

Innovative Endpoint Determination System for Antifungal Susceptibility Testing of Yeasts

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Fungal infections and antifungal resistance are increasingly recognized. Antifungal susceptibility testing remains unstandardized, and a particularly important problem is endpoint determination. In this paper we propose the yeast metabolic reduction of the tetrazolium salt 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) as a colorimetric endpoint which is quantitative and objective. Amphotericin B, fluorocytosine, and fluconazole dose-response curves were obtained, and a metabolic MIC could be determined by using precise criteria.

Yeast infections are an increasingly important problem, particularly in immunocompromised patients. The recognition of antifungal resistance stresses the need for improved susceptibility testing. Unfortunately, antifungal drug susceptibility assays have been difficult to standardize (6, 11, 15, 16, 18). While considerable progress has been made, the determination of endpoints is still not satisfactory. In most assays endpoints are determined by visually grading turbidity, a subjective operation, particularly problematic when testing azole drugs for which trailing endpoints are common (4, 7, 9). In this paper we describe an innovative colorimetric, quantitative method of endpoint determination for susceptibility testing based on the reduction of a tetrazolium salt.

Tetrazolium salts are widely used as indicators of reducing systems (2, 10). When reduced they give rise to a colored formazan crystal. Because these agents are cleaved by various dehydrogenase enzymes, active mitochondria will produce colored formazans and provide a colorimetric method to detect metabolically active cells. By using a spectrophotometer or an enzyme-linked immunosorbent assay (ELISA) plate reader, this methodology is amenable to quantitation. One tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), has been used for anticancer drug assays (1, 13, 20).

Historically, formazans were water insoluble and could not easily be quantified without performing additional steps involving centrifugation of cells and removal of media, use of an organic solvent such as dimethyl sulfoxide, and occasionally sonication. Recently a new tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), which after reduction yields a water-soluble formazan, has been synthesized (14). XTT-based assays have also been used in a variety of eukaryotic cell drug assays (3, 17, 21). We describe a drug susceptibility assay for yeasts based on XTT reduction and quantitation of the resulting colored formazan solution.

MATERIALS AND METHODS

Organisms. Most yeast strains were clinical isolates from the microbiology laboratory of the Toronto Hospital (numbers refer to the Toronto Hospital collection number). Ad-

ditional strains of *Candida lusitanae* (including *C. lusitanae* FR-8900) were obtained from R. Summerbell (Provincial Health Laboratory, Toronto, Canada). Organisms were maintained on Sabouraud agar plates at room temperature and were passaged once a month. All tests were performed using an overnight broth culture of the organism.

Broth. Buffered yeast nitrogen base (BYNB) was modified according to the method of Shadomy et al. with glucose (1%) and asparagine (0.15%) (18). A 10× solution was prepared in water and diluted with phosphate buffer, and the broth was then sterilized by filtration.

XTT. XTT was obtained from Sigma or Polysciences (catalog no. X4521 or catalog 19661). Because of the poor solubility of XTT in BYNB, the XTT stock was prepared as a saturated solution in Ringer's lactate by mixing 1 mg of XTT per ml in Ringer's lactate, resulting in almost complete dissolution of XTT. The solution was filtered through a 0.22 μm-pore-size filter, aliquoted, and stored at -70°C until needed.

PMS. Phenazine methyl sulfate (PMS) (Sigma catalog no. P9625) was prepared as a stock solution of 5 mM (1.53 mg/ml) in BYNB, filter sterilized, and kept at 4°C until needed.

MEN. Menadione (MEN) (Sigma catalog no. M5625) as a stock solution of 10 mM (1.72 mg/ml) was prepared in acetone, aliquoted, and stored at -70°C until needed.

Amphotericin B. Amphotericin B was the parenteral preparation from Squibb; 50 mg was resuspended in sterile water, and the resulting solution was diluted in BYNB to a stock solution of 0.5 mg/ml. The solution was aliquoted and stored at -70°C until needed. Unused aliquots were discarded after 1 month (12).

5-FC. 5-Fluorocytosine (5-FC) (Sigma catalog no. F-7129) was prepared as a stock solution of 2 mg/ml in BYNB, filter sterilized, aliquoted, and stored at -70°C until needed.

Fluconazole. Fluconazole powder was provided by Pfizer, Montreal, Canada. A stock solution of 2 mg/ml was prepared in BYNB, filter sterilized, aliquoted, and stored at -70°C until needed.

