Rapid Microdilution-Colorimetric Assay for Yeast Susceptibility to Fluorocytosine

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Acid production by certain yeast species through the fermentation of glucose was used as the basis of an in vitro test for measuring susceptibility of these organisms to 5-fluorocytosine. Serial dilutions of 5-fluorocytosine in yeast nitrogen base broth, with bromothymol blue indicator dye, were made on microtiter plates. A fixed-concentration suspension of yeast cells was added to successive wells of the plates, and the color change from blue to yellow, indicating generation of acid, was noted. Eighteen hours after inoculation the lowest concentration of 5-fluorocytosine that completely inhibited the production of acid was recorded as the minimum inhibitory concentration. The results were reproducible in multiple trials with organisms of the genera Candida, Torulopsis, and Saccharomyces. This test is a rapid, inexpensive alternative to current 48- to 72-h methods in which broth turbidity is used as the end point.

Candida species infections have become increasingly important causes of morbidity and mortality in recent years, especially in compromised hosts (1, 7). Whereas amphotericin B remains the major antifungal agent against these organisms, other, newer drugs may also be effective. One of these, 5-fluorocytosine (5FC), has been employed in many instances, but its usefulness is limited by variations in the susceptibility of clinical yeast isolates (2, 5). Susceptibility testing is, therefore, an important factor in the decision to use 5FC.

There are several methods for the determination of 5FC susceptibility among yeasts, but they are either complicated or slow or require sophisticated equipment (4, 9). This report describes a method that is rapid and simple enough to be applied in nearly any clinical laboratory, whether isolation of a yeast organism is common or rare. The generation of acid by the metabolism of glucose by yeasts (11) is the basis of the test.

MATERIALS AND METHODS

Media. The growth medium used was yeast nitrogen base broth (10), modified to include 3% dextrose (8). The pH was adjusted to 7.2 with 10% potassium hydroxide. Four milligrams of bromothymol blue dye powder (Fisher Scientific Co., Fairlawn, N.J.) was added to each 100 ml of this solution, resulting in a bright blue liquid called for simplicity "blue broth." This broth was filter sterilized and stored at 4°C until used. A 1% solution of 5FC powder (Hoffman-LaRoche, Inc., Nutley, N.J.) in distilled water was filter sterilized and added to an amount of blue broth to give a concentration of 650 µg/ml. This stock solution of 5FC was likewise stored at 4°C until used. Bromothymol blue yields a yellow color at pH 6 and a blue color at pH 7.6.

Test organisms. Yeast isolated from patients were incubated on Sabouraud agar slants at 36°C for 24 h. Specimens tested included 10 different Candida albicans strains, 10 Candida tropicalis strains, and 8 other yeast species, all recovered from patients at Memorial Hospital. Five C. albicans isolates from other institutions, determined elsewhere to be resistant to high concentrations of 5FC, were also tested. In addition, an isolate of C. albicans (Hoffman-LaRoche no. 66531), known to be susceptible to 5FC concentrations of 0.25 to 0.5 µg/ml, was tested simultaneously with every trial of an unknown strain.

Yeast suspensions. A loopful of yeast was transferred to 1 ml of blue broth and suspended evenly. The concentration of yeast cells was determined with a counting chamber. Budding organisms were counted as two. The original suspension was then adjusted to a concentration of 2 x 10^7 cells per ml with blue broth and used in the susceptibility test.

5FC dilutions. Serial dilutions of 5FC in blue broth were made in two rows of successive wells of sterile, flat-bottomed microtiter trays with a 50-µl diluting loop (Cooke Laboratory Products, Alexandria, Va.). Concentrations of 5FC in the wells ranged from 130 to 0.016 µg/ml. An extra well with 130 µg/ml was used as an organism-free control, and one well was prepared with no 5FC, to serve as a drug-free control. Before inoculation of the organisms, the volume in each well was 0.2 ml.

Inoculation of 5FC dilutions. A 50-µl drop of yeast suspension containing 10^5 cells was added to each well except the organism-free control, which received 50-µl of blue broth. The final volume in
each well was 0.25 ml. The microtiter trays were covered with fitted sterile plastic lids and incubated at 37°C for 18 to 20 h. The minimum inhibitory concentration (MIC) was read as the lowest concentration of 5FC in which no color change, from blue to light green or yellow, was detectable.

Turbidimetric control. A standard turbidity assay for 5FC susceptibility, modified by an incubation temperature of 26°C, was employed to measure the MIC of each yeast isolate tested and was read at 48 h (8). With this method, a smaller inoculum of yeast (5 × 10⁶ cells) was added to a larger volume of medium (1 ml) with a lower concentration of glucose (1%) and incubated at a lower temperature (26°C) for a longer period of time (48 h). The MIC was defined as the lowest concentration of 5FC in which turbidity was not visible. The MIC obtained by broth turbidity was compared with that obtained by the microdilution-colorimetric technique, to check the accuracy of the new method.

Subcultures from microtiter trays. Broths from nonturbid wells, including those with color change and those up to three dilutions above the colorimetric MIC, were subcultured onto blood agar plates and incubated at 37°C. These subcultures were read at 24, 48, and 72 h.

