofold differences until the control tube approached the plateau. After a point, which could be defined as the lowest %T of control growth that gave an $IC_{1/2}$ variation of no more than twofold over the range of inocula tested, the $IC_{1/2}$ values increased and showed more variation over this inoculum range. For this isolate, the point is 50%T. Figure 6 represents the concurrent MIC determinations and illustrates that, even with the visual end point plotted as a function of

FIG. 3. A comparison of the MIC and three calculated IC_n values for miconazole of three yeasts at different inoculum sizes.

FIG. 4. A comparison of the MIC and three calculated IC_n values of 5-FC of four yeasts at different inoculum sizes.

control growth, variability with inoculum size persists.

Table 1, derived from serial observations of several strains in a manner similar to that illustrated in Fig. 5, shows the control growth plateau. The lowest %T consistent with no more than twofold variation in $IC_{1/2}$ is shown. Although this latter point varied for each strain, all of our isolates demonstrated minimal variation when the $IC_{1/2}$ was calculated before the control tube reached a turbidity of 50%T (optical density, 0.30).

DISCUSSION

The visual end point of conventional susceptibility test methods, although convenient, entails several serious problems. First, it clearly requires a subjective judgment concerning which drug concentration is the lowest that

demonstrates "no growth." Neither variation between observers nor that which might occur between laboratories was examined in our studies, since all determinations were performed by the same investigator. However, variation would be expected to be maximal with an end point that attempted to distinguish "almost no growth" from "complete absence of growth." Second, the commonest criterion of no growth, no visible turbidity, assumes that very small changes in turbidity are always caused by cell growth. We also employed this criterion in the visual studies since it seemed the sharpest end point, but we are not convinced that the underlying assumption is always correct. In particular, suspensions that became minimally turbid during incubation with high concentrations of miconazole did not demonstrate significant increases in numbers of organisms (hemocytometer counts). It is possible that with some drugs and/or yeasts other events, such as increased adhesiveness with cell clumping or cell

FIG. 5. $IC_{1/2}$ values for 5-FC calculated from sequential measurements of four different inoculum sizes for one strain of yeast. Each point represents the $IC_{1/2}$ value for the inoculum as a function of the turbidity of the drug-free control. The shaded area is the range of least $IC_{1/2}$ variation.

FIG. 6. MIC values for 5-FC determined from sequential readings of four different inoculum sizes for the same yeast strain as shown in Fig. 5. Each point represents the MIC value for the inoculum as a function of the turbidity of the drug-free control.

swelling (1), could account for minor changes in visual clarity independent of cell multiplication. It seems impossible to control for this with visual methods. A third drawback is inoculum dependence. Block et al. (4), for example, demonstrated this for cryptococcus against 5-FC. We have demonstrated this for several species for both 5-FC and miconazole. To make visual methods reproducible one must be careful to standardize inocula. Because of different growth characteristics of various yeasts, the practice of using a standard dilution of an overnight growth would not be sufficient. Furthermore, our results suggest that in comparing the relative sensitivity of strains between different inocula, even if standardized, results can be conflicting. Thus in Fig. 1 and 2 strains that appear to have different susceptibilities at 10^4 cells/ml have identical MICs at 10^3 cells/ml. Therefore, even with standardized inocula, visual MICs have serious limitations as an in vitro parameter to correlate with clinical events.

The turbidimetric method reported here

avoids the problems just listed. Other authors have described similar turbidimetric assays and found them sensitive and reproducible (1, 7). The calculated value of $IC_{1/2}$ avoids a subjective end point. Furthermore, this turbidimetric end point is an objective measure of reduced growth. The $IC_{1/2}$ criterion obviates problems in discriminating between two similar tubes and in pondering the significance of minimal changes in tube clarity. Finally, we find that this criterion of sensitivity is free from the inoculum dependence seen with conventional methods. All of our initial inocula were below the visible range. However, Bennett, using slightly turbid suspensions and studying a narrower range of inocula, also showed inoculum independence with a similar method (1).

Other research techniques for susceptibility testing, such as killing curves constructed by colony or hemocytometer counts, may also avoid problems of the visual MIC determination. However, they require much more attention and materials. In contrast, this turbidimetric technique could readily be adapted to clinical use. Since results do not vary with inocula, a standard dilution of an overnight broth could be used. At times, for slower-growing isolates, a higher inoculum might be more convenient. The preparation of drug dilutions is identical with that used in visual techniques. We have purposely employed readily available plastic disposable tubes for incubation and have found their optical properties sufficiently uniform for the purposes of this technique. Similarly, the simple spectrophotometer used in this study is available in many clinical labs. With more sophisticated spectrophotometric equipment, this test could be automated. A few serial readings of the control tube should be performed to ensure that the drug-free mixture is

TABLE 1. Turbidity of yeasts in drug-free medium at plateau and at limit of least $IC_{1/2}$ variation

Species	%T at plateau	Lowest %T with intrastrain ^a $IC_{1/2}$ variation ≤ 2 -fold	
		Miconazole	5-FC
<i>C. albicans</i> (St strain)	30	30	50
<i>C. parapsilosis</i> (Ba strain)	27	27	NT ^b
<i>C. parapsilosis</i> (Wi strain)	29	32	32
<i>C. tropicalis</i> (lab strain)	31	32	39
<i>T. glabrata</i> (Cu strain)	16	18	27

^a Starting inocula of 10^2 to 10^5 cells/ml.

^b NT, Not tested.

actively growing at the time of the $IC_{1/2}$ determination. It appears that most strains can be read at 24 to 48 h, with a variety of initial inocula. Repeated measurements of the drug tubes appear unnecessary.

The clinically important level of susceptibility is presently unknown. Turbidimetric methods will usually yield values below concurrent visual MICs although, as noted in Fig. 4 and 5, MICs with low initial inocula tend to approximate $IC_{1/2}$ values. As one example, fungal meningitis can respond to therapy despite very low levels of antifungal activity in the cerebrospinal fluid, i.e., drug concentrations below the MIC for the organism as determined by the visual method (3, 5). At present, it seems most important to utilize a method of susceptibility testing that is reliable, so that more firm correlations can be made with clinical efficacy.

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