XTT reduction assay. XTT assays were performed on a yeast suspension by adding XTT and an electron-coupling agent and incubating the mixture at the appropriate temperatures. As with tumor cell lines, the addition of an electron-coupling agent was necessary for reduction of XTT (17). We tried two such agents, PMS and MEN, at various concen-

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trations to obtain optimum conditions. Prior to each assay, an aliquot of a stock solution of XTT (1 g/liter) was thawed, and either PMS (20 μ l) or MEN (2 μ l) was added to 4 ml of XTT. A 0.25-ml aliquot of this mixture was added to culture tubes containing 1 ml of yeast suspension (final concentrations: XTT, 0.25 mg/liter; PMS, 0.025 mM; MEN, 1 μ M). Drug assays were all performed with MEN. For drug susceptibility assays with *Candida albicans*, the stock XTT solution was diluted 1:1 with Ringer's lactate and then processed as described above. Tubes with XTT were vortexed and incubated for 3 h at 35°C (with PMS) or 30°C (with MEN). After 3 h the tubes were vortexed to homogenize the formazan solution and then centrifuged to pellet the yeast. The tubes were then read by a spectrophotometer (Quantum dual-wavelength analyzer; Abbott), and the $A_{492.6}$ was recorded.

XTT reduction curves. In order to determine the relationship between the amount of XTT reduction and the amount of live yeast, XTT reduction curves were determined for several species. Yeasts were grown overnight in BYNB at 35°C to obtain a concentrated suspension of yeast. This suspension was serially diluted in a 1:1 ratio with BYNB in a series of eight disposable culture tubes (12 by 75 mm) (the final volume in each tube was 1 ml). The most concentrated suspension used was a 1:1 dilution of the overnight growth. A CFU count was performed by diluting the remaining 1 ml 10-fold in BYNB, vortexing it, and plating 10 μ l on a Sabouraud agar plate. XTT reduction assays were performed with each tube as described above.

Antifungal susceptibility assay. The antifungal agent to be tested was thawed, diluted 10-fold in 1 ml of BYNB, and serially diluted in disposable culture tubes (12 by 75 mm). Drug-free control tubes were included in all series. Final concentrations ranged from 25 to 0.003 mg/liter for amphotericin B and 100 to 0.012 mg/liter for fluconazole and 5-FC. The inoculum was prepared from an overnight growth at 35°C of the organism in BYNB; this suspension was diluted in BYNB to obtain a 95% transmission at 530 nm (15) (approximately 2×10^6 CFU/ml). Fifty microliters of the suspension was added to each tube, 10 μ l was added to 1 ml of BYNB, and 10 μ l of this dilution was plated on Sabouraud agar for a CFU count. Tubes were incubated at 30°C with shaking for 24 h. Thereafter an XTT assay was performed. Drug susceptibility assays were performed in triplicate. For each drug concentration and for the control tubes (no drug), the mean and standard deviation were computed. Results are shown as relative optical density (OD) (i.e., mean OD of each concentration divided by the mean OD of the drug-free tubes). Error bars were computed as follows. For each drug concentration the absolute error was taken as 1 standard deviation. The relative error on the relative OD was therefore the sum of the relative errors; the absolute error on the relative OD was computed from the relative error.

XTT reduction curves of *C. albicans* TTH-156 in the presence of antifungal agents. A standard inoculum of *C. albicans* 156 was prepared as described above. Fifty microliters was inoculated into tubes containing antifungal agents at specified concentrations and into drug-free control broth. Tubes were incubated with shaking at 30°C for 24 h and serially diluted in a 1:1 ratio with media containing the same concentration of antifungal agent as the initial tubes. The last 1 ml was used to perform a CFU count. An XTT assay was performed as described above.

RESULTS

XTT alone is not reduced to formazan by yeasts. With the addition of PMS XTT is effectively reduced by yeasts, resulting in an orange supernatant. The intensity of the reaction is influenced by the concentration of XTT, the concentration of PMS or MEN, and by the length and temperature of incubation (data not shown). BYNB alone does not reduce XTT, even in the presence of PMS or MEN.

When tests included PMS as an electron-coupling agent, it was found that there was a considerable variation in the degree of XTT reduction among different yeast isolates tested. *C. albicans* gave the strongest signal, while *Candida guilliermondii* and *Torulopsis glabrata* gave signals too weak to be useful in drug assays. MEN proved to be a much more potent electron-coupling agent than PMS. Initial assays using a MEN concentration of 0.2 mM produced a rapid metabolic reduction and OD values greater than 2.0 for most cell counts. At the highest cell counts MEN resulted in the formation of a yellow supernatant. By using a final concentration of MEN of 1 μ M and a temperature of 30°C, we obtained the results shown in Fig. 1a and b which demonstrate a linear log-log relationship between the OD and the number of CFU over 2 orders of magnitude. Furthermore, all species gave a signal strong enough to be used for drug assays. With these conditions *C. albicans* often gave a signal slightly above an OD of 2.0 with the drug susceptibility assays. Because of this, one-half the usual concentration of XTT was used for *C. albicans* (Fig. 1a). Table 1 shows the slope and coefficient of determination obtained by least-square line fitting for each strain, demonstrating an excellent correlation between the intensity of the colorimetric reaction and the colony count.