RESULTS

Appearance of trays. MICs determined by the microdilution-colorimetric test were reproducible in multiple trials for a given isolate, to within one or two dilutions. From trial to trial, this slight discrepancy was observed where the MIC was low (≤4 μg/ml), and so it represents very small differences when expressed as actual concentration of 5FC. In 27 of 39 pairs of tests (69%), the MICs obtained were identical. In 10 of the 39 (26%), there was a one-dilution difference, representing 0.25 to 2.0 μg/ml. In 2 (5%), the difference was two dilutions, or 0.37 μg/ml and 3.0 μg/ml, respectively. The color change caused by acid production was distinct and easy to read. In wells with lower concentrations of 5FC, visible turbidity was present as well as the obvious bright yellow color, but the color change was invariably present at concentrations of 5FC at which there was no detectable turbidity in the well. The presence of color change without visible turbidity was noted at concentrations from one to three dilutions beyond the highest level at which there was visible turbidity in the same colorimetric test. Any change toward yellow that could be distinguished from color change in the yeast-free control wells was considered to indicate ongoing metabolism. The well in which even a slight color change was seen was interpreted as indicating resistance to that concentration of 5FC.

This interpretation was supported by examining subcultures transferred onto solid medium from nonturbid microtiter wells, whether color change was present or not. Viable yeast colonies grew from these subcultures, including those from wells in which the broth 5FC concentration had been in excess of the colorimetric MIC.

Comparison with turbidimetric method. Figure 1 illustrates the MICs determined by both methods for 28 of the yeast isolates obtained at Memorial Hospital. Comparative tests were done with an additional six C. albicans isolates not shown in the figure. The MICs by the colorimetric method were usually one or two, and at times more, dilutions higher than the MICs by the turbidimetric method. A strain was considered resistant for clinical purposes if the MIC was greater than 10 μg/ml. All isolates tested, except the Candida krusei, would therefore be considered "susceptible." For the C. krusei, the turbidimetric MIC was 33 μg/ml and the colorimetric MIC was 130 μg/ml, a difference of two dilutions. For the five resistant C. albicans isolates supplied by other inves-
tigators (not shown in the figure) the colorimetric assay showed MICs greater than 130 μg/ml in every instance. These data demonstrated that the assay worked for strains resistant to high concentrations of 5FC as well as for strains susceptible to low concentrations of the drug.

**DISCUSSION**

The conditions under which 5FC susceptibility testing is done have been shown to affect the outcome significantly (3). The inoculum size, sugar concentration, temperature, and time of incubation in the colorimetric method herein described all differ from the standard turbidimetric technique with which it is compared. These conditions were chosen because they yielded a colorimetry result with the known Hoffman-LaRoche-supplied organism (MIC = 0.5 μg/ml) that agreed with the turbidimetric result reported to us by Hoffman-LaRoche (MIC = 0.25 to 0.5 μg/ml). Our own turbidimetrically determined MIC with this isolate was also in this range. Moreover, the MIC for this known test organism, obtained by the colorimetric assay, was reproducible in the range of 0.25 to 0.5 μg/ml in 16 separate trials. Because of this, we felt justified in using the above conditions of inoculum size, sugar concentration, time, and temperature in subsequent experiments with a total of 39 unknown isolates.

The observation that MICs are higher by the colorimetric method than they are by the turbidimetric technique warrants comment. We believe the colorimetric test to be more sensitive and valid for 5FC susceptibility testing. Subcultures to solid medium, from nonturbid wells with or without color change, yielded viable yeast colonies. This is in accord with the observations that 5FC is generally not considered to be a fungicidal drug (12). However, even a microbicidal drug may allow organisms to remain metabolically active at drug concentrations which inhibit replication (6). Visible replication, as evidenced by turbidity, is inhibited by certain concentrations of 5FC. Our data show that at these same and at higher 5FC concentrations, yeast metabolic activity continues. It is, however, notable that in no case did the differences between MICs obtained by both techniques change the characterization of an isolate from "susceptible" to "resistant." Actual differences in the range of 0.25 to 2.0 μg/ml in separate trials of a given isolate are less important than the fact that the differences are very small and do not affect the clinical applicability of the test. As it would be applied in the clinical laboratory, the MIC would be expressed adequately as "less than 4 μg/ml," which would eliminate even the small discrepancies observed among the several trials with the same isolate. Where the MIC was greater than this level, trial-to-trial differences in the absolute value were not observed with either colorimetry or turbidimetry for the 5FC concentrations tested.

We believe that colorimetry offers definite advantages as a clinical tool over existing turbidimetric methods for assaying 5FC susceptibility in yeasts. Whereas this colorimetric method distinguishes between susceptible and resistant isolates as accurately as do turbidimetric techniques currently available, it is more economical of time and laboratory space and costs less than turbidimetry. These features cause colorimetry to compare favorably with turbidimetry, which Block et al. describe as "both time consuming and costly" (3). The assay is simple and rapidly set up, the end point is clear and easy to read, and the test is adaptable to diagnostic microbiology laboratories, whether the need to test for 5FC susceptibility is frequent or rare.

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**LITERATURE CITED**