To assess the magnitude of XTT reduction variation within species, four different strains of both *C. albicans* and *C. lusitaniae* were tested. Results are shown in Fig. 1c and suggest that the intraspecies variation is less than the interspecies variation; furthermore, the shapes of the curves are preserved.

For drug susceptibility assays inocula fell within the range of 0.31×10^5 to 2.7×10^5 CFU (mean, 1.24×10^5 ; standard deviation, 0.68×10^5) on the basis of a viable colony count.

Figure 2a illustrates the dose-response curve of *C. albicans* TTH-116 to increasing concentrations of amphotericin B as assayed by the XTT method. XTT reduction is essentially unaffected until a critical concentration of amphotericin is reached. Then there is a sharp drop in the OD values. The drop in CFU count parallels the drop in XTT reduction. At concentrations of amphotericin B greater than 0.78 mg/liter, no colonies were obtained at a dilution of 1:1,000 and the OD was indistinguishable from background. Visually, the amount of growth corresponded to the color intensity.

If the linear log-log relationship in Fig. 1 holds in the presence of amphotericin B, this means that at any amphotericin B concentration x we have the following: $\log OD(x) = k \log CFU(x)$, where k is a constant. Hence, at all x , $\log OD(x)/\log OD(0) = \log CFU(x)/\log CFU(0)$, where $x = 0$ represents the drug-free control tubes. For each concentration of ≤ 0.78 mg/liter, we computed the relative indices in the second equation and plotted the result as a function of the amphotericin B concentration. The results, shown in Fig. 2b, demonstrate that the XTT reduction assay remains a good indicator of the amount of live yeast in a drug susceptibility assay.

Four separate dose-response curves were determined with *C. albicans* TTH-116 over a 10-month period with three

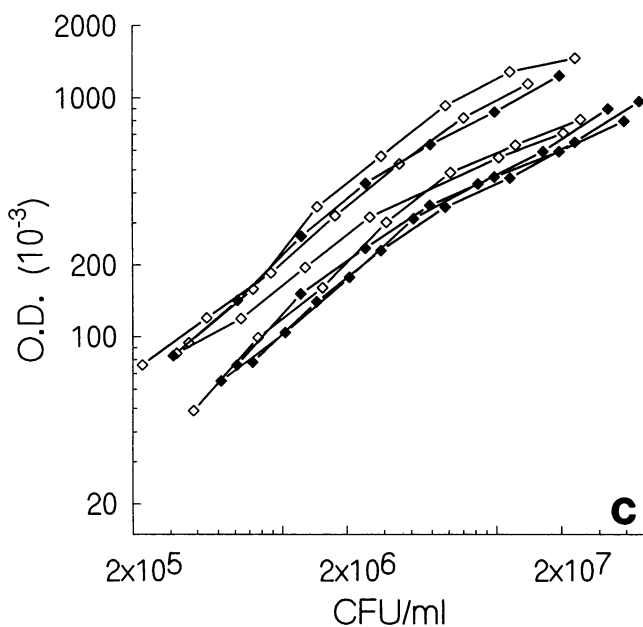
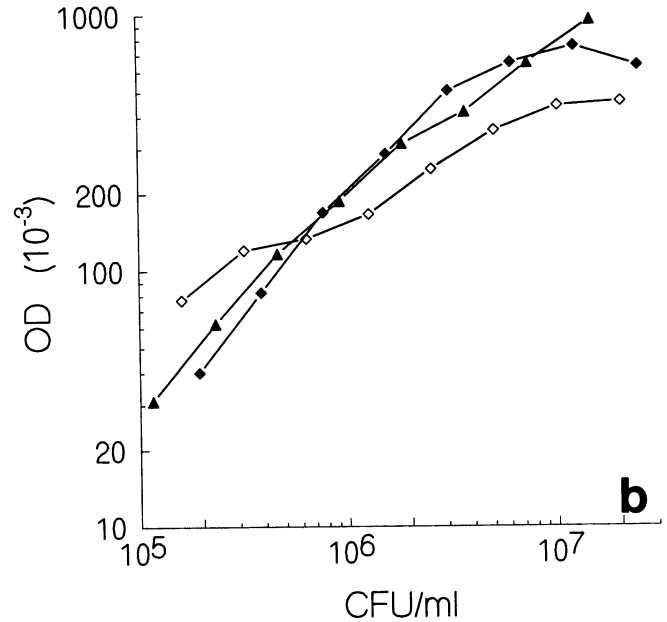
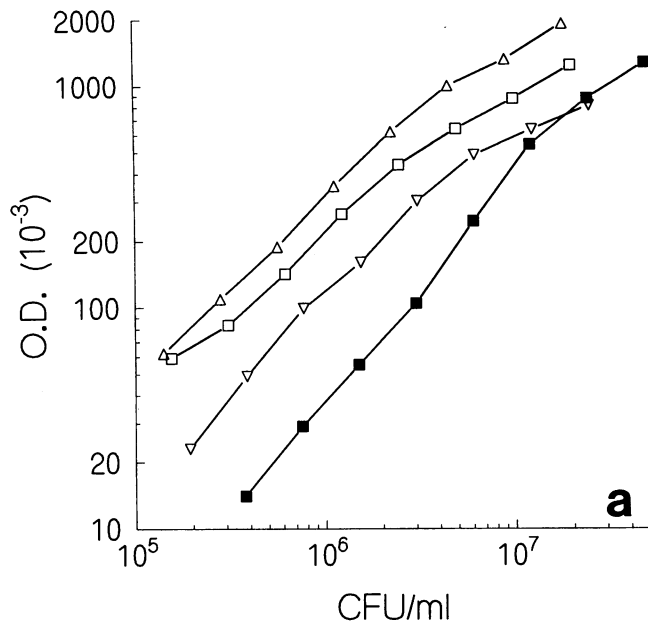


FIG. 1. (a and b) Relationship between the amount of XTT reduction (in the presence of menadione) and the amount of live yeasts for representative strains of different species. (a) Δ , *C. albicans*; \square , *C. lusitaniae*; \blacksquare , *C. guilliermondii*; ∇ , *C. albicans* (XTT at half concentration). (b) \diamond , *C. tropicalis*; \blacktriangle , *C. parapsilosis*; \blacklozenge , *T. glabrata*. (c) Variation in XTT reduction between multiple strains among two species. \blacklozenge , *C. lusitaniae* strains; \diamond , *C. albicans* strains (XTT at one-half concentration).

esophagitis relapsed during fluconazole therapy. Unlike amphotericin B, fluconazole does not produce a drop in detectable color to background levels; instead, its dose-response curve demonstrates a plateau slightly above background. This was in agreement with the visual observation of a light growth of yeast in the tubes.

Figure 5 shows dose-response curves obtained with 5-FC. Two resistant yeast isolates are readily identified. As with fluconazole, the curves drop to a plateau rather than to the background level.

The resistance of *C. albicans* TTH-156 to both fluconazole and 5-FC offered the opportunity to test whether these drugs interfere with the reduction of XTT independently of their action on yeast growth and survival. As described in Materials and Methods, XTT reduction curves for this resistant isolate were determined after the isolate was grown in the presence of fixed subinhibitory concentrations of amphotericin B, fluconazole, and 5-FC. As can be seen in Fig. 6, for each of the three antifungal agents the XTT reduction curves

different lots of amphotericin B. The results shown in Fig. 2c demonstrate excellent reproducibility.

Figure 3 demonstrates amphotericin B dose-response curves obtained from various yeast strains. *T. glabrata* required incubation for 48 h because of poor growth at 24 h as assessed by the growth in the drug-free control and the colony size on the plate used to verify the inoculum. In each case the curves drop sharply to the background level, suggesting a well-defined endpoint. Among the strains tested, the most resistant strain was a *C. lusitaniae* isolate. *C. lusitaniae* strains are known to frequently demonstrate resistance to amphotericin B (8).

Figure 4 illustrates the dose-response curves of the same strains tested with fluconazole. Two resistant yeast isolates were readily identified. Of interest, *C. albicans* TTH-156 was isolated from a patient with AIDS whose candidal

TABLE 1. Slopes and coefficients of determination of lines in Fig. 1

Species	Slope	r ²
<i>C. albicans</i>	0.72	0.99
<i>C. lusitaniae</i>	0.65	0.99
<i>C. tropicalis</i>	0.38	0.97
<i>C. parapsilosis</i>	0.69	0.98
<i>C. guilliermondii</i>	0.97	0.99
<i>T. glabrata</i> ^a	0.93	0.95
<i>C. albicans</i> (1/2 XTT concn)	0.74	0.97

^a Values computed with only six datum points.

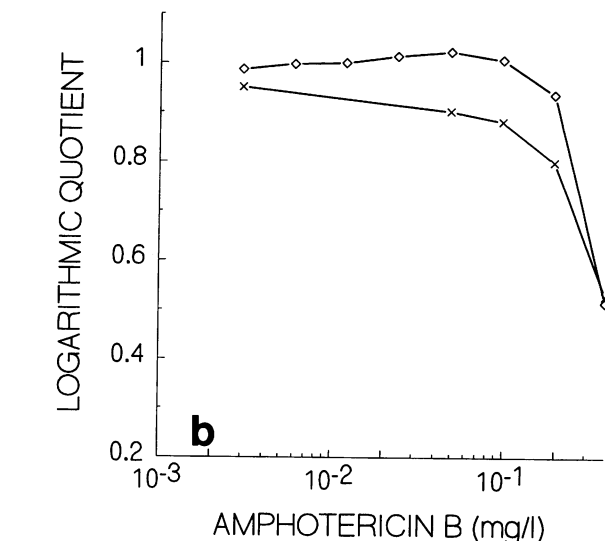
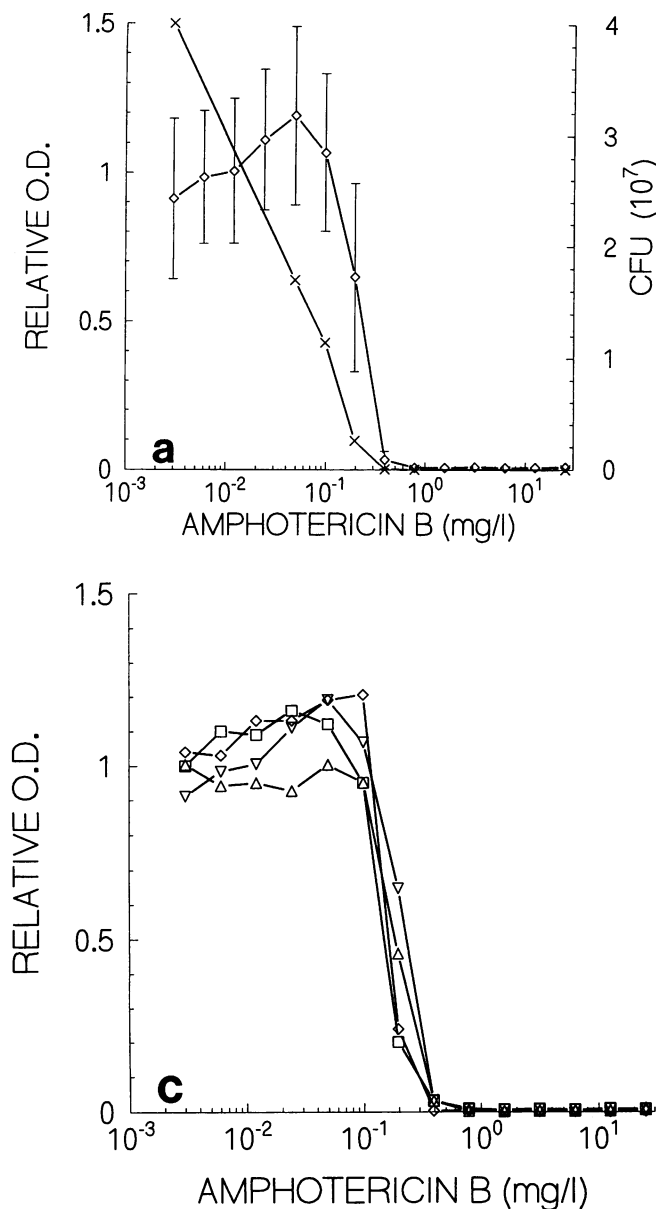


FIG. 2. (a) Dose-response curve of a strain of *C. albicans* with amphotericin B. A CFU count was also performed and is illustrated. \diamond , relative OD; \times , CFU. (b) Logarithmic quotients as a function of the amphotericin B concentration. As discussed in the text, these two curves are expected to be similar. \diamond , $\log OD(x)/\log OD(0)$; \times , $\log CFU(x)/\log CFU(0)$. (c) Four dose-response curves made with the same strain over a period of 10 months.

were similar to those obtained in media free of antifungal agents for concentrations that have little or no effect on yeast growth.

DISCUSSION

Tetrazolium salts are reduced by active mitochondria and are not reduced by dead cells or by erythrocytes (13). This reaction distinguishes between live and dead cells. For example, this property has been used in agriculture to assess seed viability (2) and has been exploited to develop anticancer drug assays based on the tetrazolium salt MTT (1, 13, 20).

The intensity of the reaction depends on several characteristics of the culture studied, including the number of live cells, the amounts of cellular proteins, and cellular metabolic activity (activated cells metabolize MTT more readily [13]). Drugs which affect cell turnover and metabolic activity will affect tetrazolium reduction. The degree of tetrazolium re-

duction is a measure of cellular metabolic activity, and it does not require cellular division, in contrast to ^3H incorporation assays. In practice there is good agreement between results of MTT assays and ^3H incorporation assays (5, 13). MTT assays, because of the insolubility of the resulting formazan, necessitated extra steps, such as centrifugation, aspiration of the media, use of an organic solvent such as dimethyl sulfoxide, and sometimes sonication, to quantitate the amount of formazan generated. The XTT tetrazolium salt differs in that it produces a water-soluble formazan (14). XTT-based assays have been used for antiviral (21), anticancer (17), and antifilarial (3) drug screening.

As with tumor cell lines (17), yeasts do not measurably reduce XTT and the use of electron-coupling agents is necessary. Electron-coupling agents such as PMS and MEN accelerate tetrazolium reduction and produce a more intense reaction, in part because they enhance the action of cellular diaphorases (2, 10). With the addition of PMS, XTT is effectively reduced by yeasts. Different species varied in their ability to metabolize XTT. This is analogous to differences between tumor cell lines (1, 17). Because the XTT reduction was too weak to be useful for some species, we tried menadione and found it to be a much more potent electron-coupling agent for yeasts than for tumor cell lines (17). The resulting signals were above the threshold of our spectrophotometric detector at the usual concentrations of MEN. At this high concentration of MEN we noticed the formation of a yellow supernatant at high CFU counts which we have been unable to explain. A similar phenomenon is known to occur with other tetrazolium salts: ditetrazolium salts can produce formazans of two different colors (2, 10), while monotetrazolium salts can produce two colors through persistence of a free-radical intermediate or metal chelation (2). By reducing the concentration of MEN, these problems disappear and we obtain a family of well-behaved curves for all species tested under the same conditions. We have not observed the formation of formazan crystals; if it does

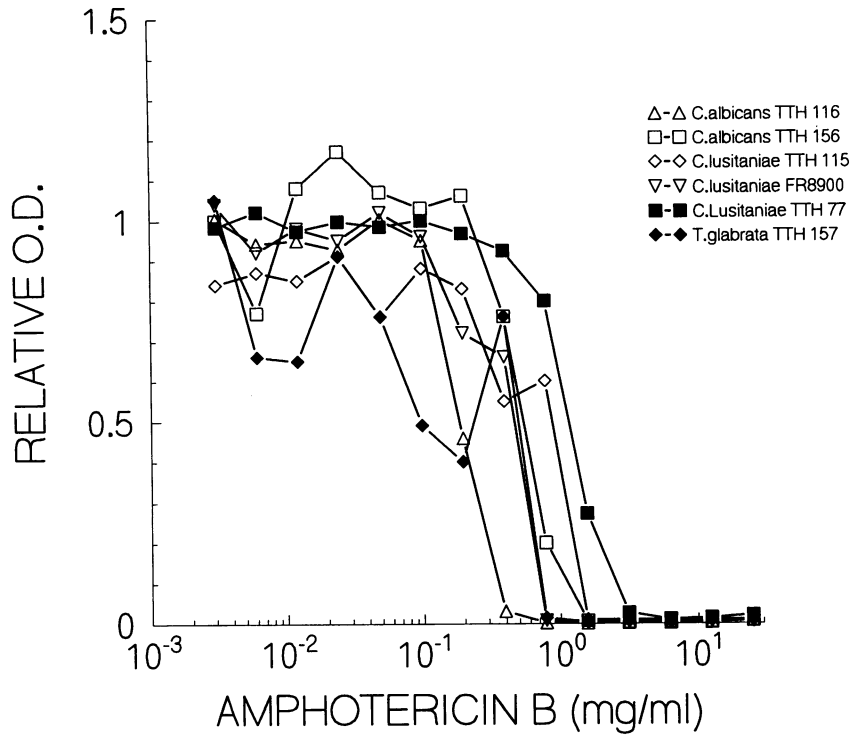


FIG. 3. Dose-response curves of several strains to amphotericin B.

occur, it does not appear to influence the reliability of XTT reduction as an index of the amount of live yeast present.

The linear relationship between CFUs and OD when plotted on a log-log graph vividly illustrates that XTT assays

are a reliable indicator of the number of live yeast cells over 2 orders of magnitude. Amphotericin B (25 µg/ml) has a slight but visible yellow color at 492 nm. It produces a signal of 10 (i.e., OD × 1,000). By using twice this signal as the

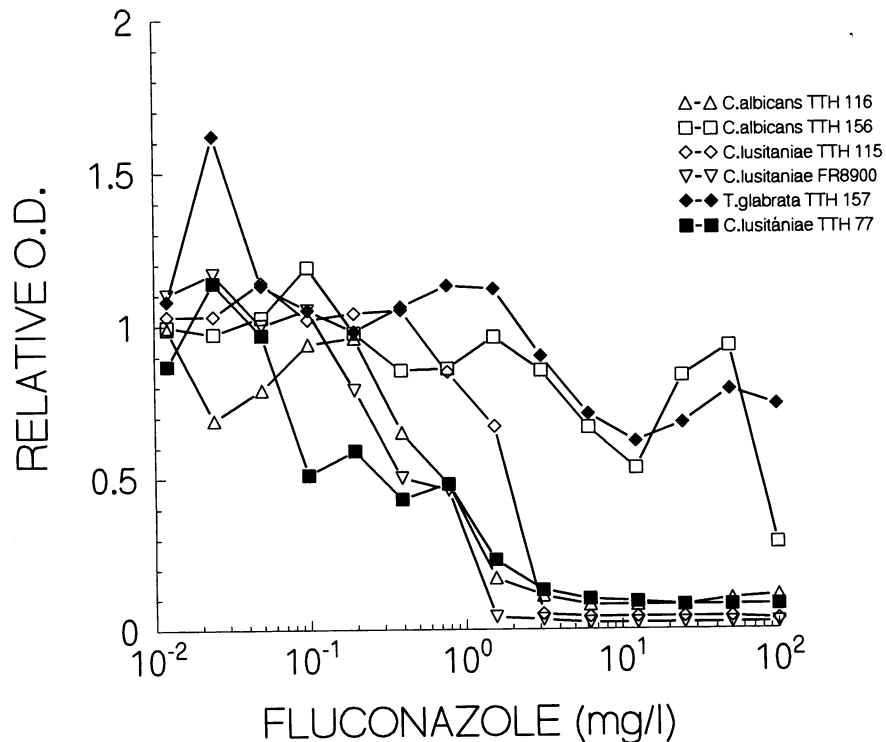


FIG. 4. Dose-response curves to fluconazole.

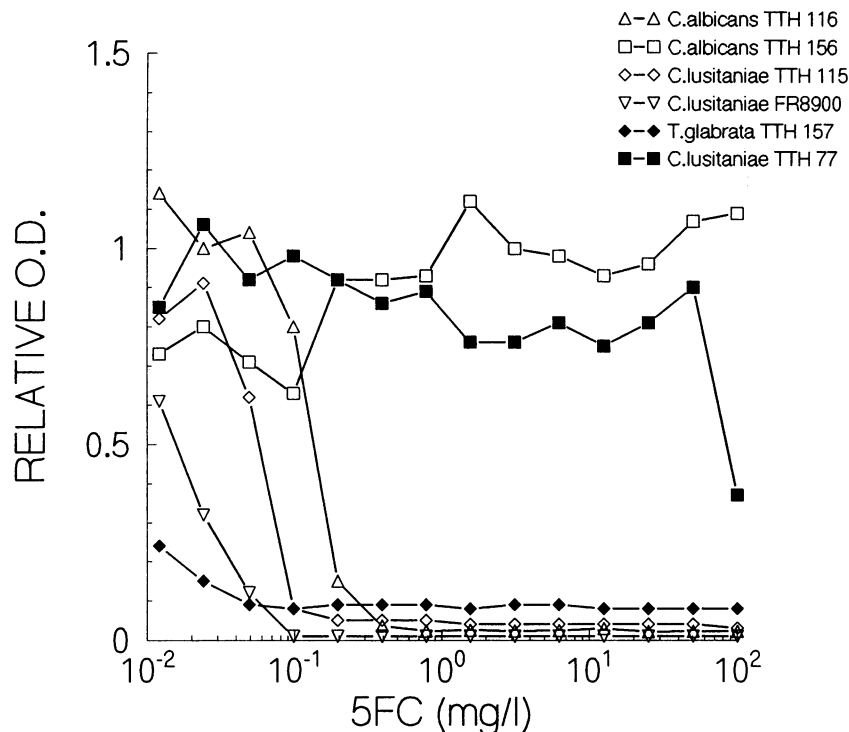


FIG. 5. Dose-response curves to 5-FC.

baseline threshold of sensitivity, we are able to detect approximately 1×10^5 to 4×10^5 CFU. Given that the inoculum used in the drug assays is about 10^5 CFUs, this suggests that the XTT methodology can detect as little as

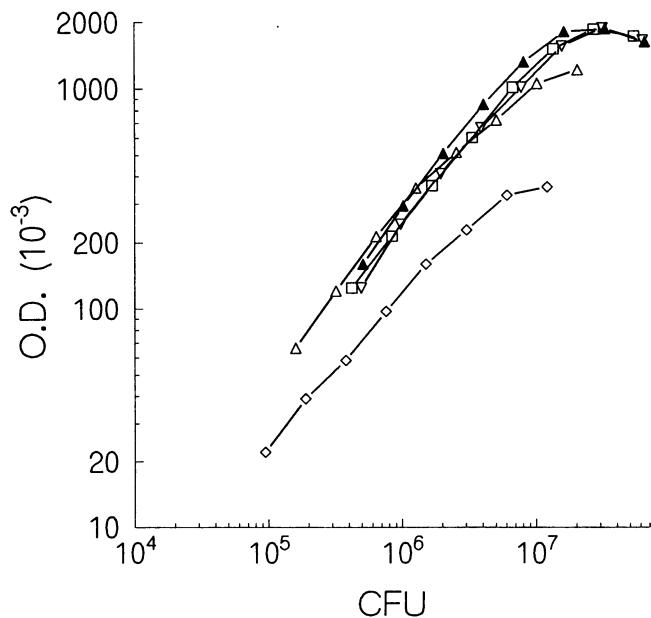


FIG. 6. By use of a resistant strain (*C. albicans* TTH-156), these XTT reduction curves done in the presence of antifungal agents demonstrate that antifungal agents per se do not inhibit XTT reduction. \blacktriangle , BYNB; \square , fluconazole (50 mg/liter); \diamond , fluconazole (100 mg/liter); ∇ , 5-FC (100 mg/liter); \triangle , amphotericin B (0.095 mg/liter).

one or two divisions occurring during the time of the assay (24 h). This is very sensitive, since under optimal conditions the doubling time of *C. albicans* is approximately 1.73 h at 30°C (19).

The dose-response curve obtained for amphotericin B essentially drops to baseline once the MIC is exceeded. This corresponds with the visual inspection of the tubes which show no growth at high concentrations of amphotericin B. This is in agreement with previous observations (4). For amphotericin B the corresponding decrease in CFU mirrors the decrease in the relative OD. As demonstrated in Fig. 2b, the linear log-log relationship appears to be preserved, suggesting that for the amphotericin B susceptibility assay, XTT reduction is a good indicator of the CFU count.

Although the XTT assay was applicable to many yeast species (Fig. 4), because of its weaker absolute signal (Fig. 1) *T. glabrata* had to be incubated for 48 h to be analyzed. For *T. glabrata* small absolute variations in OD therefore resulted in greater changes in the relative OD. More experience with slowly growing yeasts will be required before this assay can be relied upon.

Dose-response curves for fluconazole (Fig. 4) illustrate the phenomenon of trailing endpoints or "ghosting" (7, 9, 11). In general, the yeast isolates we tested exhibited a gradual rather than an abrupt decrease in XTT reduction in relation to fluconazole. These two features underline the potential benefits of a colorimetric assay which can be used to obtain a defined endpoint or MIC. It is interesting to speculate that the in vitro trailing phenomenon and shape of the susceptibility curves may have some relevance to the clinical efficacy of the drug.

A proposed definition of an antifungal MIC has been "the lowest concentration of drug that produces the greatest reduction in growth as compared to the control" (11). This entails a degree of subjectivity if the endpoint is determined

TABLE 2. MICs of three drugs for strains tested

Strain	MIC (mg/liter) of:		
	Amphotericin B	Fluconazole	5-FC
<i>C. albicans</i> TTH-116	0.78	12.5	0.39
<i>C. albicans</i> TTH-156	1.56	>100	>100
<i>C. lusitaniae</i> TTH-77	6.25	25	>100
<i>C. lusitaniae</i> TTH-115	1.56	3.13	0.2
<i>C. lusitaniae</i> FR-8900	0.78	1.56	0.1
<i>T. glabrata</i> TTH-157	0.78	>100	0.1

by visually scored turbidity. This definition can be particularly problematic with trailing endpoints or gradual decreases in growth. To be meaningful in the context of our assay, a MIC should be below a defined threshold. In view of the data reviewed here, a practical threshold appears to be a relative OD of 0.1 (90% reduction in OD). The MIC can then be defined as follows: the lowest concentration of antifungal agent which achieves the greatest difference in relative OD compared with the control, provided that the relative OD is 0.1 or less. With this definition the MICs for the yeasts tested in this study can be determined and are shown in Table 2. The development of reproducible and quantitative methods will permit greater interlaboratory agreement on MICs and is a prerequisite to correlate drug activity and clinical outcome.

The usefulness of the XTT reduction method ultimately depends on how accurately the intensity of the colorimetric reaction reflects the inhibitory capacity of the antifungal agent or the actual number of CFU in the broth. For amphotericin B there was a good correlation with visual growth, relative OD, and the CFU count. Although the conditions of the assay are suitable for the three main classes of antifungal drugs, for fungistatic agents the benefit is that one can define an MIC quantitatively. Because of its quantitative nature it could also lend itself to drug combination studies.

As pointed out by Espinel-Ingroff et al. (4), standardization of a microdilution assay would be desirable. This methodology has been used in the field of cancer research, where quantitation is provided by using an ELISA plate reader (13, 17). ELISA methodology could be used to determine yeast MICs by microdilution.

RPMI has recently been suggested as a suitable medium for antifungal susceptibility testing (4, 11, 16). XTT-based susceptibility assays for antifilarial drugs have used RPMI successfully as the testing broth (3). It is also possible that modifications in the assay could improve the range of fungi that can be assessed by this technique. The ability to provide an objective endpoint for susceptibility testing would, it is hoped, contribute to the establishment of standardized antifungal drug assays.

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